AN EXTENDED INVESTIGATION INTO THE MECHANISM AND KINETICS OF BACTERIAL SULPHATE REDUCTION

University Of Cape Town

WRC Report No. 1251/1/07

Water Research Commission

AN EXTENDED INVESTIGATION INTO THE MECHANISM AND KINETICS OF BACTERIAL SULPHATE REDUCTION

Report to the Water Research Commission

by

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ISBN 978-1-77005-447-9
Report no 1251/1/07

May 2007

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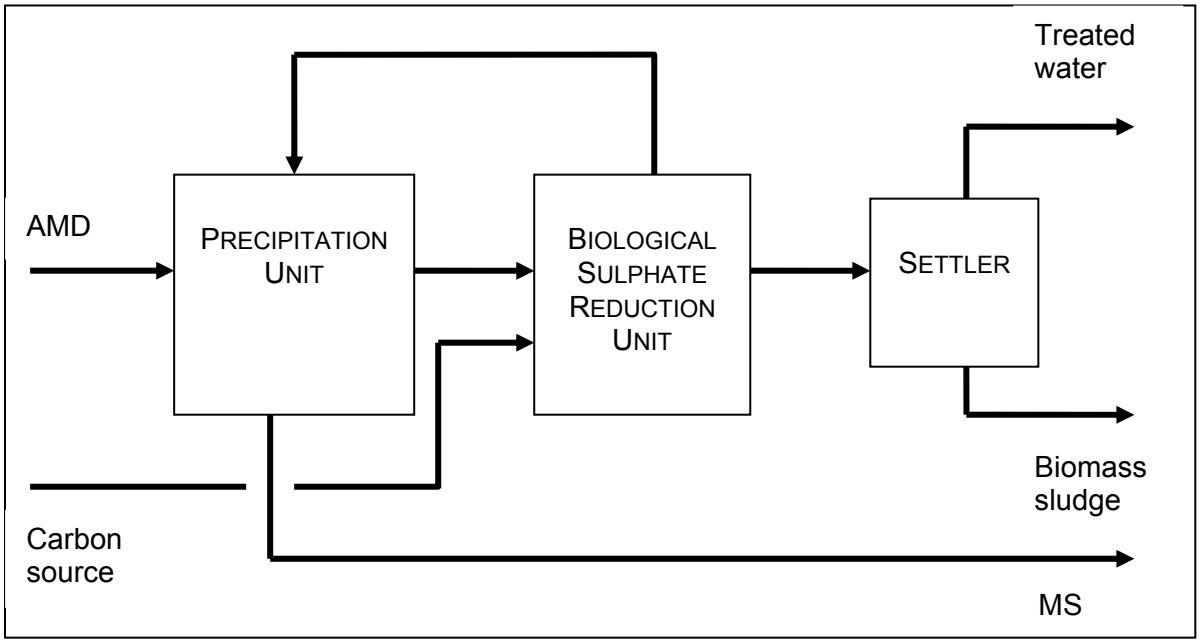
ABSTRACT

This project deals with an investigation into a remediation system based on the reduction of sulphate by sulphate reducing bacteria to treat water containing elevated concentrations of metals and sulphate typical in acid mine drainage (AMD). The process was investigated within the framework of the process flowsheet shown below, in which sulphide formed on biological sulphate reduction was recycled upstream to a precipitation reactor to precipitate metals from AMD as metal sulphides. In addition to metal sulphides, the products of the process were treated water, biological sludge and H_2S. Excess sulphide can then be converted into elemental sulphur or further metal sulphides (not included in present study).



The first aim of the project was based on the recognition of the need to extend the very simple systems studied previously to provide a deeper, more fundamental understanding of both the metal precipitation and the biological sulphate reduction processes through introducing more complexity into the operating environment, such as mixed metal precipitation, product inhibition and mixed microbial culture analysis.

The second aim was to improve the current simulation model for sulphate reduction by replacing the literature-based kinetics with kinetics measured in the laboratory in WRC project K5/1080.

The third aim was to carry out a process evaluation by generating an overall model of the biological treatment process. This would be used to investigate the viability of the process with respect to the carbon source and electron donor and to highlight bottlenecks in process application to guide future experimental work required.

Metal precipitation

- This aspect of the investigation showed that the thermodynamically based, commercial software packages used to predict metal speciation in aqueous solutions are not suitable tools to perform predictive modelling on metal precipitation from a complex stream under conditions where the system will not reach thermodynamic equilibrium.
- The copper sulphide system was extensively investigated and important differences between this and the original model system of nickel carbonate were identified. It was shown that the extremely insoluble nature of copper sulphide means that high global supersaturation must be avoided altogether if a reasonable precipitate is to be obtained. In addition, the formation of fines (a critical parameter for metal removal) must be avoided at the feed points as, unlike in the nickel carbonate system, these do not aggregate with time spent in the reactor. The sulphide to copper ratio is the most critical parameter affecting copper removal.
- The experimental studies showed that the precipitation of zinc from aqueous solution by a mixture containing sulphide and bicarbonate is dominated by the crystalline ZnS species. The iron and copper systems are characterised by the formation of amorphous metal sulphide precipitates and soluble metal polysulphide complexes. It is proposed that this phenomenon is related to sulphide species concentration, rather than total sulphide concentration, particularly in the case of copper.
- The use of anaerobic digester overflow resulted in effective copper precipitation over a wider range of metal to sulphide ratios than with synthetic solutions. This is attributed to the buffering effect of the soluble organic acids, which in turn affects the soluble sulphide speciation. While the use of digester overflow broadened the range over which significant iron precipitation occurred, the efficiency of iron precipitation did not exceed 75% under even the most favourable conditions. Efficient iron removal (>98%) was achieved in the synthetic system under a narrow range of conditions.
- It was recommended that pre-treatment of ferrous iron by oxidation be considered.
- The inability of the software packages to account for the formation of amorphous or colloidal precipitates, the absence of accurate thermodynamic data on some species and the fact that the predictions are only able to represent the system at thermodynamic

equilibrium, precluded the generation of an accurate model to predict both iron and copper precipitation in both the synthetic and biological systems.

Simulation of sulphate reduction

- This aspect of the investigation demonstrated that existing literature parameters are not applicable for simulation of sulphate reduction at high sulphate concentrations and with the carbon source in excess.
- Both the steady state and the dynamic simulations show that the measured kinetic parameters of Moosa et al. (2002, 2004) provide better predictions of the experimental data than literature data and thereby provide a contribution to the predictions required in estimating process performance and in reactor design.
- The temperature dependencies of the Moosa et al. (2004) parameters render them more applicable to a wider range of operating conditions.
- Sulphate inhibition plays a significant role in the accuracy of the dynamic prediction.
- H_2S inhibition has not been incorporated into the model, but is negligible under the conditions at which this data was collected;
- Application of the existing models to a steady state calculation for typical AMD conditions results in an underestimation in calculation of the reactor volume.
- The use of a reactor configuration supporting a long sludge retention time or high sludge age, such as the UASB reactor, is supported by the dynamic simulations.

Effect of sulphide on sulphate reduction

- This study found that, over the pH range of 6 to 7.5, the principal sulphide species inhibitory to the sulphate reducing bacteria is the undissociated H_2S. The maximum specific growth rate increased with increasing pH, owing to the changing speciation from H_2S to HS^- in the pH range of 6.0 to 7.5 studied. The operation of biological sulphate reduction reactors at pHs such that the presence of undissociated H_2S is minimised is thus recommended.
- At pH 7.8, the inhibition of sulphide with increasing concentration showed a decrease in the volumetric sulphate reduction rate with increasing sulphide addition to the feed stream at pH 7.8 over the concentration range 0 to 1.25 kg m^{-3}. The inhibition was observed through a decrease in the maximum specific growth rate and an increase in the death rate constant with increasing sulphide addition. Further the ratio of sulphate reduced to ethanol oxidised and of ethanol oxidised to acetate formed decreased with increasing inhibition. While the model based on the Contois equation could satisfactorily predict these observations for sulphide additions less than 1.0 kg m^{-3}, it failed to predict

observation at increased sulphide concentrations. The results suggest that a factor in addition to sulphide concentration and speciation requires consideration to complete the modelling satisfactorily. This may be the bioavailability of trace metals.

- Kinetic data for biological sulphate reduction using ethanol as carbon source and electron donor were gathered. These were described satisfactorily by the Contois-based model of Moosa et al. (2002). Further, the maximum specific growth rate obtained of 0.060 h^{-1} was similar to that obtained for the acetate system.
- Substrate inhibition was demonstrated at increased feed sulphate concentrations of 12.5 and 15.0 kg m^{-3}. While the latter led to reactor failure at a 10 day residence time of both the liquid and biomass fractions, the former illustrated inhibition through the onset of washout at a lower dilution rate, reduced sulphate conversion and a reduced volumetric sulphate reduction rate despite the increased sulphate loading rate. Hence, where necessary, recycle should be used to ensure feed sulphate concentrations to the biological sulphate reduction reactor are appropriate to avoid substrate inhibition.

Microbial speciation

By using the molecular technique described in this study, it was possible to detect different groups of SRB in the bioreactor across several genera. Using pure culture studies, the methodology used has been validated for the current system. The dominant genera were found to be *Desulfonema*, *Desulfobacteriaceae* and *Desulfobulbus*. *Desulfovibrionaceae* and *Desulfobacter* were the least dominant of the genera studied.

Preliminary studies suggest trends in dominant species with both changing carbon source (acetate vs ethanol) and changing sulphate concentration. On increasing the concentration of soluble sulphide added in continuous chemostat culture, a clear decrease in the prevalence of the *Desulfonema*, *Desulfobulbus* spp. and the *Desulfobacteriaceae* groups was shown. *Desulfobacter*, *Desulfotomaculum* and *Desulfovibrionaceae* were relatively unaffected by the range of sulphide concentration studied. Tools are thus in place to assist in the tailoring of microbial populations to provide increased resilience to process conditions and to overcome the negative impacts of process perturbation. The impact of microbial population dynamics on performance through process kinetics is the topic of ongoing study.

Using complex carbon sources as the electron donor

Review of the literature indicated that acidogenesis is affected by the presence of sulphide and metal ions. A limited pH range of 7.5 to 8.0 is proposed in which both acidogenesis and sulphidogenesis can operate satisfactorily, while methanogenesis is inhibited. Further, data on the effect of sulphate on acidogenesis is limited to sulphate concentrations of 4.0 kg m^{-3}

and less. Methanogenesis is inhibited in the presence of low sulphide concentrations. Hence, conditions for the optimisation of sulphidogenesis and acidogenesis with the concomitant inhibition of methanogenesis can be obtained; however, further data on impact of sulphate on acidogenesis at high sulphate concentrations will be valuable and is partially supplied by Ristow et al. (2004).

Factors governing the variable operating costs are dependent on the choice of carbon source. It was found that provision of a more complex carbon source (molasses) is cheaper than a simple carbon source (ethanol) while the disposal requirements for a complex carbon source are higher than for a simple carbon source. However, where use of the complex carbon source provides environmental burden reduction by avoiding its need for disposal, the assessment is changed. While a significant burden results on the use of primary sewage sludge (PSS), this can be offset against the reduced treatment required in the wastewater works providing a beneficial scenario. In order to assess PSS more fully, it is necessary to consider biomass retention within the anaerobic bioreactor, the use of a flocculating biomass system such as the UASB, enhanced dewatering of the biomass sludge and the potential need for subsequent treatment of the effluent water stream. It is recommended that these studies be undertaken.

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1 INTRODUCTION

Acid mine drainage (AMD) has been described as a major source of water pollution worldwide. Recent reports indicate that AMD affects over 700 km of streams and rivers in Great Britain (Jarvis and Younger, 2000) and over 3000 km in the United States (Hedin, 1997). The remediation of AMD affected waters has significant cost implications. A study by the US Environmental Protection Agency estimated the cost of remediation by current technologies at upwards of $5 billion in the state of Pennsylvania alone. Upon the cessation of mining and dewatering operations the water table reverts to its natural level, a process termed groundwater rebound. This may result in uncontrolled AMD discharge from springs or drainage adits, which are topographically below the rebound water level.

A significant portion of the AMD in these regions originates from abandoned mines sites and waste rock dumps. In South Africa, the full impact of AMD contamination is yet to be experienced. While the coal industry has been responsible for AMD pollution, this has tended to be localised and on a relatively small scale. In terms of the gold mining industry, the Witwatersrand conglomerates typically contain approximately 1.6% sulphur, as pyrite. As a result the waste rock dumps and tailings impoundments have significant AMD potential. A recent study (Naicker et al., 2003) concluded that shallow groundwater, contaminated by mining activities, contributes about 20% of the stream flow in the East Rand region. In terms of groundwater rebound, the East Rand region represents the most immediate threat of a high volume, uncontrolled AMD discharge. During the peak production period, 24 mines operated 90 shafts in the region. By 1995 only two mines remained operational (Scott, 1995) and indications are that available gold resources will be exhausted within the next five years. The interconnectedness of the historical mining operations has resulted in the entire East Rand Basin still being dewatered by the operating mines. As a result no uncontrolled discharges have yet been observed originating from the previously abandoned sites. As a result, the majority of the published research is specifically relevant to sites in the UK, Europe and North America, where longer term data is readily available.

AMD arises from the bacterially catalysed oxidation of sulphide minerals, most commonly pyrite (FeS_2). This process may occur in operating and abandoned mines, where sulphide minerals are exposed to the atmosphere, as well as in waste rock piles and tailings

generated by the processing of mined ore (Johnson, 1995). The oxidation of pyrite is written as follows:

$$FeS_2 + {}^7/_2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+$$

Additional exposure to oxygen, typically at the surface discharge point, results in the oxidation and subsequent hydrolysis of the ferrous iron, generating of additional proton acidity. Therefore, the oxidation of one mole of pyrite can ultimately produce four moles of proton acidity, making this one of the most prolific acid generating reactions in nature (Appelo and Postma, 1993). The acid generated by these reactions can be responsible for further leaching of acid labile minerals resulting in a "classical" AMD discharge, having a low pH, high sulphate and iron loading and potentially dangerous levels of other metals.

It is important to be able to predict how long the contamination will persist and what changes in the composition of the discharge can be expected over time. This information is important when considering what type of treatment strategy to implement. Historical predictions, such as those of Frost (1979) and Glover (1983), were optimistic and typically predicted a 50% decrease in iron concentration in each subsequent period equal to that required for flooding of the abandoned workings. Several UK mines continue to discharge polluted water after 70 years, which clearly attests to the inadequacy of these models (Younger, 1997).

Younger (2000) analysed data from 81 abandoned deep coal mine discharges in the UK and used the information to derive practical prediction guidelines for pollution for the initial period following complete flooding of the mine voids. He identified two features of this period that are most critical to predict. They are:

1. the peak contaminant concentrations following the dissolution of all acid generating salts (AGS) in the workings during flooding, and
2. the duration of the main period of flushing, as contaminant concentrations decline to their long-term levels.

Peak iron and sulphate concentrations in any mine system are clearly related to the availability of AGS in the mined strata. Data from the UK coalfields have suggested that total sulphur content is a good proxy measure for the availability of AGS in the worked seams and adjoining disturbed strata. Table 1.1 shows predictions for first approximation estimates of peak (immediately after mine flooding) and long-term (after complete flushing) iron concentrations from the data for 81 UK coalfield discharges.

Table 1.1: Estimates for peak and long-term iron concentrations based on total sulphur content of worked seams (Younger, 2000)

Total S content (wt %)	Peak Fe conc. (mg ℓ^{-1})	Long-term Fe conc. (mg ℓ^{-1})
S < 1	0.15 ± 0.27	0.02 ± 0.76
1 < S < 2	98 ± 14	12 ± 8
2 < S < 3	267 ± 40	34 ± 22
3 < S < 4	873 ± 163	110 ± 78
4 < S < 5	1494 ± 248	190 ± 127

The duration of the first flush depends primarily on the volume of the mined system and the rate of recharge, although hydraulics of flushing has proven to be more complex and attempts to model systems using above factors alone have resulted in optimistic predications.

This information suggests that, while a mine is being dewatered, the water quality, although poor, does not represent the situation likely to be encountered upon flushing. This must be considered when developing remediation strategies for currently dewatered mine systems.

Currently, the majority of remediation strategies involve the use of active treatment systems, which is essentially conventional wastewater engineering applied to mine waters. Active treatment has been defined as “the improvement of water quality by methods which require ongoing inputs of artificial energy and/or (bio)chemical reagents” (Younger et al., 2002). While there is no technical limit to the quality of water which can be achieved using existing technology, the economic and environmental costs can be prohibitive. For instance, the application of an energy intensive process such as reverse osmosis to treat an AMD discharge may cause more environmental damage (through the generation of electricity by combustion of fossil fuels and disposal of concentrated brines) than it prevents.

Typically, active treatment systems employ the ODAS (oxidation, dosing with alkali and sedimentation) approach, rather than more specialised techniques. Conventional ODAS systems are well suited to achieving the discharge standards typically required for treated mine water. These are:

1. 6.5 < pH < 8.5
2. Total Fe < 1 mg ℓ^{-1}
3. Total suspended solids < 50 mg ℓ^{-1}

These techniques are based on the same basic strategy, which involves the oxidation of relatively soluble cations, such as Fe^{2+} and Mn^{2+} to their less soluble oxidised forms followed by dosing with alkaline chemicals. This is done to raise the pH of acidic streams and to counteract the release of protons which occurs when Fe^{3+}, Mn^{4+} and Al^{3+} hydrolyse prior to the formation of solid metal hydroxides. A final sedimentation step is required to separate the hydroxide solids from suspension. While these steps (O, DA and S) are present in almost all systems, it is important to note that they need not be arranged in this order and duplication of various processes may occur, dictated by the nature of the effluent to be treated (Younger et al., 2002).

There is a significant degree of flexibility in the technology that can be employed to achieve the various steps in the process and the selection depends on the nature of the effluent and the surrounding environment. For example, if sufficient land area and favourable topography exist, the process can be relatively passive, utilising cascade aeration and sedimentation ponds. At the other extreme, chemical oxidants, such as H_2O_2 or sodium hypochlorite, and more active sedimentation (lamellar plate thickeners or centrifuges) may be required.

The popularity of the ODAS approach is testament to its predictability, flexibility and relative low cost. In addition, as calcium-based alkaline salts are typically used, the process adds significant hardness to the receiving water, which is generally beneficial as it reduces the ecotoxicity of most heavy metals (Kelly, 1988). However, conventional ODAS systems have a number of limitations, including (Younger et al., 2002):

- The wider environmental costs associated with lime use, including transportation and the effects of limestone mining elsewhere in order to produce the alkali dosing agents.
- Equipment maintenance costs may be high due to clogging of fittings with iron hydroxides, calcium carbonate and/or gypsum. The use of anti-scaling agents results in significant additional purchase and handling costs.
- The high pH that is required to remove metals such as manganese may result in the remobilisation of other metal hydroxides, such as aluminium.
- The usual failure to produce any net lowering of the total dissolved solids. In many cases the high TDS contents of mine waters is more detrimental to the use of receiving waters for irrigation or water supply than the presence of elevated iron

concentrations. This is particularly true in the South African context, where salinity is often a more serious concern than metal content.

- The low rate of sulphate removal is of concern. Although some sulphate removal may be achieved by the precipitation of metal hydroxyl-sulphates and gypsum, the residual concentration is typically well in excess of the drinking water limit.
- Residual concentrations of some metals can remain relatively high. This is significant where there are high initial concentrations of metals such as zinc, cadmium and manganese, which only precipitate as hydroxides at relatively high pH values.
- The cost of continuous reagent addition may become prohibitive where polluting discharges persist for several decades.
- The low density of the hydroxide sludge results in large volumes that can make handling and disposal difficult and expensive.

A number of advances have been made in recent decades that have overcome some of the limitations of conventional ODAS technologies. These include the high density sludge (HDS) and SAVMIN^{TM} processes. Notwithstanding this, there is an increasing trend towards the development of active biological processes, primarily utilising bacterial sulphate reduction as a method of reducing the TDS of the effluent. The metabolic reactions involved result in the generation of bicarbonate alkalinity and aqueous sulphide, both of which can be employed to neutralise acidic effluents and effect the precipitation of heavy metals. The use of biogenic alkalinity, rather than lime, to increase the pH overcomes a number of the limitations highlighted above. In addition, the precipitation of metals as sulphides rather than hydroxides has the potential to reduce heavy metals to lower residual concentrations and produce a less voluminous sludge. Finally, the use of a complex or “waste” carbon source significantly reduces the substrate cost of the process. There are, however, a number of areas where an improved understanding of the fundamental processes involved could translate to improved process efficiency.

South Africa, as a semi-arid region, is particularly vulnerable to contamination of water resources. While heavy metal pollution is a serious concern, the potential impact of salinity, primarily as sulphate, is equally alarming. This is less of a concern in Europe and North America where sufficient clean water is typically available to dilute the sulphate load once the heavy metals have been removed.

While a number of investigations have been carried out on the effect of mining on water quality, the majority of these have been published in limited circulation reports. In addition, early investigations, such as that of Marsden (1986), underestimated the contribution of tailings to water pollution. The steady increase in TDS of water in the Vaal barrage system during the 1980's prompted further investigation. This led to the conclusion that mine dumps were a source of serious contamination of surface and groundwater, particularly due to percolation of rainwater through the dumps (Jones et al., 1988). This finding has accelerated the rate of research into AMD treatment in South Africa.

Aside from contamination originating from tailings and abandoned mines, a second significant source of pollution is groundwater pumped from operational mines. As previously mentioned the East Rand basin is currently being dewatered by Grootvlei Propriety Mines Ltd, which pumps between 80 and 120 Mℓ of high TDS water per day to the surface. This water is currently treated on site using HDS technology to achieve neutralisation and iron precipitation. While this process effectively neutralises the water and removes the majority of the iron, the sulphate concentration is not significantly reduced and the majority of the high sulphate water is discharged into the ecologically sensitive Blesbokspruit.

A number of investigations have been conducted to assess the long term impacts of using lime treated (gypsiferous) mine water for irrigation during rehabilitation of mine sites and for agricultural purposes (Annandale et al., 1999; 2001). The results indicate that the majority of the salinity would be immobilised as stable gypsum in the soil profile, suggesting this could be an economically feasible method of utilising large quantities of gypsiferous water without causing irreparable environmental damage.

Notwithstanding these findings, elevated sulphate concentrations remain a serious concern, both in terms of environmental impact and the increased cost of treating water to potable quality. Physico-chemical desalination options (spiral reverse osmosis, electrodialysis and GYP-CIX ion-exchange) for this water have been investigated (Schoeman and Steyn, 2001), but are not economically attractive and still require the disposal of a concentrated brine. As a result, biological treatment options, based on biological sulphate reduction are an attractive option.

These systems can be divided broadly into passive and active approaches. Passive systems rely on naturally occurring biological and geochemical phenomena and allow the development of low cost, self sustaining treatment systems which require minimal maintenance and management, such as wetlands, anoxic limestone drains and reactive

barriers. Research in passive systems in South Africa has been spearheaded by Pulles and associates (Pulles et al., 1995; Pulles et al., 2001). The research identified a sulphate reduction rate of 60 g m^{-3} carbon source d^{-1} as the minimum for economic viability. Initial pilot scale systems fell well below this target, primarily due to hydrodynamic issues and variability in the performance of the sulphate reducing units (Pulles et al., 2003). Continued research has led to the development of the Integrated and Managed Passive (IMPI) treatment process, consisting of two sulphate reduction and two sulphide oxidation modules. The first module, the degrading packed bed reactor, is packed with layers of specifically selected carbon sources and is periodically supplemented with readily available carbon. Mean sulphate reduction rates in this module have been in the region of 400 g m^{-3} d^{-1}, well in excess of the original economic target (Pulles et al., 2003).

Research into active biological systems was pioneered by Maree and co-workers at the CSIR (Maree and Strydom, 1985; Maree and Hill, 1989; Maree et al., 2001). This work illustrated the potential of efficient sulphate reduction in anaerobic packed bed reactors using carbohydrate electron donors, such as sucrose and molasses, as well as short chain alcohols. This work led to the development of an integrated biological/chemical process which is currently being operated at pilot scale (Maree et al., 2004). Internationally, the development of active biological systems for the treatment of high sulphate effluents has been pioneered by the Paques group, based in the Netherlands. A demonstration plant based on their Thiopaq® technology has been operational at Anglo Coal's Navigation site in Witbank for over 24 months. The plant is capable of treating 3 Mℓ per day, with a water recovery in excess of 95% (Rein et al., 2005).

A major constraint on the viability of biotreatment systems utilising sulphate reducing bacteria is the cost of a simple electron donor source, such as alcohols or volatile fatty acids. A significant amount of research has recently been conducted in South Africa on the feasibility of utilising waste or complex carbon sources. These have included algal biomass (Boshoff et al., 2004a), tannery effluent (Boshoff et al., 2004b), primary sewage sludge and complex carbohydrate sources (Rose et al., 2002; Whittington-Jones et al., 2002; Molwantwa et al., 2004; Ristow et al., 2004). This research has culminated in the successful piloting of the Rhodes BioSURE process at the Ancor sewage works in Springs. The process treats post HDS water from the Grootvlei mine, utilising primary sewage sludge at the electron donor. A full scale plant, at the same location, was commissioned in early 2005 and is currently under construction.

The examples listed above show that a significant amount of research has been conducted on the biological treatment of AMD in South Africa. However, much of this research has been either at a laboratory scale or has been site and effluent specific. The ability to effectively model the performance of biological treatment processes, accounting for changes in physical parameters, effluent flow and composition and electron donor would represent a significant advancement in the field. Such a model requires rigorously determined kinetic and mechanistic parameters as well as a sound understanding of the metal precipitation process, which may not be predicted adequately using basic thermodynamic parameters. This report presents data which can be incorporated into a model of an integrated biological treatment process.

2 PROBLEM STATEMENT

This project deals with an investigation into a remediation system to treat water containing elevated concentrations of metals and sulphate typical in acid mine drainage (AMD), based on the reduction of sulphate by sulphate reducing bacteria. During mine operation, remediation systems are required to process drainage from rock dumps and tailings as well as effluents from metallurgical processing. On mine closure, due to the potential longevity of acid mine drainage discharges, the system should ideally require little maintenance and have low operating costs. Furthermore, the system requires a high turndown ratio, a measure of its capacity to handle fluctuating conditions typical of effluent treatment processes.

On initiation of this study through WRC project K5/1080, the process was investigated based on the holistic process flow sheet shown in Figure 2.1, in which sulphide formed on biological sulphate reduction was recycled upstream to a precipitation reactor to precipitate metals from AMD as metal sulphides. In addition to metal sulphides, the products of the process were treated water, biological sludge and H_2S, and potentially elemental sulphur. In K5/1080, the following key assumptions were made:

- The precipitation of mixed metal sulphides was considered to be governed by thermodynamics and was initially based on the generally accepted assumption of instantaneous reactions and the generation of stable, crystalline metal sulphides, including iron and copper sulphides.
- The SRB reactor was considered to function on complex carbon sources and the simulation model constructed was informed purely by literature data, much of which was generated under largely differing operating conditions or was poorly specified.

Owing to the limitations of the literature data on both metal precipitation and biological sulphate reduction, it was necessary to carry out fundamental studies into the mechanisms and kinetics of each of the individual processes. In addition, due to the complexity of the system as a whole, with poorly understood aqueous chemistry and interacting bacterial populations, the work programme was structured as follows:

- The metal precipitation system was studied in a simplified form, using divalent nickel as the model metal, and carbonate as the associated anion in the system. This

system was then extended to examine the more complex copper sulphide system. Both aqueous and gaseous sulphide sources were investigated.

- The SRB system was also studied in a simplified form, using the simple carbon source acetate to provide a framework for the fundamental understanding of the sulphidogenic process and investigating the operating parameters of sulphate loading and temperature.

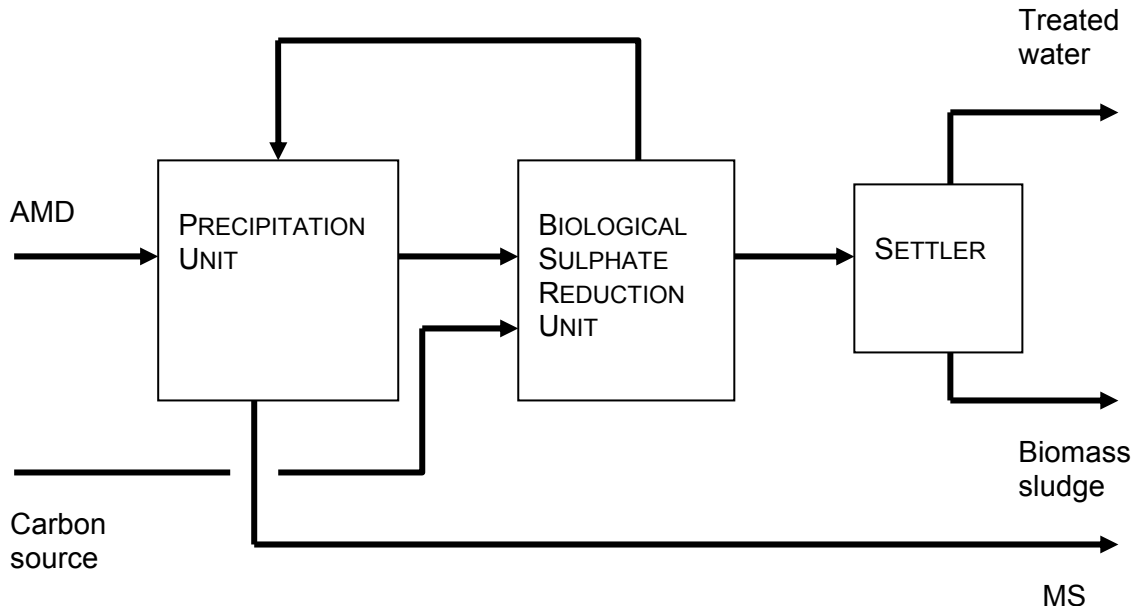

Figure 2.1: Process for the treatment of acid mine drainage investigated in K5/1080

Based on the outcomes of WRC project K5/1080, which are given in detail in Section 3, the aims and objectives of WRC project K5/1251 were compiled. These were based on the recognition of the need to extend the very simple systems studied in K5/1080 and provide a deeper, more fundamental understanding of the component processes described in Figure 2.1. This decoupling of the system was carried out to generate more sophisticated and fundamental models for the separate precipitation and SRB unit operations. An additional objective was to utilise the overall model of the biological treatment process, initially formulated in project K5/1080, albeit in a simplified form, to investigate the viability of the process with respect to the carbon source and electron donor.

Hence, in K5/1251, the sub-processes of metal precipitation and biological sulphate reduction were studied separately and at a more sophisticated level than in K5/1080, partly to provide deeper insight and partly to develop more widely applicable system models. In the

treatment processes, these unit operations are coupled only by the effect of residual metal concentrations on biological sulphate reduction, the quantity of sulphide (principally in the H_2S form) and carbonate (principally in the bicarbonate form) generated in biological sulphate reduction and the manipulation of the process to provide these anionic species in aqueous or gaseous form. Hence, at this stage, it is logical to model the two reactors separately, as unit operations in an integrated system.

The final aspect of the project sought to identify key factors in the application of the technology which may require further fundamental understanding. A significant operating cost associated with SRB systems is the provision of the carbon source and electron donor, hence it is proposed that the use of complex waste carbon sources to provide these. Hence, the suitability of a number of potential carbon sources has been assessed. In order for the process to be financially viable, it must compare favourably with existing chemical treatment strategies while providing outputs of equivalent or improved quality.

In the extension of understanding of these sub-processes to inform process modelling on which K5/1251 was focussed, the following were considered:

Metal sulphide precipitation:

- The approach to modelling of metal sulphide precipitation shifted from the copper sulphide system (a multivalent metal and single anionic species) to a mixed metal system and more than one ionic species (Section 5);
- Limitations in the thermodynamic approach were addressed and kinetic and aqueous chemistry factors were investigated; (Section 5);
- Synthetic laboratory streams were replaced with SRB overflow generated under realistic operating conditions and the effect of the presence of soluble and particulate organic matter in the sulphide stream on metal precipitation was considered (Section 5).

Biological sulphate reduction:

- The experimental results extending the kinetic data on biological sulphate reduction were incorporated into the steady state and dynamic simulation model (Section 6).
- The need for the inclusion of sulphide inhibition in the simulation model was investigated (Section 6).
- Based on literature reviewed and the simulation model developed in K5/1080, the experimental kinetic studies were extended to consider the interaction between sulphide speciation and pH, as well as sulphide loading (Section 7).
- The experimental kinetic studies of biological sulphide reduction were extended from the simplest organic carbon source acetate to ethanol which is typically metabolised by incomplete oxidation (Section 7).
- A steady state and dynamic mathematical simulation model was developed, the objective being to simulate the biological process using the kinetics measured in K5/1080 (Section 6).
- Based on a combination of literature review and experimental findings in K5/1080, molecular methods to analyse the mixed microbial population were established (Section 8.1). Key contributors to this mixed population were identified and population shifts on exposure to sulphide identified (Section 8.2).

Process evaluation:

Because of the need to identify a cheap and abundant carbon source and electron donor for large scale biological sulphate reduction, the use of complex carbon sources was investigated.

- A literature review was undertaken to assess the impact of the presence of sulphate, sulphide and other operating parameters on the sub-processes on anaerobic digestion (Section 9.2). Hydrolysis was not considered here, this being the key component of WRC project K5/1216 at the outset of K5/1251.
- A feasibility study was conducted, using a modified version of the model framework established in K5/1080, to compare the performance of three carbon sources: ethanol, molasses and primary sewage sludge, in terms of process performance, quality of outputs and economics (Section 9.3).

As outlined above, the findings of WRC project K5/1080 are detailed in Section 3. Using the approach outlined above, the detailed aims and objectives of WRC project K5/1251 are defined in Section 4.

3 PRIOR RESEARCH OUTPUTS FROM WRC CONTRACT K5/1080

An initial project, K5/1080 (Hansford et al., 2004), centred on biological sulphate reduction for treatment of acid mine drainage was conducted by this research team. This was focussed on generating a fundamental understanding of key aspects of biological sulphate reduction and metal sulphide precipitation. The following outcomes were derived:

- A comprehensive and critical review of the literature detailing the mechanism and kinetics of biological sulphate reduction was completed and used as a starting point for the modelling of the process. The rate equations and kinetic constants for the component sub-processes from the hydrolysis of sewage sludge to methanogenesis and sulfidogenesis were assessed and those of value in the modelling exercise tabulated. Areas of inadequate literature knowledge were highlighted. These included bacterial sulphate reduction and the inhibitory effect of sulphate, sulphide and dissolved metal species on this process.
- The anaerobic sulphate reduction process was modelled within the framework of the falling bed sludge reactor, using AQUASIM. The model described the steady state data of the Rhodes University BioSure process adequately.
- A more generalised model was developed in the high level mathematical language OCTAVE to describe both dynamic and steady state behaviour of the anaerobic digester. This model accounted for pH, sulphide, hydrogen and fatty acid inhibition within the constraints of the current literature understanding.
- Experimental studies using the simple carbon source, acetate, were carried out in order to establish the kinetics of biological sulphate reduction under anaerobic conditions and at neutral (or slightly higher) pH. These were conducted at steady state, based on chemostat culture in a well mixed stirred tank reactor. Detailed data as well as a kinetic model describing the sulphate reduction kinetics as a function of sulphate loading rate and temperature were presented.
- Experimental studies and thermodynamically based simulations, using ASPEN and OLI Systems Inc ESP software, were conducted using an iron-free model divalent cation system (nickel and carbonate). It was demonstrated that the thermodynamic based simulations were unable to account for inefficiencies related to fines formation during precipitation. Therefore, experimental studies focused on identifying the mechanism of fines formation and developing strategies for their minimisation.

- The experimental studies showed that the pellet reactor is an appropriate technology for the precipitation of sparingly soluble solids. Manipulating reactor conditions allows the growth of sufficiently large particles, which enhances solid/liquid separation and prevents sludge formation. A second order mathematical model identified supersaturation as the most critical variable in determining metal removal. A number of strategies were identified which could be used to control supersaturation.

4 AIMS AND OBJECTIVES OF WRC CONTRACT K5/1251

The broad aim of project K5/1251 is to develop a model for the simulation of the processes involved in the bacterial reduction of sulphate to treat acid mine drainage using different carbon sources and electron donors. The project sought to investigate the mechanism and kinetics of biological and physicochemical sub-processes of bacterial sulphate reduction, anaerobic digestion, ionic equilibria and the removal of metals by precipitation. The simulation model, developed in WRC K5/1080, is extended to enable the investigation of different process operation strategies.

This has been achieved through the following:

- The simplified systems studied in K5/1080 have been extended to more complex systems that resemble the conditions encountered in practice more closely. This leads to the generation of improved models of each of the primary sub-processes.
- The current simulation model for sulphate reduction is improved by incorporating relevant kinetics measured in K5/1080.
- Further, in assessing the applicability of the process, evaluation of the choice of carbon source and electron donor on the overall viability of an SRB treatment system was considered.

The aims specified for the project are as follows:

1. To quantify the effect of high levels of sulphate and sulphide on the bioprocesses involved in bacterial sulphate reduction,
2. To obtain rate equations and determine kinetic parameters for the various sub-processes involved in bacterial sulphate reduction through use of continuous culture and initial rate studies, using various carbon sources, in which sulphate and sulphide levels are adjusted by addition to the feed and sulphide removal through gas stripping,
3. To identify populations of methanogens and sulphidogens involved in the reduction of sulphate to sulphide and the production of methane from acetate, hydrogen and bicarbonate under different operating conditions,
4. To develop a metal removal model based on ionic equilibria and speciation chemistry using appropriate software,
5. To incorporate the experimental results into a mathematical model and dynamic simulation of the biological, physical and chemical processes which will be used for

two purposes: to direct the experimental investigation and for prediction of process performance, and

6. To analyse the current literature on the anaerobic digestion pathway to determine the effect of the presence of high sulphate or sulphide concentrations on its kinetics and to incorporate these findings into a mathematical model where possible.

The broad aims outlined in the research proposal have been refined into the following research objectives:

1. To understand the chemistry of mixed metal sulphide precipitation and determine optimal conditions for the generation of stable, crystalline precipitates.
2. To evaluate the relative importance of the kinetic and thermodynamic driving forces controlling the metal precipitation.
3. To determine the effect of the bicarbonate to sulphide ratio and the presence of soluble and particulate organic matter in the anaerobic digester overflow on metal precipitation.
4. To extend the operating range of the biological kinetic data with respect to sulphate reduction using acetate as the model carbon source through variation of the sulphate and sulphide concentrations present, and thereby extend the associated model developed.
5. To understand the effect of different concentrations of sulphide species (HS^- and $H_2S_{(aq)}$) on biological kinetics, and their interaction with pH.
6. To understand the contribution of the different microbial populations to the reaction kinetics and evaluate their relative susceptibility to inhibitory compounds.
7. To extend the biokinetics data and modelling of biological sulphate reduction to an additional carbon source representing incomplete oxidation i.e. ethanol.
8. To incorporate the experimental results into a mathematical model for dynamic and steady state simulation of the process.
9. To analyse the literature on anaerobic digestion to determine the effect of the presence of sulphate or sulphide on anaerobic digestion.
10. To link the research to real world problems and use these to evaluate the applicability of the process to a variety of situations, including the choice of carbon source and electron donor.

5 HEAVY METAL PRECIPITATION BY SULPHIDE AND BICARBONATE: EVALUATING METHODS TO PREDICT ANAEROBIC DIGESTER OVERFLOW PERFORMANCE

5.1. INTRODUCTION

The chemical precipitation of metals, primarily by lime addition, has historically been the preferred method for the treatment of acid mine drainage (Dean et al., 1972). While this method is effective and relatively cost effective, when compared to complex physico-chemical strategies, it does have disadvantages in terms of the characteristics of the resulting sludge and the fact that minimum solubilities of the specific metal hydroxides occur at different pH values.

An ambient temperature ferrite process has been developed under the auspices of the Water Research Commission (Morgan et al, 2005). The aim of the process is primarily for iron removal, with its main advantages being that it produces a fast settling, non-leaching sludge. However, it requires the addition of lime in order to raise the pH sufficiently and it is not yet proven technology for the removal of the range of metals found in acid mine drainage. As a stand alone process, it does not address both elevated sulphate levels and elevated metal concentrations, but only the latter; hence it cannot be compared directly with the process based on biological sulphate reduction. Nonetheless, the advantages of the ferrite process are numerous, and could be combined with the advantages of the sulphide processes used in combination with biological sulphate reduction, as suggested below.

The use of sulphide has become an increasingly popular option (Bhattacharyya et al., 1981; Feng et al., 2000). Sulphide precipitation occurs effectively over a broad pH range and precipitation of certain metals can be achieved at low pH values. In addition, in this case, a sulphide precipitation process has the advantage of consuming the sulphide produced in the sulphate reduction reaction.

The cost associated with regular chemical dosing over a prolonged period has resulted in a shift in research focus towards active and passive biological treatment systems. Many of these systems, such as artificial wetlands and sulphate reducing bacteria (SRB) based systems still rely heavily on precipitation as the primary mechanism for removing metals from solution. While the reactive species may be of biological origin, the precipitation

process is essentially the same as that which occurs in chemical based systems. The biological systems contain three weak acid-base systems, which are responsible for the determining the pH. They are the carbonate system, the sulphide system and the water dissociation system. Based on the pK values for the various equilibrium reactions (Stumm and Morgan, 1996), the biological sulphate reducing system, where the pH rarely exceeds 8 (Gibson, 1990), will be dominated by the HCO_3^-, HS^- and $H_2S(aq)$ species. The relative concentration of these species depends on the bacterial consortium, the electron donor and the reactor conditions, although the bicarbonate concentration will always exceed the sulphide when an organic electron donor is used (van Hille, 2001). An example of the range of reactions occurring where lactate is the primary carbon source is shown in Table 5.1. Therefore, a range of bicarbonate to sulphide ratios from 1:1 to 6:1 was employed for this work.

Table 5.1: Possible reactions associated with lactate utilisation in a SRB reactor

Oxidative reactions
2 lactate + SO_4^{2-} + H^+ → 2 acetate + $2HCO_3^-$ + $2H_2O$ + HS^-
acetate + SO_4^{2-} → $2HCO_3^-$ + HS^-
Fermentative reaction
3 lactate → acetate + 2 propionate + HCO_3^- + H^+

Active treatment systems which are dependent on chemical dosing typically achieve this by the addition of a concentrated solution/slurry of the reactive chemical from one or more discrete points at a volume significantly lower than the effluent volume. Therefore, the precipitation experiments were conducted using a metal solution to active reagent ratio of 10:1.

A long term outcome for this research would be the linking of the biological sulphate reduction model with a metal precipitation model. The biological model would predict the composition of the stream exiting the sulphate reducing reactor. Based on the composition of this stream the relative flow rates of the AMD stream and sulphide/alkaline stream could be determined to maintain optimum performance of the precipitation unit. The tools currently available for modelling the precipitation unit are based on thermodynamic equilibria. This section of the work reports on the use of the available speciation software to model metal precipitation from a complex metal stream using a mixture of sulphide and bicarbonate, as well as experimental work performed in order to validate the model.

A number of software packages exist that enable the user to estimate cation and anion speciation in aqueous media. These include MINTEQA2, PHREEQC, MINEQL, OLI Systems and CHESS. These packages utilise thermodynamic databases as a basis for the speciation calculations. For this research project, the speciation modelling was conducted using Visual MINTEQ (Gustafsson, 2003) and OLI Systems, Inc (OLI Systems, Inc, 2000). Visual MINTEQ is a windows-based translation of MINTEQA2 ver. 4.0, which has an enhanced user interface. These two packages were chosen as they are commonly used and were readily available. A detailed description of the equations, species and thermodynamic constants used for the modelling is not appropriate for this report, but more detail is presented in a paper by Lacour and associates (2005).

5.2. MODEL FOR A COMPLEX STREAM

The composition of the AMD stream used as the basis for the precipitation model is shown in Table 5.2.

Table 5.2: Composition of AMD stream used for the generation of precipitation models

Component	mg ℓ^{-1}	mM
pH	**2.38**	
Fe	**800**	**14.32**
Zn	**130**	**1.99**
Mn	**80**	**1.46**
Cu	**70**	**1.10**
Ca	**130**	**3.24**
Mg	**130**	**5.35**
Na	**300**	**13.04**
H_2SO_4	**485**	**9.92**
Total sulphate	**3900**	**40.60**

A number of scenarios were modelled, using both software packages. These were:

Heavy metal (Fe, Zn, Cu and Mn) to sulphide stoichiometric ratios of:

- 0.5, 1.0, 1.5 and 2.0:1

Bicarbonate to sulphide stoichiometric ratios of:

- 0, 1, 2, 4 and 6:1

There were no significant differences between the models generated by the two software packages. In both cases a clear affinity series between the reactivity of the metals and sulphide was established, the series being Cu > Zn > Fe > Mn. This result is based on the thermodynamic stability of the product and essentially suggests that if sulphide is limited, it will react to form the most stable precipitate with copper, followed by zinc, iron and then manganese. Therefore, the models predict that as soon as sufficient sulphide is added 100% of the copper will precipitate as covellite (CuS), followed by the zinc as sphalerite (ZnS). The models suggest that the affinity of copper and zinc for sulphide is so strong that the precipitation was not influenced by the presence of the other aqueous species. The situation was different for iron and manganese, where in the absence of a stoichiometric excess of sulphide or a significant concentration of bicarbonate, which would drive the equilibrium pH into the alkaline range, complete precipitation was not predicted. For example, at a heavy metal to sulphide ratio of 1:1 and without bicarbonate the equilibrium pH is predicted to be 4.65. In this situation complete precipitation of copper and zinc was predicted, but only 63.5% for iron and 0% for manganese. The remaining sulphide was predicted to exist predominantly as $H_2S(aq)$ and $FeHS^+$. In all cases iron precipitation was predicted to occur as Mackinawite, rather than amorphous FeS.

In any system designed to treat a significant volume of AMD by precipitation, the retention time in the precipitation/clarification unit becomes an important design parameter as this affects reactor size and capital cost. For the purposes of the experimental validation of the models a reaction time of 120 minutes was chosen, after which solid-liquid separation was achieved by filtration through a 0.45µm nylon membrane filter. Analysis of the filtrate showed that the concentration of copper and iron remaining deviated significantly from the model predictions.

Once it became apparent that, in most cases, the correlation between the model predications and the experimental data was poor, the focus of this section of the work shifted away from the development of a model based on thermodynamics, to developing a greater understanding of the reasons behind the observed discrepancies. Therefore, the complete outputs of the thermodynamic models will not be presented.

The experimental work detailed in the section that follows investigates the behaviour of individual metals (iron, copper and zinc) under a range of metal:sulphide and bicarbonate:sulphide ratios and attempts to provide explanations for the phenomena observed during the validation phase.

5.3. EXPERIMENTAL

5.3.1. Materials and methods

All experiments were performed using acid-washed borosilicate glassware. pH measurements were made using a Cyberscan 2500 pH meter, alkalinity determined by titration against 0.02N H_2SO_4 and aqueous sulphide determined using a Merck Spectroquant test kit. Metal analysis was performed using a GBC 909AA atomic absorption spectrophotometer, linked to a GBC integrator.

Metal stock solutions ($CuSO_4.5H_2O$, $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$, all Merck) were made up to a concentration of 1.574 mM. Sulphide ($Na_2S.9H_2O$) stock solutions (0.05 M and 0.1 M) and bicarbonate ($NaHCO_3$) stock solutions (0.1 M and 0.5 M) were made up using analytical grade reagents (Merck). Anaerobic digester overflow was obtained from a sulphate reducing reactor, seeded with a bacteria consortium isolated from the algal integrated ponding system at the Grahamstown sewage treatment works and maintained on a modified Postgate B growth medium. The digester overflow had a pH of 7.80, an aqueous sulphide concentration of 33 mM and a bicarbonate concentration of 133.6 mM. A portion of this overflow was centrifuged at 10 000 rpm for 10 minutes to remove the bacterial cells and particulate organic matter. The centrifugation had no effect on the pH or sulphide concentration, but the bicarbonate concentration was reduced to 126.3 mM.

Experiments with the synthetic solutions were performed, in duplicate, according to a statistical design (response surface methodology). In all cases, the bicarbonate to sulphide ratios tested were 1:1, 2:1, 4:1 and 6:1. Initially, four metal to sulphide ratios were tested, 2:1, 1:1, 1:1.5 and 1:2. Analysis of this initial data set indicated different precipitation phenomena for each metal. Therefore, a number (up to four) of additional levels were tested to provide more detailed information around these phenomena. In all cases 100 mℓ of the metal solution was added to 10 mℓ of the bicarbonate and sulphide mix. The flasks were stoppered and placed on an orbital shaker for 120 min. The pH was measured after 1, 30, 60 and 120 min. At 60 and 120 min a 10 mℓ sample was removed for metal analysis. A portion of the sample was filtered through a 0.45 µm nylon membrane filter, a portion centrifuged at 5 000 rpm for 5 min and a portion allowed to settle for 10 min, with the metal concentration determined in the filtrate and supernatants respectively. A minimum of 40 data points, for each metal, were available for statistical analysis.

The response surface methodology was used to determine the effect each individual variable (i.e. the metal to sulphide ratio (M:S) and bicarbonate to sulphide ratio (B:S)) and their combined effect on the percentage of metal removed from solution (Meyers and Montgomery, 1995). The interaction between these variables was determined through the development of a mathematical equation that describes the performance of each system. As the relationship between the variables is not necessarily linear, a higher order polynomial model, with interaction was utilised. Initially a second order model was used, but analysis of the results indicated that in certain cases a higher order model was required to more adequately describe the system. The following equation represents the second order empirical model on which the initial response surfaces were based:

$$z = \beta_0 + \beta_1 * \text{B:S ratio}^2 + \beta_2 * \text{B:S ratio} + \beta_3 * \text{B:S ratio} * \text{M:S ratio} + \beta_4 * \text{M:S ratio} + \beta_5 * \text{M:S ratio}^2$$

where z represents the measured response and $\beta_{1\text{-}5}$ represent the coefficients of the various factors. This model accounts for the individual effects of both ratios as well as the possible interaction between the B:S and M:S ratio. A t-test was performed to determine the significance of each variable in the equation. An add-in for Microsoft Excel was used to plot the three dimensional surfaces.

A similar protocol was used for the experiments testing the SRB digester overflow. However, the bicarbonate to sulphide ratio in the digester overflow was fixed, which precluded a matrix experimental design. A volume of digester overflow was calculated, such that the metal to sulphide ratio was the same as for the synthetic experiments. Deionised water was added to make the volume up to 10 mℓ, after which the metal solutions were added and the experiments performed as previously described. The experiments were repeated using overflow that had been centrifuged at 10 000 rpm for 10 minutes to remove any particulate organic matter.

5.3.2. ***Results and discussion***

For the synthetic solutions the addition of iron and copper to the bicarbonate/sulphide mixture resulted in a rapid reaction, leading to a colour change from a clear solution to an inky black and dark brown colour respectively. In both cases, a precipitate that could be easily separated from the aqueous phase was only formed in the presence of a significant stoichiometric excess of metal. The zinc experiments, however, showed a different trend. Addition of the metal solution did not result in an instant reaction, rather a distinct white precipitate became visible after two to three minutes. This precipitate could be readily

removed by filtration or centrifugation and settled within five minutes after being removed from the shaker. The three-dimensional surfaces generated from the statistical analysis of the synthetic solution data are shown in Figures 5.1-5.5. In all cases precipitate removal was achieved by filtration.

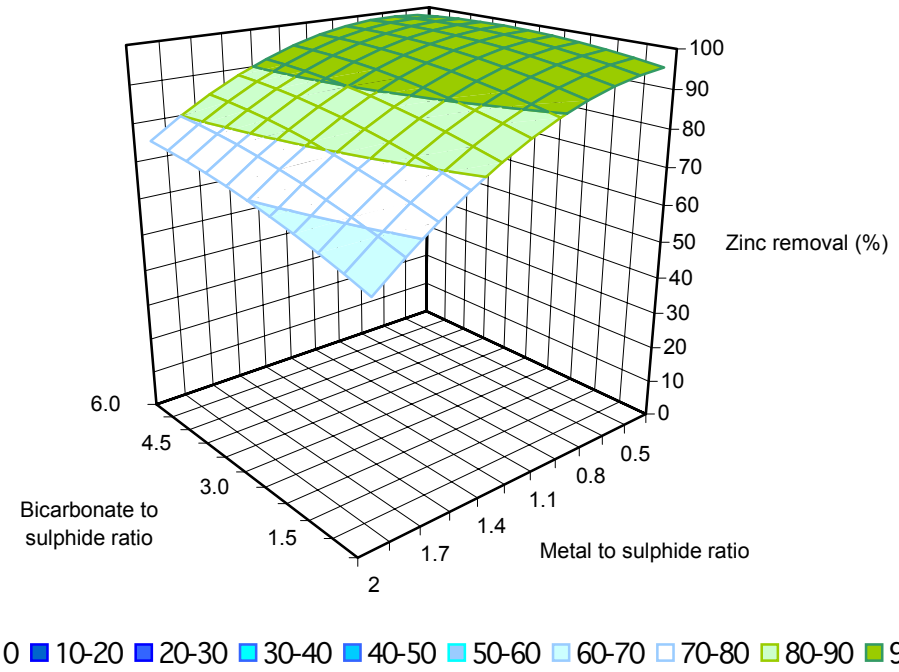

Figure 5.1: Response surface, modelling zinc precipitation by a combination of sulphide and bicarbonate. Note: Y-axis (M:S ratio) scale reversed to allow better visualisation of the surface

The model (R^2 = 0.89), with P values in parentheses, for the zinc surface is shown below:

$$z = 92.4 - 0.35*\text{B:S ratio}^2 + 2.05*\text{B:S ratio} + 1.60*\text{B:S ratio}*\text{M:S ratio} + 9.24*\text{M:S ratio} - 13.76*\text{M:S ratio}^2$$
$$\qquad\qquad (0.272) \qquad\quad (0.413) \qquad\quad (0.079) \qquad\quad (0.334) \qquad\quad (<0.001)$$

The R^2 value indicates that the model accounts for the almost 90% of the experimental variation observed, while the t-test show that the metal to sulphide ratio is the most significant variable. The P value of the interaction effect (0.079) indicates that this factor is also relatively significant. Visual analysis of the surface indicates that this is particularly true where there is an excess of zinc relative to sulphide. In this situation a portion of the zinc precipitates as $Zn(OH)_2$, with increased hydroxide precipitation as the bicarbonate

concentration increases. At a metal to sulphide ratio of 1:1 and below, ZnS precipitation dominates and the effect of the increasing bicarbonate concentration is negligible.

The response surface and model equation for the iron precipitation are shown overleaf.

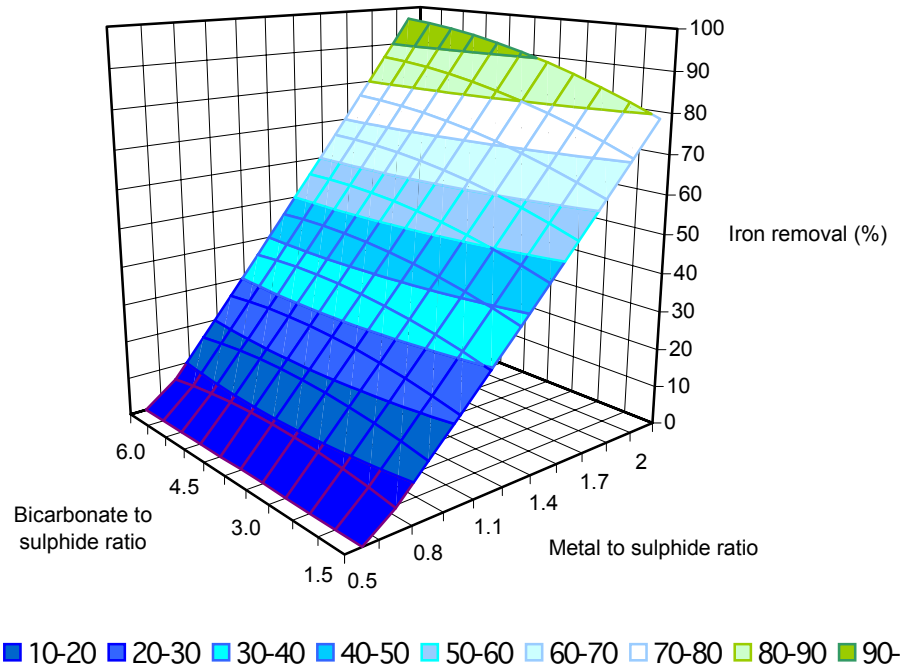

Figure 5.2: Response surface, modelling iron precipitation by a combination of sulphide and bicarbonate

The model (R^2 = 0.74), with P values in parentheses, for the iron surface is shown below:

$$z = -28.8 - 0.49*\text{B:S ratio}^2 + 1.85*\text{B:S ratio} + 2.56*\text{B:S ratio}*\text{M:S ratio} + 47.41*\text{M:S ratio} + 1.67*\text{M:S ratio}^2$$
$$(0.696) \qquad (0.846) \qquad (0.470) \qquad (0.470) \qquad (0.930)$$

The relatively low R^2 value indicates that the second order model does not adequately describe experimental data. The raw data (Table 5.3) shows that the most efficient iron removal is achieved at a metal to sulphide ratio of 1.5:1. A third order model was generated which yielded a surface (Figures 5.3 and 5.4) that accounted for over 90% of the variation. In addition, the statistical analysis was able to identify the variables that had a significant influence on the system. In this case the three terms representing the metal to sulphide ratio (i.e. y, y^2 and y^3) all returned P values smaller than 3×10^{-8}, while the next most significant factor, an interaction factor, had a P value of 0.71. This clearly indicates that the system is dominated by the reaction between dissolved iron and aqueous sulphide.

Table 5.3: Mean iron precipitation values (% removal) at various M:S and B:S ratios (n=2)

	M:S ratio					
B:S ratio	2:1	1.5:1	1.2:1	1:1	1:1.5	1:2
1:1	68.61	82.12	14.48	5.95	5.26	9.64
2:1	77.91	87.03	17.21	8.31	9.87	11.54
4:1	84.61	95.18	22.78	11.36	10.35	6.77
6:1	88.07	97.87	18.08	12.31	9.57	8.68

Figures 5.3 and 5.4 show different three-dimensional views of the surface generated by the third order model. The shape of the surface is significantly different to that of the zinc surface, with efficient precipitation only achieved where the iron to sulphide molar ratio is 1.5:1 or greater. The view of the surface illustrated in Figure 5.4 shows the effect of increasing bicarbonate concentration on the iron precipitation at iron to sulphide ratios of 1.5:1 and greater. Below this ratio the product of the reaction could not be removed by either filtration or centrifugation. The most likely explanation for this is the formation of an amorphous or colloidal precipitate, due to excessively high local supersaturation. The supersaturation ratio given by

$$SS = \frac{\alpha_{Fe^{2+}} \alpha_{S^{2-}}}{K_s{}^{0}{}_{,FeS}}$$

where $K_s{}^{0}{}_{,\,FeS}$ is the thermodynamic solubility product for FeS. Harmandas and Koutsoukos (1996) investigated the formation of ferrous sulphides in aqueous solutions at an iron to sulphide ratio of 1:1. They found that at near neutral pH (7.1-7.4) the precipitate formed was mackinawite, while at pH 5.4-5.6 amorphous FeS was formed. However, the concentrations of iron and sulphide under the conditions where mackiniwite formed were between 1.0 and 5.0 x 10^{-5} M, while the concentrations used at the lower pH were at least an order of magnitude higher (5.0-7.0 x 10^{-4} M), resulting in a significantly higher SS. This, rather than the pH could account for the formation of the amorphous precipitate. The current work was performed at iron and sulphide concentrations in the millimolar (10^{-3} M) range, which is consistent with the predicted iron loading in the groundwater pumped from the Grootvlei mine (Schoeman and Steyn, 2001). In addition, Harmandas and Koutsoukos (1996) found that the amorphous precipitates were very sensitive to oxidation by atmospheric oxygen. This is consistent with the current results and previous experimental work (van Hille, 2001).

This result highlights the importance of hydrodynamics in precipitation systems, particularly where the reaction kinetics of the precipitation reaction are rapid. In many precipitation reactions, such as those between iron and copper and sulphide, the nucleation times are of

the order or milliseconds, or less (Sohnel and Garside, 1992). In contrast, the time scales for the various mixing and hydrodynamic processes are much larger. Therefore, it cannot be assumed that the system is homogeneous with respect to the distribution of the reacting species prior to the initiation of the precipitation reaction. In situations such as this, the use of global supersaturation values is of little value. However, in situations where there is a significant induction time prior to nucleation, such as the zinc sulphide system, hydrodynamic issues become less important and the system tends towards homogeneity.

As with the zinc surface, it can be seen that an increase in bicarbonate concentration only has an effect where the metal to sulphide ratio is greater than 1.5:1. In that region some of the iron would precipitate as a hydroxide or carbonate.

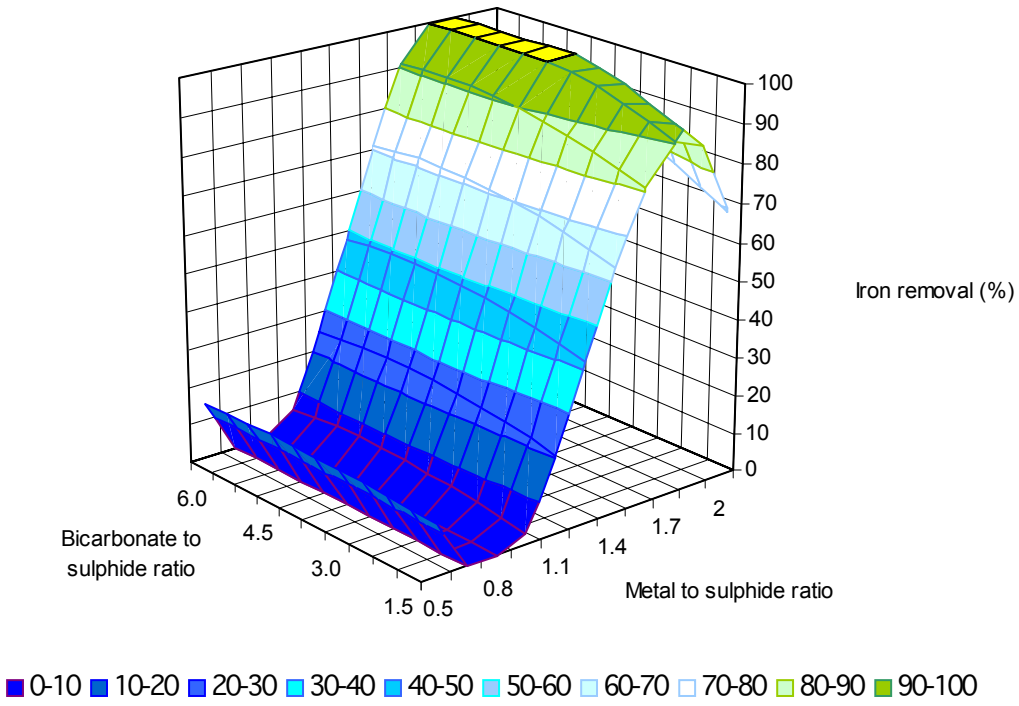

Figure 5.3: Response surface, modelling iron precipitation by a combination of sulphide and bicarbonate, generated using a 3rd order model (R^2 = 0.93)

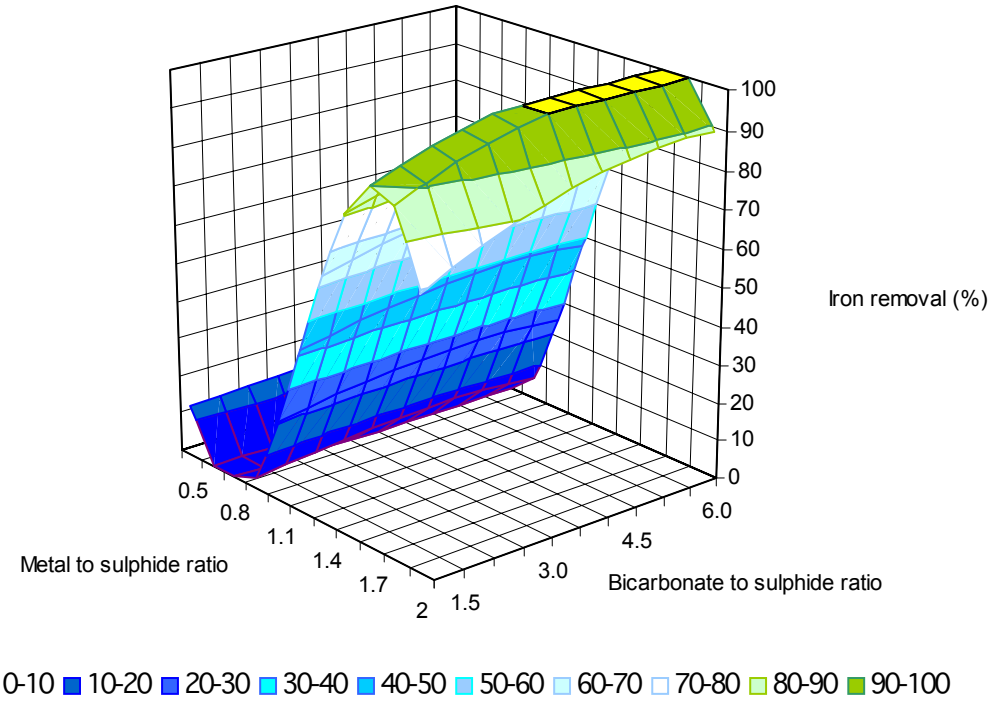

Figure 5.4: Response surface, modelling iron precipitation by a combination of sulphide and bicarbonate, generated using a 3rd order model (R^2 = 0.93)

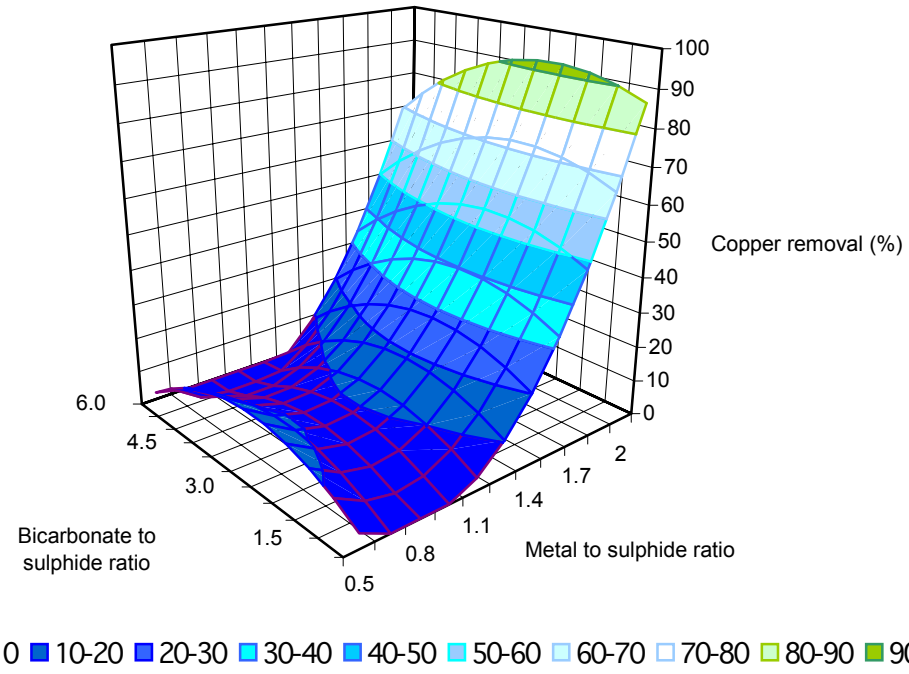

Figure 5.5: Response surface, modelling copper precipitation by a combination of sulphide and bicarbonate

The response surface for the copper precipitation is shown in Figure 5.5. The model equation (R^2 = 0.79), with P values in parentheses, for the copper surface is shown below:

$$z = 26.2 - 2.06*(\text{B:S ratio})^2 + 15.09*\text{B:S ratio} - 2.11*\text{B:S ratio}*\text{M:S ratio} - 99.34*\text{M:S ratio} + 62.74*(\text{M:S ratio})^2$$

(0.062) (0.076) (0.462) (<0.005) (<0.001)

Copper presented the most complex situation and also highlighted a deficiency of the model. As with iron, an easily removable precipitate was only formed under a narrow set of conditions. Unlike the situation for iron, where the bicarbonate concentration had a negligible effect once sulphide dominated the system, the bicarbonate concentration had a significant effect on the product formed. This is highlighted in Table 5.4.

Table 5.4: Mean copper precipitation values (% removal) at various M:S and B:S ratios (n=2)

	M:S ratio							
B:S ratio	2:1	1.8:1	1.75:1	1.6:1	1.5:1	1:1	1:1.5	1:2
1:1	67.69	78.51	78.08	25.67	10.36	4.30	8.85	7.56
2:1	78.53	84.21	86.23	72.30	16.10	8.24	8.70	7.29
4:1	89.99	91.23	92.74	14.89	5.02	9.50	8.84	8.60
6:1	92.29	9.78	8.56	7.88	7.22	7.30	7.66	7.38

Table 5.4 shows that at a M:S ratio of 2:1 the results are consistent with those for zinc and iron, with increasing precipitation achieved as the bicarbonate concentration increases. The same trend occurs at the 1.8 and 1.75:1 ratios, except where the bicarbonate concentration is six times that of the sulphide, at which point the resulting product cannot readily be removed from solution. At a metal to sulphide ratio of 1.6:1 an easy to handle precipitate was only formed when the B:S ratio was in the region of 2:1. The consequence of this for the model is that at a B:S ratio of 6:1 the surface is less accurate, particularly at M:S ratios between 1.6 and 2:1. This would account for the lower R^2 value as well the increased significance of the B:S ratio factors. A third order model did not improve the R^2 value, while a fourth order model increased the R^2 value to 0.87, but introduced additional errors into the surface.

Supersaturation is again a key factor in understanding the precipitation behaviour, however the situation is more complex. The equation for calculating supersaturation ratio described previously yields similar results irrespective of the bicarbonate concentration, which does not adequately describe the observed results. It is proposed that the HS^- species, rather than

total aqueous sulphide species, controls supersaturation and precipitation. Previous work (Hammack et al., 1993; van Hille, 2001) has shown that copper can effectively be removed from solution, as a readily settling precipitate, using gaseous hydrogen sulphide. This process is rate limited by the mass transfer of sulphide from the gaseous to the liquid phase. Due to the rapid reaction kinetics the supersaturation ratio remains low and precludes the formation of an amorphous precipitate.

The zinc, copper and iron precipitation achieved using the digester overflow, as well as the pH after 120min is graphically depicted in Figures 5.6 to 5.8. In each case results are compared to those obtained using the synthetic solutions at bicarbonate to sulphide ratios of 4:1. The zinc data (Figure 5.6) shows no significant difference between the metal removals achieved using the biological or synthetic stream. There is little difference in the pH between the synthetic and biological systems at M:S ratios of 2:1 to 1:1, which is expected as all the available sulphide has reacted with zinc. The slightly lower pH observed in the biological systems is due to the presence dissolved organic acids, such as lactate and acetate, which originate from the growth medium. At M:S ratios below 1:1 an excess of aqueous sulphide is present, which accounts for the significant increase in the pH of the synthetic system. The presence of the organic acids buffers the biological systems, although this has a negligible effect on the zinc precipitation.

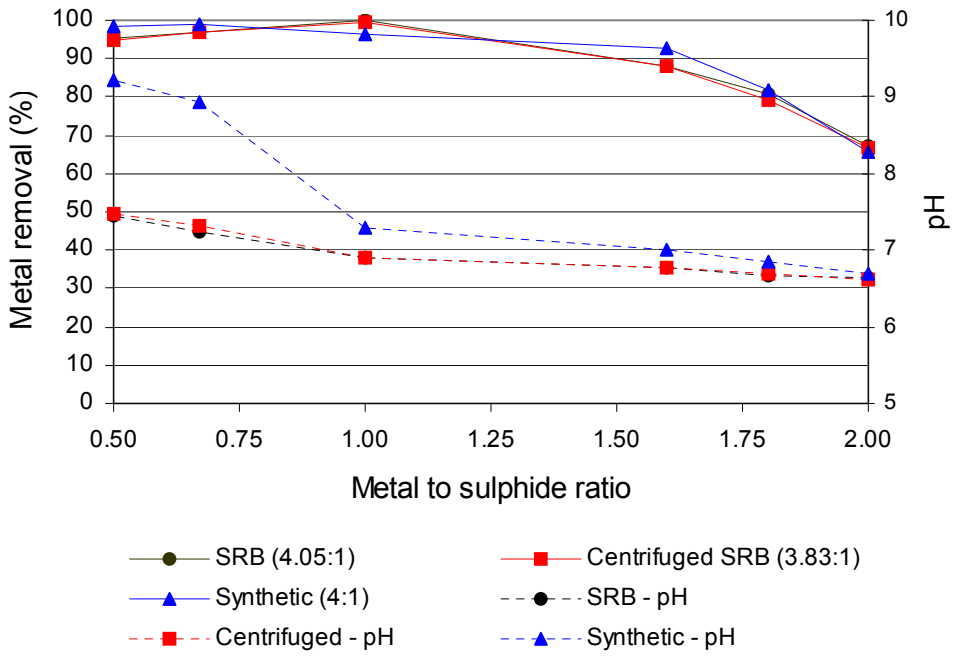

Figure 5.6: A comparison of zinc precipitation and pH achieved by mixing of the metal solution with a synthetic sulphide/bicarbonate mixture and anaerobic digester overflow (T = 120 min). The bicarbonate to sulphide ratios are shown in parentheses

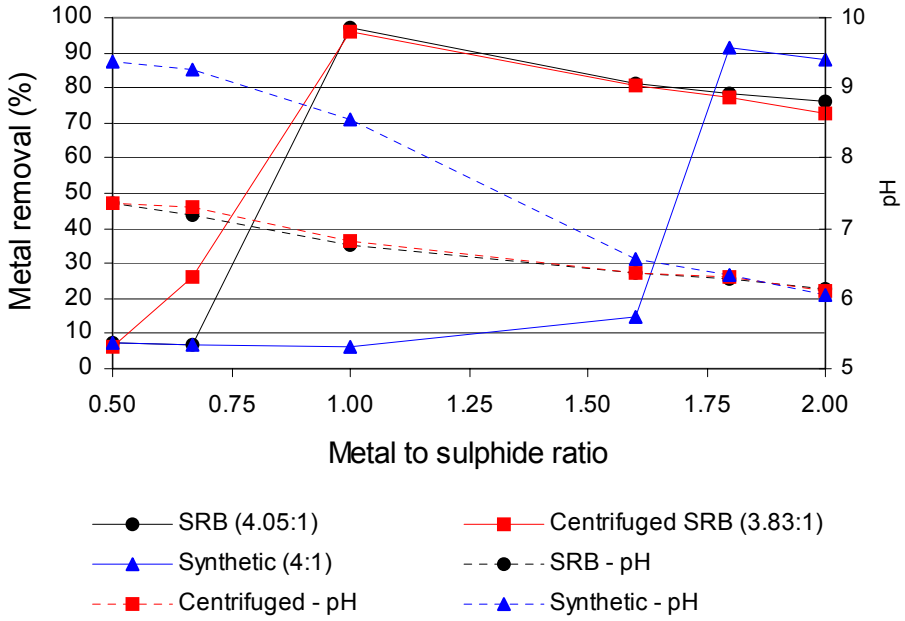

Figure 5.7: A comparison of copper precipitation and pH achieved by mixing of the metal solution with a synthetic sulphide/bicarbonate mixture and anaerobic digester overflow (T = 120 min). The bicarbonate to sulphide ratios are shown in parentheses

The trends for copper removal and pH (Figure 5.7) are significantly different from those for zinc (Figure 5.6), despite the fact that the concentrations of metal, bicarbonate, sulphide and organic acids are identical. This provides further evidence of the differences in the precipitation mechanisms. The differences in the pH and percentage metal removal at the M:S ratio of 2:1 is explained by the fact $Cu(OH)_2$ precipitates at a lower pH than the corresponding zinc precipitate. As a result a greater portion of the alkalinity is used for metal precipitation and the pH is lower. The presence of the organic acids reduces the available alkalinity, explaining why the copper precipitation is lower in the biological systems. This effect is negligible in the case of zinc because there is not enough alkalinity, even in the synthetic system, to raise the pH high enough to effect significant Zn precipitation.

At a M:S ratio of 1.6:1 a significant decrease in copper removal results in the synthetic system. This can be attributed to the formation of an amorphous copper sulphide precipitate as a result of high supersaturation. This is consistent with results obtained by Pattrick et al. (1997). In the biological systems, the organic acids buffer the pH at a lower value, effectively maintaining a lower HS^- species concentration. It is proposed that this species plays a key role in determining the supersaturation.

At an M:S ratio of 1:1 the copper removal in the synthetic system remains poor, but the pH increases significantly to 8.55. If all the sulphide reacted with copper to form the amorphous precipitate, the bicarbonate would buffer the system between pH 8.2 and 8.3. This, along with the fact that some copper is still precipitated suggests the formation of some polysulphide complexes, such as $CuS(HS)_2^{2-}$ and $CuS(HS)_3^{3-}$. Shea and Helz (1988) have confirmed the presence of these complexes in anoxic waters. In this case some copper remains free to precipitate as $Cu(OH)_2$.

In the presence of excess sulphide the organic acids are no longer able to buffer pH effectively, resulting in an increase in the HS^- species concentration. Formation of amorphous CuS and a decrease in copper removal results. This is consistent with the trend observed in Figure 5.7. Further experimental work is underway to quantitatively confirm the presence of the various copper-sulphide precipitates and complexes.

The precipitation of iron by the digester overflow (Figure 5.8) shows a similar trend to copper, although the metal precipitation at the higher metal to sulphide ratios is lower. The most significant difference between the iron and copper data occurred at M:S ratios between 2:1 and 1:1. In the case of copper the metal removal increased from 75% to over 95%, but for iron there was little increase in precipitation. Aqueous speciation modelling suggests the differences can be accounted for by the behaviour of the different metals in an aqueous medium containing free bicarbonate ions. Figure 5.9 shows the predicted concentration of metals remaining in solution at increasing equilibrium pH values, while Figure 5.10 predicts metal precipitation as a function of increasing bicarbonate ion concentration. The graphs indicate that copper will begin to precipitate as a hydroxide in the presence of free bicarbonate, or when the pH of the solution approaches pH 6. Iron precipitation, as siderite ($FeCO_3$), is predicted to begin once the free bicarbonate concentration exceeds 2 mM (Figure 5.10). The precipitation of ferrous hydroxide in significant quantities only occurs at pH values in excess of pH 8.5 and therefore cannot be considered a factor. The presence of dissociated organic acids in the aqueous medium would reduce the concentration of free bicarbonate by driving the carbonate species equilibrium towards carbonic acid. This may account for decreased iron precipitation between the synthetic and biological systems up to a M:S ratio of 1:1. Below this value, the effect of the organic acids on sulphide speciation accounts for the differences.

A second explanation could be a possible direct interaction between the iron and the soluble organic material. This possibility is currently being investigated.

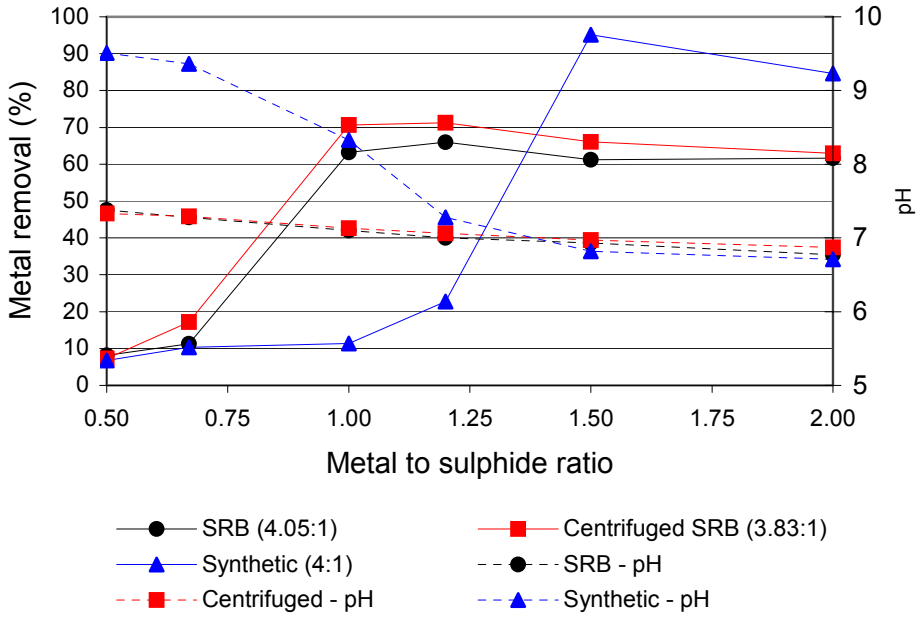

Figure 5.8: A comparison of iron precipitation and pH achieved by mixing of the metal solution with a synthetic sulphide/bicarbonate mixture and anaerobic digester overflow (T = 120 min). The bicarbonate to sulphide ratios are shown in parentheses

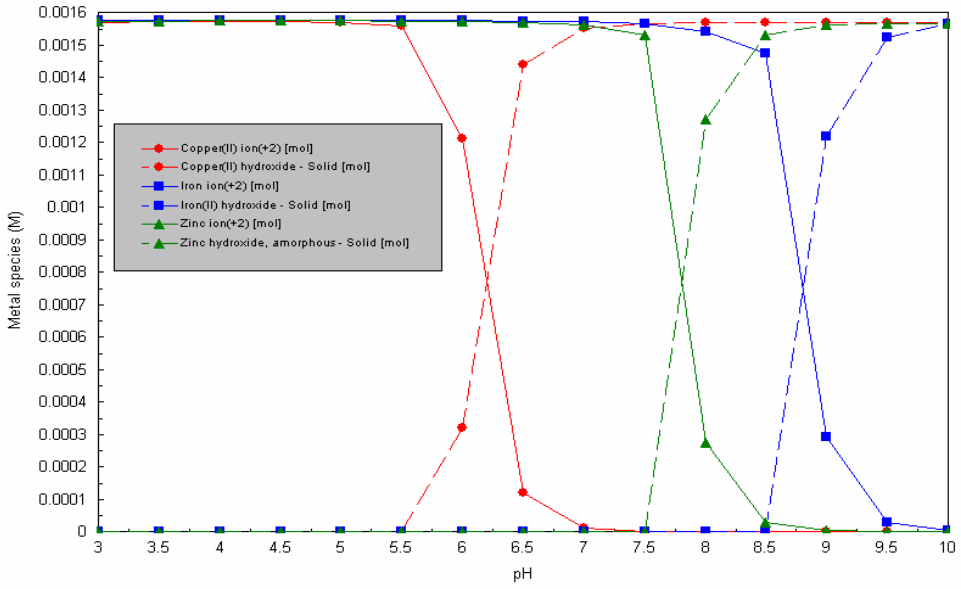

Figure 5.9: Metal hydroxide precipitation as a function of increasing equilibrium pH. Prediction generated using OLI Systems Inc., Stream Analyzer software. Initial metal ion concentration = 1.574 mM

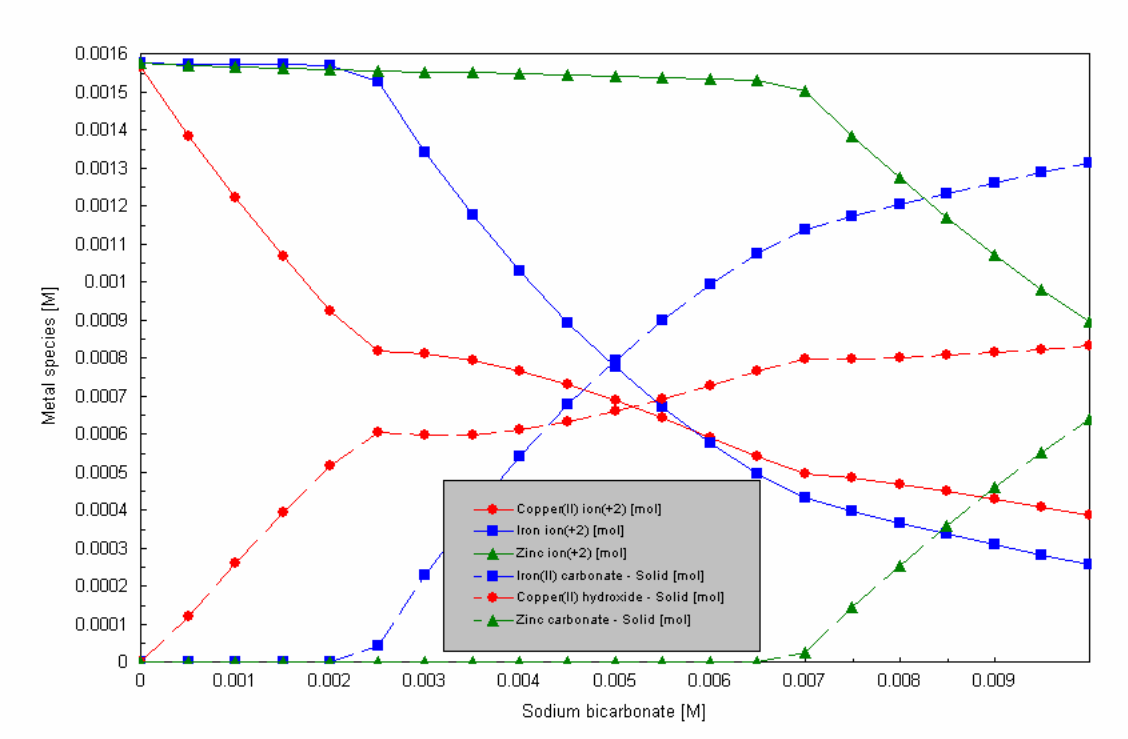

Figure 5.10: Metal precipitation as a function of increasing bicarbonate ion concentration. Prediction generated using OLI Systems Inc., Stream Analyzer software. Initial metal ion concentration = 1.574 mM

Iron precipitation may be enhanced by the oxidation of ferrous iron to ferric iron, which readily hydrolyses and precipitates as $Fe(OH)_3$ or FeOOH, even under moderately acidic conditions. Oxygen ingress into reaction flasks was minimised under the current experimental conditions.

5.4. AQUEOUS SPECIES MODELLING

The two speciation packages used previously to model the complex stream were again used to model the experiments performed on the individual metals.

5.4.1. Materials and methods

Single point simulations were performed to predict metal precipitation and equilibrium pH for each experimentally tested condition (i.e. M:S and B:S ratio). This was performed for each metal. The precipitation prediction data was statistically analysed in the same way as the experimental data, to generate second order models and the corresponding surfaces.

5.4.2. ***Results and discussion***

Thermodynamic modelling of the synthetic precipitation systems yielded similar results for all three metals. In all cases, at M:S ratios of 1:1 and below at least 99.9% of the metal was predicted to precipitate as the metal sulphide. At ratios above 1:1, all available sulphide reacted with metal to form a metal sulphide and some additional hydroxide or carbonate precipitation occurred due to the presence of the bicarbonate. In addition, there was no significant difference between the models generated using Visual MINTEQ and OLI Systems Inc. As all the generated surfaces were similar, the copper surface generated using Visual MINTEQ is shown in Figure 5.11 as a representative example.

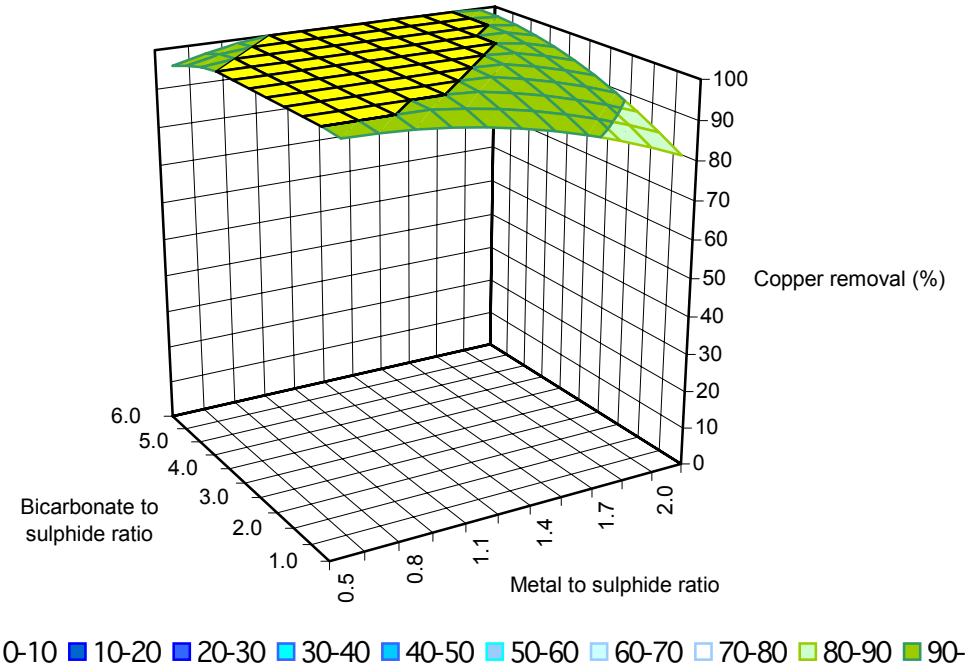

Figure 5.11: Response surface obtained using data points generated using the aqueous species modelling software Visual MINTEQ. The data points were obtained by modelling the 32 conditions tested experimentally

The surface in Figure 5.11 is significantly different from those obtained for the experimental copper and iron systems. The fact that the predictions are based only on thermodynamic data and represent an equilibrium situation means that amorphous or colloidal precipitates, which occur due to high supersaturation, cannot be accounted for. In addition, the thermodynamic databases tend to be incomplete or inaccurate. For instance, Shea and Helz (1988) presented stability constants for five copper sulphide and polysulphide complexes of importance in anoxic waters. Neither of the applications tested included data

for these complexes. The two software packages were able to generate predictions for zinc that closely resembled the experimental surface. The chemistry of zinc sulphide precipitation is relatively simple, with crystalline sphalerite (ZnS) forming under a wide range of conditions.

The software packages tested were able to accurately predict zinc precipitation under a range of experimental condition, but were unable to accurately predict copper and iron precipitation. This is primarily due to the inability of the packages to account for local supersaturation effects, kinetic factors and the formation of amorphous or colloidal precipitates.

The CHESS modelling package claims to be able to model the formation and dissolution of colloidal phases and allow extended kinetic control of dissolution and precipitation reactions. It may be worthwhile to investigate this option further.

5.5. CONCLUSIONS

Based on the evidence presented above, for this reaction system, it is possible to draw a number of conclusions:

1. Reaction kinetics may be more important than thermodynamics in determining the efficiency of metal precipitation. For iron and copper, the reaction kinetics are so rapid that nucleation occurs prior to complete mixing having been achieved. As a result, areas of very high local supersaturation exist around the reagent introduction points and primary nucleation is the dominant phenomenon. This can lead to amorphous or colloidal precipitates which are difficult to remove from suspension. Furthermore, due to the rapid nucleation rate, the amorphous precipitates are formed in regions of high aqueous sulphide concentration. There is sufficient evidence to indicate that amorphous copper sulphide resolubilises in aqueous sulphide to form metastable polysulphide complexes. In the case of zinc, the induction time prior to nucleation is sufficiently long to ensure effective mixing, resulting in lower supersaturation and a stable, crystalline precipitate.

2. The software packages based on thermodynamic databases may not accurately predict metal removal in situations where the retention time in the system is limited, as thermodynamic equilibrium is not achieved. In addition, the models assume homogeneous distribution of the aqueous species prior to nucleation so

supersaturation is determined based on global values. The experimental data shows that hydrodynamic and local supersaturation phenomena cannot be ignored.

3. The empirical models, developed from the experimental data, are able to better account for the inhomogeneity of the reactor system. However, the models will only be valid for a particular set of hydrodynamic parameters.

4. The characteristics of the iron and copper sulphide precipitates are dependent on sulphide speciation, not only stoichiometry. This is confirmed by the significant differences observed in certain instances where total sulphide remained constant, but differences in the bicarbonate concentration or the presence of soluble organic acids affected the equilibrium between the H_2S_{aq} and HS^- species. It is proposed that the HS^- species plays the most significant role and additions work is underway to test this hypothesis.

5. The use of anaerobic digester overflow increased the range of stoichiometric ratios over which effective metal removal could be achieved. The presence of soluble organic acids buffered the pH at a lower value, which shifted the sulphide species equilibrium toward aqueous H_2S. The lower HS^- concentration reduced the supersaturation, which discouraged the formation colloidal precipitates.

6. The presence of particulate organic matter did not have a significant impact on metal precipitation in any of the systems investigated. This may not be the case in situations where the superaturation is significantly lower and secondary nucleation becomes the dominant process and particulate matter could act as sites for nucleation.

The most important implications of this research for the development on integrated biological treatment systems are that, where metal precipitation by sulphide is considered hydrodynamic and local supersaturation phenomena become critical. In order to form a precipitate that can readily be separated from the aqueous component it is necessary to limit supersaturation. This may be achieved by innovative reactor design, improved mixing, increasing the number of reagent addition points or manipulating the sulphide species equilibrium.

6 SIMULATION OF BIOLOGICAL SULPHATE REDUCTION

A steady state and dynamic mathematical model has been formulated with the objective of simulating the dominant biological processes occurring in the SRB reactor system. The dynamic model has been extended to take into account:

- The biological kinetics of sulphate reduction measured in the laboratory under contract K5/1080;
- Sulphate inhibition of sulphate reduction;
- Sulphide inhibition has not been included in the model, since it has not been possible to develop a mathematical formulation of the process of sulphide inhibition from the experimental data.

6.1. STEADY STATE MODEL

6.1.1. ***Conservation equations***

Assuming steady state operation of a continuous bioreactor, the following mass balances apply:

Substrate balance:

$$D \cdot (S_o - S) = r_s \qquad 6.1$$

The substrate in this case is sulphate, since it has been reported as the limiting substrate in both the work of Moosa et al. (2002) and Ingvorsen et al. (1984). The acetate has been fed in excess.

Biomass balance:

$$D.x = r_x \qquad 6.2$$

$$D = \mu \qquad 6.3$$

*6.1.2. **Kinetic expressions and parameters***

The rate expression for biomass production is as follows:

$$r_x = \mu \cdot [x] \qquad 6.4$$

The rate expression for substrate utilisation (in this case, the sulphate reduction rate) is as follows:

$$r_s = \frac{\mu \cdot [x]}{Y_{x,s}} \qquad 6.5$$

In the work of Moosa et al. (2002), kinetic parameters for acetate utilising sulphate-reducing bacteria (SRB) were determined. These parameters are applicable for the Contois equation and the standard kinetic parameters μ_{max} and K_s were determined as functions of both initial sulphate concentration and temperature. The Contois form of the specific growth rate used by Moosa et al. (2002, 2004) is as follows:

$$\mu = \frac{\mu_{\max}[s]}{K_s[x]+[s]} - k_d \qquad 6.6$$

An examination of the literature has shown that the only comparable parameters available are those of Ingvorsen et al. (1984), also collected in a continuous system with sulphate as the limiting reactant. Other parameters in the literature were determined using batch or UASB systems at low sulphate concentrations and with acetate (the carbon source) as the limiting substrate.

The standard Monod form used by Ingvorsen et al. (1984) is:

$$\mu = \frac{\mu_{\max}[s]}{K_s+[s]} - k_d \qquad 6.7$$

The kinetic parameters proposed by Moosa et al. (2002) and Ingvorsen et al. (1984) are presented in Table 6.1. The parameters of Moosa et al. (2002) are both temperature (T) and initial sulphate concentration (S_o) dependent.

Table 6.1: Kinetic parameters from Moosa et al. (2002) and Ingvorsen et al. (1984)

	Moosa et al. (2002)	Ingvorsen et al. (1984)
μ_{max} (h^{-1})	0.061	0.013
K_s (kg m^{-3})	$6.52*10^{36}e^{(198/RT)}S_o$	0.024
$Y_{x,s}$ (kg kg^{-1})	0.568	0.158
k_d (h^{-1})	$8.8*10^{11}e^{(-78.8/RT)}$	

Simultaneous solution of the conservation and kinetic equations for the three variables S, X and r_s is required. For the steady state case, this can be achieved using the Solver function in MS Excel.

6.2. DYNAMIC MODEL

6.2.1. ***Conservation equations***

For the dynamic case, the kinetic expressions remain unchanged. However, it is necessary to rewrite the conservation equations in the form of differential equations.

Substrate balance:

$$\frac{dS}{dt} = D \cdot (S_o - S) + r_s \qquad 6.8$$

Biomass balance:

$$\frac{dX}{dt} = D \cdot X + r_x \qquad 6.9$$

6.2.2. ***Inhibition***

The effect of total sulphide inhibition has been included in the simulator for the dynamic case. This has been achieved using the inhibition function equation and inhibition parameters from literature. This function represents uncompetitive inhibition due to total sulphide. K_I used in this case is 35 mg ℓ^{-1} as reported in Maillacheruvu et al. (1993).

$$EUI = S/(K_s + S \cdot (1 + I/K_I)) \qquad 6.10$$

The rate expressions are the same as in the steady state calculations but the inhibition function is included in the expression.

$$r_x = \mu \cdot [x] \cdot EUI \qquad 6.11$$

Due to the presence of differential equations, mathematical software is required and thus g-PROMS, a high level mathematical programming language, was used to carry out these simulations.

6.3. RESULTS

Moosa et al. (2002) reported experiments at 4 different initial sulphate concentrations: 1, 2.5, 5 and 10 kg m^{-3}. All the data sets apart from the 2.5 kg m^{-3} set were used to determine the parameters and the 2.5 kg m^{-3} data set was used to validate the predictions. In this work, the 2.5 kg m^{-3} data set will be used as the experimental data against which the comparison will be made.

6.3.1. ***Steady State Case***

Figure 6.1 illustrates the steady state predictions of the volumetric sulphate reduction rate at a temperature of 35°C and various dilution rates. It can be observed that the predicted sulphate reduction rate more closely matches the experimental data when the parameters of Moosa et al. (2002) are used.

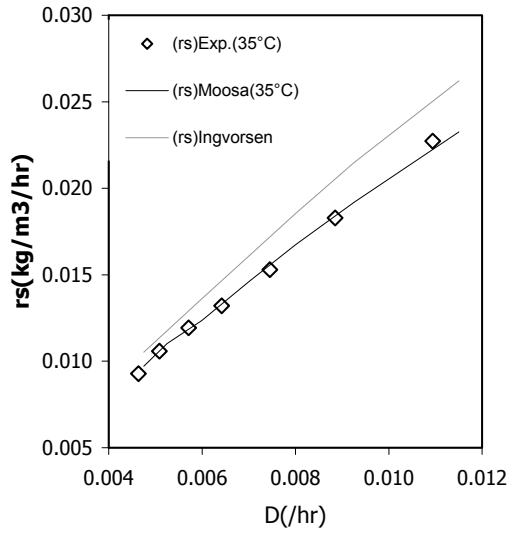

Figure 6.1: Comparison of steady state volumetric sulphate reduction rate at 35°C and dilution rates between 0.004 and 0.012hr^{-1}

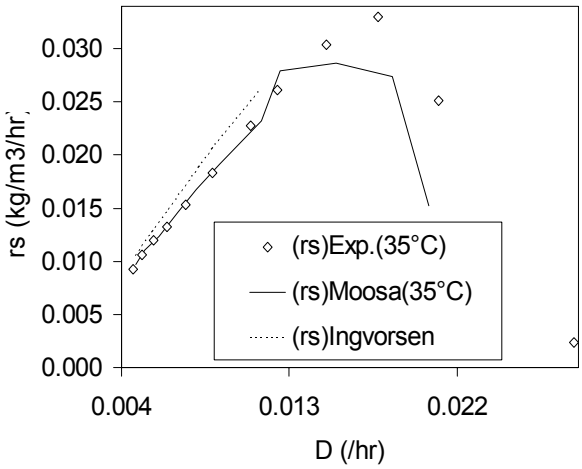

Figure 6.2: Steady state volumetric sulphate reduction rate at 35°C and dilution rates up to 0.02hr^{-1}

If higher dilution rates are investigated, the r_s using the parameters of Ingvorsen et al. (1984) cannot be determined since the maximum specific growth rate, μ_{max} along with the biomass balance limits the maximum dilution rate. Figure 6.2 shows the predictions at higher dilution rates using the parameters of Moosa et al. (2002). Up to a dilution rate of approximately 0.012hr^{-1}, the predictions are excellent. However, deviation from the experimental data is observed at higher dilution rates.

6.3.2. ***Dynamic case***

6.3.2.1. With sulphate inhibition

In Figure 6.3, the outlet sulphate concentration is plotted as a function of time and a comparison is made between the predictions obtained when the different parameter sets are used as well as against experimental data collected by Moosa et al. (2002).

It can be clearly seen that the prediction when the parameters of Moosa et al. (2002) are used is significantly better than that of the Ingvorsen et al. (1984) parameter prediction. This can be attributed to the fact that the kinetic equation for sulphate reduction generated by Moosa et al. (2002) includes a parameter that accounts for the inlet sulphate concentration which is a form of sulphate inhibition. This explains the almost immediate and sudden decrease in the exit sulphate concentration when using the parameters of Ingvorsen et al. (1984). There is no substrate inhibition and therefore no limit to how quickly the sulphate levels can drop.

Despite the fact that the calculated parameters of Moosa et al. (2002) were obtained in the steady state region, the prediction in the dynamic region is also reasonable. This provides evidence that the model can be used with relative confidence in the dynamic as well as the steady state region.

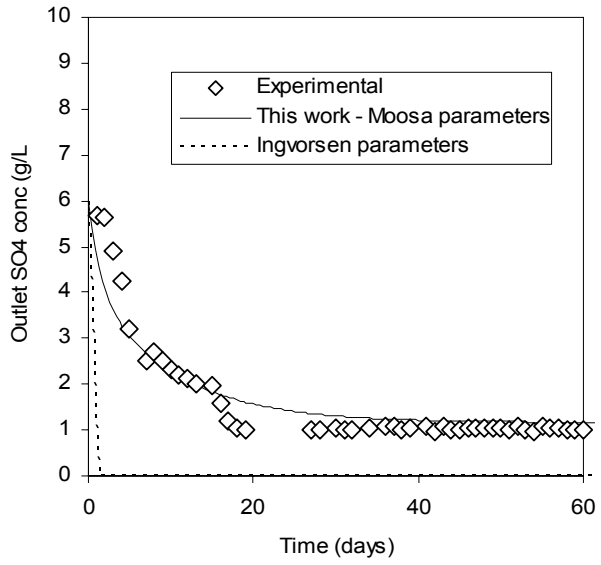

Figure 6.3: Dynamic simulation of the outlet SO_4 concentrations following perturbation of a continuous flow process

6.3.2.2. With sulphide inhibition

Figure 6.4 shows the model prediction both including and excluding sulphide inhibition. When sulphide inhibition is included, as given in Eq 6.10, the inhibition is calculated on the basis of the total sulphide concentration. If total sulphide concentration is used as the basis for inhibition, the outlet sulphate concentration will be significantly higher than that measured (at pH = 7.8) by Moosa et al. (2000).

However, according to the findings presented in Section 7.2 the inhibition is only due to the soluble portion of the undissociated H_2S. Therefore, under the conditions at which this data was collected, the effect of inhibition due to H_2S will be minimal. This is illustrated in Figure 6.5, with the experimental conditions indicated on the graph at pH = 7.8. Under these conditions, the sulphide speciation is shifted towards the HS^- species whereas the H_2S species is chiefly responsible for inhibition. This would explain why the kinetic expression without sulphide inhibition in Figure 6.4 provides a better fit to the experimental data than the expression that includes the sulphide inhibition based on total sulphide concentration.

Incorporation of the measured effects of sulphide inhibition into the simulation has not yet been possible, owing to the need to identify the factor in addition to sulphide concentration affecting the rate of sulphide reduction at increased sulphide concentrations (Section 7.2.6).

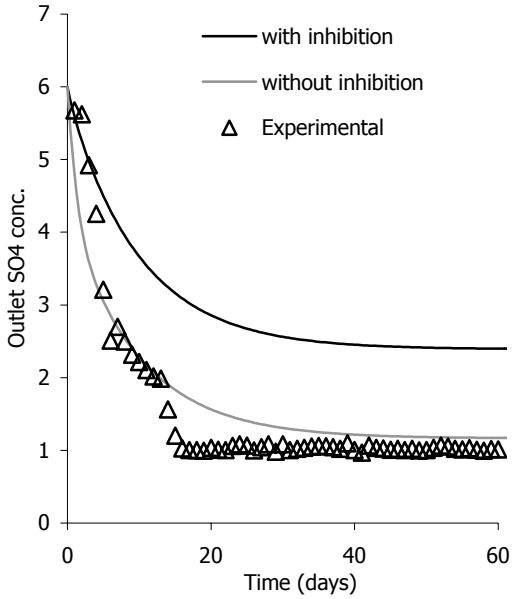

Figure 6.4: Dynamic simulation of the outlet sulphate concentrations following perturbation of a continuous flow process. The data is compared to modelling including and excluding sulphide inhibition

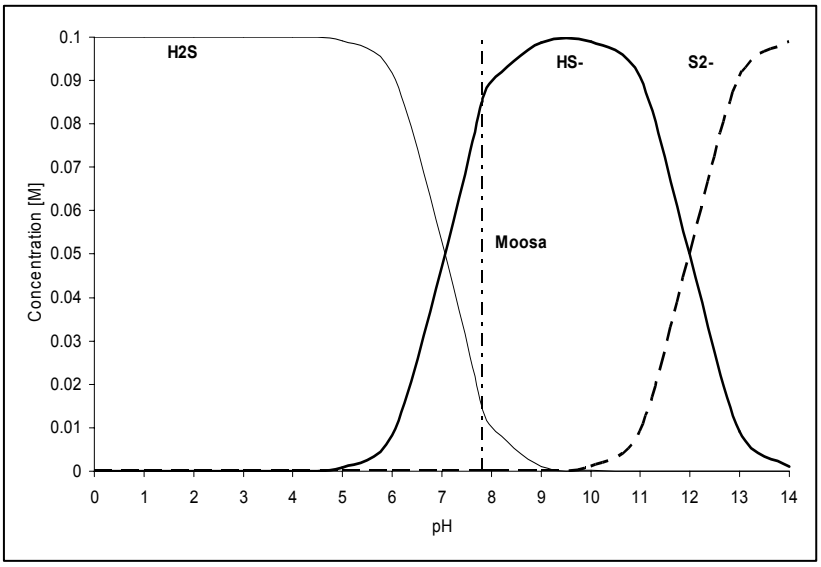

Figure 6.5: Sulphide equilibrium and speciation showing the dependence on pH

6.4. DESIGN CASE STUDIES

6.4.1. ***Steady State case***

Using the parameters for a typical AMD stream with a flow-rate of 10 Mℓ day^{-1}, a sulphate concentration of 1500 mg ℓ^{-1} and a dilution rate (D) of 0.01hr^{-1}, the reactor volume using the two different models was calculated. (This dilution rate is the one at which the greatest deviation in predictions was observed.) Table 6.2 illustrates the calculated sulphate reduction rates and reactor volumes for the two data sets.

Table 6.2: Calculated sulphate reduction rates and reactor volumes

	r_s (kg m^{-3} hr^{-1})	V (m^3)
Ingvorsen et al. (1984)	0.026	$2.4x10^4$
Moosa et al. (2002)	0.023	$2.7x10^4$

From Table 6.2, it can be seen that there is an 11% difference in the calculated reactor volume when the two different data sets are used, with the Ingvorsen et al. (1984) model leading to an underestimation of the reactor volume by approximately 3000 m^3.

6.4.2. ***Dynamic case***

From the dynamic case study, it can be seen that the estimation of the time required to reach steady state would be considerably underestimated had the model of Ingvorsen et al. (1984) been used. In this case, the reactor retention time would have been estimated at approximately 1 day, as opposed to the nearly 40 days required to reach steady state in the Moosa et al. (2002) data. While the well mixed continuous stirred tank reactor has been used in these studies (Ch. 7) to enable accurate kinetic data of microbial growth and performance to be generated, the data of Moosa et al. (2002) illustrates clearly that reactor configurations in which liquid phase and solid phase residence times are uncoupled and in which a long solids retention time is facilitated are preferred for BSR processes. The traditional continuous stirred tank reactor is not a viable reactor configuration for sulphate reduction systems, but is a very useful research tool. These findings corroborate the current thinking in reactor design for sulphate reduction, where UASB type reactor systems are favoured.

6.5. CONCLUSIONS

The conclusions from this aspect of the study are:

- Existing literature parameters are not applicable for simulation of sulphate reduction at high sulphate concentrations and with the carbon source in excess.
- Both the steady state and the dynamic simulations show that the kinetic parameters obtained from the work of Moosa et al. (2002) provide better predictions of the experimental data than those obtained when using the kinetic parameters of Ingvorsen *et al.* (1984). This indicates the importance of provision of fundamental kinetic data in the provision of appropriate modelling tools for process design and optimisation.
- The temperature dependencies of the Moosa et al. (2002) parameters render them more applicable to a wider range of operating conditions.
- Sulphate inhibition plays a significant role in the accuracy of the dynamic prediction.
- H_2S inhibition has not been incorporated into the model.
- Application of the existing models to a steady state calculation for typical AMD conditions results in an underestimation in calculation of the reactor volume.
- The simulations show that separation of the solid and hydraulic retention times is a significant issue in reactor design. For this reason, a reactor configuration yielding a long sludge retention time, such as the UASB reactor, would be suitable. The combined experimental and modelling validation of this approach is proposed

7 THE KINETICS OF BIOLOGICAL SULPHATE REDUCTION: INHIBITION BY SULPHIDE AND SULPHATE AND COMPARISON OF CARBON SOURCE

7.1. INTRODUCTION

The effects of sulphate concentration and its volumetric loading on the kinetics of sulphate bioreduction and activity of sulphate reducing bacteria were investigated at a constant temperature (35°) in our previous work (Moosa et al., 2002). These studies, and their extension in this investigation, were carried out in a well mixed continuous stirred tank reactor at stable state using acetate as carbon source and electron donor. Choice of the CSTR allows the generation of rigorous kinetic data under defined conditions in which the effect of culture history is minimised. The use of the simple carbon source acetate allows a kinetic understanding of the complete oxidisers of the sulphate reducing bacteria to be established without superimposition of effects of culture conditions on the performance of the fermentative, acidogenic or acetogenic populations essential in metabolism of more complex carbon sources.

In our previous work (Moosa et al., 2002), an increase in initial concentration of sulphate in the range 1.0 to 10.0 kg m^{-3} enhanced the reaction rate from 0.007 to 0.170 kg m^{-3} h^{-1}. For a given initial concentration of sulphate and for dilution rates below the wash out value, an increase in volumetric loading of sulphate led to a linear increase in volumetric reduction rate of sulphate. Using the experimental data and an approach based on fundamental relationships between the kinetics of bacterial growth and bioreduction of sulphate, a kinetic model for anaerobic reduction of sulphate was developed. The developed model, given by Equation 7.1, incorporated terms for the effects of initial and residual concentrations of sulphate, as well as biomass concentration:

$$r_s = \left(\frac{\mu_{max}[S]}{K'_s[S_0][x]+[S]} - K_d \right) \frac{[x]}{Y} \qquad 7.1$$

where r_s = reaction rate (kg m^{-3} h^{-1}),

[x] = biomass concentration (kg dry weight m^{-3}),

[S] = residual concentration of sulfate (kg m^{-3}),

$[S_0]$ = initial concentration of sulfate in the feed (kg m^{-3}),
μ_{max} = maximum specific growth rate (h^{-1}),
K'_s = apparent saturation constant (kg dry $weight^{-1}$ m^3),
K_d = decay coefficient (h^{-1}),
Y = biomass yield coefficient (kg bacteria kg $sulphate^{-1}$)

The values of μ_{max}, K'_s , k_d and Y are 0.061 (h^{-1}), 0.015 (kg dry $weight^{-1}$ m^3), 0.035 (h^{-1}) and 0.567(kg bacteria kg $sulphate^{-1}$), respectively at a temperature of 35°C and across a sulphate loading rate of 0.006 to 0.8 kg m^{-3} h^{-1}. Furthermore, should the sulphate converted have resided in aqueous solution, total sulphide concentrations (calculated based on sulphate reduction) in the range 1.54 to 2.99 kg m^{-3} (pH 7.8) can be postulated. No effect of this calculated sulphide concentration on the kinetics of anaerobic sulphate reduction was evident. It must be noted that these sulphide concentrations were not confirmed by measurement in the previous study.

The effect of sulphide on SRBs has been studied by various researchers but the exact mechanisms of toxicity have not been elucidated. Hydrogen sulphide speciates in water according to the following equations (Garrels and Christ, 1965):

$$H_2S \Leftrightarrow H^+ + HS^-$$

$$HS^- \Leftrightarrow H^+ + S^{2-}$$

Sulphide toxicity is regarded as being pH dependent (as shown in the previous section). The neutral undissociated H_2S can easily pass through the cell membrane (Speece, 1983). However this relationship is not straightforward. Isa et al. (1986) reported that a mixed culture of SRBs grown in a fixed film reactor was not inhibited by high concentrations of sulphide (concentrations of total sulphide in excess of 0.8 kg m^{-3}). Using a pure culture of *Desulfotomaculum acetoxidans,* Widdel (1988) concluded that at H_2S concentrations in excess of 0.085 kg m^{-3} inhibition occurred. During the degradation of lactate Hilton and Oleskiewiscz (1988) found that a mixed SRB culture was inhibited by total sulphide concentrations of 0.40 kg m^{-3} (H_2S concentration of 0.20 kg m^{-3}) whereas Reis et al. (1992) reported complete inhibition of lactate utilising SRBs at H_2S concentrations of 0.55 kg m^{-3} (pH 6.2 to 6.6). Okabe et al. (1992) found 50% inhibition of lactate utilising *Desulfovibrio desulfuricans* H_2S concentrations of 0.25 kg m^{-3} at pH 7.0.

Process failure was reported by Stucki et al. (1993) at H_2S concentrations of 0.05 kg m^{-3} in a fixed bed sulphate reducing reactor fed acetate. Visser (1995) showed the total sulphide concentration for 50% inhibition of a mixed SRB culture grown batchwise in a UASB using acetate as the organic source to vary from 0.6 to 1.1 kg m^{-3} when the pH varied between 7.2 and 8.3. Sulphide inhibition has been shown by O'Flaherty et al. (1998) to be culture specific. They found that inhibition of all bacterial groups studied correlated to the total sulphide concentrations (0.6 to 1.5 kg m^{-3}) in the pH range 7.2 to 8.5. Propionate utilising SRBs were shown to be the most sensitive of all the bacterial groups.

Sulphide has been shown to have an impact on the operation of sulphate reducing systems and it was against this background that the objectives of this study were formulated. In Section 7.2 of this chapter results are presented from experiments to determine the effect of sulphide on the kinetics of anaerobic sulphate reduction. In order to study this effect, it was necessary to confirm the sulphide species leading to inhibition of bacterial sulphate reduction as well as the effect of sulphide concentration.

Further to this, as sulphide is the product of sulphate reduction and previous work (Moosa, 2000) had suggested inhibition at high sulphate loadings in the absence of accumulation of sulphide, the effect of sulphate loading as a potential inhibitor is considered in Section 7.4. Section 7.3 broadens the applicability of the kinetic data and kinetic model of sulphate reduction developed by extending the study from the use of acetate as carbon source and electron donor to ethanol, a more complex carbon source that undergoes either incomplete oxidation to acetate or complete oxidation to CO_2.

7.2. THE EFFECT OF THE CONCENTRATION AND SPECIATION OF THE SOLUBLE SULPHIDE PRODUCT ON THE KINETICS OF BIOLOGICAL SULPHATE REDUCTION

7.2.1. Introduction

The K_a value of the dissociation equilibrium of hydrogen sulphide is 9.1×10^{-8} at 18°C (Weast and Astle, 1988). Hydrogen sulphide dissociation follows the equilibrium reaction:

$$H_2S \Leftrightarrow HS^- + H^+$$

In the pH range 6 – 8, hydrogen sulphide exists in one of these forms (H_2S or HS^-). Below pH 6, hydrogen sulphide is found predominantly in the undissociated form (H_2S). Dissociated hydrogen sulphide dissociates further at higher pH:

$$HS^- \Leftrightarrow S^{2-} + H^+$$

the dissociation constant for this reaction is 1.1 $\times 10^{-12}$ at 18°C (Weast and Astle, 1988). Hydrogen sulphide dissociation to S^{2-} occurs near pH 12. Total hydrogen sulphide can be determined at any time by the relationship:

$$H_2S_{total} = H_2S_{(aq)} + HS^-$$

or, knowing that all hydrogen sulphide is produced from the reduction of sulphate to sulphide:

$$H_2S = (SO_4^{2-})_{\text{inf}\,luent} - (SO_4^{2-})_{effluent}$$

Figure 7.1 shows the relationship between the species of sulphide and pH.

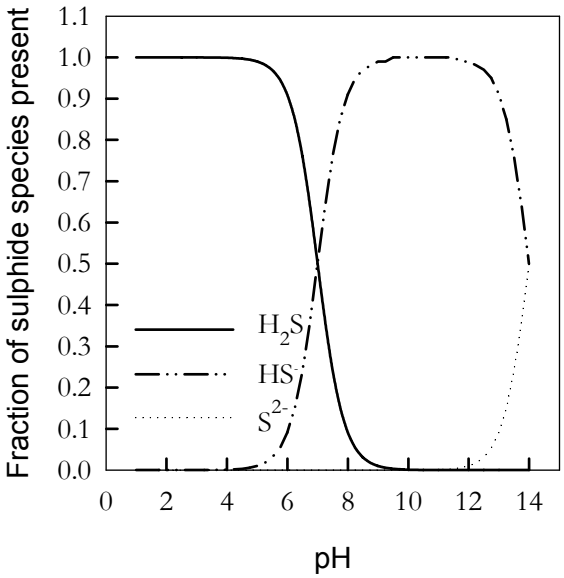

Figure 7.1: Relationship between the species of hydrogen sulphide and pH

7.2.2. ***Effect of sulphide concentration and speciation on biological sulphate reduction – literature review***

The sulphate reducing bacteria (SRB) have the highest tolerance, compared to other anaerobic micro-organisms, to sulphide. Their activity is nonetheless inhibited by the presence of sulphide (Reis et al., 1991a, b; Maillacheruvu et al., 1993; Konishi et al., 1996; O'Flaherty et al., 1997, 1998; O'Flaherty and Colleran, 1998, 2000). Table 7.1 shows the inhibitory levels of sulphide on the activity of SRB reported by independent researchers. The mechanism of sulphide inhibition has not been understood but two hypotheses are proposed. The one is that the undissociated sulphide in the environment precipitates any metals present, the result being that the SRB are deprived of essential trace metals that are required as cofactors for the activation of their enzyme systems (Bharathi et al., 1990). The

second hypothesis is that the sulphide is absorbed into the cells and denatures proteins, by acting as a cross-linking agent between the polypeptide chains (Postgate, 1984). The sulphide could also interfere with the metabolic coenzymes through the formation of sulphide bonds. However this theory was challenged by Parkin and Owen (1986). Sulphide inhibition has been shown to be reversible (Reis et al., 1992; Okabe et al., 1992). For example, after exposure of *Desulfovibrio* to inhibitory concentrations of sulphide, bacterial growth ceased. Once the sulphide was removed bacterial growth proceeded again and reached the same concentration as a control. In contrast to other micro-organisms where only the undissociated form of hydrogen sulphide (H_2S) has been shown to be inhibitory, there is debate as to whether total sulphide or only dissociated hydrogen sulphide (H_2S) is inhibitory (Stucki et al., 1992; Visser, 1993).

Hilton and Oleskiewicz (1988) investigated the degradation of lactate by anaerobic sludge containing sulphate reducers over a pH range of 6.5 to 8.0. From 30 day batch experiments they found that even under alkaline conditions the SRB were inhibited. They concluded that a direct relationship existed between the extent of inhibition and the total sulphide concentration (H_2S and HS^-). In contrast Reis et al. (1992) found that only the undissociated form was inhibitory over a pH range of 6.2 to 6.6. The same conclusion was drawn by Visser (1995), whose results show that loss of activity has a better correlation with undissociated hydrogen sulphide than with total sulphide. This is in accord with the theory that only undissociated H_2S is able to penetrate the cell membrane (Speece, 1983). O'Flaherty et al. (1998) studied the pH dependency of sulphide inhibition on various strains of SRB as well as mixed SRB. The general trend showed that as the pH increased from 6.8 to 8.5, the SRB were able to tolerate higher concentrations of total sulphide (Figure 7.2). This is in agreement with Visser (1995) who showed that the tolerated level of sulphide increased by about two fold when the pH was increased from 7.1 to 8.1. This further indicates that the undissociated sulphide, which accounts for some 60% of the aqueous sulphide at the lower pH range and only 10% at pH 8.1, and not total sulphide is inhibitory to SRB.

Table 7.1: Inhibitory levels of sulphide on SRB populations as reported in literature

Reference	Microorganisms	Organic source	Temperature (°C)	pH	Sulphide (kg m^{-3})	Reactor and mode of operation	Observations
Karhardkar et al. (1987)	*Desulfotomaculum*	Lactate	35	7.0	0.064 TS[1]	Shake flask Batch	Bacterial growth ceased
Hilton & Oleszkiewicz (1988)	Mixed SRB (suspended sludge)	Lactate	35	7.0	0.400 TS[1]	Shake flask Batch	50% decrease in sulphate reduction
McCartney & Oleszkiewicz (1991)	*Desulfovibrio desulphuricans*	Lactate	35	7.2 – 7.6	0.089 TS	Shake flask Batch	50% inhibition of sulphate reduction
McCartney & Oleszkiewicz (1993)	Mixed SRB (suspended sludge)	Lactate	35	7.1 – 7.3	0.300 H_2S	Stirred tank reactor Batch	50% inhibition of sulphate reduction
				8.0	0.185 H_2S	Stirred tank reactor Batch	50% inhibition of sulphate reduction
Reis et al. (1992)	*Desulfovibrio*	Lactate	37	6.2 – 6.6	0.550 HS[1]	Shake flask Batch	Complete inhibition of bacterial growth
Okabe et al. (1992)	*Desulfovibrio desulphuricans*	Lactate	35	7.0	0.600 TS[1]	Stirred tank reactor Continuous	Cellular yield decreased 95% Lactate utilisation decreased by 69%
	Desulfovibrio desulphuricans	Lactate	35	7.0	0.440 TS[1]	Stirred tank reactor Batch	Cellular yield decreased by 70% No effect on lactate utilisation
	Mixed SRB (suspended sludge)	Lactate	35	7.2 – 7.6	0.08 H_2S[1]	Stirred tank reactor Batch	50% inhibition of sulphate reduction

Reference	Microorganisms	Organic	Temperature (°C)	pH	Sulphide (kg m^{-3})	Reactor and mode of operation	Observations
Visser (1995)	Mixed SRB (granular)	Acetate	35	7.2-7.4	0.171 H_2S	Shake flask Batch	50% decrease in bacterial activity
	Mixed SRB	Acetate	35	8.1-8.3	0.057 H_2S	Shake flask Batch	50% decrease in bacterial activity
O'Flaherty et al. (1998)	Mixed SRB adapted to sulphate	Acetate	30-37	6.8-8.5	0.374-1.011 TS[1]	Upflow fixed film Continuous	50% decrease in bacterial activity
		Propionate	30-37	6.8-8.5	0.328-0.559 TS[1]	Upflow fixed film Continuous	50% decrease in bacterial activity
		Butyrate	30-37	6.8-8.5	0.593-2.059 TS[1]	Upflow fixed film Continuous	50% decrease in bacterial activity
		Ethanol	30-37	6.8-7.5	0.561-1.164 TS[1]	Upflow fixed film Continuous	50% decrease in bacterial activity
		Butyrate	30-37	6.8-7.5	0.467-0.988 TS[1]	Shake flask Batch	50% decrease in bacterial activity
		Ethanol	30-37	6.8-7.5	0.500-1.004 TS[1]	Shake flask Batch	50% decrease in bacterial activity
	Mixed SRB not adapted to sulphate	Butyrate	30-37	6.8-8.5	0.489-0.960 TS[1]	Shake flask Batch	50% decrease in bacterial activity
		Ethanol	30-37	6.8-7.5	0.544-1.124 TS[1]	Shake flask Batch	50% decrease in bacterial activity

Furthermore care should be taken in interpreting the inhibition kinetics obtained from batch tests in which pH, sulphide and the limiting substrate cannot be controlled. Total sulphide has been shown to be inhibitory at concentrations as low as 0.08 kg m^{-3} in batch systems (McCartney and Oleskiewicz, 1991).

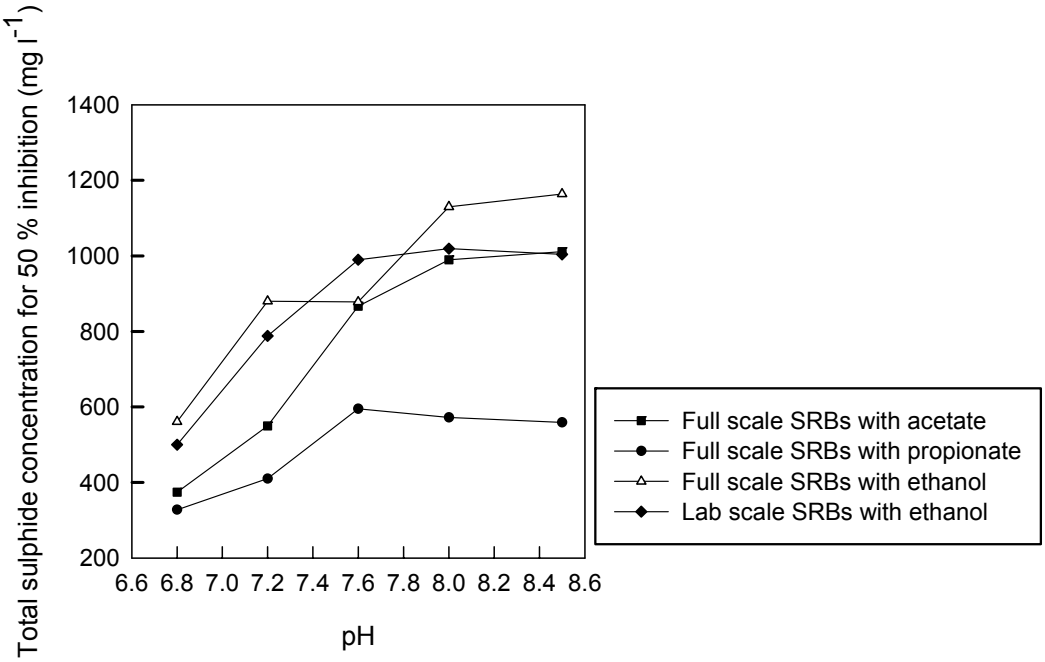

Figure 7.2: Tolerance levels of a mixed SRB culture to total sulphide as a function of pH (O' Flaherty et al*.,* 1998)

In contrast to this Okabe et al. (1992) found when using *Desulfovibrio desulphuricans* in a continuous bioreactor at pH 7.0 and a dilution rate of 0.2 h^{-1}, that for total sulphide concentrations up to 0.60 kg m^{-3} both lactate utilisation and cellular production were strongly inhibited. Further work by Okabe et al. (1995) using *Desulfovibrio desulphuricans* in a continuous bioreactor with lactate as the organic source at pH 7.0 showed that the maximum specific growth rate (μ_m) decreased from 0.33 to 0.21 h^{-1} as the total sulphide concentration was increased from 0.026 to 0.438 kg m^{-3}. They also noted that at the higher sulphide concentrations, cell yield decreased from 0.043 g cell g $lactate^{-1}$ at a total sulphide concentration of 0.108 kg m^{-3} to 0.01 g cell g $lactate^{-1}$ when the total sulphide was 0.437 kg m^{-3}. The lactate utilisation, however, remained unchanged. They offered two explanations for this phenomenon. Firstly, the decrease in bacterial concentration was attributed to lysis as a result of sulphide presence. Secondly, the energy was used to overcome the inhibition i.e. for maintenance and not for growth. However, above a total sulphide concentration of 0.50 kg m^{-3} both lactate utilisation and cellular production were inhibited as observed by their

earlier work (Okabe et al., 1992). Supporting the trend that sulphate reducers can acclimatise to sulphide in continuous system, Omil et al. (1998) noticed that in the presence of acetate as an organic source, SRB are able to acclimatise to a total sulphide concentration of 0.80 kg m^{-3}. In addition, Okabe et al. (1995) showed that maximum specific growth rates calculated for batch experiments were lower than those calculated for the chemostat at corresponding sulphide concentrations. Grady et al. (1996) note that in a continuous culture selection pressure results in an adaptation of the chemostat culture to changing environmental conditions.

7.2.3. ***Objectives and experimental programme***

A review of the literature has revealed that despite considerable research in the area of biological sulphate reduction to sulphide, the effect of sulphide speciation on the kinetics of the reaction have not been studied rigorously. Furthermore, the effect of sulphide concentration on sulphate reduction kinetics has not been modelled. Owing to the successful implementation of anaerobic sulphate reduction on a large scale being dependent on the understanding of the reaction kinetics and the effect of inhibitory compounds on the process kinetics, a rigorous study of the effects of sulphide speciation and concentration on the rate and extent of anaerobic sulphate reduction was carried out.

The objectives of this study were two-fold, centred on providing a rigorous understanding of the sulphide species causing sulphide inhibition of bacterial sulphate reduction and on determining the critical aqueous sulphide concentration and the concentration of the pertinent sulphide species at which this inhibition was mediated. These were achieved by investigating the kinetics of anaerobic sulphate reduction in continuous stirred tank reactors. To achieve a good spread of the ratio of soluble sulphide species present, four pH values were chosen, based on appropriate literature, to allow the interconnectedness between pH and sulphide speciation to be realised. These pH values of 6.0, 6.5, 7.0 and 7.5 were selected over the range in which the most significant transition from the undissociated form of H_2S to the dissociated form HS^- is observed (Figure 7.1) and used at a fixed sulphide concentration. Under these conditions, the S^{2-} concentration was negligible.

Further, the range of aqueous sulphide concentration was investigated at a fixed pH of 7.8 ± 0.2 through the addition of sulphide to the feed stream to the continuous stirred tank reactors at a concentration of 0.50, 0.75, 1.00 and 1.25 kg m^{-3} (as sulphide). The addition was made in the form of sodium sulphide. Previously, it has been shown that the varying sodium concentration resulting does not affect the bacterial sulphate reduction kinetics (Moosa

2000). As a sulphate feed concentration of 2.5 kg m^{-3} was used, these additions had potential to provide a sulphide concentration range in the reactor of 1.33 to 2.08 kg m^{-3} if all sulphate available was converted to aqueous sulphide.

7.2.4. ***Materials and methods***

7.2.4.1. Microorganisms

A mixed bacterial culture, enriched with respect to sulphidogens and from which methanogens had been eliminated as described in the preceding study (Hansford et al*.,* 2004), was used as the inoculum for all experiments. The stock culture was maintained in continuous mode at a retention time of 6 days. The feed sulphate and acetate concentrations were 2.5 and 10 kg m^{-3} respectively. The reactor pH of the stock culture was maintained at pH 7.8 and the temperature at 35°C.

7.2.4.2. Medium

A soluble complex medium, detailed in Table 7.2, was used. The medium is based on that developed by Postgate (1984) and modified by Visser et al. (1993). All reagents were analytical grade. Sulphate was added in the form of sodium sulphate and acetate was used as the organic source.

Table 7.2: Composition of the medium used for growth and maintenance of the anaerobic mixed culture (Visser et al., 1993)

Components	Weight (g)
Peptone	0.400
Lab-Lemco[1]	0.133
K_2HPO_4	0.040
$NaHCO_3$	1.250
Deionised Water	500 mℓ
Trace Metals	
$CoCl_2.6H_2O$	0.0119
$FeCl_2.4H_2O$	0.0785
$MnCl_2.4H_2O$	0.0038
$NaMoO_4.2H_2O$	0.0038
$NiCl_2.6H_2O$	0.0045
Deionised Water	500 mℓ

[1] An extract used for general bacteriological purposes containing 12.4% N and 5.7% NaCl

7.2.4.3. Experimental apparatus

The experiments were carried in one-litre Quickfit vessels. The glass lids of the vessels and the Quickfit adapters were sealed with vacuum grease to ensure that an anaerobic system was maintained. The reactor was kept mixed by Heidolph overhead stirrers driving two bladed impellers at a speed of 400 rpm. Fresh medium was fed into the reactor by a variable speed peristaltic pump. To avoid channelling, the feed was introduced close to the base of the reactor. The effluent discharged by gravity through a U-shaped overflow tube, designed to maintain the volume in the reactor at 1 ℓ. A constant temperature of 35°C was maintained by placing the reactor in a constant temperature waterbath and the pH was manually controlled. When the pH deviated from the set point, concentrated sodium hydroxide or hydrochloric acid was added to the reactor. The experimental apparatus is shown schematically in Figure 7.3.

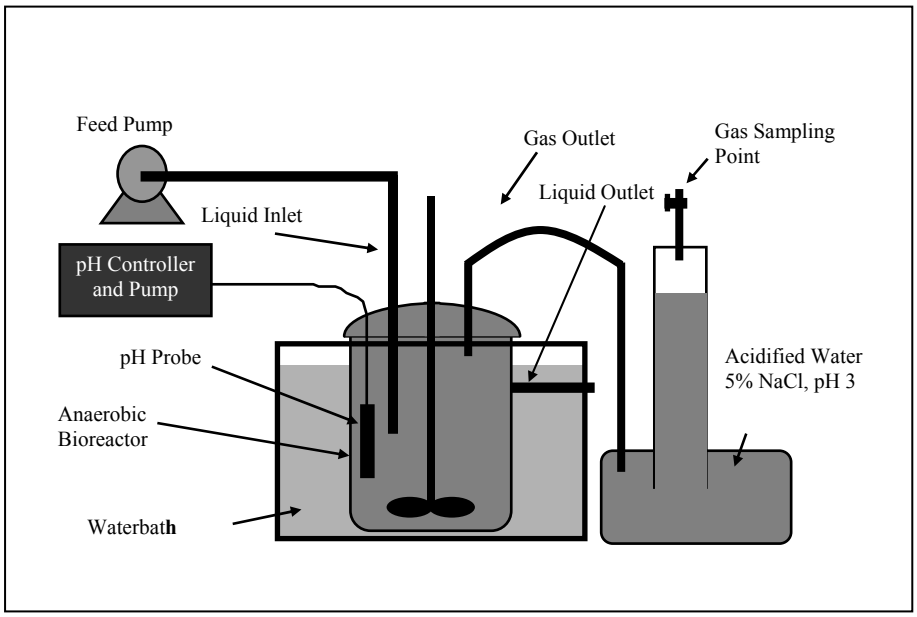

Figure 7.3: Schematic diagram of the experimental set up

7.2.4.4. Operation of the bioreactor

A volume of 800 mℓ media was equilibrated to the operating temperature in the reactor prior to adding 200 mℓ inoculum. The reactor was operated in batch until the concentration of sulphate decreased to a stable value. Thereafter the reactor was switched to continuous mode of operation. Feed addition was increased stepwise during the course of the experiment to affect a range of dilution rates. Flow rates in the range 0.004 and 0.021 ℓ h^{-1} were applied, representing dilution rates in the range 0.004 and 0.021 ℓ h^{-1}. 'Stable' state

conditions were achieved at each flow rate to provide data to estimate the kinetics of sulphate reduction and bacterial growth. 'Stable' conditions were assumed when the residual sulphate concentration and bacterial concentration varied by no more than 12 and 15% respectively for a period of operation equal to one retention time. Liquid samples were taken from the reactor on a daily basis and analysed for sulphate, sulphide, bacterial and acetate concentrations.

7.2.4.5. Speciation of soluble sulphide, mediated through varying pH

The effect of sulphide speciation on the sulphate reduction process was studied at a temperature of 35°C, sulphate concentration of 2.5 kg m^{-3} and an acetate concentration of 10.0 kg m^{-3}. Four pH values (6.0, 6.5, 7.0, and 7.5) were used, providing information over a range of sulphide species in which the fraction of aqueous sulphide present in the undissociated form varied from 60 to 10%.

7.2.4.6. Concentration of soluble sulphide present: sulphide addition to feed

The effect of soluble sulphide concentration on the sulphate reduction process was studied at a temperature of 35°C, sulphate concentration of 2.5 kg m^{-3} and an acetate concentration of 2.3 kg m^{-3}. Sulphide was added to the feed stream at concentrations of 0.0, 0.50, 0.75, 1.00 and 1.25 kg m^{-3}, using sodium sulphide.

7.2.4.7. Analytical procedures

A turbidimetric method was used to measure the concentration of sulphate (APHA, 1992). Prior to sulphate determination, suspended solids were removed from the sample by centrifugation. After addition of 0.25 mℓ conditioning reagent[2] to 5 mℓ of sample, an excess amount of finely ground $BaCl_2$ was added and the sample was stirred for 1 minute on a vortex mixer. The absorbance of the sample was then measured at a wavelength of 420 nm. The absorbance of the sample was used to calculate the concentration of sulphate. A calibration curve for dependency of adsorption on sulphate concentration was obtained using a similar procedure. Sulphide was determined as described by APHA (1992). Acetate concentration was determined with a High Performance Liquid Chromatograph (HPLC, Beckmann, System Gold), with a UV detector (Detector no 168) and a glass lined Wakosil column. Phosphoric acid solution, 20 mM, with a pH of 2.5 was used as the mobile phase. The pH of the mobile phase was adjusted with sodium dihydrogen orthophosphate. The bacterial concentration was determined by measurement of dry weight following drying in an oven at 80°C for 2 days.

[2] 50 mℓ glycerol, 30 mℓ concentrated HCl, 75 g NaCl, 100 mℓ ethanol and 300 mℓ deionised water

7.2.5. ***Results and discussion: sulphide speciation***

7.2.5.1. Kinetic data

The kinetic data for bacterial growth and bioreduction of sulphate at pH values of 6.0, 6.5, 7.0 and 7.5 are shown as Figures 7.4 to 7.7 respectively. The volumetric reduction rate calculated on the basis of initial concentration of sulphate, flow rate of the feed, working volume of the reactor and conversion, was used to ascertain the kinetics of the reaction.

In the reactor with a pH of 6.0 the maximum bacterial concentration of 2.3 g ℓ^{-1} was observed a dilution rate of 0.0042 h^{-1}. As can be seen from Figure 7.4, a sulphate conversion of 40% was obtained at volumetric loading rates in the range 0.021 to 0.010 kg m^{-3} h^{-1}. Increasing the volumetric loading rate in the range 0.021 to 0.052 kg m^{-3} h^{-1} led to a linear decrease in volumetric reduction rate from 0.008 to 0.0016 kg m^{-3} h^{-1} and a decrease in sulphate conversion from 40 to 3.2%. The maximum reduction rate for this set of experiments was 0.0019 kg m^{-3} h^{-1} achieved at a retention time of 5 days. The corresponding conversion of sulphate was 39%. The trend of acetate utilisation was similar to that for sulphate reduction. At low volumetric loadings, corresponding to higher sulphate conversions, a higher level of acetate was utilised than at the higher loading rates. The ratio of sulphate reduced to acetate utilised was constant over the range of applied loading rates and was 0.72 mol sulphate mol $acetate^{-1}$.

On operation at pH 6.5, the bacterial concentration decreased from 2.3 to 1.5 g ℓ^{-1} as the dilution rate was increased from 0.004 to 0.014 h^{-1}. The decreasing trend was more pronounced as the dilution rate was increased from 0.014 to 0.029 h^{-1}. Applying loading rates ranging from 0.010 to 0.026 kg m^{-3} h^{-1}, the sulphate conversion remained constant at 50%. The maximum reduction rate of sulphate in this set of experiments was 0.014 kg m^{-3} h^{-1}, achieved at a retention time of two days, corresponding to 27% sulphate conversion. The molar ratio of reduced sulphate to utilised acetate was 0.72.

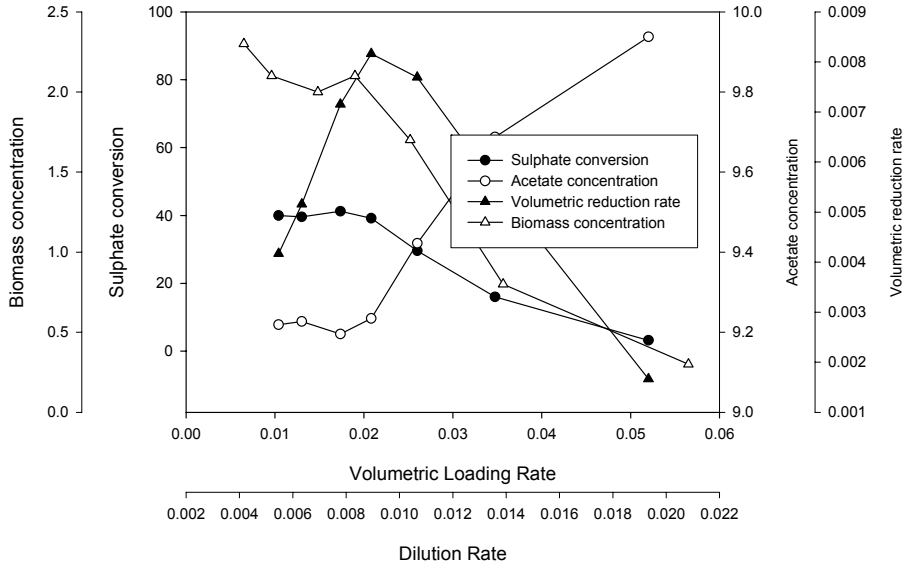

Figure 7.4: Stable state kinetics of continuous sulphate reduction at pH 6.0.

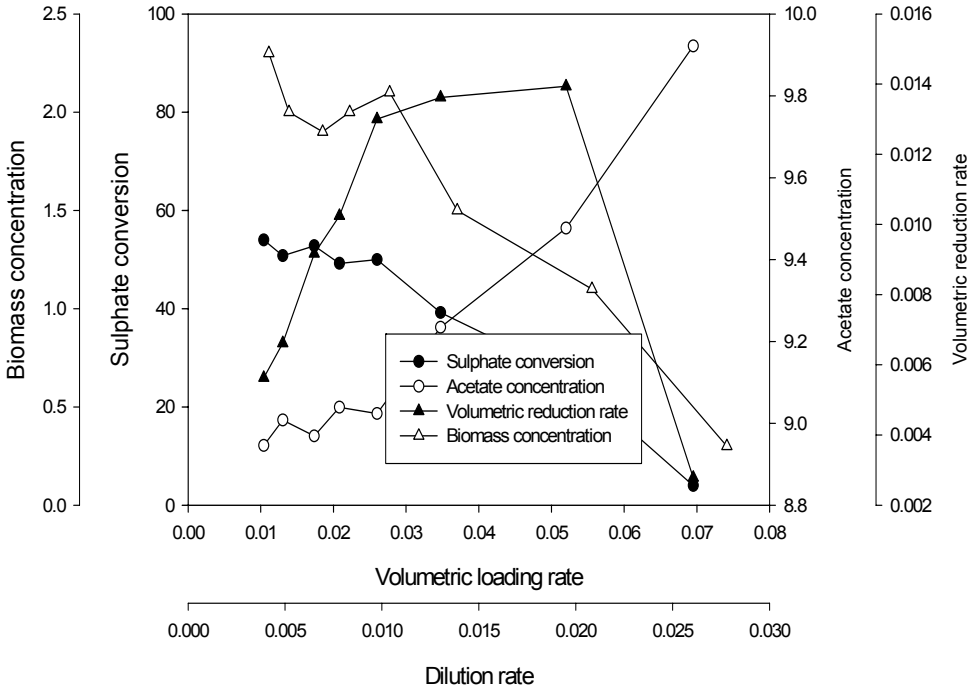

Figure 7.5: Stable state kinetics of continuous sulphate reduction at pH 6.5.

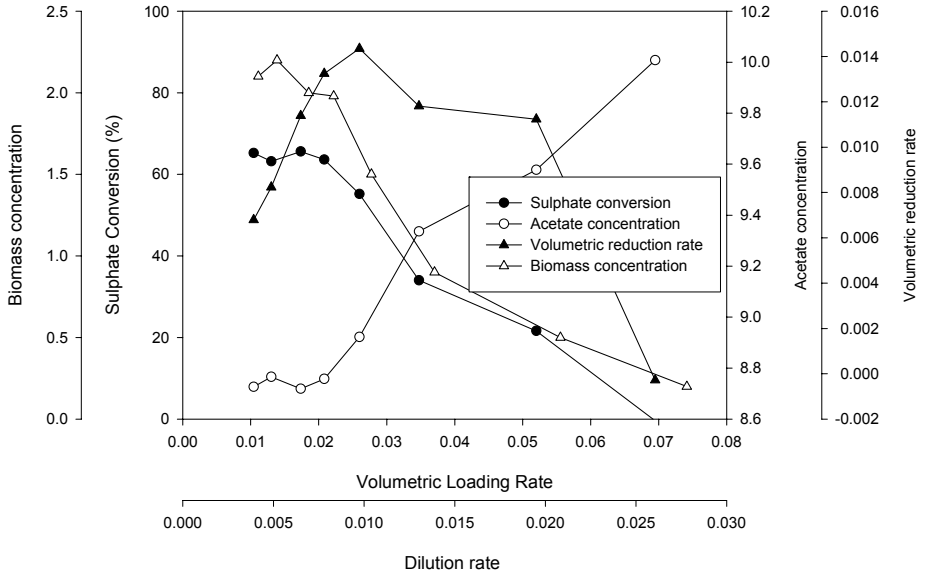

Figure 7.6: Stable state kinetics of continuous sulphate reduction at pH 7.0**.**

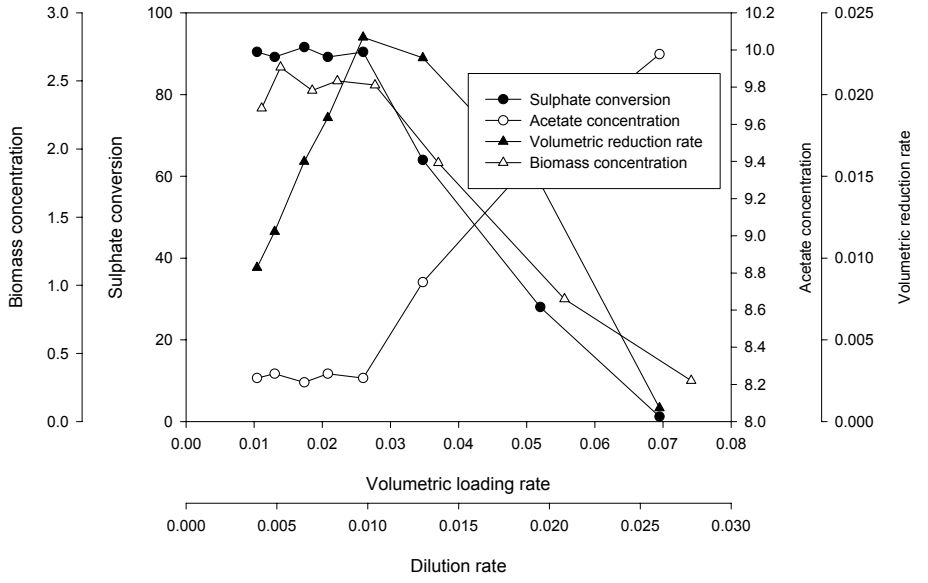

Figure 7.7: Stable state kinetics of continuous sulphate reduction at pH 7.5

The maximum bacterial concentration when the reactor pH was 7.0 was 2.2 g ℓ^{-1}, observed at a dilution rate of 0.005 h^{-1}. For volumetric loadings in the range 0.010 to 0.0208 kg m^{-3} h^{-1} a relatively constant conversion of sulphate to sulphide (63–65%) was observed. The maximum reduction rate for this set of experiments was 0.014 kg m^{-3} h^{-1} achieved at a retention time of 4 days. The corresponding sulphate conversion was 55%. Increasing the loading rate beyond 0.026 kg m^{-3} h^{-1} resulted in a

decrease in both sulphate conversion and volumetric sulphate reduction rate. The coupling of acetate utilisation and sulphate reduction was again observed. For the whole range of applied volumetric loading rates, the molar ratio of reduced sulphate to utilised acetate was 0.72.

When the reactor pH was 7.5 the maximum bacterial concentration was 2.6 g ℓ^{-1} (Figure 7.7) observed at a dilution rate of 0.005208 h^{-1}. Applying loading rates in the range 0.010 to 0.026 kg m^{-3} h^{-1} led to sulphate conversions around 90%. In this run the maximum reduction rate was 0.024 kg m^{-3} h^{-1} at a retention time of 4 days. The conversion of sulphate at this retention time was 90%. The dependency of acetate utilisation and sulphate reduction was similar to the previous run with a constant ratio of 0.72 mol of sulphate reduced/mole of acetate utilised.

7.2.5.2. Sulphide speciation

Figure 7.8 presents profiles of maximum sulphate conversion as a function of undissociated sulphide concentration on reduction of 2.5 kg m^{-3} sulphate at pH across the range pH 6.0 to 7.5. Both the extent of conversion and volumetric rate of sulphate reduction are given. These data clearly illustrate a decrease in both the extent of conversion achieved and the rate of sulphate reduction on increasing the undissociated H_2S concentration from 0.18 to 0.30 kg m^{-3} with decreasing pH. These results indicate that the sulphate reducing consortia present are inhibited at the lower pH values by undissociated hydrogen sulphide. Correlation of the performance with respect to sulphate reduction and aqueous sulphide is not appropriate in this study owing to the constant amount of sulphate and sulphide added to each experiment. Where the majority of sulphate is reduced to sulphide, a direct correlation between these must result without providing input with respect to the mechanism.

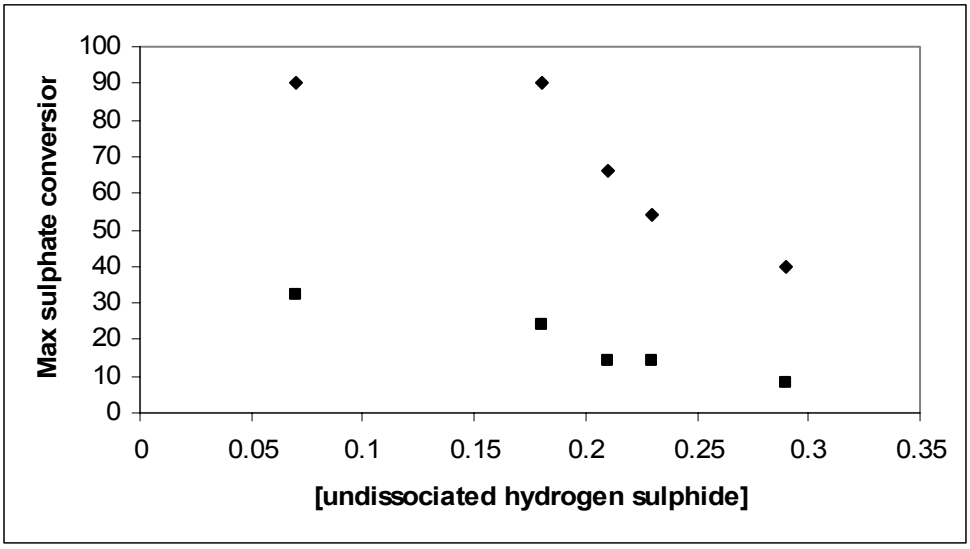

Figure 7.8: Maximum extent and rate of sulphate conversion as a function of the undissociated hydrogen sulphide concentration, manipulated through varying pH across the range pH 6.0 to 7.8. ♦ Extent of sulphate conversion (%), ■ vol. sulphate reduction rate (10^{-3} kg m^{-3} h^{-1})

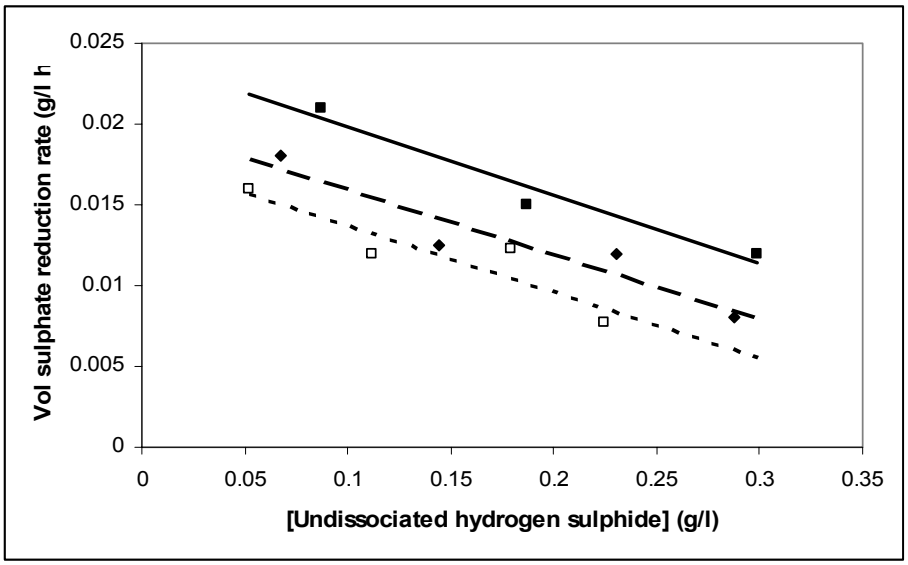

Figure 7.9: Volumetric sulphate reduction rate as a function of undissociated H_2S concentration. The undissociated H_2S concentration is varied as a function of pH (pH 6.0 to 7.5) and % sulphate conversion: □ 30% conversion of sulphate feed, ♦ 40% conversion of sulphate feed, ■ 50% conversion of sulphate feed. Linear trend lines are provided

In Figure 7.9, the rate of sulphate reduction is given as a function of undissociated H2S concentration across a range of levels of sulphate conversion. To achieve 40% reduction in the reactors operating at a pH of 6, 6.5, 7 and 7.5, the corresponding H_2S concentrations are 0.30, 0.25, 0.18 and 0.05 kg m^{-3} respectively. At these conditions, the rate of sulphate reduction increased on decreasing concentration of undissociated H_2S while the concentration of aqueous sulphide remained constant. These data support that H_2S is the dominant sulphide species causing inhibition of sulphate reduction, hence increased inhibition occurs with decreasing pH across this range. At the three aqueous sulphide concentrations given, the rate of sulphate reduction decreased similarly with increasing concentration of undissociated H2S, yielding a slope of -0.041 kg SO_4^{2-} kg^{-1} undissociated H_2S per hour.

Table 7.3 provides an outline of the maximum sulphate reduction observed for the range of pH values as well as the average aqueous sulphide and H_2S concentration observed. It is clear that the lowest H_2S concentrations were observed at the highest pH where the sulphate conversion was the highest. These results are in agreement with those observed by O'Flaherty et al. (1998) and Visser (1995). They attributed the increase in bacterial activity with increasing pH to the lower concentrations of undissociated H_2S observed at the higher pH values.

Table 7.3: Average aqueous sulphide and H_2S concentrations as a function of pH

pH	Maximum Sulphate Reduction (%)	Average [Aqueous Sulphide] (kg m^{-3})	Average [H_2S] (kg m^{-3})
6	40	0.32	0.29
6.5	54	0.43	0.23
7	66	0.52	0.21
7.5	90	0.73	0.18
7.8	90	0.75	0.07

7.2.5.3. Growth parameters

For this study the modified Chen and Hashimoto model as described in Moosa et al. (2002) was used to determine the microbial growth parameters. The model was developed to account for high biomass concentrations that result in mass transfer limitations. The kinetic parameters are presented in Table 7.4.

It is evident that the maximum specific growth rate increases with increasing pH and then remains relatively constant at 0.060 h^{-1}. This indicates that at the lower pH when the H_2S

species is dominant the bacteria grow at a slower rate than at the higher pH values. This implies that the bacteria present at the lower pH will not tolerate high dilution rates, unless biomass retention is achieved. The same trend was observed by O'Flaherty et al. (1998) when studying the effect of pH in the range 6.8 to 9.8 on pure SRB cultures grown in batch. The substrate affinity constant, K_s, remained relatively constant for the range of pH values.

Table 7.4: Growth parameters calculated using the modified Chen and Hashimoto model (Moosa et al., 2002)

pH	μ_m h^{-1}	K_s kg m^{-3}	k_d h^{-1}
6.0	0.021	0.131	0.037
6.5	0.052	0.138	0.040
7.0	0.059	0.131	0.034
7.5	0.061	0.135	0.036

7.2.6. ***Results and discussion: Sulphide concentration***

7.2.6.1. Kinetic data

Figures 7.10 to 7.14 present the steady state data for the continuous stirred tank reactors receiving 0.00, 0.50, 0.75, 1.0 and 1.25 g ℓ^{-3} of sulphide. Data collected on the conversion of sulphate on the continuous feed of medium containing increasing concentrations of added sulphide is compared in Figure 7.10. Here, it is clearly seen that the conversion decreases with increasing added sulphide concentrations of 1.0 g L^{-1} and more. Further, at low retention times the negative effect of increasing sulphide addition on sulphate conversion is seen.

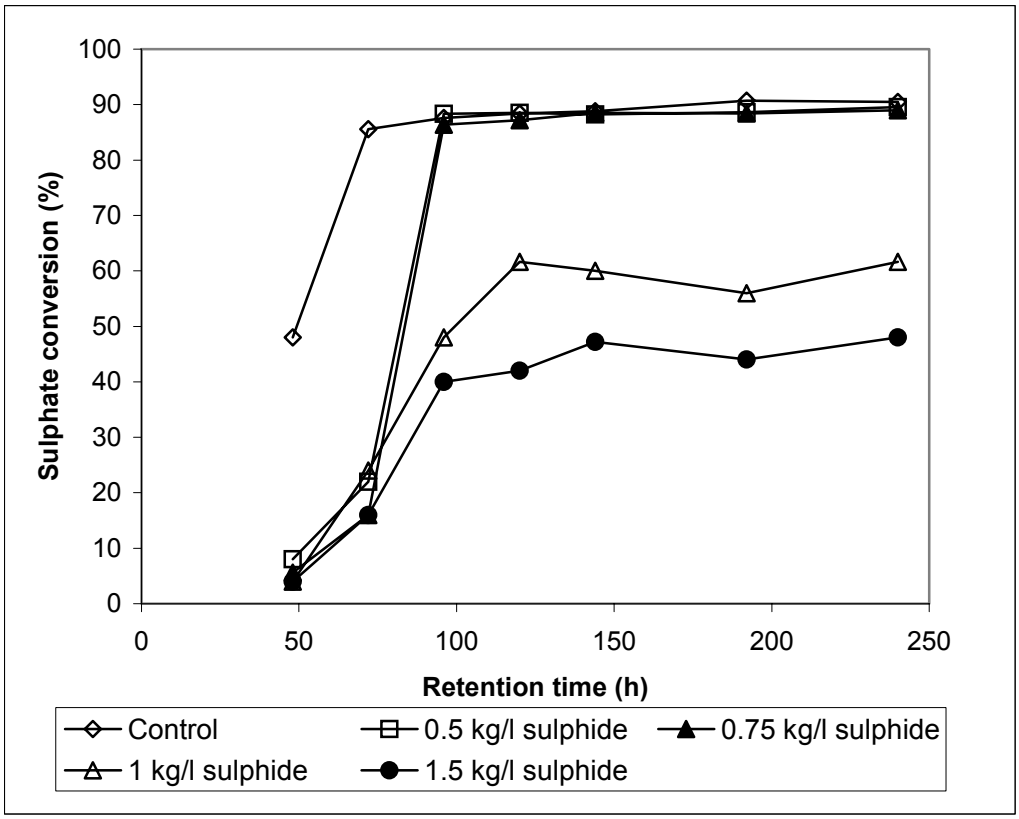

Figure 7.10: Conversion of sulphate at increasing sulphide feed concentrations

On no sulphide addition to the feed and a feed sulphate concentration of 2.5 kg m^{-3} (data not shown), the bacterial concentration showed a consistent decrease with increasing of dilution rate above 0.004 h^{-1}. The maximum bacterial concentration achieved was 2.6 g ℓ^{-1}, observed at a dilution rate of 0.005 h^{-1}. High conversion of sulphate in the range, 80% to 90%, was observed for loading rates in the range 0.010 to 0.040 kg m^{-3} h^{-1}. The increasing of volumetric loadings in this range was accompanied by an enhancement in reduction rate from 0.009 to 0.032 kg m^{-3} h^{-1}. The maximum reduction rate, 0.032 kg m^{-3} h^{-1}, was obtained at a retention time of 2.5 days (volumetric sulphate loading rate of 0.015 kg m^{-3} h^{-1}). The conversion of sulphate at this retention time was 80%. Applying higher volumetric loading rates led to a substantial decrease in the reduction rate of sulphate. The ratio of sulphate reduced to acetate utilised was relatively constant at 0.77 mole reduced sulphate/mole utilised acetate.

On increasing the feed sulphide concentration to 0.5 g ℓ^{-1} (Figure 7.11), the maximum bacterial concentration was 2.3 g ℓ^{-1} at a dilution rate of 0.004 h^{-1}. Biomass concentration decreased with increasing dilution rates above 0.008 h^{-1}. The sulphate removal efficiency remained relatively constant at 89% as the volumetric loading rate was increased from 0.010 to 0.026 kg m^{-3} h^{-1} (dilution rates of 0.010 to 0.015 h^{-1}). In this range of loading rates the

volumetric reduction rate increased from 0.009 to 0.023 kg m^{-3} h^{-1}, with the maximum reduction rate being observed at a retention time of 4 days (volumetric sulphate loading rate of 0.026 kg m^{-3} h^{-1}). The sulphate reduction and biomass concentration at this retention time were 88.4% and 1.7 g ℓ^{-1} respectively. Increasing the volumetric loading rate above 0.026 kg m^{-3} h^{-1} resulted in a decrease in the sulphate reduction to 22% and the biomass concentration to 0.80 g ℓ^{-1} at a loading rate of 0.035 kg m^{-3} h^{-1} (dilution rate of 0.014 h^{-1}) indicating the onset of washout. For this set of experiments washout was complete at a retention time of 2 days. The ratio of sulphate reduced to acetate utilised was relatively constant at 0.86 mole reduced sulphate/mole utilised acetate.

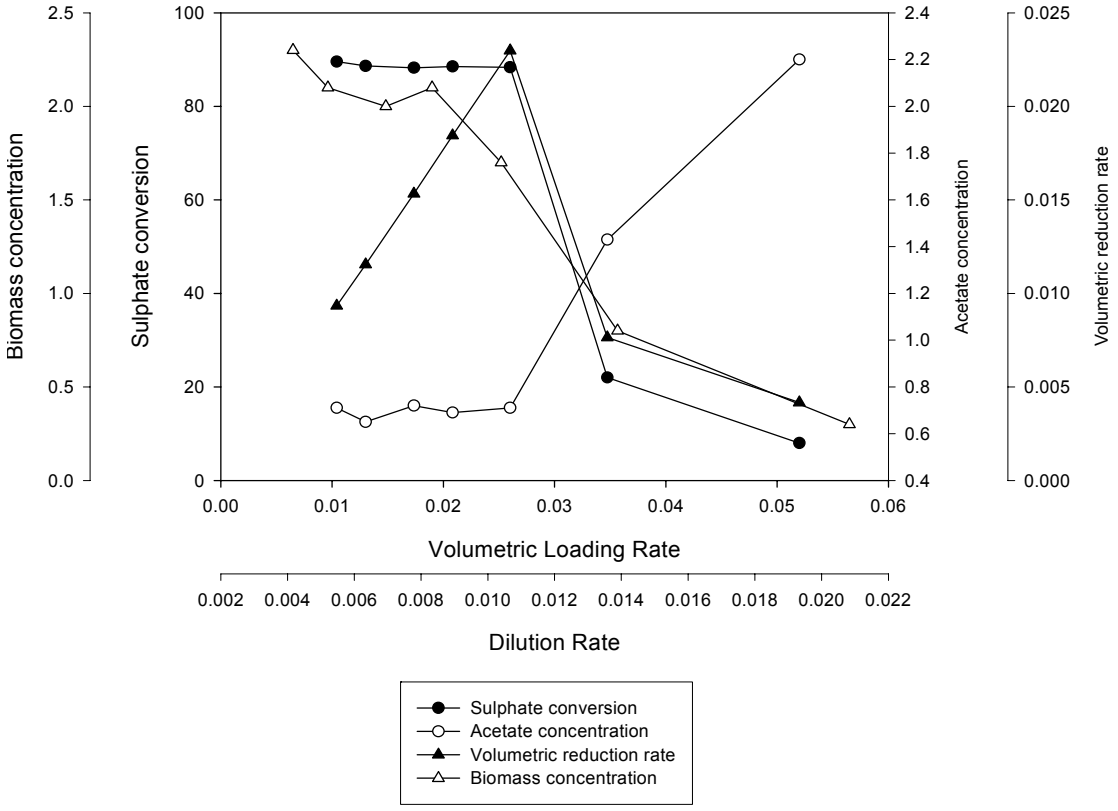

Figure 7.11: Stable state kinetics of continuous sulphate reduction at a feed sulphide concentration of 0.5 g ℓ^{-1}

When the inlet sulphide concentration was 0.75 g ℓ^{-1} (Figure 7.12), a maximum bacterial concentration of 2.4 g ℓ^{-1} was observed at dilution rate of 0.004 h^{-1}. The percentage sulphate removal varied between 89 and 86% for loading rate between 0.010 and 0.026 kg m^{-3} h^{-1}. The volumetric reduction rate increased from 0.009 to 0.023 kg m^{-3} h^{-1} as the retention time was decreased from 10 to 4 days. The maximum reduction rate occurred at a retention time of 4 days. Decreasing the retention time below 4 days resulted in a decrease in sulphate

removal, bacterial concentration and volumetric reduction rate as well as an increase in residual acetate concentration indicating washout. The acetate removal profile displayed the inverse trend to the sulphate reduction profile. The ratio of sulphate reduced to acetate utilised was 0.9 mole reduced sulphate/mole utilised acetate.

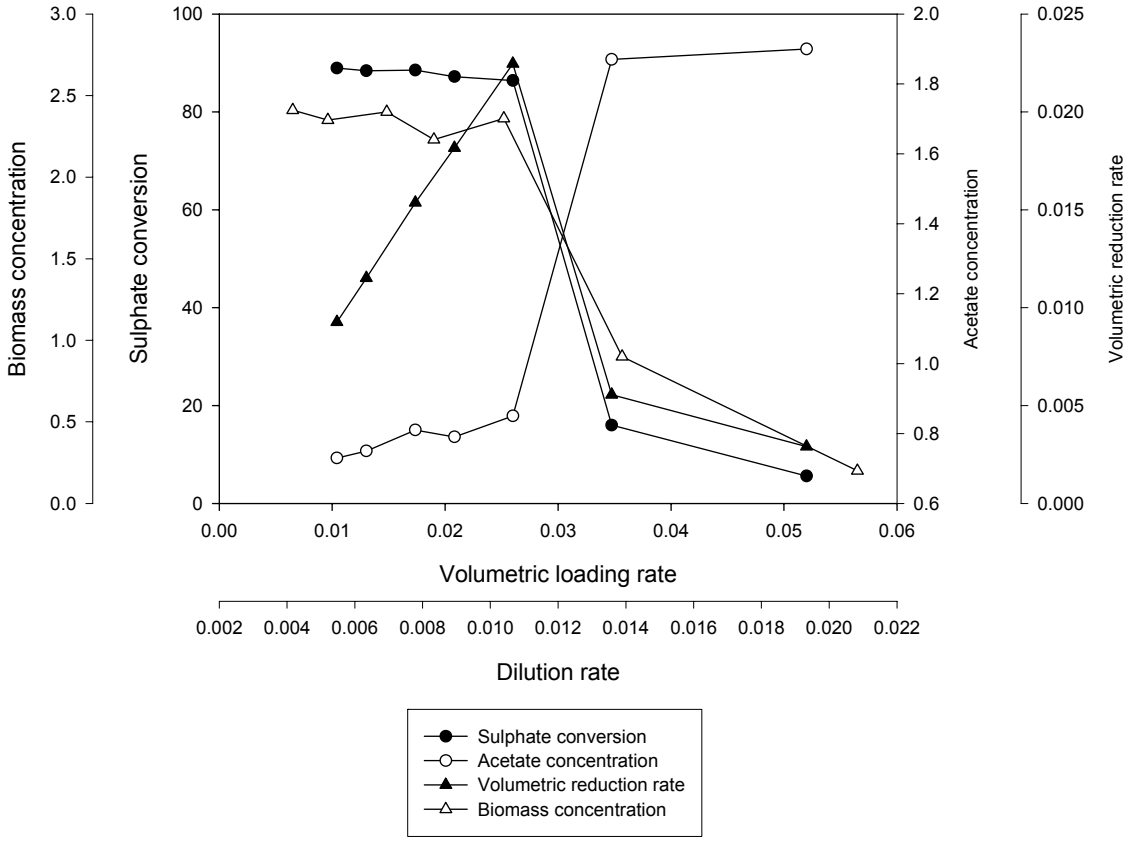

Figure 7.12: Stable state kinetics of continuous sulphate reduction at a feed sulphide concentration o f 0.75 g ℓ^{-1}

Figure 7.13 presents data collected at an inlet sulphide concentration of 1.0 g ℓ^{-1}. The maximum biomass concentration under these conditions was 2.9 g ℓ^{-1}, observed at a dilution rate of 0.005 h^{-1}. The bacterial concentration remained relatively constant in the range 2.7 g ℓ^{-1} over the dilution rate range of 0.005 to 0.010 h^{-1}. Thereafter it decreased to 1.2 g ℓ^{-1} at a dilution rate of 0.014 h^{-1} indicating the onset of washout. Maximum sulphate conversion of 56 to 62% occurred at volumetric sulphate loading rates in the range 0.010 to 0.021 kg m^{-3} h^{-1}. The volumetric sulphate reduction rate displayed a steady increase from 0.006 to 0.013 kg m^{-3} h^{-1} as the retention time was decreased from 10 to 6 days and remained relatively constant at 0.013 kg m^{-3} h^{-1} at retention times of 6 and 4 days (volumetric sulphate loading rates of 0.021 and 0.026 kg m^{-3} h^{-1}. On further reduction in retention time to 3 days, a significant decrease in sulphate reduction rate biomass concentration with concomitant

increase in residual acetate concentration indicated on of washout. The sulphate reduced to acetate utilised was 0.79 mole reduced sulphate/mole utilised acetate.

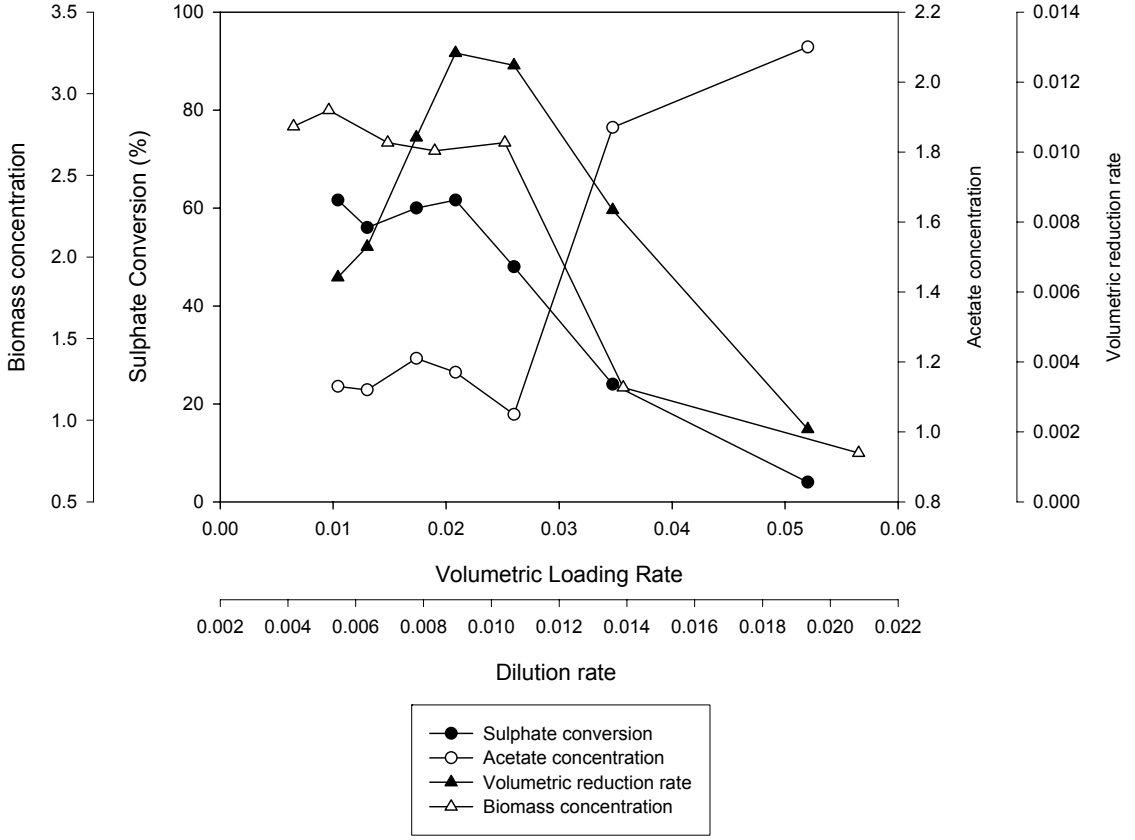

Figure 7.13: Stable state kinetics of continuous sulphate reduction at a feed sulphide concentration o f 1.0 g ℓ^{-1}

On further increasing the feed sulphide concentration to 1.25 g ℓ^{-1} sulphide (Figure 7.14) the maximum bacterial concentration achieved lay in the range 2.9 to 3.2 gℓ^{-1}, across a dilution rate range of 0.004 to 0.010 h^{-1}. The maximum sulphate conversion in this experiment was 48%, observed at the lowest volumetric sulphate loading rate of 0.0104 kg m^{-3} h^{-1}, the sulphate reduction rate at this loading was 0.005 kg m^{-3} h^{-1}. The volumetric sulphate reduction rate increased to 0.010 kg m^{-3} h^{-1} linearly as the retention time was decreased from 10 to 4 days (volumetric sulphate loading rate increased from 0.010 to 0.026 kg m^{-3} h^{-1}). Over these conditions, sulphate conversion remained above 40%. On further decrease of retention time below 4 days, a decrease in volumetric reduction rate of sulphate, bacterial concentration and sulphate conversion was observed indicating the onset of washout. The acetate removal followed the same trend as the sulphate removal with sulphate reduced per acetate utilised being 0.61 reduced sulphate permole utilised acetate.

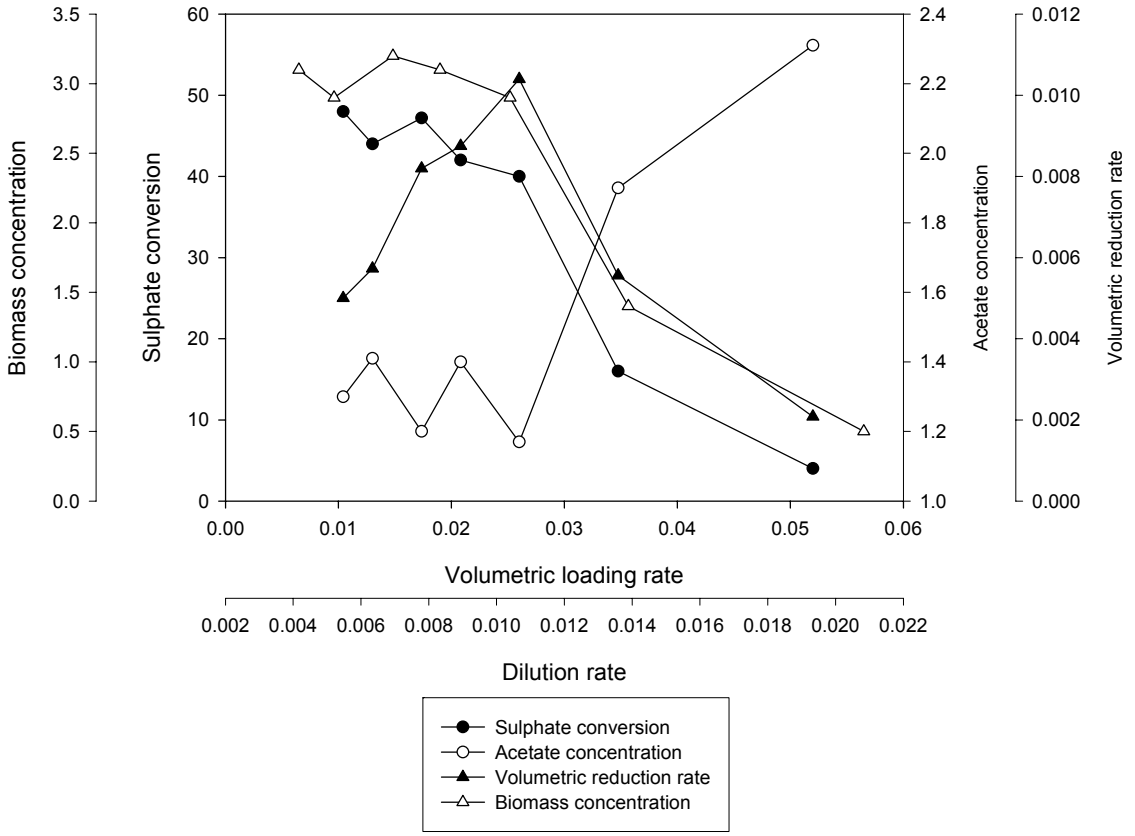

Figure 7.14: Stable state kinetics of continuous sulphate reduction at a feed sulphide concentration o f 1.25 g ℓ^{-1}

7.2.6.2. Sulphide concentration and speciation

From the results presented it is apparent that sulphate removal and volumetric reduction rate of sulphate were affected by increasing the feed sulphide concentration (Table 7.5 and Figure 7.15). The maximum bacterial concentration remained relatively constant at 2.68 ± 0.37 g ℓ^{-1} for the range of applied feed sulphide concentrations (0.0 to 1.25 g ℓ^{-1}). Similarly, the maximum sulphate conversion remained constant at 89% as the sulphide addition was increased from 0.00 to 0.75 g ℓ^{-1}. However, on sulphide addition to the feed of greater than 0.75 g ℓ^{-1}, the maximum conversion of sulphate and the volumetric sulphate reduction rate decreased steadily. This indicated that there was a critical sulphide concentration above which inhibition occurred. In this system, the critical sulphide concentration lay between 0.75 and 1.00 g ℓ^{-1}. This observed decrease could not be correlated to the aqueous sulphide measured in the reactors. For the reactors with a feed concentration of 0.50, 0.75 and 1.00 g ℓ^{-1}, the maximum aqueous sulphide concentration plateaued at approximately 1.16 g ℓ^{-1}, indicating that the inhibition of sulphate conversion observed at a feed sulphide concentration of 1.00 g ℓ^{-1} could not only be attributed to the aqueous sulphide present in the reactor. The undissociated sulphide (H_2S) followed the same trend as the total soluble

sulphide concentration since the reactor pH was constant at 7.8 in all cases. Consequently the inhibition effect could not be accounted for by a changing H_2S concentration over this range. The maximum aqueous sulphide concentration observed using 1.00 and 1.25 g ℓ^{-1} sulphide in the feed was lower than that calculated in previous work that investigated the effect of feed sulphate concentration on the kinetics of sulphate conversion (2.99 g ℓ^{-1} biogenic aqueous sulphide, Moosa, 2000). Work by O'Flaherty et al. (1998) reinforces the values of the added sulphide at which inhibition is observed. Growing a mixed SRB culture at pH between 7.2 and 8.5, they observed the inhibition of sulphide conversion when sulphide in excess of 0.60 g ℓ^{-1} was added to the reactors. Further comparison with literature is not possible since the majority of studies available in the open literature refer to the effects of the formation of sulphide rather than the addition of sulphide. The ratio of sulphate converted to acetate utilised displayed a significant decrease as the feed sulphide concentration was increased above 0.75 g ℓ^{-1}. The theoretical ratio is 1.00, indicating that for every mole of sulphate converted to sulphide one mole of acetate is utilised as the electron donor. A ratio below 1.00 indicates that the metabolic efficiency of acetate for the conversion of sulphate is not 100% efficient with the balance being attributed to either endogenous respiration or maintenance in the absence of methanogenesis (Moosa, 2000). For these experiments no methane was detected, indicating that as the inlet sulphide was increased above 0.75 g ℓ^{-1}, the energy requirement for maintenance or endogenous respiration increased.

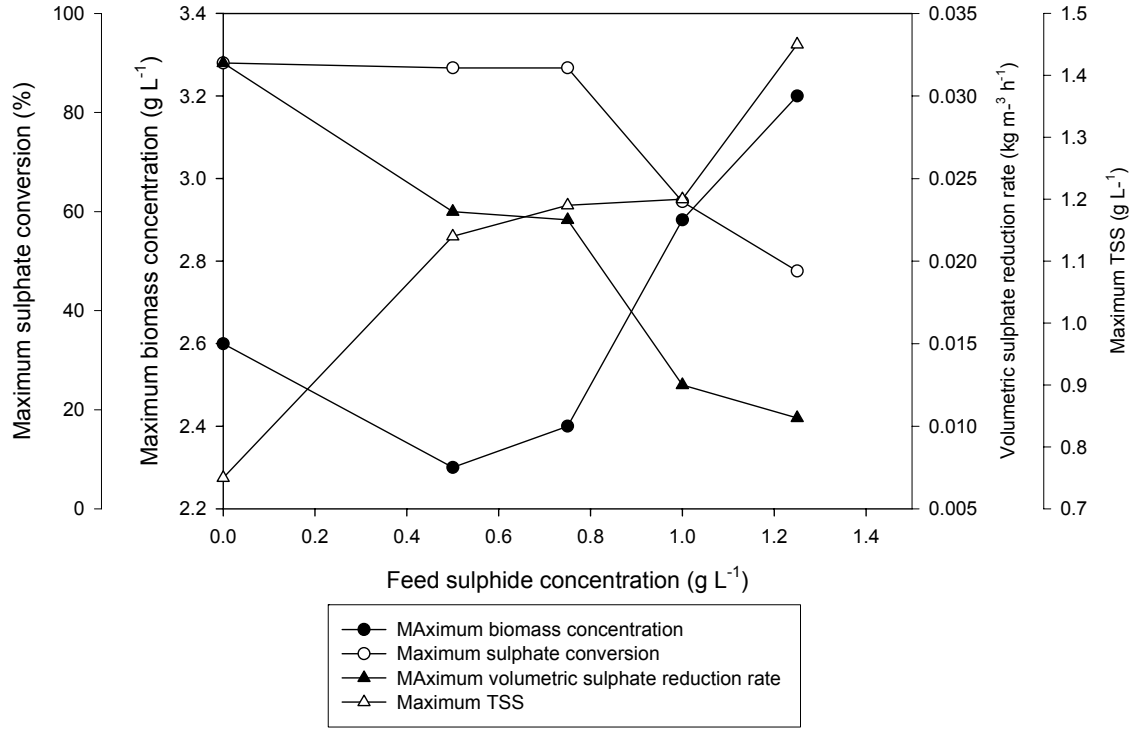

Figure 7.15: Maximum bacterial concentration, sulphate conversion, volumetric reduction rate, aqueous sulphide concentration, H_2S concentration and moles sulphate/moles acetate

Table 7.5: Effect of sulphide concentration in feed on bacterial concentration, sulphate conversion, aqueous sulphide concentration, H_2S concentration and ratio of sulphate reduced to acetate oxidised

Feed Sulphide Concentration g ℓ$^{-1}$	Maximum Bacterial Conc. g ℓ$^{-1}$	Maximum Sulphate Removal %	Maximum VRR of Sulphate kg m^{-3} h^{-1}	Maximum total sol. sulphide conc. g ℓ$^{-1}$	Maximum H_2S Conc. g ℓ$^{-1}$	Moles sulphate per moles acetate
0.00	2.6	90	0.0320	0.75	0.07	0.77
0.50	2.3	89	0.0230	1.14	0.11	0.86
0.75	2.4	89	0.0225	1.19	0.11	0.92
1.00	2.9	62	0.0125	1.20	0.11	0.79
1.25	3.2	48	0.0105	1.45	0.14	0.69

7.2.6.3. Growth parameters

The Chen and Hashimoto model, selected to represent biological sulphate reduction kinetics and given by Equation 7.1, was used to determine the kinetic parameters μ_{max}, K_s and k_d. These parameters are presented in Table 7.6. The maximum specific growth rate, μ_{max} displays a decrease with increasing feed sulphide concentration, the saturation constant K_s

remains constant, and the decay coefficient k_d displays a increase. The values for μ_{max} compare well with those presented by Moosa et al., (2001, 2002) and O'Flaherty et al. (1998). Moosa et al. (2001) presents values ranging between 0.050 and 0.063 h^{-1} with the minimum occurring when sulphate conversion was inhibited by an inlet sulphate concentration of 15.0 g ℓ^{-1}. For *Desulfotomaculum acetoxidans* grown in batch culture stirred tanks, O'Flaherty et al. (1998) found μ_{max} to be 0.063 h^{-1}. The half saturation constant values are of the same order of magnitude to values obtained from literature. The literature values range from 0.024 kg m^{-3} for a continuous culture of *Desulfobacter postgatei* (Ingvorsen et al., 1984) to 0.033 kg m^{-3} for a continuous UASB with a mixed SRB culture (Visser, 1995). The values for k_d remained lower than those for μ_{max} for feed sulphide concentration between 0.00 and 0.75 g ℓ^{-1}, thereafter it was greater than μ_{max}. This indicates that the Chen and Hashimoto model is not appropriate at feed sulphide concentrations greater than 0.75 gL-1 as steady state is not attainable under conditions where the death rate exceeds the specific growth rate, as suggested in Table 7.6.

Table 7.6: Growth constants as a function of feed sulphide concentration

Feed Sulphide g ℓ^{-1}	μ_{max} h^{-1}	K_s kg m^{-3}	k_d h^{-1}
0.00	0.061	0.050	0.035
0.50	0.057	0.054	0.037
0.75	0.055	0.052	0.043
1.00	0.049	0.049	0.064
1.25	0.041	0.051	0.079

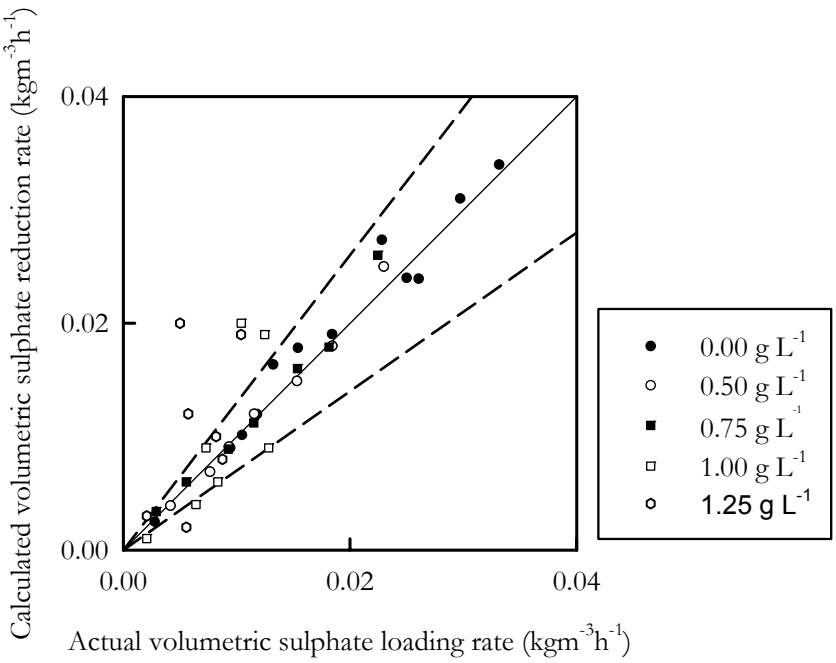

Figure 7.16: Parity chart for sulphide concentration of 0.00, 0.50, 0.75, 1.00 and 1.25 g L^{-1} sulphide

To assess further validity of the model, a parity chart was prepared and is presented as Figure 7.16. The percentage error of 24% was associated with the model developed (Moosa 2000). From the parity chart it is again apparent that the model can be used to represent the volumetric sulphate reduction rates when the feed inlet sulphate in below 1.00 g L^{-1}. For sulphide concentrations of 1.00 g L^{-1} and above, the model fails. The competitive, uncompetitive and non competitive inhibition growth models were tested but failed to represent the data. This is in accordance with the observation of an additional factor impacting kinetics of sulphate reduction indicated by Figure 7.15. This factor remains to be elucidated.

Since endogenous metabolism is accounted for in the specific growth rate expression, the rate of sulphate utilisation can be represented by the following equation where $Y_{x/S}$ is a constant value.

$$\frac{r_S}{[X]} = \frac{1}{Y_{x/s}}\mu$$

The r^2 value for the determination of $Y_{x/S}$ and $Y_{x/acetate}$ ranged between 0.84 and 0.91. The values for $Y_{x/S}$ and $Y_{x/acetate}$ are presented in Table 7.7. $Y_{x/S}$ and $Y_{x/acetate}$ did not vary significantly as a function of inlet sulphide concentration in the range 0.00 to

0.75 g L^{-1}. When the feed sulphide concentration was increased above 0.75 g L^{-1}, there was a significant decrease in both $Y_{x/S}$ and $Y_{x/acetate}$. These values compare well with those previously been observed by Moosa (2000). Values ranging between 0.57 and 0.58 (kg bacteria (kg sulphate)$^{-1}$) and 0.56 and 0.58 (kg bacteria (kg acetate)$^{-1}$) were calculated for feed sulphate concentrations in the range 1.0 to 10.0 kg m^{-3}. When the feed sulphate concentration was further increased to 15.0 kg m^{-3} and inhibition occurred, $Y_{x/S}$ and $Y_{x/acetate}$ decreased to 0.32 (kg bacteria (kg sulphate)$^{-1}$) and 0.33 (kg bacteria (kg acetate)$^{-1}$) respectively.

Table 7.7 Yield coefficients based on sulphate and acetate as a function of feed sulphide concentration

Feed Sulphide gL^{-1}	$Y_{x/S}$ (kg bacteria (kg sulphate)$^{-1}$)	$Y_{x/acetate}$ (kg bacteria (kg acetate)$^{-1}$)
0.00	0.57	0.56
0.50	0.57	0.56
0.75	0.56	0.55
1.00	0.51	0.49
1.25	0.46	0.41

7.3. KINETIC ANALYSIS ON USE OF ETHANOL AS CARBON SOURCE AND ELECTRON DONOR AND A BROAD RANGE OF SULPHATE CONCENTRATIONS

7.3.1. Introduction

Ethanol has been used industrially by Paques Bioprocesses (The Netherlands) as carbon source and electron donor for the treatment of AMD. Despite this, there is a lack of rigorous kinetic data for microbial growth in the sulphidogenic process using ethanol as carbon source and electron donor in the literature. Limited pure culture studies have been conducted on ethanol-oxidising species, leading to the publication of postulated degradation mechanisms (Schink et al., 1985), microbial growth rates (Szewzyk and Pfennig, 1990) and microbial yields on ethanol (Laanbroek et al., 1984, 1982). There are several mixed culture studies involving ethanol as an organic intermediate by Colleran and co-workers (O' Flaherty and Colleran, 1999; O'Flaherty et al., 1998 a, b; Colleran et al., 1995, 1994). These studies are focussed on characterising and optimising industrial process performance rather than kinetic analysis. Saturation constants for sulphate and kinetic parameters for ethanol utilising

species in mixed cultures are not widely available in the literature (Moosa, 2000; O'Flaherty et al., 1998b). The constants that are available have been presented in WRC report K5/1080 (Hansford et al., 2004).

The objectives of this study were to determine the kinetics of anaerobic sulphate reduction using ethanol as the carbon and electron donor source. Further, the effect of substrate inhibition by sulphate was studied by extending the range of sulphate concentrations studied to 15 kg m^{-3}.

7.3.2. ***Materials and methods***

7.3.2.1. Micro-organisms, medium and bioreactor operation

The microbial culture, growth medium, reactor configuration and operation used were in accordance with those described in Sections 7.2.4.1 to 7.2.4.4.

7.3.2.2. Sulphate and ethanol concentrations used

The growth medium used was as outlined earlier (Section 7.2.4.2), with ethanol and sulphate added as detailed in Table 7.8. Based on the ratio of sulphate to ethanol added, an excess of the carbon source and electron donor was maintained.

Table 7.8: Sulphate and ethanol concentration used

SO_4^{2-} concentration kg m^{-3}	Ethanol kg m^{-3}
1.0	1.9
2.5	4.9
5.0	9.8
10.0	19.6
12.5	24.5
15.0	29.4

7.3.2.3. Analytical methods

Analytical methods used are outlined in Section 7.2.4.7.

7.3.3. ***Results***

For the feed medium containing 1.0 kg m^{-3} sulphate (Figure 7.17), the average bacterial concentration of 0.48 g ℓ^{-1} was observed over the dilution rate range 0.0041 to 0.0208 h^{-1} (volumetric sulphate loading rate range of 0.0041 to 0.0208 kg m^{-3} h^{-1}). Increasing dilution rates in the range 0.042 to 0.083 h^{-1} resulted in a decrease in bacterial concentration. At a dilution rate of 0.083 h^{-1} (volumetric sulphate loading rate of 0.083 kg m^{-3} h^{-1}), the bacterial concentration was 18% that of the average observed over the dilution rate range 0.004 to 0.021 h^{-1}. As can be seen in Figure 7.17, 85 to 90% conversion of sulphate was observed at volumetric sulphate loading rates in the range 0.004 to 0.021 kg m^{-3} h^{-1}. The corresponding sulphate reduction rate increased across this range from 0.004 to 0.018 kg m^{-3} h^{-1} (corresponding dilution rates in the range 0.004 to 0.021 h^{-1}). The volumetric sulphate reduction displayed a consistent increase from 0.004 to 0.025 kg m^{-3} h^{-1} as the volumetric loading rate was increased from 0.004 to 0.042 kg m^{-3} h^{-1}. The corresponding conversion of sulphate varied between 90% (volumetric sulphate loading rate of 0.004 kg m^{-3} h^{-1}) and 60% (volumetric sulphate loading rate of 0.042 kg m^{-3} h^{-1}). Further increase in sulphate loading rate (0.083 kg m^{-3} h^{-1}) led to a sharp decrease in reduction rate of sulphate. The trend of ethanol utilisation mimicked that of sulphate conversion. At low volumetric sulphate loading rates, corresponding to high conversion sulphate, ethanol utilisation was high. The decrease in the conversion of sulphate at higher volumetric sulphate loadings led to a decrease in ethanol utilisation. The ratio of sulphate converted to ethanol utilised was 0.213 ± 0.014 moles sulphate moles $ethanol^{-1}$. The formation of acetate mirrored the ethanol utilisation profile. The ratio of ethanol utilised to acetate formed was 1.09 ± 0.005 moles ethanol moles $acetate^{-1}$, indicating predominant incomplete oxidation of ethanol.

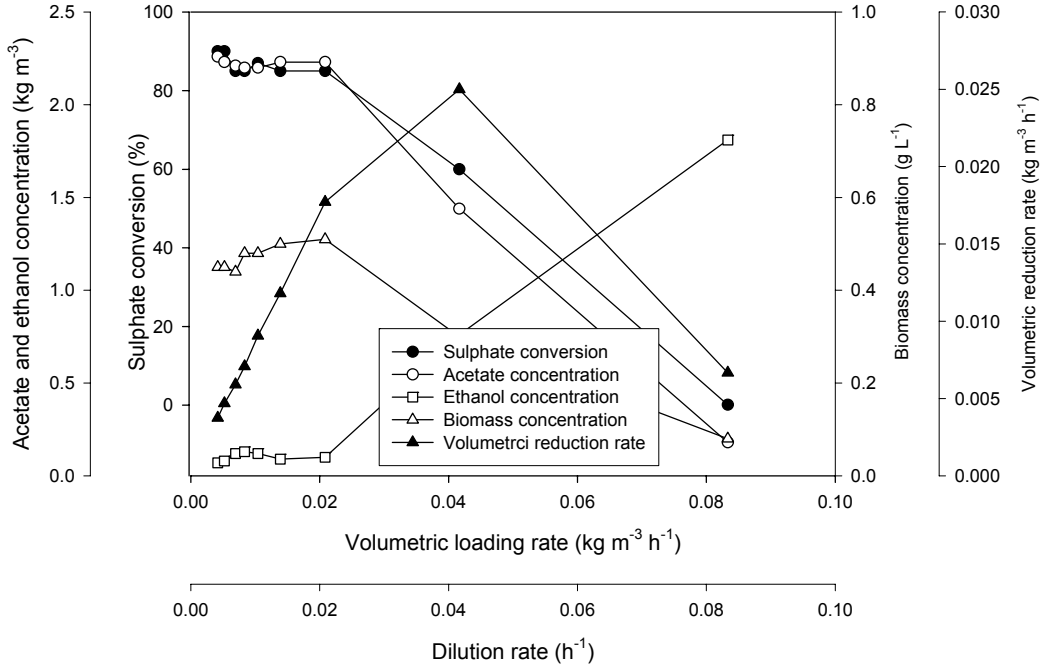

Figure 7.17: Stable state kinetics for feed sulphate concentration of 1.0 kg m^{-3}

The data collected with a feed medium containing 2.5 kg m^{-3} sulphate are presented in Figure 7.18. The bacterial concentration remained constant at 0.383 ± 0.008 g ℓ^{-1} over the range of dilution rates 0.004 to 0.021 h^{-1}. Increasing the dilution rate to 0.042 h^{-1} resulted in a marked decrease in bacterial concentration to 0.14 g ℓ^{-1}. Sulphate conversion of 93 ± 2% was observed for volumetric sulphate loading rates in the range 0.010 to 0.052 kg m^{-3} h^{-1} (dilution rates of 0.004 to 0.021 h^{-1}). The volumetric sulphate reduction rate increased from 0.010 to 0.047 kg m^{-3} h^{-1} with increasing volumetric sulphate loading rates in this range. The maximum reduction rate of 0.047 was observed at a volumetric sulphate loading rate of 0.052 kg m^{-3} h^{-1} (dilution rate of 0.021 h^{-1} and retention time of 48 h). The conversion of sulphate at this retention time was 90%. Applying higher volumetric sulphate loading rates led to a substantial decrease in the reduction rate of sulphate. The dependency of ethanol utilisation and sulphate conversion was similar to the previous run with a constant ratio of 0.212 ± 0.034 moles sulphate moles $ethanol^{-1}$. The ratio of ethanol utilised to acetate formed was 1.08 ± 0.07 moles ethanol moles $acetate^{-1}$.

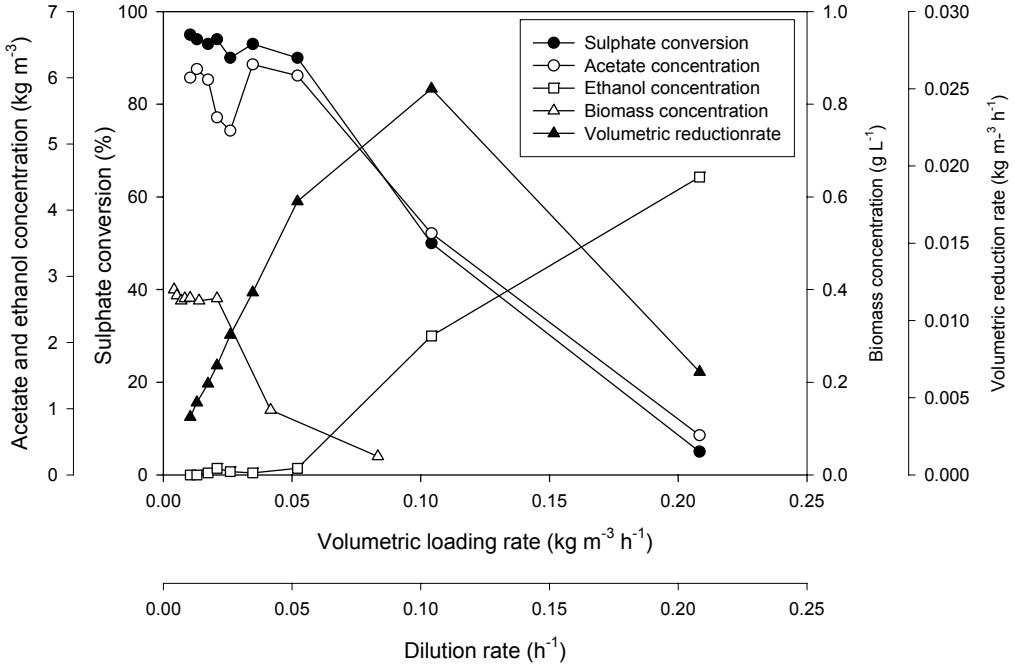

Figure 7.18: Stable state kinetics for feed sulphate concentration of 2.5 kg m^{-3}

For a feed medium containing 5.0 kg m^{-3} sulphate, shown in Figure 7.19, the bacterial concentration remained constant at 0.554 ± 0.022 g ℓ^{-1} as the dilution rate was increased from 0.004 to 0.021 h^{-1} (volumetric sulphate loading rate of 0.021 to 0.104 kg m^{-3} h^{-1}). Increasing the dilution rate to 0.042 h^{-1} (volumetric sulphate loading rate of 0.208 kg m^{-3} h^{-1}) resulted in a decrease in bacterial concentration to 0.260 g ℓ^{-1} indicating the onset of washout. Further increasing the dilution rate to 0.083 h^{-1} (volumetric sulphate loading rate of 0.417 kg m^{-3} h^{-1}) led to complete washout, with bacterial concentrations reaching 0.070 g ℓ^{-1}. Applying loading rates from 0.021 to 0.104 kg m^{-3} h^{-1} (dilution rates between 0.004 and 0.021 h^{-1}) the conversion of sulphate varied from 95 to 87%. Over this range of volumetric sulphate loading rates a linear increase in reduction rate of sulphate from 0.0208 to 0.091 kg m^{-3} h^{-1} was observed. Further increase of volumetric sulphate loading rate up to 0.208 kg m^{-3} h^{-1} (dilution rate of 0.042 h^{-1}) led to a decrease in conversion of sulphate to 43% and the reduction rate to 0.090 kg m^{-3} h^{-1}. The maximum volumetric reduction rate of sulphate was 0.091 kg m^{-3} h^{-1} achieved at a volumetric loading rate of 0.104 kg m^{-3} h^{-1} (dilution rate of 0.021 h^{-1}), the corresponding conversion of sulphate was 87%. The ratio of sulphate conversion to ethanol utilised was 0.231 ± 0.011 moles sulphate moles $ethanol^{-1}$. The acetate formation mimicked that of ethanol utilisation. The ratio of ethanol utilised to acetate formed was 1.044 ± 0.042 moles ethanol moles $acetate^{-1}$.

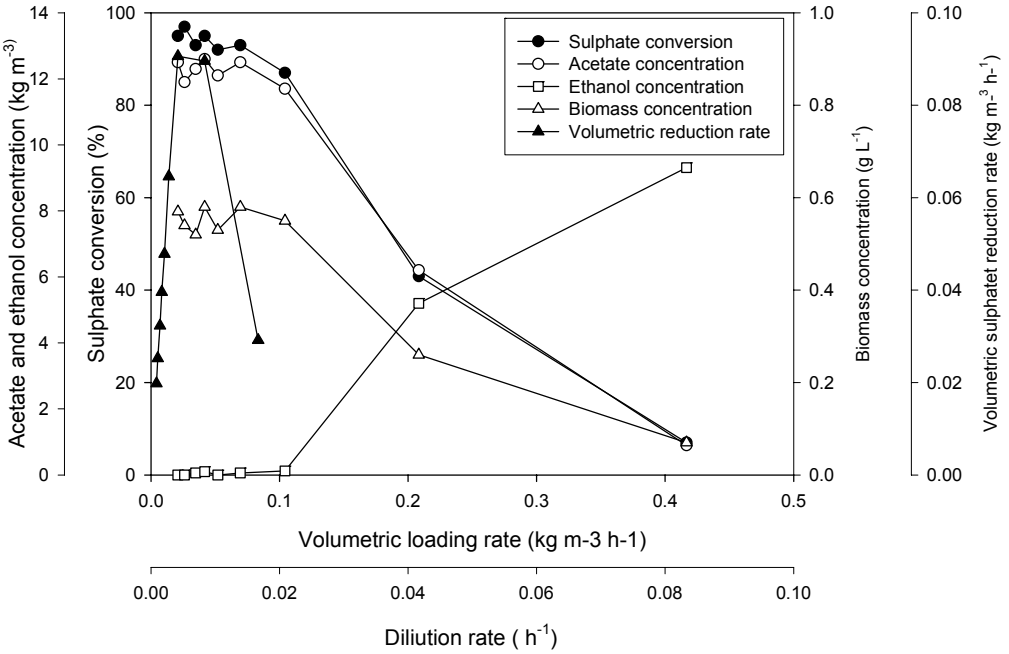

Figure 7.19: Stable state kinetics for feed sulphate concentration of 5.0 kg m^{-3}

With the feed medium containing 10.0 kg m^{-3} sulphate, illustrated in Figure 7.20, the bacterial concentration remained relatively constant at 0.696 ± 0.011 g ℓ^{-1} for dilution rates in the range 0.004 to 0.021 h^{-1} (volumetric sulphate loading rates between 0.042 to 0.208 kg m^{-3} h^{-1}). Further increase of dilution rate from 0.0208 to 0.010 h^{-1} (volumetric sulphate loading rate of 0.104 kg m^{-3} h^{-1}) led to a decrease in bacterial concentration 0.69 to 0.15 g ℓ^{-1}. For the volumetric sulphate loading rate range 0.042 to 0.208 kg m^{-3} h^{-1} (dilution rate range of 0.004 0.021 h^{-1}), a relatively constant and high conversion of sulphate (81 ± 2%) was observed. The increase in volumetric sulphate loading rate in this range, however, led to a noticeable enhancement of sulphate reduction rate. The maximum reduction rate achieved was 0.171 kg m^{-3} h^{-1} at a volumetric sulphate loading of 0.208 kg m^{-3} h^{-1} (dilution rate of 0.0201 h^{-1}). The corresponding sulphate conversion was 82%. Increasing the volumetric sulphate loading rate beyond 0.208 kg m^{-3} h^{-1} resulted in a decrease in sulphate conversion to 20% and a reduction in the volumetric sulphate conversion to 0.083 kg m^{-3} h^{-1}. The coupling of sulphate conversion and ethanol utilisation was observed. Across the range of volumetric sulphate loading rates used, the ratio of sulphate reduced to ethanol utilised was 0.196 ± 0.067 moles sulphate per mole ethanol. The ratio of ethanol utilised to acetate formed was 1.571 ± 0.246 moles ethanol per mole acetate.

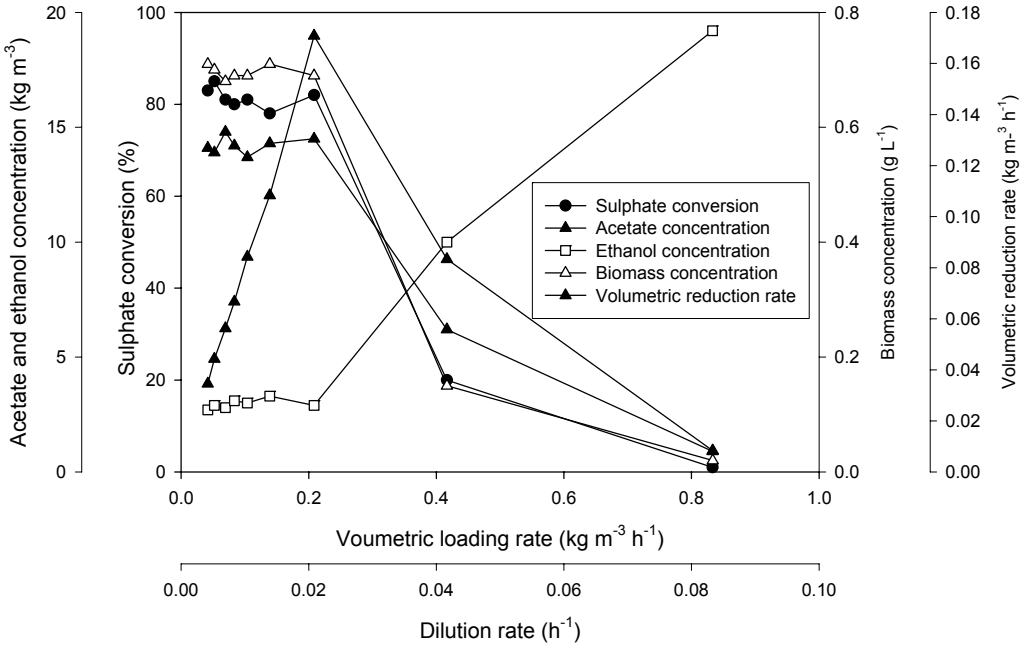

Figure 7.20: Stable state kinetics for feed sulphate concentration of 10.0 kg m^{-3}

When the feed medium contained 12.5 kg m^{-3} sulphate (Figure 7.21), the bacterial concentration remained relatively constant at 0.802 ± 0.04 g ℓ^{-1} for dilution rates over the range 0.004 to 0.010 h^{-1} (volumetric sulphate loading rate range 0.052 to 0.130 kg m^{-3} h^{-1}). Further increase in dilution rate up to 0.083 h^{-1} (volumetric sulphate loading rate of 1.041 kg m^{-3} h^{-1}) led to a steady decrease in bacterial concentration. The conversion of sulphate was 58.4 ± 4.1% for the range of loading rates between 0.052 to 0.130 kg m^{-3} h^{-1} (dilution rates of between 0.004 to 0.010 h^{-1}). For loading rates above 0.1300 kg m^{-3} h^{-1} the conversion of sulphate displayed a descending trend with increasing loading rate. For volumetric sulphate rates in the range 0.052 to 0.260 kg m^{-3} h^{-1} (dilution rates between 0.004 to 0.0211 h^{-1}), the volumetric reduction rate of sulphate displayed an increasing trend with the maximum of 0.108 kg m^{-3} h^{-1} being observed at a loading rate of 0.260 kg m^{-3} h^{-1} (dilution rate of 0.0211 h^{-1}). When the loading rate was increased above 0.260 kg m^{-3} h^{-1}, the reduction rate and sulphate conversion decreased. The ratio of sulphate converted to ethanol utilised was 0.195 moles sulphate moles $ethanol^{-1}$ and the ratio of ethanol utilised to acetate formed was 1.53 moles ethanol moles $acetate^{-1}$.

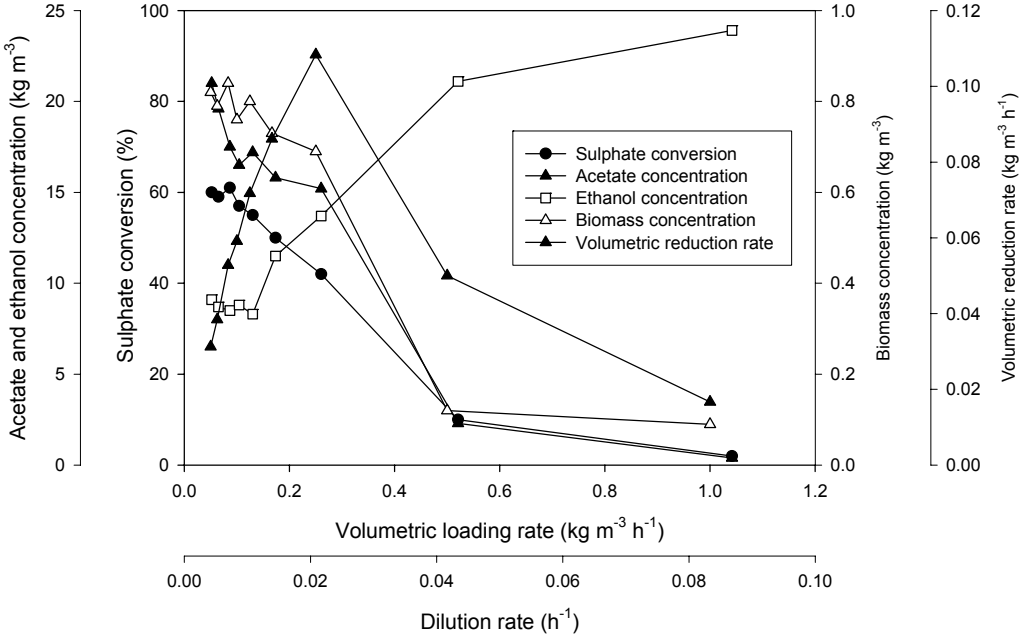

Figure 7.21: Stable state kinetics for feed sulphate concentration of 12.5 kg m^{-3}

With 15.0 kg m^{-3} sulphate in the feed, the bacterial concentration and sulphate conversion displayed a rapid decrease at a retention time of 10 days and did not reach stable state. This indicated washout and reactor failure.

7.3.4. ***Discussion – ethanol as carbon source and electron donor***

A similar trend in sulphate conversion with respect to volumetric sulphate loading rate was observed for the five feed sulphate concentrations. Sulphate conversion remained relatively constant, and in the range 80 to 95%, for the lower range of volumetric sulphate loadings. As the sulphate loading was increased, the sulphate conversion decreased. The volumetric sulphate loading rate at which this decline occurred was unique for each feed sulphate concentration (Table 7.9). The dilution rate corresponding to the decline in sulphate conversion was 0.0208 h^{-1} for feed sulphate concentrations of 1.0, 2.5, 5.0 and 10.0 kg m^{-3} and 0.010 h^{-1} when the feed sulphate concentration was 12.5 kg m^{-3}. Similar trends were observed by Moosa (2002) studying the effect of feed sulphate concentration in the range 1.0 to 15.0 kg m^{-3}. Stucki et al. (1992), studying the degradation of sulphuric acid by SRBs with acetate as the organic source in a continuous packed bed reactor, observed a similar trend.

As the inlet sulphate concentration was increased from 1.0 to 10.0 kg m^{-3}, the maximum percentage sulphate conversion observed varied between 81 and 93%. Work by Grady et

al. (1972) on *Aerobacter aerogenes* in a continuous system degrading glucose (COD ranging from 0.5 to 1.5 kg m^{-3}) showed that the maximum percentage COD removal remained relatively constant at 99% regardless of feed COD concentration. Further work by Grady and Williams (1975) using a mixed culture in a continuous system degrading a mixed organic feed with COD ranging from 0.5 to 2.0 kg m^{-3} showed that the maximum percentage conversion of COD was constant at 98% regardless of the feed COD. Stucki et al. (1992) using a continuous packed bed reactor observed a maximum sulphate conversions of 98%, at a dilution rate of 0.021 h^{-1}, for initial sulphate concentration of 0.5 and 8.0 kg m^{-3}.

Table 7.9: Maximum sulphate conversion and corresponding volumetric sulphate loading rates as a function of feed sulphate concentration

Sulphate Concentration (kg m^{-3})	Maximum Sulphate Conversion (%)	Corresponding Volumetric Sulphate Loading Rate (kg m^{-3} h^{-1})	Corresponding Dilution Rate (h^{-1})
1.0	86 ± 3	0.004 to 0.021	0.004 to 0.021
2.5	93 ± 2	0.010 to 0.052	0.004 to 0.021
5.0	93 ± 5	0.021 to 0.104	0.004 to 0.021
10.0	81 ± 2	0.042 to 0.210	0.004 to 0.021
12.5	58 ± 4	0.052 to 0.130	0.004 to 0.021

For all the feed sulphate concentration studies, volumetric sulphate reduction rate was an increasing linear function of volumetric sulphate loading rates below washout. As the volumetric sulphate loading rate was increased for all the experiments washout was approached. The onset of washout was gradual, due to the diversity of the SRB population. Where washout was significant, the linear increasing relationship between volumetric sulphate loading and reduction rate ceased.

For the case of all five feed sulphate concentrations, the ethanol utilisation profile followed a similar trend to that of sulphate conversion, indicating a direct relationship between ethanol utilised and sulphate converted. In all cases, the ratio of sulphate reduced to ethanol utilised was calculated to determine whether the sulphate concentration had any effect on the stoichiometry of the reaction and to ascertain the metabolic efficiency of ethanol (Table 7.10). The ratio varied between 0.195 and 0.233 indicating that the feed sulphate concentration did not have a significant effect on the stoichiometry of the sulphate reduction reaction nor on the metabolic efficiency. This observation is reinforced by the published literature. Characklis et al. (1989) states that the stoichiometry of the biological reaction

should not be affected by the feed substrate concentration. The ratio observed lay significantly below the theoretical ratio of 0.5 indicating that for every 1 mole of ethanol utilised, between 0.38 and 0.46 moles goes to the provision of electrons for the sulphate of reduction and since no active methanogens were present, the balance went elsewhere, e.g. cell growth, maintenance. The average metabolic efficiency of acetate was 44% for the microbial reduction of sulphate by the mixed SRBs population employed for this work.

The ratio of ethanol utilised to acetate formed was calculated to determine the presence of complete or incomplete oxidisers. In the presence of complete oxidisers, the presence of acetate is minimised indicated by the ratio of ethanol utilised to acetate formed above 1. Table 7.10 presents the data for the 5 sulphate concentrations. At sulphate concentrations below 10.0 kg m^{-3}, the ratio of ethanol utilised to acetate formed is relatively constant at 1. At 10.0 kg m^{-3} and above, the ratio is greater than 1, indicating that all the ethanol reduced is not converted to acetate. This suggests either the presence of complete oxidisers converting the ethanol to via acetate to the bicarbonate ion or the presence of methanogens; however, methane production was not observed.

Table 7.10: Dependency of molar ratio of sulphate utilised to ethanol utilised as well as ethanol utilised to acetate formed on feed sulphate concentration

Feed Sulphate Concentration (kg m^{-3})	Moles sulphate used per moles ethanol used	Moles ethanol used per mole acetate formed
1.0	0.213 ± 0.014	1.090 ± 0.005
2.5	0.212 ± 0.034	1.080 ± 0.070
5.0	0.233 ± 0.011	1.044 ± 0.042
10.0	0.196 ± 0.067	1.571 ± 0.246
12.5	0.195 ± 0.047	1.53
Theoretical	0.50	

Table 7.11: Growth constants as a function of feed sulphate concentration using ethanol as carbon source

Feed sulphate concentration (kg m^{-3})	μ_m (h^{-1})	K_s (kg m^{-3})	k_d (h^{-1})
1.0	0.058	0.069	0.021
2.5	0.061	0.093	0.025
5.0	0.059	0.124	0.027
10.0	0.063	0.175	0.031
12.5	0.057	0.213	0.030

Table 7.11 presents the kinetics constants for growth of sulphate reducing bacteria as a function of feed sulphate concentration calculated using the model proposed by Moosa et al. (2002). Using a t-test it was shown that μ_m and k_d does not vary significantly as a function of feed inlet concentration across the range 1.0 to 10.0 kg m^{-3} at the 95% confidence interval. The constant value for μ_m is supported by the constant range of dilution rates at which the maximum bacterial concentration occurs (0.00417 to 0.0208 h^{-1}). These results are consistent with previous work presented by Moosa et al. (2001). A constant μ_m with increasing feed substrate was also observed by Grady et al. (1972), Grady and Williams (1975) and Morris (1976). Grady et al. (1972) observed a constant μ_m of 0.512 h^{-1} with increasing feed COD concentrations from 0.50 to 1.50 kg m^{-3} for a pure batch culture (*Aerobacter aerogenes*) aerobically degrading glucose. When using a mixed culture to degrade glucose in batch, Grady et al. (1972) found a constant μ_m of 0.455 h^{-1} as the feed COD concentration was increased in the range 0.5 to 2.0 kg m^{-3}. Studying the degradation of a mixed feed substrate (glucose, fructose, sorbital and lysine) in batch, Grady and Williams (1975) found that μ_m remained constant at 0.274 h^{-1} as the feed COD was increased from 0.50 to 2.00 kg m^{-3}. A constant μ_m of 0.467 h^{-1} was observed by Morris (1976) studying the anaerobic degradation of dairy manure with increasing feed volatile solids concentration in the range 0.035 to 0.088 kg m^{-3}. The constant μ_m with increasing feed sulphate concentration in the range 1.0 to 12.5 kg m^{-3} indicated that increasing the feed sulphate in this range has no significant effect on the maximum specific growth rate of the microbial population. This is consistent with Bailey and Ollis (1986) who report that μ_m is not expected to change with increasing substrate concentrations to a certain maximum value after which the system will react unfavourably due to substrate inhibition. The average μ_m value obtained for this work (0.060 h^{-1}) is similar to the value obtained for a pure culture of *Desulfotomaculum acetoxidans* (Widdel & Pfenning, 1986; O' Flaherty et al. 1998).

From Table 7.11, it is evident that the K_s term is an increasing function of the inlet sulphate concentration. The higher K_s values observed at the higher feed sulphate concentrations indicate that the affinity of the microbes for sulphate is decreased as the feed sulphate concentration is increased. From the definition of K_s, the residual sulphate concentration at which the specific microbial growth rate is half the maximum specific growth rate is an increasing function of feed sulphate concentration. This is in agreement with Chen & Hashimoto (1980). Studying the fermentation of methane using livestock waste they found that as the COD was increased from 0.50 to 1.50 kg m^{-3}, K_S was constant while above a COD of 1.50 kg m^{-3} K_S increased with increasing COD. They attribute the increase in K_S to the system becoming saturated with substrate.

7.3.5. ***Discussion – sulphate inhibition***

The data presented considers the kinetic performance of the mixed bacterial population of sulphate reducers over the inlet sulphate concentration range from 1 to 15 kg m^{-3}. The reactor failed at a 10 day residence time on receiving a sulphate feed concentration of 15 kg m^{-3}. Further, observing the dilution rate corresponding to the onset of washout and the maximum sulphate conversion obtained (Table 7.9), it is clearly seen that reactor performance was compromised at a sulphate feed concentration of 12.5 kg m^{-3} over the performance observed at feed concentrations of 1 to 10 kg m^{-3}. When the inlet sulphate concentration was increased to 12.5 kg m^{-3}, the maximum sulphate conversion decreased from the range 81 to 95% to 58%. This is further confirmed on observation of the maximum sulphate reduction rates observed in Table 7.12 which illustrates a maximum at a feed concentration of 10 kg m^{-3}. Similar trends were reported by Moosa (2000) on studying the effect of sulphate feed concentration in the range 1.0 to 15 kg m^{-3} in an acetate system. Further, Stucki et al. (1992) reported a decrease in sulphate conversion from 98% at a feed sulphate concentration of 8 kg m^{-3} and volumetric loading rates of 0.330 kg m^{-3} h^{-1} and below to 74% on increasing the volumetric sulphate loading rate to 0.670 kg m^{-3} h^{-1}. A similar decrease occurred on increasing the feed concentration to 160 kg m^{-3}. During the continuous reduction of sulphate with in a UASB treating a VFA mixture, Omil et al. (1998) observed a decrease in sulphate conversion form 70.4 to 60.7% as the sulphate concentration in the feed was increased from 2.9 to 3.5 kg m^{-3}. They proposed sulphide, resulting from the conversion of sulphate, to be responsible for the decrease in sulphate conversion at the higher feed sulphate concentrations.

Table 7.12: Effect of sulphate feed concentration on maximum volumetric sulphate reduction rate observed

Sulphate concentration (kg m^{-3})	Maximum volumetric sulphate reduction rate (kg m^{-3} h^{-1})
1.0	0.025
2.5	0.047
5.0	0.100
10.0	0.171
12.5	0.108
15.0	no stable state

7.4. CONCLUSIONS

From the study presented, the data collected across the pH range 6.0 to 7.5 supports that undissociated H_2S is the dominant species in the product-mediated inhibition of bacterial sulphate reduction. This indicates the importance of operating the biological sulphate reduction process at a sufficiently high pH to minimise the presence of undissociated H_2S. While the sulphide concentration was shown to affect the sulphate reduction performance, the kinetic model developed was only able to predict these findings for sulphide additions in the feed stream of 1 kg m^{-3} and less. Further, the performance at higher sulphide concentrations was not modelled adequately by the incorporation of inhibition terms of the form used for competitive, non-competitive or un-competitive inhibition, suggesting the role of an as yet unidentified factor in performance of sulphate reduction under high sulphide concentrations.

The kinetic model, utilising the Contois form of equation, previously developed to describe bacterial sulphate reduction using acetate as carbon source and electron donor is appropriate for its description where the electron donor is ethanol. The maximum specific growth rate of 0.060 h^{-1} obtained is approximately similar in both cases. At sulphate feed concentrations below 10 kg m^{-3}, incomplete oxidation of the ethanol predominated. At sulphate concentrations of 10 kg m^{-3} and higher, the presence of complete oxidation of ethanol became evident.

On increase of the feed sulphate concentration above 10 kg m^{-3}, the onset of substrate inhibition owing to sulphate was evident. This was illustrated by a decrease in the maximum sulphate reduction rate as well as the onset of washout of the bacterial population at a lower

dilution rate. At a sulphate feed concentration of 15 kg m^{-3}, the continuous reactor system failed, further confirming substrate inhibition. This is in accordance with similar results collected with acetate as the carbon source and electron donor (Moosa, 2000). It confirms the need to utilise recycle on treating high sulphate AMD streams to ensure an appropriately low sulphate feed concentration for efficient biological sulphate reduction.

8 MICROBIAL SPECIATION

8.1. ANALYTICAL APPROACHES TO MICROBIAL POPULATION STRUCTURE – A LITERATURE REVIEW

8.1.1. Introduction

The SRB are a diverse group of anaerobic bacteria that have the ability to use sulphate as terminal electron acceptor in the consumption of organic matter, with the concomitant production of H_2S. They are ubiquitous in the environment and have pivotal roles in the biogeochemical cycling of carbon and sulphur. Sulphate reduction could be responsible for up to 50% of organic matter degradation in high-sulphate environments such as estuarine and marine sediments (Jorgensen, 1982); however, active sulphate reduction has also been reported in low-sulphate environments such as soils and freshwater sediments (Postgate, 1984; Jones and Simon, 1984; Bak and Pfenning, 1991a, b). This group of bacteria are the key active agents in biological sulphate reduction, hence form the focus in the analysis of microbial populations contributing to active sulphate reduction processes in effluent treatment and AMD remediation. The detection of sulphate reducers and sulphate-reducing activity in sediments, wastewater treatment plants, and fouling biofilms is thus of practical and scientific relevance.

Conventional microbial techniques based on selective culturing are of limited usefulness for quantification and characterisation of environmental populations, as many strains do not grow in the laboratory environment, owing to cultivation media poorly resembling natural growth conditions or the interdependence of different strains of micro-organisms (Brock, 1987; Wagner et al., 1993). Techniques based on the analysis of bacterial DNA and RNA may complement the conventional microbiological approach and can be used to determine the presence and distribution of individual bacterial species, including SRB, in complex bacterial communities (Amann et al., 1992; Ramsing et al., 1993; Raskin et al., 1996).

The microbial world is subject to physicochemical constraints that differ from those met by larger organisms. Here, the relevant spatial scale is micrometers, viscous forces predominate, and diffusion rather than advection is the relevant transport mechanism for the solute exchange between bacteria and their biotic and abiotic surroundings (Dusenbery, 1996; Koch, 1996; Jorgensen, 1995, 1998). Many microbes stick to each other or to surfaces

rather than existing planktonically. Microbial biofilms, microcolonies and aggregates are hot spots of activity in which microbes can influence their environment. Typically, steep gradients of physicochemical parameters are present in such microbial communities over distances ranging from a few micrometers up to some tenths of a millimetre. Microbial life is thus a life in constantly changing gradients, which are affected by changes in environmental variables, their effect on microbial activity and *vice versa*. Tools and techniques to directly monitor the microenvironment and activity of microbes in their natural habitats have become available and are largely based on the use of microsensors (Revsbech and Jorgensen, 1986; Klimant et al., 1997; Kuhl and Revsbech, 1998). These tools have potential application in bioprocesses for effluent treatment such as sulphate reduction systems.

Studies relating population analysis to community function are scarce, partially because of difficulties in monitoring microbial activities. Within biofilms, the convection of compounds is hindered, and consequently mass transfer to the cells often limits conversion rates. Because of this resistance to mass transfer, biofilms develop various microenvironments, which differ from the bulk liquid (Bungay et al, 1969; de Beer et al., 1994; Kuhl and Jorgensen, 1992). This complicates the interpretation of community function analysis, because extrapolation of community behaviour to that of individual cells is impossible without knowledge of their microenvironment.

The efficiency and robustness of a wastewater treatment plant depends on the composition and activity of the microbial communities present. Although biological wastewater treatment has been used intentionally for more than a century, absence of detailed knowledge of the microbiology of this process led to the microbial communities being considered as a “black box”. Progress in the design and control of wastewater treatment plants was derived mainly from empirical research. Following the introduction of molecular techniques in microbial ecology, the composition and dynamics of microbial communities in these systems can be determined and key microbial players identified. Through extending our knowledge of the microbiology of the sulphate reducing bacterial populations in this manner, we plan to improve approaches to both process control and process robustness of the active sulphate reduction system. In this project, we seek to exploit molecular methods to complement traditional microbial methods to study the population structure in active sulphate reduction reactors, recognising that these populations include a mixture of planktonic and aggregated biomass. While the relation of biological function to microbial diversity at a micro-scale is not a constituent part of this study, it will be relevant to the extension of this research.

*8.1.2. **Traditional approaches to microbial culture analysis***

Traditionally, two approaches to investigate the micro-organisms in wastewater treatment plants were applied: light microscopy and microbial culture. Samples were analysed by standard light microscopy to assess the abundance of floc forming and filamentous bacteria. Due to the importance of the latter organisms for sludge bulking and foaming, key indicators were developed for a provisional identification of filamentous bacteria using their reaction to Gram- and Neisser-staining and morphological characteristics (Eikelboom, 1975; Jenkins et al., 1993). Recent molecular approaches revealed that micro-organisms affiliated with different bacterial domains are identified as the same filament type using these keys. Furthermore, polymorphism has been described (Wagner et al., 1993).

The second approach is based on cultivation and isolation of bacteria from wastewater treatment plants. The number of active bacteria is estimated by most probable number (MPN) techniques or total plate counts. After isolation, bacteria can be identified using either physiological parameters or chemotaxonomic markers such as cellular fatty acids or respiratory quinone profiles. The latter two biomarkers can be used directly for profiling activated sludge microbial communities (Liu et al., 2000) but provide relatively low resolution information. Numerous bacteria were isolated and identified from wastewater treatment plants by using cultivation-based techniques. From culture-based methods, pseudomonads, enteriobacteriaceae, and acinobacters were determined as key components of the microbial flora of these systems. After the introduction of molecular techniques for community analyses, it was realised that less than 20% of the micro-organisms can be isolated from activated sludge by standard cultivation and that those bacteria forming colonies on the respective media are generally of minor numerical importance *in situ* (Wagner et al., 1993, 1994; Manz et al., 1994; Kampfer et al., 1996). Therefore, cultivation offers a biased and incomplete view on the bacterial diversity in wastewater.

The application of molecular biological methods to investigate the occurrence and distribution of bacteria in the environment has the advantage of providing direct information on community structure. 16S rRNA-targeted oligonucleotide probes have been designed to detect groups of SRB (Devereux et al., 1992) and used successfully to demonstrate the presence of SRB in such diverse habitats as anaerobic biofilms (Kane et al., 1993; Raskin et al., 1996), marine, estuarine and freshwater sediments (Sahm et al., 1999; Devereux et al., 1996b; Purdy et al., 1997; Trimmer et al., 1997), activated sludge flocs (Manz et al., 1998) and salt marshes (Devereux et al., 1996a; Rooney-Varga et al., 1997). In this report, we review methodology available to select an appropriate approach to analysis of the SRB

population in the active sulphate reduction process. The selected methods are applied to our SRB-based effluent treatment system.

8.1.3. ***Microbial diversity in sulphate reduction environments***

SRB are a complex physiological bacterial group, and various properties have been used in traditional classification schemes. The most important of these properties were cell shape, motility, GC content of DNA, presence of desulfovirin and cytochromes, optimal temperature, and complete versus incomplete oxidation of acetate, some of which are detailed in Table 8.1. For classification within a particular genus, different electron donors are tested. SRB species may be organised into four distinct groups: the Gram-negative mesophilic SRB, Gram-positive spore forming SRB, thermophilic bacterial SRB and thermophilic archaeal SRB. All of these groups are characterised by their use of sulphate as a terminal electron acceptor during anaerobic respiration. Assignment of individual species into appropriate groups based on rRNA analysis is in general agreement with those obtained by traditional taxonomy, although some exceptions exist.

Phylogenetic classification of SRB by rRNA sequence analysis has a variety of advantages, including providing insights into the evolutionary origins of sulphate reduction in distantly related species, and in facilitation of development of group-specific phylogenetic probes and PCR primers for use in ecological studies. Phylogenetic analysis based on 16S rRNA sequence comparisons has classified the major SRB genera into a number of distinct lineages (Devereux et al., 1989). While sections of the phylogenetic tree describing the SRB have been published, a comprehensive tree is not yet available.

Table 8.1: Morphological characterisation of key species of SRB (Postgate, 1994)

Species	Form	Flagella	Motility	Spores
Desulfovibrio	vibrio	single polar/lophotrichous	+	-
Desulfobacter	ellipsoidal rod	-	-	-
Desulfobulbus	lemon/onion shape	-	-	-
Desulfococcus	spheres	-	-	-
Desulfotomaculum	rod/fat vibrio	peritrichous	tumbling/+	+
Desulfonema	long filament	-	gliding/rolling	
Desulfosarcina	packages, irregular	a	a	-

[a] signifies transient packages of irregular non-motile cells which release motile ellipsoid rods.

Although SRB have generally been considered to be obligatory anaerobic bacteria, a widespread occurrence of SRB has been reported in oxic environments. While several SRB species are known to be oxygen tolerant (Postgate, 1984; Widdel, 1988; Dannenberg et al., 1992), it is not clear whether they will survive or proliferate under oxic conditions via respiration with nitrate or oxygen as an electron acceptor.

A combined approach of traditional and molecular methods of culture identification together with perturbation of the environment can be used to provide insight into population structure, as indicated in the case study below.

8.1.3.1. Case study on SRB population analysis: UASB granules

Methanogenic and sulphidogenic granular sludge consists of well-settling microbial aggregates that develop by the mutual attachment of bacterial cells in the absence of a carrier material (Lettinga, 1995). These aggregates contain a variety of bacterial species involved in the anaerobic degradation of organic matter, including hydrolytic, fermentative, acidogenic, acetogenic, homoacetogenic, sulphate-reducing, and methanogenic bacteria. These aggregates develop spontaneously in wastewater treatment systems of the upflow anaerobic sludge bed (UASB) reactor under a variety of operation conditions (Lettinga, 1995).

The formation, composition, and functioning of UASB granules has been investigated by a variety of analytical techniques, including activity, molecular, microbial, and physiological assays (Verstraete et al., 1996). The spatial distribution of the bacterial species present within UASB aggregates has been studied by using light, electron, and laser-scanning microscopy on either intact or sectioned individual aggregates (MacLeod et al., 1990). Based on these observations, conceptual models have been postulated to describe the distribution of acidogens, syntrophic and methanogenic bacteria within UASB aggregates (MacLeod et al., 1990; Guiot et al., 1992; Fang et al., 1994). These models proposed a multilayered structure with H_2-consuming bacteria located at the outside of the aggregate, methanogens located in the inner part, and H_2-producing bacteria located between the two layers.

The use of molecular techniques has been particularly useful for the identification and localisation of the different microbial populations present in methanogenic granular sludge. Slot-dot blot hybridisation of the 16S rRNA extracted from granules is used to both identify and quantify the methanogenic, syntrophic, and sulphate-reducing populations (Raskin et al., 1996; Oude Elferink et al., 1998a, b). The application of FISH with specific probes for a bacterial species or population further allows their detection and localisation within granular

sludge (Harmsen et al., 1996b; Sekiguchi et al., 1999). By using the FISH technique, different layered population structures have been determined in different UASB aggregates cultivated on different substrates (Harmsen et al., 1996a, b; Sekiguchi et al., 1999).

Although these investigations considerably improved our understanding of the granular sludge composition and anatomy, there is still a lack of knowledge about the distribution of microbial activities within granular sludge. Population distributions determined by molecular probes do not necessarily correspond to microbial activity distributions, since bacterial populations can have very low or unusual activities. Activity distributions in anaerobic granular sludge are poorly documented, since *in situ* activity measurements require specific analytical tools, e.g., microsensors. The activity distribution of fermentative and methanogenic populations in UASB aggregates has been measured with micrometer resolution by using microsensors for pH and glucose (de Beer et al., 1992; Lens et al., 1993, 1995). An inhomogeneous activity distribution has been reported, with acetogenic and methanogenic activity being predominantly located in the outer layer and the centre of the aggregates respectively (Lens et al., 1993).

8.1.4. ***Molecular techniques for population analysis***

A variety of tools are reported in the literature in which a molecular biological approach is used for analysis of the diversity of the microbial population present. These are reviewed in Appendix A2 to provide context for the selection of the tool 16S rRNA fluorescent in situ hybridisation (FISH) in this study.

The FISH method involves application of oligonucleotide probes to permeabilised whole microbial cells. Probes can be designed to specifically target narrow to broad phylogenetic groups (from species to domain) by virtue of variable evolutionary conservation within the 16S rRNA molecule. The major steps in probe design are identifying short regions (usually 15-25 nucleotides in length) in a sequence alignment unique to the target group of interest, centralising mismatches to nontarget organisms (where possible), and modifying the sequence to meet probe design criteria such as minimum melting temperature. The probes enter the cells and specifically hybridise to their complementary target sequence in the ribosomes. If no target sequence is present in the ribosomes of the cells, the probes are unable to hybridise and the unbound probe is removed by a subsequent wash step. Hence only specifically targeted cells retain the probes under appropriate stringency conditions in the hybridisation and wash steps. Probes are typically labelled at the 5’ end with fluorochrome reporters such as fluorescein and sulfoindocyanine (Cy3, Cy5) dyes. Microbial cells containing hybridised probes can be directly observed under epifluorescence

microscopy owing to the natural amplification of the fluorescent signal by large numbers of ribosomes in any given target cell. An advantage is that multiple probes with varying target specificity can be used in the same preparation providing they are labelled with clearly distinguishable fluorochromes.

The first demonstration of FISH method in its modern form was by DeLong and co-workers (1989), using a simple artificial microbial consortium. Subsequently, FISH has been applied in a variety of natural and artificial ecosystems confirming the value of the method (Amann et al., 1995). However, there are also number of limitations associated with the method such as poor cell permeability, ribosome accessibility and content, and sample autofluorescence. FISH probes have been designed mainly to target 16S rRNAs but 23S rRNA targets are also used (Amann et al., 1995).

8.2. USE OF 16S RRNA-TARGETED OLIGONUCLEOTIDE PROBES TO INVESTIGATE THE COMMUNITY STRUCTURE OF ANAEROBIC SULPHATE-REDUCING BACTERIA IN CONTINUOUS BIOREACTOR

8.2.1. ***Introduction***

The application of direct *in situ* molecular methods enables microbial communities to be characterised in their natural habitat. Due to the fact that environmental conditions influence the cellular rRNA content (Flardh et al., 1992; Kemp et al., 1993), the amount of rRNA is considered to correlate with the growth rate (Poulsen et al*.,* 1993; Schaechter et al., 1958), and *in situ* probing using rRNA-targeted oligonucleotides may therefore be a powerful tool for the identification of bacteria and potential assessment of their activities in their natural habitats. Fluorescent *in situ* hybridisation (FISH) provides potential important advantage over other indirect molecular tools, such as PCR and immunofluorescence, as quantification is possible and potential exists for distinction between metabolically active and largely inactive cells.

16S rRNA directed oligonucleotide probes have been designed for specific groups of SRB (Devereux et al., 1992; Amann et al., 1990; Rabus et al., 1996; White and Gadd, 2000; Purdy et al., 2001; Bade et al., 2000) and have been demonstrated to represent valuable tools for group- and species-specific hybridisation studies of SRB populations in complex communities such as biofilms (Amann et al., 1992; Ramsing et al., 1993), marine sediments (Devereux and Mundfrom, 1994) and microbial mats (Ramsing et al., 1993; Risatti et al*.,* 1994) without prior isolation of the target organisms. Most studies to date relating the distribution of SRBs and their metabolic activity, have been aimed at characterisation and

stratification of natural environments or gaining insight into the survival of SRBs in natural environments Devereux et al., 1996b; Santegoeds et al., 1998; Okabe et al., 1999; Ito et al., 2002a, b; Ravenschlag et al., 2000). The application of these studies to active bioremediation processes is not reported.

In the present study, the community structure of sulphate-reducing bacteria in an anaerobic continuous bioreactor containing mixed culture used for treatment of a sulphate-containing wastewater was investigated using 16S rRNA-targeted oligonucleotide probes. The bioreactors were used to treat an influent containing 2.5 kg m^{-3} of sulphate and acetic acid as a carbon source at a concentration of 2.3 kg m^{-3} or greater. The kinetics of anaerobic sulphate reduction of the culture were studied in this laboratory (Moosa, 2000; Moosa et al. 2002, 2004; Hansford et al., 2004; Section 7 of this report). The maximum sulphate reduction rate observed was 0.032 kg m^{-3} h^{-1}, obtained at a retention time of 2.5 days with sulphate conversions around 80% to 90%.

A fluorescence *in situ* hybridisation (FISH) technique, based on binding of a fluorescent-labelled oligonucleotide probe to 16S rRNA, was used to estimate the numbers of ribosome-rich bacteria in samples taken from the bioreactor operated under steady state conditions. Hybridisation to a universal rRNA probe, EUB338, for the domain Bacteria was performed, and this was followed by a non-sense probe, NON338 as a control for non-specific staining. Counting and size classification were performed by fluorescent microscopy. Sulphate-reducing consortia were identified by using five genus-specific probes (129 for *Desulfobacter*, 221 for *Desulfobacterium*, 228 for *Desulfatomaculum*, 660 for *Desulfobulbus,* and 657 for *Desulfonema*) and four group-specific probes (385 as a general SRB probe, 687 for *Desulfovibrionaceae*, 804 for *Desulfobacteriaceae* and 814 for the *Desulfococcus* group) probes. The contribution of these bacterial groupings to the population structure was determined at an influent sulphate concentration of 2.5 kg m^{-3} and a residence time of 6 days, corresponding to a volumetric loading rate of 0.017 kg m^{-3} h^{-1} in a well mixed continuous stirred tank reactor in which the liquid and biomass residence times were identical. The impact of increased dissolved sulphide concentration, through sulphide additions of 0.00 to 1.25 kg m^{-3} to the feed stream, on the bacterial population structure was determined.

8.2.2. ***Materials and methods***

8.2.2.1. Culture, culture conditions and bioreactor configuration

The steady state sulphate reduction bioreactors were operated at a 1 litre scale using a mixed culture of anaerobic bacteria enriched in sulphidogenic species, previously described in Section 7.2.6. Bioreactor configuration, medium and operating conditions are given in Section 7.2.4.

8.2.2.2. Bioreactor operating conditions

In order to define the dominant bacterial groups within the mixed sulphidogenic culture, the bioreactor was operated at steady state at a 6 day residence time, a pH of 7.8 and an influent sulphate concentration of 2.5 kg m^{-3}.

In order to investigate the effect of increasing concentrations of dissolved sulphide on the bacterial population structure, dissolved sulphide was included in the feed to the reactor at a concentration of 0.00 to 1.25 kg m^{-3}, resulting in residual dissolved sulphide concentrations at steady state of 0.75 to 1.45 kg m^{-3}. Performance of these reactors is reported in Section 7.2.6.

8.2.2.3. Pure cultures

The pure cultures of SRB used in this study included *Desulfobacter latus* DSM 3381, *Desulfotomaculum ruminis* DSM 2154, *Desulfobulbus propionicus* DSM 2554, *Desulfobacterium autotriphicum* DSM 3382, *Desulfococcus multivorans* DSM 2059, *Desulfonema limicola* DSM 2076 and *Desulfovibrio gigas* DSM 496, which were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). Pure culture controls were used to calibrate the response of the target genera to the oligonucleotide probes used. SRB cultures were grown according to Widdel and Bak (1992). Non-SRB control (*E.coli* NCTC 09001) was grown in nutrient broth.

8.2.2.4. Oligonucleotide probes

16S rRNA-targeted oligonucleotide probe sequences were deposited at probeBase (Loy et al., 2003). The following 16S rRNA-targeted oligonucleotide probes were used: (i) EUB338, (ii) NON338 as a negative control, (iii) five genus-specific probes (SRB660, 221, 129, 228, 657), and (iv) four group-specific probes (SRB385, 687, 814 and 804). Oligonucleotide probes used, their specificities, pure culture controls, hybridisation conditions, and references are given in Table 8.2. All probes were commercially synthesised and labelled

with fluorescein isothiocyanate (FITC) at the 5' end by University of Cape Town, Department of Molecular and Cellular Biology (Cape Town, South Africa).

8.2.2.5. Cell fixation and hybridisation

Mixed culture samples were fixed by incubation in paraformaldehyde (4% [wt/vol] in phosphate-buffered saline) at 4°C for 1-2 h. These were subsequently washed in phosphate-buffered saline and stored in phosphate-buffered saline-ethanol (1/1) at -20°C for *in situ* hybridisation (Amann 1995). All *in situ* hybridisations were performed according to the procedure described by Amann (1995) in 8 µl of hybridisation buffer containing 0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.01% SDS and variable formamide concentrations as described in Table 8.2 with 1 µl probe solution. Hybridisations were carried out at 46°C for 2 h in an equilibrated sealed moisture chamber. The final probe concentration was approximately 50 ng μl^{-1}. Subsequently, a stringent washing step was performed at 48°C for 20 min in 50 ml of pre-warmed washing solution (variable NaCl concentration as described in Table 8.2, 20 mM Tris-HCl [pH 7.2], 0.01% SDS). The washing buffer was removed by rinsing the slides with double-distilled H_2O. Samples were simultaneously stained with the prokaryote stain 4', 6-diamidino-2-phenylindole (DAPI). The slides were then rinsed briefly with double-distilled H_2O and allowed to air dry prior to microscopic observation.

8.2.2.6. Optimisation of in situ stringency conditions

For *in situ* application, optimal stringency conditions were adjusted for each FITC- labelled probe performing whole cell hybridisations using target and non-target organism(s). The hybridisation stringency was gradually increased by the addition of formamide to the hybridisation buffer in concentration steps of 10% (v/v) across the concentration range 0 to 60% formamide, according to Manz et al. (1992). The sodium chloride concentration was adjusted according to the formamide concentration used in the hybridisation buffer (Table 8.2).

Table 8.2: 16S rRNA-targeted oligonucleotide probes used in this study

Probe[a]	Specificity	Pure culture controls	Sequence of probe (5'→3')	Target site[b]	FA[c] con. (%)	NaCl[d] con. (mM)	Reference
p338 (S-D-Bact-0338-a-A-18)	Most bacteria	SRBs, *E. coli* NCTC 09001	GCTGCCTCCCGTAGG AGT	338-355	10	0.386	Amann et al. 1990
non-Bact338	Non (negative control)	-	ACTCCTACGGGAGGC AGC	338-355	0	0.9	Manz et al. 1992
p385 (S-*-Srb-0385-a-A-18)	Some SRBs	SRBs	CGGCGTCGCTGCGTC AGG	385-402	30	0.071	Amann et al. 1992
p129 (S-G-Dsb-0129-a-A-18)	*Desulfobacter*	*Desulfobacter latus* DSM 3381	CAGGCTTGAAGGCAG ATT	129-146	10	0.386	Devereux et al. 1992
p221 (S-G-Dsbm-0221-a-A-20)	*Desulfobacterium*	*Desulfobacterium autotrophicum* DSM 3382	TGCGCGGACTCATCT TCAAA	221-240	10	0.386	Devereux et al. 1992
p660 (S-G-Dsbb-0660-a-A-20)	*Desulfobulbus*	*Desulfobulbus propionicus* DSM 2056	GAATTCCACTTTCCCC TCTG	660-679	30	0.071	Devereux et al. 1992
p687 (S-G-Dsv-0687-a-A-16)	*Desulfovibrionaceae*	*Desulfovibrio gigas* DSM 496,	TACGGATTTCACTCCT	687-702	10	0.386	Devereux et al. 1992
p804 (S-*-Dsb-0804-a-A-18)	*Desulfobacteriaceae*	*Desulfobacter latus* DSM 3381	CAACGTTTACTGCGT GGA	804-821	10	0.386	Devereux et al. 1992
p228 (S-*-Dfm-0228-a-A-15)	*Deuslfotomaculum* spp.	*Desulfotomaculum ruminis* DSM 2154	GGGACGCGGAYCCAT	228-242	50	0.019	Daly et al. 2000
P657 (S-*-Dsn-0657-a-A-20)	*Desulfonema* spp.	*Desulfonema limicola* DSM 2076	TTCCGCTTCCCTCTC CCATA	657-676	30	0.071	Fukui et al. 1999
p814 (S-*-Dscoc-0814-a-A-18)	*Desulfococcus* group	*Desulfococcus multivoranns* DSM 2059	ACCTAGTGATCAACG TTT	814-883	10	0.386	Devereux et al. 1992

[a]nomenclature according to Alm et al. (1996), [b]16S rRNA position according to *E. coli* numbering (Brosius et al. 1981),

[c] formamide concentration in the hybridisation buffer, [d]sodium chloride concentration in the washing buffer

8.2.2.7. Total cell counts

For the determination of total cell counts, the culture liquid was diluted to appropriate concentrations and filtered through polycarbonate membranes (0.2 µm pore size, Millipore, Germany). After air drying, the membranes were mounted on a glass slide with 2 µl DAPI (1 µg ml^{-1}) and stained in the dark with additional 10 µl DAPI for at least 15 min (Christensen and Poulsen, 1994; Porter and Feig, 1980). Before microscopy, a drop of anti-fading reagent AF1 (Citifluor Ltd., London, UK) was added. Differential cell counts were made directly by averaging the cell numbers obtained in a minimum of 10 randomly chosen microscopic fields

of view per well from three to six wells. The total number of hybridised cells was estimated by counting the cells that were hybridised with the probe EUB338. All data are shown as the proportion of cells within a group (domain or genus) relative to DAPI stained cells.

8.2.2.8. Photomicroscopy

All microscopic observations and image acquisition were performed with a model BX40 microscope fitted for epifluorescence (Olympus, Japan) with the blue filter set U-MWB (excitation wavelength 460-490 nm) and UV filter set U-MWU (excitation wavelength 330-385 nm). Slides were mounted in anti-fading solution AF1 and viewed under oil immersion with UV fluorite flat-field objectives (X100). Exposure times were approximately 0.5 s under phase contrast and 15 to 40 s for epifluorescence micrographs. All image combining, processing, and analysis were performed with the software package and CCD camera (ColorView II) provided by Soft Imaging System (Germany).

The highest fluorescence intensity of each background was used as a threshold low value. All pixels with fluorescence intensity above the threshold value were counted as probe-stained area. Results were corrected by subtracting signals observed with the nonsense probe NON338. In all cases, cell numbers were obtained from a minimum of 10 fields of view per well and from three to six wells per sample. The total number of cells present was estimated by both the DAPI count and the number of cells that hybridised with probe EUB338. All data are shown as the proportion of cells within the group (domain, genus or species) i.e. hybridised to the probe representing that group relative to the sum of cells hybridised with the probe SRB385.

8.2.3. ***Results and Discussion***

8.2.3.1. Specificity and selectivity of 16S rRNA probes under specified hybridisation conditions

Probes were hybridised to pure cultures of target organisms and non-target organisms at a range of stringencies. Stringency was varied using formamide concentration at set hybridisation (46°C) and wash temperatures (48°C). A range of formamide concentrations (0-60%) in 10% increments was used. This required seven replicate slides, one for each formamide concentration. The objective was to determine the range of stringencies (formamide concentrations) at which the probe specifically hybridised to the target organisms but not to the non-target organisms. The optimal stringency usually was taken as the highest formamide concentration before specific hybridisation signal was lost. The window of specific hybridisation stringencies was between the lowest formamide concentration at which the non-target organism showed no fluorescence and the optimal probe stringency. The optimal stringency found for each probe is given in Table 8.2.

8.2.3.2. Total prokaryotic count and prevalence of SRB

In this investigation, *in situ* probing was performed to determine the occurrence and relative abundance of SRB in the anaerobic bioreactor in the absence of added sulphide. By this approach, individual members of the SRB could be shown to be present. The total prokaryotic population was determined as 1.8×10^7 cells ml^{-1} by DAPI staining. A lower cell count was obtained with probe EUB338 (for the domain Bacteria) (1.3×10^7 cells ml^{-1}) after subtracting the counts obtained with nonsense probe NON338. Microbial cells were counted only if auto-fluorescence was absent, which was ascertained by placing the UV filter immediately behind the FITC filter without moving the microscopic field (Figure 8.1a,b). The concentration of cells hybridising with the SRB385 probe was approximately 1.1×10^7 cells ml^{-1} (86% of cells recognised by the EUB338 probe) (Figure 8.1c,d), illustrating the dominance of the SRB in this population.

8.2.3.3. Composition of SRB cultures: major groups

FISH analysis of the steady state culture in the absence of added soluble sulphide revealed that *Desulfobacteriaceae* group hybridised with probe SRB804 were the most dominant SRB group in the continuous reactor and made up 47% of the cells hybridised with the probe SRB385. Unlike the *Desulfobacteriaceae* group, *Desulfovibrionaceae* was relatively less abundant in the continuous bioreactor, accounting for about 16% of the total SRB385

hybridised cells (approximately 2.08 x 10^6 cells ml^{-1}) through hybridisation to probe SRB687. The distribution of SRB and their cell numbers in the bioreactor are shown in Figure 8.2.

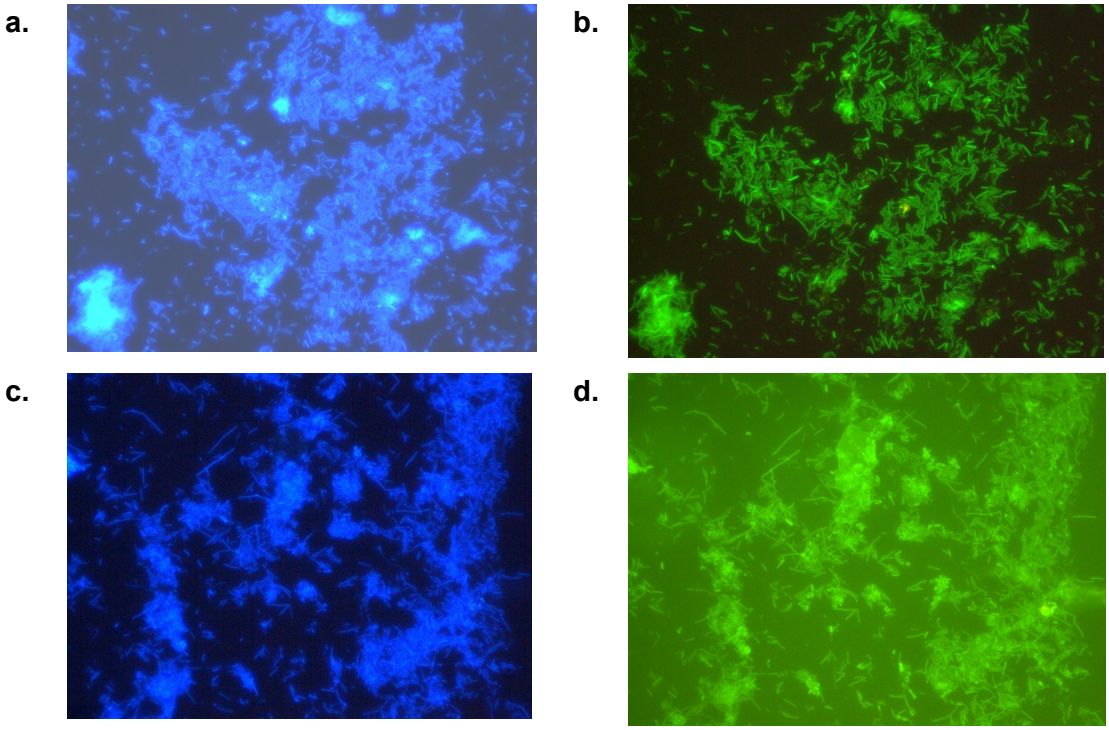

Figure 8.1: Determination of total cell number (micrographs correspond to same field): a. staining with DAPI; b. staining with EUB338 general probe; c. staining with DAPI; d. hybridisation with SRB385 probe

8.2.3.4. Composition of SRB cultures: major genera

FISH analysis revealed that *Desulfonema* hybridised with probe SRB657 and *Desulfobulbus* hybridised with probe SRB660 were the most dominant SRB genuses in the continuous reactor at 2.5 kg m^{-3} without soluble sulphide addition. These accounted for 62% and 44% of the cells hybridised with the probe SRB385, respectively (Figure 8.2). Probe SRB814, which hybridises with *Desulfococcus*, *Desulfosarcina*, and *Desulfobotulus* accounted for 24% of the total SRB385 hybridised cells. An abundance of cells hybridising with the nominally genus-specific SRB228 probe for *Desulfotomaculum* (approximately 3.77 x 10^6 cells ml^{-1} corresponding to 29% of SRB385 hybridised cells) was found in all microscopic fields, and their fluorescence signals were strong. Another prevalent SRB present, accounting for about 29% of the total SRB385 hybridised cells, was detected by probe SRB221 which hybridised with *Desulfobacterium*. *Desulfobacter* hybridised with probe SRB129 were accounted only for about 22% of the total SRB385 hybridised cells (approximately 2.86 x 10^6 cells ml^{-1}).

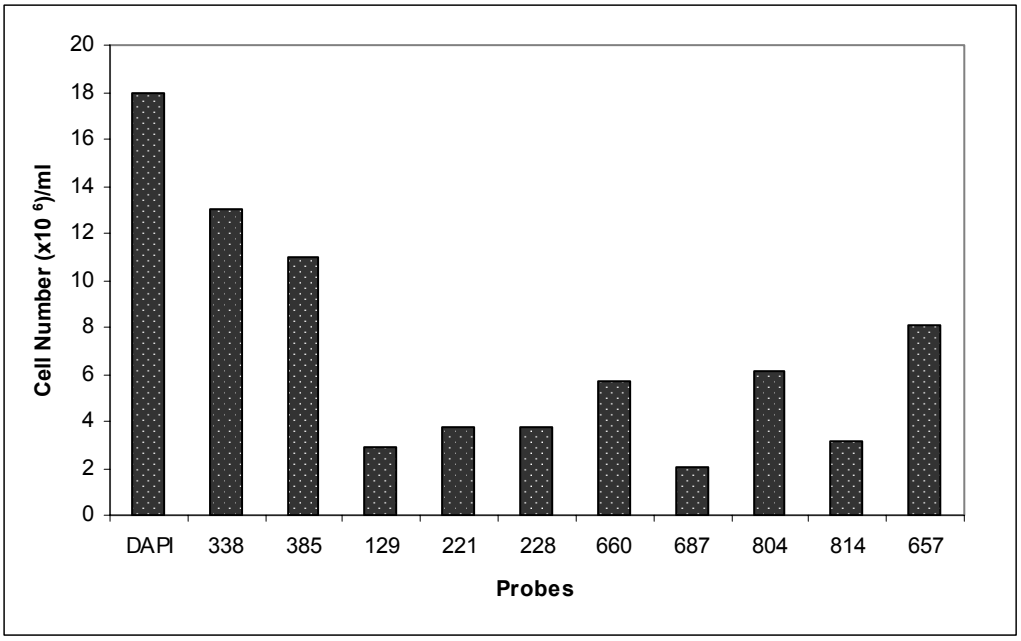

Figure 8.2: *In situ* distribution and abundance of SRB population in the bioreactor. The probe data is expressed relative to the number of SRB385 hybridised cells

While exact quantification has not been achieved, identification of the major groupings is evident through this study. In order to improve the accuracy of quantification, it is necessary to refine the use of FISH to eradicate clumping of cells during their analysis and to maintain the fluorescent signal over expanded time ranges. It is clear from the sum of the individual probe counts, that probe SRB385 does not account for all sulphate reducing bacteria. This has been reported previously by Rabus et al. (1996), who illustrated the SRB385 probe sequence is not complementary to most of the *Desulfobacteriaceae* group. To improve the accuracy of the estimation of the SRB, SRB385 should be used in combination with the modified version of the probe SRB385Db. However, this may result in the detection of a broader range of anaerobic bacteria (Manz et al., 1998). Further, based on the selection of genus- and group-based probes used, it can be expected that some bacterial species will bind to more than one of these probes.

8.2.3.5. Shift in SRB community structure on exposure to sulphide loadings.

Sulphide exposure was varied through the addition of soluble sulphide at concentrations of 0.50, 0.75, 1.00, 1.25 kg m^{-3} to the feed medium to the continuous reactors (Section 7.5.6). The relative abundance and distribution of SRB in the bioreactors with varying sulphide loadings was determined under steady state conditions at a residence time of 6 days by hybridisation of seven phylogenetically distinct groups of SRB (*Desulfobulbus*,

Desulfobacterium, *Desulfobacter*, *Desulfovibrio*, *Desulfococcus*, *Desulfonema* and *Desulfotomaculum*) genus-specific and group-specific fluorescein labelled 16S rRNA-targeted oligonucleotide probes.

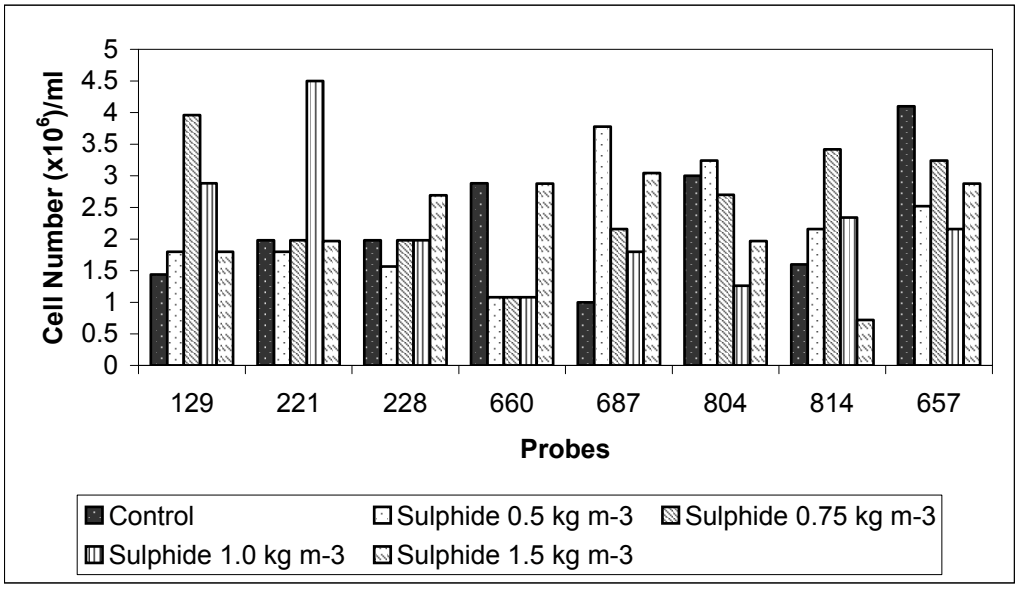

Figure 8.3: *In situ* distribution and abundance of SRB population at different sulphide loadings. Control does not include any sulphide. Data are expressed relative to the number obtained from DAPI-stained cells from the control reactor

Figure 8.3 illustrates the results of the *in situ* distribution and abundance of SRB population at different sulphide loadings. The control does not include any added sulphide; however a dissolved sulphide concentration of 0.75 kg m^{-3} results as the product of anaerobic sulphate reduction. Effective sulphide concentrations in the experiment reactors and sulphate reduction performance are detailed in Section 7.7. Prior to sulphide addition, SRB belonging to the *Desulfonema* and *Desulfobulbus* genera (SRB657 and SRB660 respectively) with *Desulfobacteriaceae* group (SRB804) were present at high proportions. Following the addition of sulphide to the bioreactors, FISH analysis results showed that sulphide inhibited the *Desulfonema*, *Desulfobulbus* spp. and *Desulfobacteriaceae* groups, reducing their prevalence. Increased sulphide addition did not influence the *Desulfobacter*, *Desulfotomaculum*, *Desulfobacterium* spp. or *Desulfovibrionaceae* groups. Only the highest concentration of added sulphide of 1.25 kg m^{-3} was found to inhibit the *Desulfococcus* group (SRB814). An increase in total biomass and total cell number (determined by DAPI staining) was observed with increasing addition of soluble sulphide to the feed (Figure 8.4).

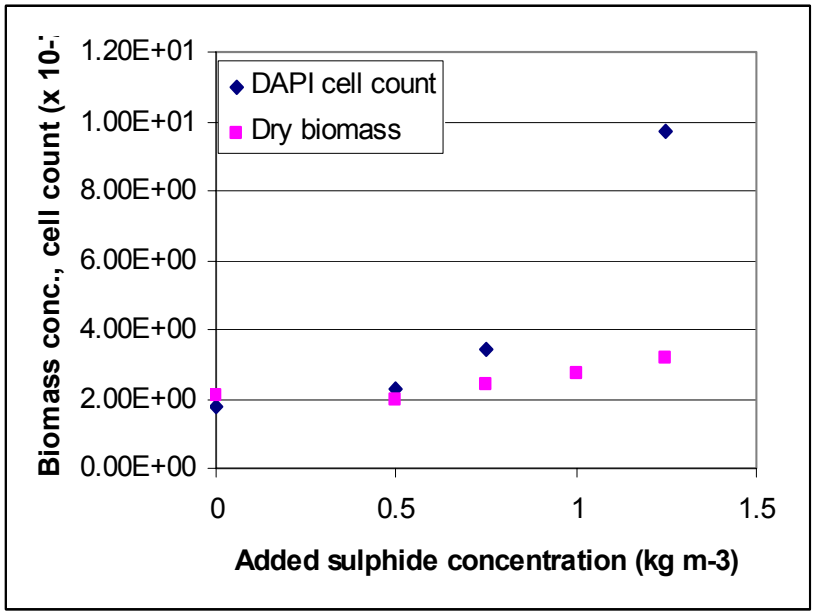

Figure 8.4: Effect of added sulphide concentration on biomass concentration

Desulfobacteriaceae group was only present at high numbers (with an average percentage of 14%) at the lowest sulphide concentration. *Desulfobacterium* showed dominance with an average percentage of 25% at 1.0 kg m^{-3} sulphide concentration. *Desulfonema* was found to be highly susceptible to varying sulphide concentrations. This genus was not a significant member of the microbial community at high sulphide concentration. *Desulfobulbus* was also poor competitor in the presence of varying sulphide concentrations. An abundance of cells hybridising with SRB814 for *Desulfococcus* group with average percentages 10% and 13%, SRB129 for *Desulfobacter* with average percentages 12% and 16% was found only at 0.75 and 1.0 kg m^{-3} sulphide loadings, respectively. On the other hand, *Desulfovibrionaceae* group was highly competitive at all sulphide loadings. *Desulfobacter* and *Desulfotomaculum* were also highly competitive at the highest sulphide concentration.

8.2.4. ***Conclusions***

By using the molecular technique described in this study, we were able to detect different groups of SRB in the bioreactor across several genera. *In situ* hybridisation with fluorescent-labelled rRNA-targeted probes has reached a level which allows the direct, cultivation-independent analysis of most members of the natural microbial communities present.

Using pure culture studies, the methodology used has been validated for our system. The mixed culture of sulphate reducing bacteria present under steady state conditions in the continuous reactor operated at a 2.5 day residence time at 35°C and pH 7.8 with a feed containing 2.5 kg m^{-3} sulphate and 2.3 kg m^{-3} acetate showed a dominance of *Desulfonema*, *Desulfobacteriaceae* and *Desulfobulbus*. *Desulfovibrionaceae* and *Desulfobacter* were the least dominant of the genera studied.

Our ongoing work to define the shift in community structure with varying environmental conditions such as carbon source, sulphide concentration, and sulphate concentration is in progress. Preliminary studies suggest trends in dominant species with both changing carbon source (acetate vs ethanol) and changing sulphate concentration. These studies will be reported in detail later. Completed studies on the shift in population structure on increasing concentrations of soluble sulphide added in continuous chemostat culture clearly show a decrease in the prevalence of *Desulfonema*, *Desulfobulbus* spp. and the *Desulfobacteriaceae* group with increasing sulphide. *Desulfobacter*, *Desulfotomaculum* and *Desulfovibrionaceae* were little affected by the range of sulphide concentration studied. It is recognised that further method development, especially with respect to the handling of microbial aggregation and enhancing of fluorescent signal stability, will enhance the accuracy of the data generated. Such studies could assist in the optimisation of industrial water treatment processes through allowing the establishment of population structures tailored to provide robust and efficient functioning within the varying conditions found in effluent treatment processes as well as the ability to modify populations to enable rapid response to process perturbation.

9 USING COMPLEX CARBON SOURCES AS THE ELECTRON DONOR

9.1. INTRODUCTION

While the kinetic modelling of biological sulphate reduction, reported in Section 7, is focused on small organic molecules used directly by the sulphate reducing bacteria as the carbon source and electron donor to enable a fundamental understanding of the sulphidogenic sub-process to be generated, it is well recognised that the cost-effective implementation of biological sulphate reduction may require the use of complex waste carbon sources. In order to inform the extension of this study to complex carbon sources, the following key features need to be considered:

- In the presence of a complex carbon source, the bacterial population requires functional diversity, including hydrolysis of particulate carbon sources, fermentation of large soluble carbon sources, acidogenesis and acetogenesis as well as sulphidogenesis. In order to understand the availability of carbon sources and electron donors for sulphidogenesis, the effect of sulphate on the other functional groupings of bacteria requires understanding. While the effect of sulphate concentration on hydrolysis of particulate COD has been a key consideration of the work of Ristow et al. (2004) under WRC project K5/1216, a review of the effect of sulphate, sulphide and metals on acidogenesis and acetogenesis was defined as a specific outcome of this study. This is provided as a literature review in Section 9.2 to define whether further experimental study or more detailed inclusion in the simulation model is required.
- Further, prior to the recommendation of a particular carbon-based raw material as carbon source and electron donor, it is necessary to have a framework in which to assess the feasibility of provision of this source in sufficient quantity to meet the AMD treatment needs. Further, the impact of different raw materials on process performance and economics is required. Section 9.3 provides a framework for this discussion through the review of 3 potential complex raw materials.

9.2. REVIEWING THE EFFECT OF SULPHATE ON GENERAL ANAEROBIC DIGESTION

9.2.1. ***Introduction***

Anaerobic digestion is well known for treatment of organic wastes. The process involves fermentative bacteria, acidogens, acetogens and methanogens (MPB) (McCarty, 1964). The following processes occur during the breakdown of insoluble carbon-based materials:

- Breakdown of insoluble compounds by external hydrolytic enzymes secreted by the bacteria.
- Acidogenesis of sugars and amino acids to hydrogen, CO_2 and short chain fatty acids (SCFA's) such as acetic acid, propionic and lactic acids.
- β-oxidation of long chain fatty acids to yield acetate, propionate and hydrogen.
- Acetogenesis of propionate and other SCFA's to acetate, hydrogen and carbon dioxide.
- Methanogenesis using hydrogen and acetate as substrates.

Where sulphate is present, biological sulphate reduction utilising hydrogen, acetate, propionate and other SCFA's as substrates may also occur. SRBs have been shown to compete with the MPB for carbon and energy sources (Barton, 1995). A representation of the degradation of insoluble compounds in the presence of sulphate is shown as Figure 9.1. Depending on the nature of the organic substrate, certain pathways may not be active.

The occurrence of sulphate reduction during the anaerobic process has been considered by many researchers as undesirable (Winfrey and Zeikus, 1977; Banat, 1981; Smith and Klug, 1981; Banat and Nedwell, 1983; Mulder, 1984; Isa et al., 1986). for two reasons:

- The Sulphate Reducing Bacteria (SRBs) utilise the organic compounds that would otherwise be used by the methanogens.
- The SRBs produce sulphide which is toxic to the methanogens and imposes negative impacts on the environment in which it is produced.

However, in recent work the utilisation of the sulphate reduction process to reduce the sulphate content of the effluent streams is recognised. The removal of sulphates from mine water, groundwater and industrial effluents have been studied extensively (Maree *et. al*., 1987; Barnes et al., 1991; Dvorak, 1992; Du Preez and Maree, 1994; Oude Elferink et al., 1994; Visser, 1995; Colleran et al., 1996; Genschow et al., 1996; Dries et al., 1998; Elliot et al., 1998). The effect of sulphate *per se* on the anaerobic digestion process has not been

addressed in any great detail. It is the objective to present here the available literature knowledge of the effect of sulphate on the anaerobic digestion process. For completeness, the inhibition of various compounds and environmental factors affecting the sub-processes are presented.

As shown in Figure 9.1, the degradation of insoluble organic compounds consists of several steps. These include hydrolysis, acidogenesis, methanogenesis and oxidation (Zehnder, 1988). The hydrolysis step is important during the degradation of insoluble organic compounds (Eliosov and Argaman, 1995). The products formed during acidogenesis provide the carbon and energy source for the SRBs methanogens. Consequently, in the presence of long chain organic compounds the acidogenic phase plays an important role in providing substrates for the anaerobic reduction of sulphate (Widdel, 1988). Methanogenic bacteria compete with SRBs for the carbon and electron source, in most cases acetate, H_2 and CO_2. This has direct consequences for the sulphate reduction process. In order to understand these interactions, acidogenesis and methanogenesis are reviewed in subsequent sections with emphasis placed on the inhibition caused by S-containing and other compounds. Hydrolysis is not reviewed here, as it forms the focus of WRC project K5/1216.

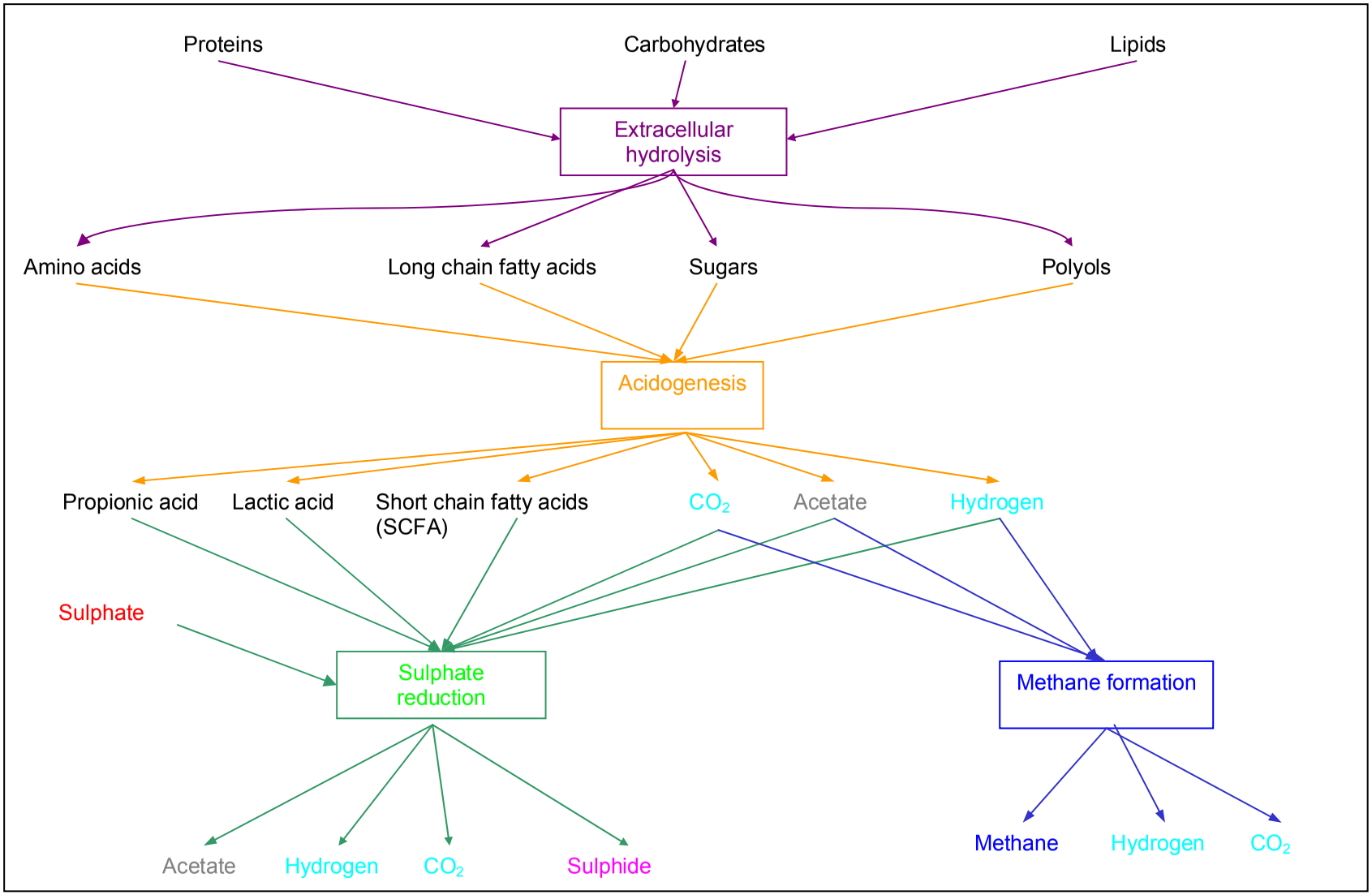

Figure 9.1: Representation of the general anaerobic digestion process. Sub processes include: hydrolysis, acidogenesis, sulphate reduction and methane formation

Table 9.1: Performance of various bioreactors used for the treatment of sulphate containing waste streams using long chain organics

Reference	Effluent treated	Organic utilised	Origin of inoculum	Reactor type and mode	Temp. (°C)	pH	Influent sulphate (kg m^{-3})	HRT (h)	Volumetric reduction rate (kg m^{-3} hr)
Maree & Strydom (1985)	mine water	sugar	activated sludge	UASB (1 ℓ), C	30	7	2.00	11	0.164
	Mine water	pulp mill effluent (COD = 2.9 kg m^{-3})	activated sludge	UASB (1 ℓ), C	30	7	1.35	11	0.082
	Mine water	pulp mill effluent (COD = 3.6 kg m^{-3})	activated sludge	UASB (1 ℓ), C	30	7	1.35	11	0.107
	Mine water	raw sewage sludge (COD = 5 kg m^{-3})	activated sludge	UASB (1 ℓ), C	30	7	1.35	11	0.095
	Mine water	raw sewage sludge (COD = 5.6 kg m^{-3})	activated sludge	UASB (1 ℓ), C	30	7	1.35	11	0.118
Maree (1987)	synthetic	molasses (2 mℓ $ℓ^{-1}$)	-	PBR (1 ℓ) dolomite pebbles), C	31	7.0	0.90	20	0.019
	synthetic	molasses (2 mℓ $ℓ^{-1}$)	-	PBR (1 ℓ) (dolomite pebbles), C	31	7.0	0.90	15	0.004
	synthetic	molasses (3 mℓ $ℓ^{-1}$)	-	PBR (1ℓ) dolomite pebbles), C	31	7.0	0.90	20	0.035
	synthetic	molasses (2 mℓ $ℓ^{-1}$)	-	SBR(1 ℓ), C	31	7.0	2.50	15	0.113

Reference	Effluent treated	Organic utilised	Origin of inoculum	Reactor type and mode	Temp.	pH	Influent sulphate	HRT	Volumetric reduction rate
					(°C)		(kg m^{-3})	(h)	(kg m^{-3} hr)
	synthetic	beet molasses	-	UASB (lab), C	-	-	4.00	33.6	0.112
Christensen et al. (1996)	mine water	whey	cow manure	CR (2 ℓ), B	15		-3.31	B 2d	0.0002
Sanchez et al. (1997)	synthetic	dairy plant effluent	sewage sludge	UASB (13 ℓ), C	31	7	0.84	19	0.043
	synthetic	acetic acid	UASB (10 ℓ)	UASB (1.7 ℓ), C	30	8	3.5	3.4	0.625

Reactor types: STR, stirred tanks; UASB, upflow anaerobic sludge bed reactor; PBR, packed bed reactor; SBR, sludge bed reactor; CR, column reactor, RSB, racked sludge bed, C; continuous; B, batch; EGSB, expanded granular sludge bed reactor.

9.2.2. ***Acidogenesis***

9.2.2.1. Review of acidogenesis

Previously, acidogenesis has been viewed by some researchers as a two step process: hydrolysis and acid formation (Eastman and Ferguson, 1981; Dinopoulou et al., 1988). Recently, however, the general consensus is that the hydrolysis phase is separate from acidogenesis and carried by different bacterial groups (Speece, 1996). For the purposes of this review, acidogenesis comprises the use of soluble substrates or intermediates as the energy source for synthesis and growth of acid-producing bacteria, resulting in the formation of acetate, propionate, butyrate, hydrogen and CO_2 and cellular material (Eastman and Ferguson, 1981).

Using glucose as a model energy source, the acidogenic phase can be represented by the following reactions (Zehnder, 1984):

$$\text{glucose} \rightarrow 2\ \text{pyruvate}$$
$$C_6H_{12}O_6 \rightarrow 2CH_3COCOO^- + 2H^+ + 2H_2 \qquad \text{9-1}$$
$$\Delta G^{o\prime} = -112.1\text{kJ / mole glucose consumed}$$

$$\text{pyruvate} \rightarrow \text{propionate}$$
$$CH_3COCOO^- + 2H_2 \rightarrow CH_3CH_2COO^- + H_2O \qquad \text{9-2}$$
$$\Delta G^{o\prime} = -123.6\text{kJ / mole pyruvate consumed}$$

$$2\ \text{pyruvate} + 2H_2O \rightarrow \text{butyrate} + 2HCO_3^-$$
$$2CH_3COCOO^- + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + HCO_3^- + H^+ \qquad \text{9-3}$$
$$\Delta G^{o\prime} = -142.7\text{kJ / mole pyruvate consumed}$$

$$\text{pyruvate} + H_2O \rightarrow \text{acetate} + \text{formate}$$
$$CH_3COCOO^- + H_2O \rightarrow CH_3COO^- + HCOO^- + H^+ \qquad \text{9-4}$$
$$\Delta G^{o\prime} = -52.1\text{kJ / mole pyruvate consumed}$$

From Figure 9.1, it can be seen that carbohydrates and nitrogen compounds are the only compounds that can be fermented in the acid-producing phase. Further degradation of these acids and the H_2 produced occurs by the SRBs and methanogens. In the absence of SRBs and methanogens, the accumulation of short chain fatty acids and H_2 has been shown to be toxic to the acidogenic bacteria (Zoetemeyer et al., 1982a)

9.2.2.2. Sulphate toxicity

It has been shown that sulphate reducing reactors fed with long chain organics (indicating the need for acidogenesis) are not inhibited by sulphate concentrations over the range 0.90 to 4.00 g ℓ^{-1} (Table 9.1). As the sulphate reducers require the products of the acidogenic phase as an organic and electron donor source, this indicates that acidogenesis is not inhibited in this range of sulphate concentrations. Data on the effect of higher sulphate concentrations were not found.

9.2.2.3. Sulphide toxicity

Hilton and Oleskiewicz (1988) investigated the inhibitory effect of biogenic sulphide on acidogenesis. Lactose uptake was examined over a pH range of 6 to 8. They concluded that unionised H_2S concentrations above 0.076 g ℓ^{-1} were inhibitory to the acidogenic micro-organisms.

9.2.2.4. Heavy metal toxicity

The inhibition of the acidogenic bacteria by metals has been reported by Lin (1993) and Yenigun et al. (1995). Previous work focussed on the inhibition effect of various organic substances on the acid-producing phase (Zoetemeyer et al., 1982a). Lin (1993) showed the following toxicity of metals in the production of acetic acid and butanol respectively: copper > zinc > chrome > cadmium > lead > nickel and copper > zinc > chrome > cadmium > nickel > lead. The experiments were carried out in batch at 35°C using sewage sludge. A copper concentration of 0.0018 g ℓ^{-1} caused a 50% decrease in the formation of acetic acid. The inhibitory concentrations of zinc, cadmium and nickel for a 50% decrease in formation of acetic acid were 0.0042 g ℓ^{-1}, 0.03 g ℓ^{-1} and 0.6 g ℓ^{-1} respectively. Yenigun et al. (1995) confirmed these results for cadmium and nickel, using batch tests with an anaerobic culture obtained from the acidogenic section of an industrial plant. They observed a 50% reduction in acetic acid production at a cadmium and nickel concentration of 0.78 g ℓ^{-1} and 0.056 g ℓ^{-1} respectively.

9.2.2.5. pH

Zoetemeyer et al. (1992b) measured the maximum specific growth rate of acidogenic bacteria on glucose over a pH range of 4.5 to 7.9. They found that the optimum pH was around 6.0 with the maximum specific growth rate rapidly decreasing to 50% at pH 5 and gradually decreasing to 25% at pH 8.0. Hilton and Oleszkiewicz (1988) report a similar trend over a pH range of 6.0 to 8.0 for the utilisation of lactose.

9.2.3. ***Methanogenesis***

9.2.3.1. Review of methanogenesis

In the anaerobic system, carbon is finally reduced to methane. Methanogenic bacteria use acetic acid, formic acid or carbon dioxide and hydrogen as substrates, through the following reactions (Zehnder et al., 1982):

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^{o'} = -31\text{kJ / mole acetate consumed} \qquad 9\text{-}5$$

$$4HCOO^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^- \qquad \Delta G^{o'} = -134\text{kJ / mole methane formed} \qquad 9\text{-}6$$

The methane produced leaves the system as a gas, because of its low solubility. The CO_2 formed is either converted to bicarbonate or leaves the system as a gas. Zinder (1984) showed that in the absence of sulphate reduction, approximately 70% of the biogas produced by methanogenic bacteria is due to conversion of acetic acid by a group of methanogens known as acetoclastic methanogens. The majority of the remaining 30% of the biogas is produced from the reduction of CO_2 by hydrogen:

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \qquad \Delta G^{o'} = -136\text{kJ / mole methane formed} \qquad 9\text{-}7$$

The free energy of formation drives the reaction towards the right and therefore methanogens have a high affinity for hydrogen.

According to Mosey (1983), the utilisation of hydrogen by the methanogens is only possible at hydrogen partial pressures below 10^{-4} atm. Above this partial pressure, H_2 is toxic to the methanogens. Zeikus et al. (1975) indicated the importance of the methanogens in a mixed culture in maintaining the pH homeostasis of the ecosystem by removing the acetic acid.

Methanogens are strict anaerobes, as they lack the enzymes catalase and superoxide dismutase (Schlegal et al., 1997), and are very sensitive to any change in the environmental conditions. The influential parameters on the activity of methanogens include: pH, temperature, nutrient requirements and toxicity or inhibition by organic and inorganic substances.

9.2.3.2. Effect of sulphate

The effect of sulphate on methanogenesis has not been presented in the open literature, however the effect of biogenic sulphide as a consequence of the presence of sulphate is reviewed below.

9.2.3.3. Effect of sulphide

In the absence of metals, unionised sulphide concentrations in the range 0.20 kg m^{-3} (McCarty, 1964) to 0.70 kg m^{-3} (Mulder, 1984) have been shown to have an inhibitory effect on the activity of methanogens. Hilton and Oleskiewicz (1986) studied the effect of sulphide on methanogenesis utilising acetate as a substrate over a pH range of 6.8 to 8.0. They concluded that the unionised hydrogen sulphide, rather than the total sulphide, is inhibitory. They confirmed that methanogenesis is severely inhibited above 0.20 kg m^{-3} hydrogen sulphide (H_2S). Visser (1995) showed that hydrogen sulphide concentrations in the range 0.05 kg m^{-3} to 0.25 kg m^{-3} result in 50% inhibition of methanogenesis.

9.2.3.4. Effect of pH

In contrast to acidogenic bacteria which have optimal growth at pH 6.0, methanogenic micro-organisms are inhibited below pH 6.5 (Speece, 1996). The formation of methane is confined to a very narrow pH range of 6.8 to 7.4 (McCarty, 1964). This has been confirmed by studies on pure cultures carried out by Zeikus et al. (1975) and Zehnder et al. (1982). In the anaerobic system, the pH is controlled by the carbon dioxide – bicarbonate buffer system. The methanogens thus become inactive when insufficient buffering is accompanied with the presence of high concentrations of volatile fatty acids (Zehnder, 1988).

9.2.3.5. Effect of temperature

Traditionally, anaerobic reactors are run at temperatures ranging from 35 to 40°C using mesophilic methanogens. However, thermophilic methanogens functioning at temperatures between 50 and 60°C offer the advantage of a significantly shorter generation time than mesophilic methanogens (Parkin and Owen, 1986). According to Zinder (1984), thermophilic *Methanosarcina* have a generation time of 12 h while mesophilic *Methanosarcina* have a generation time of 24 h (Speece, 1996). The population of thermophilic *Methanotrix* cultures doubles in 24 to 36 h whereas mesophilic *Methanosaeta* has a doubling time of 4 to 9 d. Additional investigation of the thermophilic methanogens and quantification of the accompanying energy requirement is required prior to the use of thermophilic methanogenesis on a commercial scale.

9.2.3.6. Nutrient requirements and their toxicity or inhibition

The nutrient requirements of bacteria involved in anaerobic digestion are relatively simple (Grobicki and Stuckey, 1992), including organic carbon, nitrogen and phosphorous compounds and trace amounts of magnesium, manganese, potassium, calcium, iron, nickel and cobalt. Parkin and Owen (1986) reported that the phosphorous requirement for bacterial growth and maintenance is approximately $\frac{1}{7}$ to $\frac{1}{5}$ of the nitrogen requirement. Some 4 to 10% of the organic compound is converted to cell mass (McCarty, 1964).

Of the entire consortium of anaerobes, the methanogens are commonly considered to be the most sensitive to toxicity (Kugelman and Chin, 1971; Speece, 1983). Toxic substances influence the kinetics of organic decomposition during anaerobic digestion (McCarty, 1964), the extent of the effect being dependent on the nature and concentration of the toxic substance (Parkin and Owen, 1986).

Sodium, potassium, calcium and magnesium at concentrations of 8, 12, 8 and 3 g ℓ^{-1} respectively are shown to be toxic to methanogens (McCarty, 1964). The degree of toxicity was not quantified. Kugelman and Chin (1971) ran continuous experiments using digested sewage sludge to determine the toxicity levels of various cations to methanogens. The toxicity was determined in terms of the utilisation rate of the organic source relative to a control containing no toxic substances. The utilisation rate of the organic compound was reduced by 50% in the presence of sodium, ammonium, potassium, calcium, and magnesium at concentrations of 7.32, 4.50, 5.85, 4.40 and 1.94 g ℓ^{-1} respectively. Direct comparison of the work of McCarty (1964) and Kugelman and Chin (1965) is not possible since McCarty (1964) did not detail the nature of the toxicity measurements.

Lawrence and McCarty (1965) and Lawrence et al. (1966) demonstrated that the toxicity of metals could be overcome by their precipitation as metal sulphides. This was shown by adding sodium sulphide to the media, as a precipitating agent. To investigate the effect of soluble metals on the process, sodium sulphide was excluded from the medium and the metals were added as chlorides. The results were compared to a control, which contained no metals. At a copper concentration of 0.397 g ℓ^{-1} there was 76% decrease in gas production in the absence of sodium sulphide. For 0.409 g ℓ^{-1} zinc and 0.367 g ℓ^{-1} nickel in the absence of sulphide, the decrease in gas production was 95% and 78% respectively. In the presence of sodium sulphide, the metals formed precipitates and no effect on the

production of gas was observed. From this they deduced that the metals in solution are toxic to methanogens but do not impose any toxic effect in the precipitated form.

Batch tests were carried out by Mosey et al. (1971, 1975) to ascertain factors which affect the toxicity of metals. They defined toxicity as a 20% drop in gas production over 24 h relative to a control system. It was found that pH, and the availability of soluble sulphide affected the extent of metal toxicity. By increasing the pH from 6.1 to 8.0, the soluble sulphide concentration increased and an increase in metal tolerance was observed as the metals were precipitated. The amount of copper tolerated by the bacterial system increased by 73%, while the zinc, nickel and cadmium tolerated increased by 31, 59 and 82% respectively. The metals were rendered harmless when enough soluble sulphide was present for their precipitation. According to their results the order of toxicity was copper > lead > cadmium > zinc.

The distribution of metals in a continuous anaerobic bacterial system at a ten day retention time was studied by Hayes and Theis (1978). Experiments were performed in continuous reactors at a ten day retention time. For the metals studied (nickel, copper, chromium, lead and zinc), they found less than 1% of the metals in solution extracellularly, between 30 and 60% located intracellularly and the remainder precipitated. The toxic limits (defined as the concentration at which total gas production was reduced to 70% of that in the control system) determined were: nickel 0.030 g ℓ^{-1}, copper 0.070 g ℓ^{-1}, lead 0.34 g ℓ^{-1}, chromium^{3+} 0.26 g ℓ^{-1}, chromium^{6+} 0.42 g ℓ^{-1} and zinc 0.6 g ℓ^{-1}. The reported order of toxicity was found to be: nickel > copper > chromium^{3+} > lead > chromium^{6+} > zinc. This is consistent with the trend reported by Mosey et al. (1971). Subsequent work by Hickey et al. (1989) confirmed the same trend. They found the relative toxicity to be: copper > cadmium > zinc. Using a batch system in which toxicity was defined as a 50% inhibition of gas production after 24 h, copper was toxic at 0.075 g ℓ^{-1}, cadmium at 0.1375 g ℓ^{-1} and zinc at 0.325 g ℓ^{-1}. A consistent order of toxicity, of nickel > copper > zinc was also reported by Tijero et al. (1991), based on a continuous study with a retention time of 40 d in which toxicity was defined as a 50% decrease in gas production.

Table 9.2 compares the level of toxicity of compounds reported by independent researchers. Clearly the order of toxicity of the metals is similar even though the concentrations tolerated vary as a result of the toxicity definition. Furthermore it is apparent that the calcium, potassium, magnesium and sodium concentrations tolerated are much higher than the tolerance limits of the metals Cd, Cu, Cr, Ni, Pb and Zn.

Table 9.2: Comparison of toxicity limits of different metals on activity of methanogens as reported in independent works

Concentration ($g\ \ell^{-1}$)	McCarty (1964)	Kugelman and Chin (1971)	Lawrence and McCarty (1965)	Hayes and Theis (1978)	Mulder (1984)	Hickey et al. (1989)	Tijero et al. (1991)
Criteria for Toxicity	Not defined	Not defined	Not defined	70%↓ gas*	Not defined	50%↓ gas*	50% ↓ gas*
Calcium	8.00	4.40	-	-	-	-	-
Potassium	12.00	5.85	-	-	-	-	-
Magnesium	3.00	1.94	-	-	-	-	-
Sodium	8.00	7.32	-	-	-	-	-
Sulphide	0.20	-	-	-	0.70	-	-
Cadmium	-	-	-	-	-	0.14	-
Copper	-	-	0.39	0.07	-	0.08	0.04
Chromium	-	-		0.42	-	-	-
Nickel	-	-	0.37	0.03	-	-	0.01
Lead	-	-	-	0.34	-	-	-
Zinc	-	-	0.41	0.60	-	0.33	0.45

*70%, 50% ↓ indicates that toxicity was determined by a 70% or 50% decrease in gas production relative to the control

The specific mechanism of heavy metal toxicity has not been fully understood. Interference in transmembrane potentials, electron transport chains and substrate translocation systems due to disruption of metabolic enzyme activities or changes in ionic gradients have been implicated. Several enzymes such as alcohol dehydrogenase and coenzyme A are susceptible to inactivation by metals, which react with the enzymic sulfydryl (-SH) groups (Mosey et al., 1975). The reversibility of metal toxicity is indicative of competitive inhibition. Hence it is the dissociated metal in solution that exhibits toxicity. Research into the toxicity of metals to methanogenesis has highlighted their precipitation as metal sulphides to reduce their toxicity.

9.3. A FEASIBILITY STUDY OF THREE COMPLEX CARBON SOURCES, BASED ON AVAILABILITY, COST AND ENVIRONMENTAL IMPACT

9.3.1. Introduction

The provision of adequate sources of carbon and electron donors for the cost effective and efficient implementation of biological sulphate reduction for treatment of acid mine drainage

is frequently suggested as a limitation of this process. To investigate the development of a framework with which to assess the feasibility of provision of sufficient affordable carbon source for the treatment of AMD streams within South Africa, 3 potential carbon sources are reviewed. These 3 sources are applied across 3 sites of application representing typical AMD streams in South Africa. The quality of AMD from typical SA sites is illustrated in Table 9.3. In developing the framework for the evaluation of potential carbon sources, this study addresses their availability, impact on final water quality and disposal requirements, process complexity and process economics. The study is simulation based, utilising the simulation framework described in WRC project K5/1080 and extended in Chapter 6 of this report.

Table 9.3: Composition of sample AMD streams in South Africa.

	South Africa[1]			
	Site 1 Gold	Site 2 Gold	Site 3 Coal	Site 4 Coal
pH	6.3	2.4	2.6	2.2
TDS	4200	38448	11512	4490
Sulphate	2200	22556	8122	3947
Sodium	291	77	1893	129
Potassium	12.9	-	-	-
Iron	187	6674	-	-
Zinc	-	12	-	-
Copper	-	2	-	-
Nickel	3	13	-	-
Aluminium	-	-	-	-
Manganese	-	5	-	-

All units in mg ℓ^{-1} except pH

9.3.2. Composition of Organic Substrates

Based on their availability in southern Africa, three carbon sources have been selected for evaluation as electron donors for AMD treatment: ethanol, molasses and primary sewage sludge (PSS). Typically, industrial low grade ethanol is 92% ethanol by mass. The composition of the cane molasses was estimated to have a sugar content of 48.3% of which 91.5% was considered fermentable (The United States Sugar Corporation 2004). The predominant sugar is the disaccharide sucrose. The composition for primary municipal

sludge given by Eastman and Ferguson (1981) was used for this project and consists of mole percentages of 52% proteins, 25.4% carbohydrates, 5.4% lipids and 17.4% acetate. This has been confirmed by Ristow (1999) who showed that PSS has a 33.45% undegradable fraction (Ristow et al., 2004).

*9.3.3. **The Process Flowsheet and Methodology***

A schematic diagram of the process used in this study is presented as Figure 9.2. The process consisted of: three holding tanks for AMD storage, substrate storage, and hydrochloric acid; two continuously stirred reactors, one anaerobic and one aerobic; a mixer for pH alteration; and two settlers, one to separate biomass and the other to remove sulphur from the system. Although the simplified flowsheet was not fully optimised, it provided a useful comparison of the carbon substrates. It was assumed that metals in the AMD stream (stream 1) were precipitated prior to entering the treatment system analysed here. A portion of the sulphide formed in the process was used to achieve this. The metal sulphide precipitation is not included in the analysis; rather the required sulphide leaves the system analysed as H_2S for further processing to precipitate incoming metals. The fraction of sulphide reporting to the gaseous phase for subsequent metal precipitation and that converted to sulphur can be manipulated through manipulation of the pH attained in the mixer. The mass balance was performed using a basis of 1 000 m^3 AMD day^{-1} as feed into the system with an influent sulphate concentration of 1 833 mg ℓ^{-1} (selected from field data gathered in SA). The quantity of organic substrate used was calculated to produce a water quality meeting EPA (Environmental Protection Agency, 2000) standards of 250 mg ℓ^{-1} sulphate.

Anaerobic Reactor Specification

A simple anaerobic CSTR was used to compare the organic substrates. The operating conditions were set at standard temperature and pressure of 25^{o}C and 1 atm and pH 7.4. This pH represents the mid point of the optimum pH range for sulphate reduction of pH 7.0-7.8 reported by Visser (1995). The reactor volume of 10 000 m^3 gave a residence time of 10 days, chosen to accommodate the slow rates of reaction for hydrolysis. The effect of residence time as a function of the carbon source was considered further in the sensitivity analysis.

The mass balance, equilibrium calculations and development of the simulation model were based on the work of Knobel and Lewis (Knobel 1999, Knobel and Lewis 2002, Hansford et al. 2004), extended to include ethanol as a substrate. Stoichiometric equations used in the

model can be found in Appendix A3. The simulation model was developed in MATLAB. The protocol to solve the mass balance of the reactor is presented in Figure 9.3. The equations and constants used in the model are presented in Appendix A3. Further detail is presented in Gopal (2005).

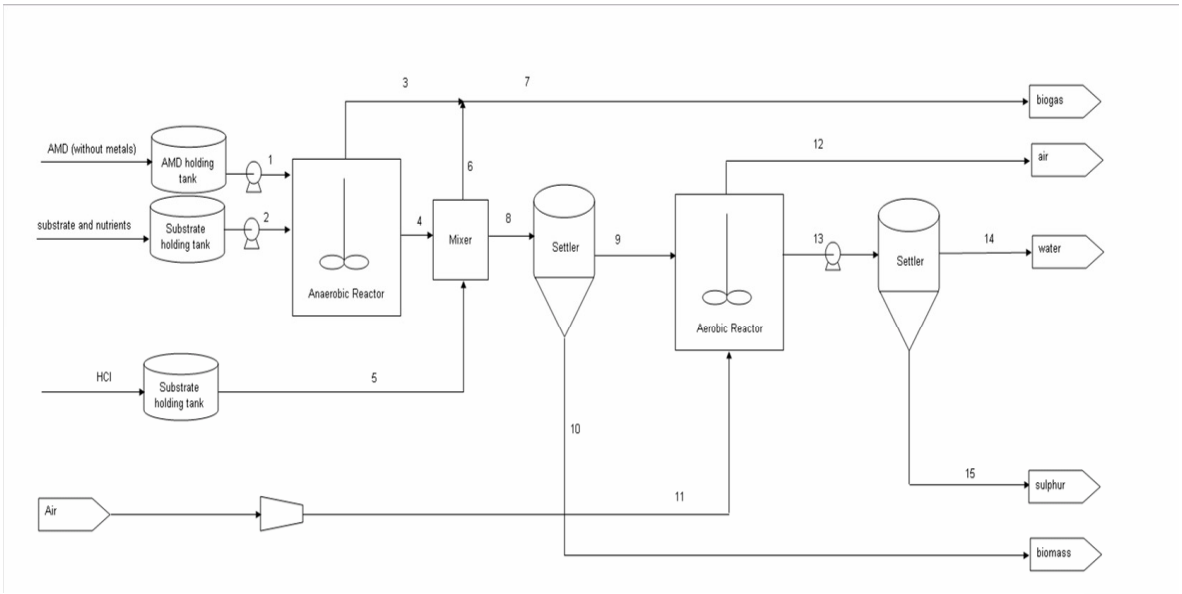

Figure 9.2: A schematic representation of the biological treatment process used in this study

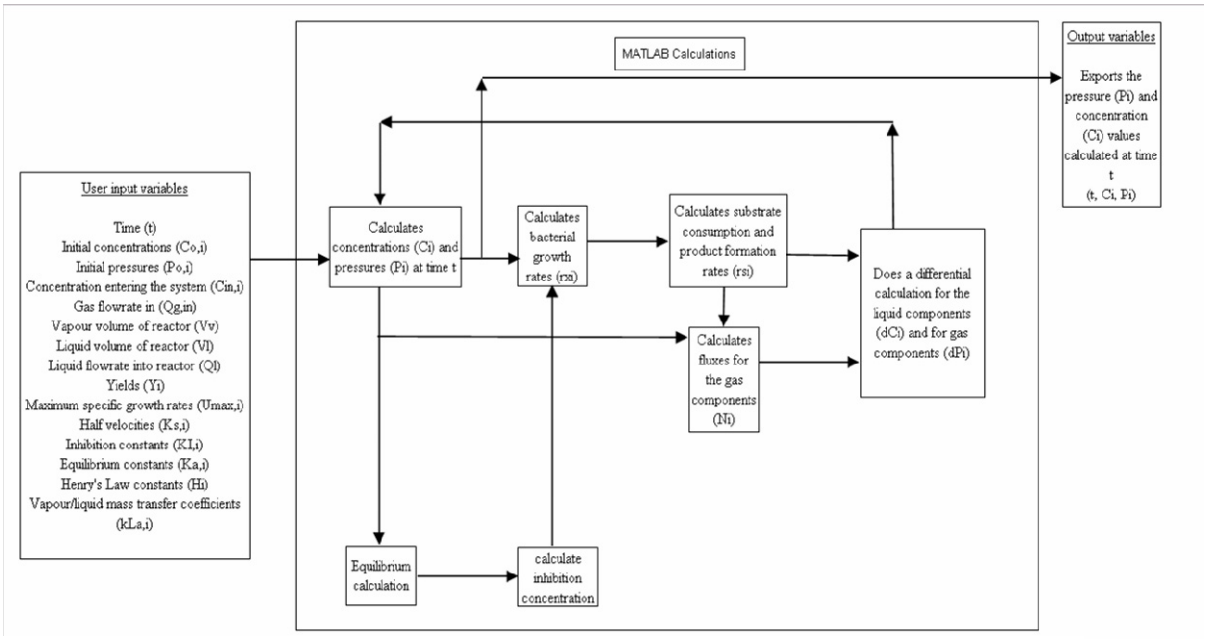

Figure 9.3: Schematic representation of the calculations performed by MATLAB

Aerobic Reactor

Following the anaerobic reactor, an aerobic reactor was included to convert the excess sulphide not required for metal precipitation. This insoluble sulphur can be removed easily from the system through solid-liquid separation. Again, a simple CSTR was employed.

The desired reaction for the production of elemental sulphur is:

$$2\ H_2S + O_2 \rightarrow 2\ S^o + 2\ H_2O \qquad 9\text{-}8$$

As shown in Figure 9.4, this forms the initial step in re-oxidation of sulphide along the oxidation chain to sulphate. Kuhn et al. (1983) suggested that the degree of oxidation depended on the ratio of molecular oxygen and sulphide concentrations. At higher levels of O_2, H_2S is completely oxidised to produce SO_4^{2-}.

$$H_2S \xrightarrow{O_2} S^o \xrightarrow{O_2} S_2O_3^{2-} \xrightarrow{O_2} SO_3^{2-} \xrightarrow{O_2} SO_4^{2-}$$

Figure 9.4: Schematic representation of possible valence states of sulphur in aqueous media (Kuhn et al., 1983)

The operating conditions for the aerobic reactor were standard temperature and pressure of 25°C and 1 atm and pH at 7.05. Chen and Morris (1972) reported the pH maximum of approximately pH 7.00. The oxygen requirement, calculated using Equation 9-8, was supplied by compressed air (21% oxygen). Sublette (1990) found that the composition of the oxygen exiting the reactor was 20.9%. This was confirmed by unpublished data of Searby (2004). It was assumed that the reactor was perfectly mixed, at steady state with a constant liquid flow rate and that Monod bacterial kinetics apply.

Kinetic data were taken from Mamashela (2002) who used a mixed culture of sulphide oxidising bacteria to convert sulphide to elemental sulphur. The values found for K_s, Y and μ_{max} were 2.941 mmole ℓ^{-1}, 0.0512 mmole mmole^{-1} and 1.272 day^{-1} respectively. Setting the exit sulphate concentration at 8.5 mg ℓ^{-1}, a dilution rate of approximately 10 days was calculated. The volume of liquid entering the reactor is approximately 1 000 m^3, hence the calculated tank volume was 10 000 m^3.

Mixer

The mixer was placed between the two reactors to lower the pH of the effluent from pH 7.41 in the anaerobic reactor to pH 7.05 by the addition of hydrochloric acid (stream 5) to achieve optimal conditions for the aerobic reactor. The pH reduction caused some H_2S to enter the gas phase. The H_2S in the gas phase (stream 6) was combined with the biogas produced from the anaerobic reactor (stream 3) and sent to the precipitation unit where it is used to remove metals from the AMD stream. By variation of the pH setpoint, the fraction of S reporting to this stream may be manipulated (not included in this analysis). A species balance was used to calculate the amount of H_2S converted to gas in the mixer. The gas leaving the system was then calculated using the standard flux equation. The mixer was sized as 1 m^3, with a residence time of 86.4 seconds, assuming the change in pH occurs rapidly.

Settling Tanks

Two settling tanks are employed in this system to effect solid-liquid separation. The first settler is placed after the anaerobic reactor to remove the insoluble components (proteins, lipids, carbohydrates and biomass) leaving the anaerobic reactor. The second settler is placed after the aerobic reactor, primarily to remove the sulphur from the effluent stream. Ristow et al. (2004) tested the settleability of PSS following digestion in a sulphate reducing system or methanogenic system. The authors found that 71 and 44% of the particulate COD settled in the methanogenic system and the sulphate reducing system respectively. Janssen et al. (1999) found a removal efficiency of 90% elemental sulphur at a velocity greater than 25 m h^{-1}. Biologically produced sulphur is hydrophilic. The buoyant density of S^o produced by *Chromatium* is reported as 1.22 g cm^{-3} (Janssen et al., 1999). The sizing of the settling tank was based on the approach of Sincero and Sincero (1996).

Sizing of holding tanks

Holding tanks were needed for storage of AMD, carbon substrate and hydrochloric acid. To ensure continuous flow of AMD to the system, the AMD holding tank was set to 6 000 m^3 allowing operation for six days at an inlet flowrate of 1 000 m^3 day^{-1}, should disruption of AMD flow occur. The size of the substrate tank was based on the calculated substrate requirement and the density of the substrate. For PSS, the substrate holding tank volume was sized to hold a three day feed volume. The molasses and ethanol substrate holding tanks were sized to hold a feed volume for 28 days, based on delivery of these substrates to site at least once every four weeks. The hydrochloric acid requirement was always less than 200 kg day^{-1}. Using a density of 1 193 kg m^{-3} for HCl (Sinnott, 2000), and HCl delivery every four weeks, the tank volume was calculated as 4.7 m^3.

9.3.4. Costing

The most accurate estimate of the purchased cost of a piece of major equipment is provided by a current quote from a suitable vendor (Turton et al., 1998). The next best alternative is to use cost data on previously purchased equipment of the same type. Cost estimates for similar units, provided by 2 equipment manufacturers in South Africa were available (Ball and Schroeder, 2001). These differed by less than half a percent and were used as a basis for costing the vessels. Scaling and inflationary effects were taken into account using the method presented in Turton et al. (1998). This method was used to calculate the cost of the holding tanks, reactors, settlers, mixer and pumps. The cost of the compressor was calculated based on the factorial method presented in Sinnott (2000). Following costing of major and ancillary equipment, the remainder of the total capital cost was calculated using costing factors provided in Turton et al. (1998) and Sinnott (2000). These factors are reproduced in Table 9.4.

Sinnott (2000) states that an estimate of the operating cost is needed to judge the viability of the project. In terms of this project, the operating cost is calculated based on an operating performance, which allows the sulphate concentration to be reduced to below EPA standards of 250 mg ℓ^{-1}. The fixed operating costs consist of insurance, patents and royalties, maintenance and repairs as well as labour. These, with the exception of labour, were calculated using the factorial method. Factors defined by Turton et al. (1998) and Sinnott (2000) are given in Table 9.4. Two major variable operating costs not yet considered are the cost of utilities (primarily electricity) and disposal costs. The other major variable operating costs are the cost of the substrate and the hydrochloric acid. The power calculations for the compressor, pumps and the agitator were based on the method of Sinnott (2000). The estimated efficiency of the pump and compressor was 0.65 (Sinnott, 2000).

Table 9.4: Factors Used For Capital and Operating Cost Estimation
(1 – Sinnott (2000), 2 – Turton et al. (1998))

Capital costs	
Factor	Multiplying factor used in economic evaluation
Total Equipment Cost	TEC
Erection, foundations and minor structural work[1]	0.45TEC
Piping and fitting[1]	0.5TEC
Instrumentation[1]	0.15TEC
Electrical power[1]	0.1TEC
Site development[2]	0.1TEC
Process buildings[2]	0.1TEC
Total Physical Plant Cost (PPC)	2.4 TEC
Design and engineering[1]	0.25PPC
Contractors fee[1]	0.05PPC
Contingency[2]	0.2PPC
Total Fixed Capital required (FRC)	1.5 PPC
Cost of Land[2]	0.06FCR
Total Capital (FCI)	1.06FCR
Operating Costs	
Factor	Multiplying factor used in economic evaluation
Total Fixed Capital Investment	FCI
Insurance[2]	0.03FCI
Patents and royalties[1]	0.02FCI
Maintenance and repairs[1]	0.01FCI
Labour	COL
Total Fixed Operating Costs (FOC)	0.06FCI+COL

9.3.5. Results of Study

Impact of carbon source

The nature of the substrate used in BSR affects the amount and quality of biomass sludge produced, the amount and quality of sulphur product formed and final effluent water quality. Table 9.5 summarises the amount of biomass sludge produced (stream 10) as a function of

the carbon source. Further the COD content of the biomass sludge, bacteria present in the sludge and amount of sulphate reporting to the sludge are compared.

The use of a complex carbon source which is not fully biodegradable is seen to increase the quantity of biomass sludge for disposal. Further the COD content of this sludge is increased, as illustrated by the increase from a 28% COD content based on the ethanol process to a 35% COD content based on the PSS process. However, it must be noted that the use of PSS does contribute to the further processing of the waste from the sewage process hence these data must be reviewed in terms of a composite analysis. The sulphate removed with the biomass sludge product is seen to increase with the complexity of the carbon source.

The excess sulphide formed is partially oxidised to elemental sulphur and recovered in its solid form (Figure 9.4, Stream 15). The solid sulphur product reclaimed is described in Table 9.5. The residual COD material in the water stream treated following use of complex carbohydrate as carbon source and electron donor results in a much reduced sulphur content of this sulphur product while approximately the same mass of elemental sulphur is recovered.

The amount of COD found in the final liquid effluent when using ethanol, molasses and PSS as carbon sources are 73, 336 and 803 kg day^{-1} respectively. The use of a complex carbon source, while providing cost benefit and waste burden reduction, results in the increased level of residual COD in the treated effluent. Under the process conditions used in this simulation, the need for subsequent treatment of the water released from the AMD treatment system would be required. However, it is recommended that the reduction of COD concentration in the anaerobic reactor be achieved by decoupling the solids residence time from the hydraulic residence time through solids retention in the reactor.

Table 9.5: Quality of the biomass sludge and elemental sulphur product formed (kg day^{-1})

Carbon source	Ethanol	Molasses	PSS
	Biomass sludge (Stream 10)		
Sludge produced	187	752	9 480
COD in sludge	53	213	3 350
Bacteria	32	62	303
SO_4	0.1	0.2	2
	Sulphur Sludge (Stream 15)		
Total	260	550	3 460
COD	76	157	1220
S^0	29	24	25

Economic considerations

Due to the nature of the calculations, the capital cost for the three plants were similar and hence the operating cost of the system was compared. To place the emphasis of the economics on the comparison of the costs rather than the actual costs calculated, an arbitrary monetary unit (AMU) was introduced.

The operating cost of molasses and ethanol are relatively similar, 86 500 AMU/year and 85 100 AMU/year respectively. PSS was the most expensive option with an operating cost of 135 000 AMU/year when the burden reduction to the wastewater treatment works (i.e. reduction in the sludge disposal requirements of this plant) was not included. This can be attributed to the high waste disposal cost. However, when the burden reduction was taken into account, the operating cost of the PSS-based system was lowest at 54 500 AMU/year.

The disposal costs of the systems using ethanol, molasses and PSS (without burden reduction) are 1 390 AMU/year, 5 560 AMU/year and 70 000 AMU/year respectively. The substrate costs for ethanol and molasses systems are 17 300 AMU /year and 16 600 AMU/year respectively. These results show that the disposal costs dominate the operating costs for complex carbon sources and the substrate costs dominate the operating costs when using simple carbon sources.

Availability of the carbon source

The availability of the carbon source is a major factor in the choice of a carbon feedstock for the biological treatment of AMD. In this study, the potential use of industrial grade ethanol, molasses and PSS were considered. The availability of the carbon source directly affects the

cost of the substrate. This impacts on the operating costs of the treatment system. Examples explaining the effect of the availability of the carbon source are presented.

Ethanol was sourced from a company in Germiston, South Africa. An alternative source for ethanol is located in Durban. The latter sells a higher quality ethanol at a 60% higher cost. Its use resulted in a 55% increase to the substrate cost. This difference in price shows the importance of the location and grade of the carbon feedstock.

A similar argument could be raised for molasses. Molasses is a by-product from the sugar manufacturing industry and one of its major uses is as animal feed. Hence, the price of this product is subject to market related fluctuations.

For PSS to be a sustainable option, a minimum population in the surrounding area would be required. The calculated amount of sludge required was 4 070 kg day^{-1} to treat the 1 000 m^3 AMD day^{-1} containing 1 833 mg SO_4^{2-} ℓ^{-1}. Based on an average production of 73 g of PSS per person per day (National Research Council, 1996), this equates to a population requirement of 55 800 people. Currently most mining towns in South Africa have populations above 150 000 people (Helders, 2004) which is significantly more than the required population thus PSS is a viable option. However, if there were a sudden decrease in the population owing to mine closures or the like, or a sudden increase in sulphate concentration or the volume of AMD requiring treatment, sewage sludge may become inadequate as a carbon source to treat AMD.

Since the treatment of AMD will continue for many years, a continuous supply of the carbon source and electron donor for this duration is required. While purchasing low cost ethanol may seem preferable in the low waste produced, market related fluctuations in the cost of this substrate over time may cause it to be an uneconomical option. Further, following mine closure minimal expenditure on AMD remediation is sought. The most sustainable option of carbon source would be PSS. PSS is available as long as there is a population above the defined threshold in the area. The potential burden relief to wastewater treatment works by using PSS increases the attractiveness of this carbon source.

Sensitivity Analysis

Critical factors considered to affect the overall feasibility of the treatment plants are the hydraulic residence time, disposal costs and sulphate loading. To take into account both capital and operating costs of the system, a 10 year cost analysis was performed. A straight

line depreciation method was used for the major equipment pieces. A salvage value of 10% of the major equipment was assumed.

The effect of hydraulic residence time on the system was analysed at hydraulic residence times (HRT) of 9, 8 and 5 days by lowering the volume of the anaerobic reactor while maintaining the flowrate into the system constant. The primary factors considered in the analysis are the amount of substrate required, amount of sludge disposed, final COD concentration in the effluent and total cost of the system. Table 9.6 presents the amount of each carbon substrate required at the various HRT. The ethanol needed remained constant as ethanol is a simple carbon compound for which a 10 day residence time is not required. The maximum rate of consumption of ethanol was reached at a HRT of less than 5 days. The amount of molasses required remained relatively constant as the HRT decreased to 8 days. Further reduction to 5 days led to an increase in the substrate requirement. Hence, the maximum rate of consumption for molasses lay between HRT of 5 and 8 days. At 5 days residence time, the feed rate exceeded the consumption rate, hence incomplete conversion resulted, increasing the substrate required. The amount of PSS required increased as the HRT decreased. Because of the slow rates of reaction for hydrolysis and the high fraction of non-degradable matter (33.45%), the majority of the PSS remained undegraded. Because of the impact of the amount of sludge being sent to landfill and its effect on the operating cost of the system, this criterion needs to be considered. Table 9.7 shows the amount of sludge disposed to landfill at the various HRT. These trends are complementary to the substrate usage.

Table 9.6: Substrate required as a function of residence times (all results are in kg day^{-1})

Hydraulic residence time	Ethanol	Molasses	PSS
10	557	2 800	4 070
9	557	2 810	4 280
8	557	2 810	4 540
5	557	3 060	6 040

Table 9.7: Sludge disposal as a function of residence times (AMD site 2, results in kg day^{-1})

Hydraulic residence time	Ethanol	Molasses	PSS
10	187	752	9 480
9	187	754	10 100
8	187	756	10 900
5	187	811	15 200

Table 9.8: Effects of decreasing hydraulic residence time on COD concentration in the final effluent (AMD site 2, results in kg day^{-1})

Hydraulic residence time	Ethanol	Molasses	PSS
10	139	393	851
9	139	399	900
8	139	407	978
5	142	526	1 450

Table 9.8 presents the COD of the final effluent (Stream 14). This COD increased as the HRT decreased for all the carbon substrates. The more complex carbon sources produced a higher COD and showed stronger dependence on HRT. All the systems had failed to meet the COD water quality standards of 75 mg ℓ^{-1} (DWAF, 1996) with the current process configuration, further illustrating the need for biomass retention.

Table 9.9 shows the effect of HRT on the 10 year cost analysis. As the HRT decreased, the cost of the systems using ethanol as the carbon substrate decreased. The effect of a 50% reduction in anaerobic reactor volume with ethanol as substrate only resulted in a 1.1% savings. For molasses, a 0.3% savings could be made by using a residence time of 8 days, however using a residence time of 5 days increased the cost by 1.6% over a residence time of 10 days. Using PSS at a residence time of 5 days, the cost increased by 31.1% in comparison with a residence time of 10 days when burden reduction was not taken into account. This increase was only 7.4% when the burden reduction was taken into account, greatly decreasing the sensitivity of systems that utilise PSS as the carbon source. PSS

systems remained the most sensitive of the three carbon sources to changes in the HRT even when the burden reduction potential of PSS was taken into account. These results show that the more complex the carbon source, the higher the HRT required. Further, choosing the correct residence time resulted in minimal cost.

Table 9.9: Percentage change in cost as a function of hydraulic residence time relative to cost at an HRT of 10 days

Hydraulic residence time	Ethanol	Molasses	PSS (no burden reduction)	PSS (with burden reduction)
9	-0.5	-3.6	3.3	0.8
8	-0.6	-0.3	7.4	2.1
5	-1.1	1.6	31.1	7.4

The sensitivity of the analysis to disposal cost was investigated by increasing these by 10, 20 and 50% on comparison to the base-case system of the 10 year cost analysis (Table 9.10). PSS was the most sensitive to an increase in disposal costs when burden reduction was not considered and ethanol the least. When the burden reduction was taken into account, the cost of using PSS as the carbon substrate decreased by 36% when the disposal costs was increased by 50%. The monetary savings achieved through avoiding the need to dispose of PSS generated in the wastewater treatment system through its alternate use in BSR appeared as a negative cost to the system. Since more than half the carbon substrate that entered the system was utilised, an increase in the disposal cost would result in an increase in the burden reduction potential (a higher negative value). In this case, it was shown that the system became more sensitive to disposal costs as the carbon source became more complex.

Table 9.10: Percentage change in cost by increasing the disposal costs

Disposal Increases	Ethanol	Molasses	PSS (no burden reduction)	PSS (with burden reduction)
10%	0.2	0.7	5.3	-7.2
20%	0.3	1.3	10.5	-14.4
50%	0.8	3.3	26.1	-36.0

Table 9.11: Percentage change in cost by increasing the sulphate loading

Sulphate increases	Ethanol	Molasses	PSS (no burden reduction)	PSS (with burden reduction)
10%	3.8	3.7	6.2	-5.4
20%	7.5	7.5	12.4	-11.2
50%	17.9	18.1	30.3	-29.0

The sensitivity of the analysis to sulphate loading was studied by increasing the sulphate concentration entering the system (Stream 1) by 10, 20 and 50% and comparing this to the base-case system using 10 year cost analysis (Table 9.11). On increasing sulphate concentration, an increase in the substrate requirement and sludge disposal resulted. PSS, in the absence of burden reduction, showed the highest sensitivity (30% increase on 50% increase in SO_4^{2-}concentration). Molasses and ethanol showed similar sensitivities (18% increase on 50% increase in SO_4^{2-} increase). When the burden reduction of PSS was taken into account, a reduction of 29.0% in the cost was found on increasing the SO_4^{2-} concentration by 50%. Here the PSS utilised was a negative cost to the system, due to its burden reduction potential. PSS experienced the highest sensitivity to influent sulphate concentration when the burden reduction was taken into account.

Changes to the sulphate loading also affected the COD concentration of the final liquid effluent leaving the system (Table 9.12). As expected, the COD concentration in the final effluent increased as the influent sulphate concentration increased. This was due to the increased COD concentration accompanying the increased substrate concentration required

and this was most marked with complex carbon sources. This will be overcome by use of a reactor with high biomass retention, separating hydraulic and solids residence times.

Table 9.12: Effect of sulphate loading on COD concentration of the final effluent

Sulphate increases	Ethanol	Molasses	PSS
0%	139	393	851
10%	147	426	911
20%	153	461	985
50%	180	564	1 190

9.3.6. Conclusions

This study has provided a framework in which the potential of carbon and electron donors for biological sulphate reduction can be assessed. While the data presented has focussed on the comparative analysis of carbon sources, further discussion of these systems and validation of the approach is presented by Gopal (2005).

In the BSR process, more complex carbon sources produced higher amounts of biomass sludge for disposal. Where biomass and hydraulic residence times are coupled, the use of a complex carbon source, while providing cost benefit, results in the increased residual COD in the treated effluent. Use of biomass retention systems in which hydraulic and biomass retention times are uncoupled is essential.

The economic analysis showed that the operating costs of the system using molasses and ethanol as carbon substrates are relatively similar. Due to the high amounts of solid waste requiring disposal on using PSS, the operating cost of this system proved to be the most expensive where burden reduction for the process generating PSS was not considered. However, when the burden reduction to the wastewater treatment works is taken into account, PSS was the most favoured economically.

If a different complex carbon source was used that did not incur disposal costs, the burden reduction benefit attained by using PSS would fall away. If the carbon source was non-hazardous, then alternate means of disposal could be found (e.g. agricultural uses) and the

cost of disposal removed. It was shown that for complex carbon sources, the disposal costs dominate on the operating costs of the system.

The results show that PSS systems were the most sensitive to process changes. Scenarios considered in the sensitivity analysis have clearly illustrated the benefits of the burden reduction when using PSS, and hence the benefits of PSS as a carbon source in AMD treatment.

10 CONCLUSIONS AND RECOMMENDATIONS

10.1. INTRODUCTION

In this project, the sub-processes of metal precipitation and biological sulphate reduction were studied separately and at a more sophisticated level than previously reported, partly to provide deeper insight and partly to develop more widely applicable system models. In the treatment processes, these unit operations are coupled only by the effect of residual metal concentrations on biological sulphate reduction, the quantity of sulphide (principally in the H_2S form) and carbonate (principally in the bicarbonate form) generated in biological sulphate reduction and the manipulation of the process to provide these anionic species in aqueous or gaseous form. Hence, at this stage, the two reactors have been modelled separately, as unit operations in an integrated system.

The final aspect of the project sought to identify key factors in the application of the technology which may require further fundamental understanding. A significant operating cost associated with SRB systems is the provision of the carbon source and electron donor, hence the use of complex carbon sources has been addressed from a feasibility approach.

10.2. METAL PRECIPITATION

Experimental studies considered the precipitation of the metals zinc, copper and iron from mixed systems containing both sulphide and bicarbonate. The precipitation of zinc from aqueous mixture was dominated by the crystalline ZnS species, hence both an empirical model based on experiments using the synthetic systems and the models based on available software packages are able to predict the precipitation of zinc sulphide from the anaerobic digester overflow accurately.

The iron and copper systems are more complex, characterised by the formation of amorphous metal sulphide precipitates and possibly metal polysulphide complexes. Their formation appears to be a function of the sulphide species available rather than aqueous sulphide concentration. As a consequence of this complexity, copper precipitation was found to occur over a wider range of Cu:S ratios in the anaerobic reactor overflow than in synthetic solution. This was attributed to the buffering capacity of the soluble organic acids and bicarbonate altering sulphide speciation. A similar behaviour was observed for iron;

however, reduced precipitation efficiency in the complex system was found with a maximum of 75% iron removal from the anaerobic reactor overflow being observed.

The inability of the software packages to account for the formation of amorphous or colloidal precipitates, the absence of accurate thermodynamic data on some species and the fact that the predictions are only able to represent the system at thermodynamic equilibrium resulted in their use to provide an accurate model of copper and iron precipitation in both the synthetic system and anaerobic reactor overflow not being feasible. Iron precipitation in the synthetic system was predicted satisfactorily by an empirical third order polynomial.

It is recommended that improved iron precipitation may be attained by the prior oxidation of iron present to ferric iron or the provision of a more reactive alkaline species. Further it is recommended that research be extended to account for the effect of the organic acids on metal precipitation where the system is not governed by the crystalline sulphide species or equilibrium conditions.

10.3. BIOLOGICAL SULPHATE REDUCTION

In WRC project K5/1080, we presented a kinetic model for the sulphidogenic phase of the biological process for AMD treatment, based on use of acetate as the carbon source and electron donor. In this project, we have extended this kinetic approach to consider the use of ethanol as carbon source, as well as to consider inhibition of sulphidogenesis through both elevated sulphate concentrations (substrate inhibition) and sulphide concentrations (product inhibition). While the kinetic inhibition has been considered using a mixed culture approach, the component microbial groups within the mixed culture and their predominance as a function of inhibitor concentration has been addressed through the establishment of fluorescence *in situ* hybridisation techniques within our laboratory. Further, through integration of the kinetic data obtained into the simulation model, we have investigated its improved prediction ability.

Kinetic data for the use of ethanol as carbon source and electron donor has been collected in continuous culture across sulphate feed concentrations of 1 to 15 kg m^{-3} and retention times of 10 to 2 days. The data collected in the range 1 to 12.5 kg m^{-3} has been shown to be described well by the Contois equation developed by Moosa et al. (2002). Over the sulphate feed concentration range 1 to 10 kg m^{-3}, the maximum specific growth rate and death rate k_d remained constant at 0.060 h^{-1} and 0.026 h^{-1} respectively. Sulphate conversion of 81 to 96%

was found under these conditions. At sulphate feed concentrations below 10 kg m^{-3}, incomplete oxidation of ethanol to acetate was dominant.

On increasing sulphate feed concentration to 12.5 kg m^{-3}, the sulphate conversion was reduced to 58%. Further, a decrease in maximum sulphate reduction rate was observed and washout was initiated at a lower dilution rate indicating substrate inhibition. At 15 kg m^{-3} sulphate feed concentration, the process failed confirming substrate inhibition. These findings illustrate the need to utilise recycle in the process design to manipulate the feed sulphate concentration where high sulphate loadings occur in the effluent stream to be treated.

The sulphide species responsible for sulphide inhibition was elucidated by varying solution pH on addition of a constant feed sulphide concentration. Over the pH range 6.0 to 7.5, the speciation of total dissolved sulphide changes from H_2S to HS^-. On increasing the H_2S concentration with decreasing pH from 7.5 to 6.0, the sulphate conversion decreased from 90% to 40%, the maximum sulphate reduction rate decreased from 0.023 to 0.008 kg m^{-3} h^{-1} and the specific growth rate decreased from 0.06 to 0.02 h^{-1}. These results indicate the value of operating the BSR process at a sufficiently high pH to minimise the concentration of undissociated H_2S, thereby reducing inhibition.

The effect of sulphide concentration was investigated by the addition of 0 to 1.25 kg m^{-3} soluble sulphide into the feed. While an aqueous sulphide concentration in solution of 0.73 kg m^{-3} was not inhibitory in the absence of H_2S, an H_2S concentration of 0.23 kg m^{-3} was shown to be inhibitory.

By using the molecular technique FISH and a selection of molecular probes based on literature study, it was shown that SRBs were dominant in the continuous reactors. In chemostat culture using acetate as carbon source and electron donor and a sulphate concentration of 2.5 kg m^{-3}, the dominant bacterial groups were *Desulfonema*, *Desulfobacteriaceae* and *Desulfobulbus*. On increase in soluble sulphide concentration, a decrease in the prevalence of *Desulfonema*, *Desulfobulbus* spp. and *Desulfobactericeae* was observed. *Desulfobacter*, *Desulfotomaculum* and *Desulfovibrionaceae* were little affected by the range of sulphide concentration studied. These studies provide a starting point through which to investigate the desired profile of the microbial population and the potential to remediate reduced performance following process perturbation through manipulation of the microbial population.

The simulation studies conducted illustrated that the kinetic model and data of Moosa et al. (2002, 2004) provided a better prediction of experimental data than kinetic data reported in the literature previously. Further, the temperature dependencies provided by Moosa et al. (2004) extend the applicability of the simulation model. The outputs of the simulation model have illustrated the potential of inappropriate kinetic data to lead to an underestimation of the reactor size. The value of appropriate kinetic data for prediction and optimisation of process performance is demonstrated. Further, process optimisation requires study of reactor configurations incorporating sludge retention.

10.4. COMPLEX CARBON SOURCES FOR AMD TREATMENT

Owing to the significant contribution to the carbon source and electron donor to the process costs of biological AMD treatment, much effort is being expended on the use of complex carbon sources. In this study, we undertook to examine two aspects of this:

- the effect of sulphate, sulphide and other components associated with biological sulphate reduction on the bacterial populations responsible for acidogenesis and acetogenesis in the form of a literature review, and
- a feasibility study investigating the impact of 3 carbon sources of increasing complexity on the cost and outputs of the AMD treatment process.

Literature has reported studies in which the presence of sulphate at concentrations below 4 kg m^{-3} did not impact acidogenesis. Studies at higher sulphate concentrations were not found. Contrary to this, dissolved H_2S was shown to be inhibitory to acidogenesis at concentrations of 0.076 kg m^{-3} and greater. While the pH optimum of acidogenesis of pH 6.0 was reported, an increase in pH to pH 8.0 showed only a 25% decrease in acidogenic activity whereas the process was severely inhibited under acidic conditions. Metal inhibition was observed, although the concentrations at which this inhibition is reported are study specific, represented by a range from 30 to 780 g m^{-3} for cadmium, 56 to 600 g m^{-3} for Ni and 1.8 g m^{-3} for 50% inhibition by copper.

Methanogens are very strict anaerobes, affected by trace oxygen concentration. While specific reports on sulphate inhibition were not found, severe inhibition by the product of sulphidogenesis, dissolved sulphide species, was observed at concentrations of 200 to 700 g m^{-3} and some inhibition at 50 g m^{-3}. The pH window for methanogenesis is reported as pH 6.8 to 7.4 and inhibition by cations and metal ions is reported.

Hence, on use of complex carbon sources for sulphidogenesis, operating conditions need to be selected to maximise the acidogenic process. Inhibition of methanogenesis is desirable.

Further to this, the simulation model developed in K5/1080 was modified for use in comparison of carbon sources for AMD treatment. The simulation was applied to ethanol, molasses and primary sewage sludge. It was shown that sufficient carbon source was attainable. The cost of substrate decreased with increasing complexity; however the quantity of sludge generated increased. Further, the quality of the effluent water decreased and the COD content of the sulphur product increased with carbon sources of increasing complexity. Within the framework of the simple simulation approach used and the continuous reactor without biomass retention, the simple carbon source ethanol appeared most favourable in terms of both cost and effluent quality. However, it is necessary to extend this study to include the effect of the use of a biomass retention system in which hydraulic and solids residence times are decoupled. Further, the settleability of sludge to achieve improved dewatering over that approximated requires attention as does the burden relief in the sewage treatment works on use of a portion of the primary sewage sludge in further processing.

11 REFERENCES

ABR: a software environment for sequence data on World Wide Web URL: http://www.mikro.biologie.tu-meuenchen.de.

APHA (1992) *Standard Methods for the Examination of Water and Wastewater*. 14th edition, American Public Health Association, Washington DC, USA.

ALM EW, OERTHER DB, LARSEN N, STAHL DA AND RASKIN L (1996) The oligonucleotide probe database. *Applied and Environmental Microbiology* **62** 3557-3559.

ALPHANAAR PA, VISSER A and LETTINGA G (1993) The effect of liquid upward velocity and hydraulic retention time on granulation in USAB reactors treating wastewater with high sulphate content. *Bioresource Technology* **43** 249-258.

AMANN RI (1995) *In situ* identification of microorganisms by whole cell hybridisation with rRNA-targeted nucleic acid probes. In: ADL Akkerman, JD van Elsas and FJ de Brujin (eds.), *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Dordrecht, The Netherlands pp 1-15.

AMANN R, BINDER I, OLSEN BJ, CHISHOLM RJ, DEVEREUX SW and STAHL DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. *Applied and Environmental Microbiology* **56** 1919-1925.

AMANN R, SPRINGER N, SCHONHUBER W, LUDWIG W, SCHMIDT EN, MULLER KD and MICHEL R (1997) .Obligate intracellular bacterial parasites of acanthamoebae related to *Chlamydi* spp. *Applied and Environmental Microbiology* **63** 115-121.

AMANN R, STROMLEY J, DEVEREUX R, KEY R and STAHL DA (1992) Molecular and microscopic identification of sulphate-reducing bacteria in multi-species biofilms. *Applied and Environmental Microbiology* **58** 614-623.

ANDREASON K and NIELSON PH (1997) Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. *Applied and Environmental Microbiology* **63** 3662-3668.

ANNANDALE JG, JOVANOVIC NZ, BENADÈ N and TANNER PD (1999) Modelling the long-term effect of irrigation with gypsiferous water on soil and water resources. *Agriculture, Ecosystem and Environment* **76** 109-119.

ANNANDALE JG, JOVANOVIC NZ, PRETORIUS JJB, LORENTZ SA, RETHMAN NFG and TANNER PD (2001) Gypsiferous mine water use in irrigation on rehabilitated open-cast mine land: Crop production, soil water and salt balance. *Ecological Engineering* **17** 153-164.

APPELO CAJ and POSTMA D (1993) *Geochemistry, Groundwater and Pollution.* Balkema, Rotterdam, 536pp.

ARNHEIM N and ERLICH H (1992) Polymerase chain reaction strategy. *Annual Review of Biochemistry* **61** 131-156.

BADE K, MANZ W and SZEWZYK U (2000) Behavior of sulphate reducing bacteria under oligotrophic conditions and oxygen stress in particle-free systems related to drinking water. *FEMS Microbioly. Ecology* **32** 215-223.

BAK F and PFENNIG N (1991a) Microbial sulfate reduction in littoral sediment of Lake Constance. *FEMS Microbioly Ecology* **85** 21-42.

BAK F and PFENNIG N (1991b) Sulfate-reducing bacteria in littoral sediment of Lake Constance. *FEMS Microbioly Ecology* **85** 43-52.

BALL H and SCHROEDER M (2001) Feasibility study on biological treatment of acid mine drainage. Final year project, Department of Chemical Engineering, University of Cape Town, Cape Town, South Africa

BANAT IM (1981) Evidence for coexistence of two distinct functional groups of sulfate reducing in salt marsh sediments. *Applied and Environmental Microbiology* **42** 985-992.

BANAT IM and NEDWELL DB (1983) Mechanisms of turnover of C_2-C_4 fatty acids in high sulphate and low sulphate anaerobic sediments. *FEMS Microbiology Letters* **17** 107-110.

BARNES LJ, SCHEEREN PJM and BUISMAN CJN (1994) Microbial removal of heavy metals and sulphate from contaminated ground-water. In: Means JL and Hinchee RE (Eds), *Emerging Technology for Bioremediation of Metals*. Lewis Publishers, Boca Raton, pp. 38-49.

BARTON LL (Editor) (1995) *Biotechnology Handbooks: Sulphate Reducing Bacteria***,** Plenum Press, New York

BELSER LW (1979) Population ecology of nitrifying bacteria. *Annual Reviews of Microbiology* **33** 309-333.

BHARATHI PAL, SATHE V and CHANDRAMOHAN D (1990) Effect of lead, mercury and cadmium on a sulphate-reducing bacterium. *Environmental Pollution* **67** 361-3774.

BHATTACHARYYA D, SUN G, SUND-HAGELBERG C and SCHWITZGEBEL K (1981) Precipitation of heavy metals with sodium sulphide: bench-scale and full-scale experimental results. *AIChE Symposium Series* **77(209)** 31-38.

BOSHOFF G, DUNCAN J and ROSE PR (2004a) The use of micro-algal biomass as a carbon source for biological sulphate reducing systems. *Water Research* **38** 2659-2666.

BOSHOFF G, DUNCAN J and ROSE PR (2004b) Tannery effluent as a carbon source for biological sulphate reduction. *Water Research* **38** 2651-2658.

BRIGMON RL, BITTON G, ZAM SG and O'BRIAN B (1995) Development and application of a monoclonal antibody against *Thiothrix* spp. *Applied and Environmental Microbiology* **61** 13-20.

BROCK TD (1987) The study of microorganisms in situ: progress and problems. *Symposia of the Society for General Microbioly* **41** 1-17.

BROSIUS J, DULL TL, SLEETER DD and NOLLER HF (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *Journal of Molecular Biology* **148** 107-127.

BUNGAY HR, WHALEN WJ and SANDERS WM (1969) Microrobe techniques for determining diffusivities and respiration rates in microbial slimes. *Biotechnology and Bioengineering* **11** 765-772.

CANFIELD DE and DES MARAIS DJ (1991) Aerobic sulfate reduction in microbial mat. *Science* **251** 1471-1473.

CARY SC, COTTRELL MT, STEIN JL, CAMACHO F and DESBRUYERES D (1997) Molecular identification and localisation of filamentous symbiotic bacteria associated with the hydrothermal vent annelid Alvinella pompejana. *Applied and Environmental Microbiology* **63** 1124-1130.

CASTRO HF, WILLIAMS NH and OGRAM A (2000) Phylogeny of sulphate-reducing bacteria. *FEMS Microbioly Ecology* **31** 1-9.

CHEN K and MORRIS J (1972) Kinetics of oxidation of aqueous sulphide by O_2. *Environmental Science and Technology* **6(6)** 529-537.

CHEN F, GONZALEZ JM, DUCTMAN WA, MORAN MA and HODSON RE (1997) In situ reverse transcriptase, an approach to characterise genetic diversity and activity of prokaryotes. *Applied and Environmental Microbiology* **61** 4074-4082.

CHRISTENSEN H and POULSEN KL (1994) Detection of *Pseudomonas* in soil by rRNA-targeted *in situ* hybridisation technique. *Soil Biology & Biochemistry* **26** 1093-1096.

CLOETE TE and STEYN PL (1987) In: Ramadori R (ed.) *Biological phosphate removal from wastewater.* Pergamon Press, Oxford, England, pp 335-338.

COSTELLO DJ, GREENFIELD PF and LEE PL (1991a) Dynamic Modelling of a single stage high rate anaerobic reactor –I. Model derivation. *Water Resource* **25** 847-858.

COSTELLO DJ, GREENFIELD PF and LEE PL (1991b) Dynamic Modelling of a single stage high rate anaerobic reactor –II. Model Verification. *Water Resource* **25** 859-871.

DABERT P, Sialve B, Delgenes J-P, Moletta R and Godon JJ (2001) Characterization of the microbial 16S rDNA diversity of an aerobic phosphorus-removal ecosystem and monitoring of its transition to nitrate respiration.. *Applied Microbiology and Biotechnology* **55** 500-509.

DALY K, SHARP RJ and MCCARTHY AJ (2000) Development of oligonucleotide probes and PCR primers for detecting phylogenetic subgroups of sulphate –reducing bacteria. *Microbiology* **146** 1693-1705.

DAMGAARD LR and REVSBECH NP (1997) A microscale biosensor for methane containing methanotrohpic bacteria and an internal oxygen reservoir. *Analytical Chemisrty* **69** 2262-2267.

DANNENBERG S, KRODER M, DILLING W and CYPIONKA H (1992) Oxidation of H_2, organic compounds and inorganic sulphur compounds coupled to reduction of O_2 or nitrate by sulphate-reducing bacteria. *Archives of Microbioly* **158** 93-99.

DEAN JG, BOSQUI FL and LANOUETTE KH (1972) Removing heavy metals from waste water. *Environmental Science and Technology* **6** 518-522.

DE BEER D, GLUD A, EPPING E and KUHL M (1997) A fast responding CO_2 microelectrode for profiling in sediments, microbial mats and biofilms. *Limnology and Oceanography* **42** 1590-1600.

DE BEER D, HUISMAN JW, VAN DEN HEUVEL JC and OTTENGRAF SPP (1992) The effects of pH profile in methanogenic aggregates on the kinetics of acetate conversion. *Water Research* **26** 1329-1336.

DE BEER D, SCHRAMM A, SANTEGOEDS CM and KUHL M (1997) A nitrite microsensor for profiling environmental biofilms. *Applied and Environmental Microbiology* **63** 973-977.

DE BEER D, STOODLEY P, ROE F and LEWANDOWSKI Z (1994) Effect of biofilm structures on oxygen distribution and mass transfer. *Biotechnology and Bioengineering* **43** 1131-1138.

DE LOS REYES FL, RITTER W and RASKIN L (1997) Group-specific small-subunit rRNA hybridisation probes to characterise filamentous foaming in activated sludge systems. *Applied and Environmental Microbiology* **63** 1107-1117.

DE LOS REYES MF, DE LOS REYES FL, HERNANDEZ M and RASKIN L (1998) Quantification of *Gordona amarae* strains in foaming activated sludge and anaerobic digester systems with oligonucleotide hybridization probes. *Applied and Environmental Microbiology* **64** 2503-2512.

DELONG EF, WICKHAM GS and PACE NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells. *Science* **243** 1360-1363.

DEVEREUX R, DELANEY M, WIDDEL F and STAHL DA (1989) Natural relationships among sulfate-reducing eubacteria. *Journal of Bacteriology* **171** 6689-6695.

DEVERUEX R, KANE MD, WINFREY J and STAHL DA (1992) Genus- and group-specific hybridisation probes for determinitive and environmental studies of sulphate-reducing bacteria. *Systematic and Applied Microbiology* **15** 601-609.

DEVEREUX R and MUNDFROM GW (1994) A phylogenetic tree of 16S rRNA sequences from sulphate-reducing bacteria in a sandy marine sediment. *Applied and Environmental Microbiology* **60** 3437-3439.

DEVERUEX R, HINES ME and STAHL DA (1996a) S cycling characterization of natural communities of sulfate-reducing bacteria by 16s rRNA sequence comparison. *Microbial Ecology* **32** 283-292.

DEVEREUX R, WINFREY MR, WINFREY J and STAHL DA (1996b) Depth profile of sulphate-reducing bacterial ribosomal RNA and mercury methylation in an estuarine sediment. *FEMS Microbioly Ecology* **20** 23-31.

DINOPOULOU G, STERRITT RM and LESTER JN (1988) Anaerobic acidogenensis of a complex wastewater: II. Kinetics of growth, inhibition, and product formation. *Biotechnology and Bioengineering* **31** 969-978.

DRYDEN SC and KAPLAN S (1990) Localization and structural analysis of the ribosomal RNA operons of *Rhodobacter sphaeroides. Nucleic Acids Research* **18** 7267-7277.

DU PREEZ LA and MAREE JP (1994) Pilot-scale biological sulphate and nitrate removal utililizing producer gas as energy source. *Proceedings of the seventh international symposium on anaerobic digestion, Cape Town, South Africa*. pp 190-204.

DUSENBERY DB (1996) *Life at small scale: the behaviour of microbes*. New York: WH Freeman and Co..

EASTMAN JA and FERGUSON JF (1981) Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *Journal of the Water Pollution Control Federation* **53** 352-366

EBERT A and BRUNE A (1997) Hydrogen concentration profiles at the oxic-anoxic interface: A microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). *Applied and Environmental Microbiology* **63** 4039-4046.

EIKELBOOM DH (1975) Filamentous Organisms Observed in Activated Sludge. *Water Research* **9** 365-388.

ELIOSOV B and ARGAMAN Y (1995) Hydrolysis of particulate organics in activated sludge systems. *Water Research* **29** 155-163.

ENVIRONMENTAL PROTECTION AGENCY (2004) Sulphate in drinking water. [online]. Available from: http://www.epa.gov/safewater/sulfate.html [Accessed 3 February 2004].

ERASMUS CL, (2000) A preliminary investigation of the kinetics of biological sulphate reduction using ethanol as carbon source and electron donor. Masters Thesis, Department of Chemical Engineering, University of Cape Town, Cape Town, South Africa.

ERHART R, BRADFORD D, SEVIOUR RJ, AMANN RI and BLACKALL LL (1997) Development and use of flouresence in situ hybridisation probes for the identification and emuneration of *Microthrix parvicella* in activated sludge. *Systematic and Applied Microbioly* **20** 310-318.

FANG HHP, CHUI HK and LI YY (1994) Microbial structure and activity of UASB granules treating different wastewaters. *Water Science and Technology* **30** 87-96.

FENG D, ALDRICH C and TAN H (2000) Treatment of acid mine water by use of heavy metal precipitation and ion exchange. *Minerals Engineering* **13** 623-642.

FLARDH K, COHEN PS and KJELLEBERG S (1992) Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. Strain CCUG 15956. *Journal of Bacteriology* **174** 6780-6788.

FROST RC (1979) Evaluation of the rate of decrease in the iron content of water pumped from a flooded shaft mine in County Durham, England. *Journal of Hydrology* **40** 101-111.

FUKUI M, TESKE A, ASSMUS B, MUYZER G and WIDDEL F (1999) Physiology, phylogenetic relationships, and ecology of filamentous sulphate-reducing bacteria (genus *Desulfonema*). *Archives of Microbioly* **172** 193-203.

GADD GM and WHITE C (1993) Microbial treatment of metal pollution – a working biotechnology? *Trends in Biotechnology* **11** 353-359.

GHIGLIAZZA R, LODI A and ROVATTI M (2000) Kinetic and process considerations on biological reduction of soluble and scarcely soluble sulphates. *Resources, Conservation and recycling* **29** 181-194.

GIBSON GR (1990) Physiology and ecology of the sulphate-reducing bacteria. *Journal of Applied Bacteriology* **69** 769-797.

GIOVANNONI SJ, MULLINS TD and FIELD KG (1995) In: Joint J (ed.) *Molecular Ecology of Aquatic Microbes.* Springer-Verlag, Berlin, Heidelberg.

GLOCKNER FO, AMANN R, ALFREIDER A, PERNTHALER J, PSENNER R, TREBESIUS K. and SCHLEIFER K-H (1996) An optimised in situ hybridisation protocol for planktonic bacteria. *Systematic and Applied Microbioly* **19** 403-406.

GLOVER HG (1983) Mine water pollution - an overview of problems and control strategies in the United Kingdom. *Water Science and Technology* **15** 59-70.

GOPAL H (2004) An evaluation of three carbon sources for the treatment of acid mine drainage. MSc dissertation, Dept. Chemical Engineering, University of Cape Town.

GRADY CPL (1996) Variability in kinetic parameter estimates: A review of possible causes and a proposed terminology. *Water Research* **30** 724-748.

GRAY ND, HOWARTH R, PICHUP RW, JONES JG and HEAD IM (2000) Use of combined microautoradiography and fluorescence in ssitu hybridisation to determine carbon metabolism in mixed natural communities of uncultured bacteria from the genus *Achromatium. Applied and Environmental Microbiology* **66** 4518-4522.

GROBICKI A and STUCKEY DC (1992) Hydrodynamic characteristics of the anaerobic baffled reactor. *Water Research* **26** 371-378.

GROTENHUIS JTC, SMIT M, PLUGGE CM, YUANSHENG X, VAN LAMMEREN AAM, STAMS AJM and ZEHNDER AJB (1991) Bacteriological composition and structure of granular sludge adapted to different substrates. *Applied and Environmental Microbiology* **57** 1942-1949.

GUIOT SR, PAUSS A and COSTERTON JW (1992) A structured model of the anaerobic granule consortium. *Water Science and Technology* **25** 1-10.

GUJER W and ZEHNDER AJB (1983) Conversion process in anaerobic digestion. *Water Science and Technology* **15** 127-167.

GUSTAFSSON JP (2003) Visual MINTEQ ver. 2.14. Department of Land and Water Resources Engineering, Stockholm, Sweden.

HAMMACK RW, DVORAK DH and EDENBORN HM (1993) The use of biogenic hydrogen sulphide to selectively recover copper and zinc from severely contaminated mine drainage. In: Torma AE, Wey JE and Lakshmanan VL (eds) *Biohydrometallurgical Technologies*. The Minerals, Metals & Materials Society, pp 631-639.

HANSFORD GS, MOOSA S, LEWIS AE, NEMATI M, KOSSEVA M and HARRISON STL (2004) The mechanisms and kinetics of biological treatment of metal-containing effluent. *Water Research Commission Report* No. 1080/1/04.

HARMANDAS NG and KOUTSOUKOS PG (1996) The formation of iron(II) sulphides in aqueous solutions. *Journal of Crystal Growth* **167** 719-724.

HARMSEN HJM, KENGEN HMP, AKKERMANS ADL, STAMS AJM and DE VOS WM (1996a) Detection and localisation of syntrophic proportionate-oxidizing bacteria in granular sludge by *in situ* hybridisation using 16S rRNA-based oligonucleotide probes. *Applied and Environmental Microbiology* **62** 1656-1663.

HARMSEN HJM, AKKERMANS ADL, STAMS AJM and DE VOS WM (1996b) Population dynamics of proportionate-oxidizing bacteria under methanogenic and sulfidogenic conditions in anaerobic granular sludge. *Applied and Environmental Microbiology* **62** 2163-2168.

HARMSEN HJM, PRIEUR D, and JEANTHON C (1997a) Group-specific 16S rRNA-taargeted oligonucleotide probes to identify thermophilic bacteria in marine hydrothermal vents. *Applied and Environmental Microbiology* **63** 4061-4068.

HARMSEN HJM, PRIEUR D, and JEANTHON C (1997b) Distribution of microorganisms in deep-sea hydrothermal vent chimneys investigated by whole-cell hybridisation and enrichment culture of thermophilic subpopulations. *Applied and Environmental Microbiology* **63** 2876-2883.

HARRISON TD (2004) Physico-chemical characteristics of South African Estuaries in relation to the zoogeography of the region. *Estuarine Costal and Shelf Science* **61**, 79-87.

HAYES TD and THEIS TL (1978) The distribution of heavy metals in anaerobic digestion. *Journal of the Water Pollution Control Federation* **50** 61-72.

HECK KL, VAN BELLE G and SIMBERLOFF D (1975) Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. *Ecology* **56** 1459-1461.

HEDIN RS (1997) Passive mine water treatment in the eastern United States. In: Younger PL (ed.) *Minewater Treatment Using Wetlands.* Chartered Institution of Water and Environment Management, London, pp 1-15.

HELDERS S (2004) The World Gazetteer [online]. Leverkusen. Available from: www.world-gazetteer.com [Accessed 12 October 2004].

HESS A, ZARDA B, HAHN D, HAHNER A, STAX D, HOHENER P and ZEYER J (1997) *In situ* analysis of denitrifying toluene- and m-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer. *Applied and Environmental Microbiology* **63** 2136-2141.

HICKEY RF, VANDERWIELEN J and SWITZENBAUM MS (1989) The effect of heavy metals on methane production and hydrogen and carbon monoxide levels during batch anaerobic sludge digestion. *Water Research* **23** 207-218.

HILTON BL and OLESZKIEWIEZ JA (1988) Sulphide-induced inhibition of anaerobic digestion. *Journal of Environmental Engineering* **114** 1377-1391.

HODSON RE, DUSTMAN WA, GARG RP and MORAN MA (1995) *In situ* PCR for visualization of microscale distribution for specific genes and gene products in prokaryotic communities. *Applied and Environmental Microbiology* **61** 4074-4082.

HUGENHOLTZ P, GOEBEL BM and PACE NR (1998) Impact of colture-indipendent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* **18** 4765-4774.

INGVORSEN K, ZEHNDER AJB and JØRGENSEN BB (1984) Kinetics of sulphate and acetate uptake by *Desulfobacter postgatei*. *Applied and Environmental Microbiology* **47** 403-408.

ISA Z, GRUSENMEYER Y and VERSTRAETE W (1986) Sulfate reduction relative to methane production in high-rate anaerobic digestion: Microbiological aspects. *Applied and Environmental Microbiology* **51** 580-587.

ITO T, NIELSEN JL, OKABE S, WATANABE Y and NIELSEN PH (2002a) Phylogenetic identification and substrate uptake patterns of sulphate-reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined

by combining microautoradiography and fluorescent *in situ* hybridisation. *Applied and Environmental Microbiology* **68** 356-364.

ITO T, OKABE S, SATOH H and WATANBE Y (2002b) Succesful development of sulphate reducing bacterial populations and their activities in a wastewater biofilm growing under microaerophilic conditions. *Applied and Environmental Microbiology* **68** 1392-1402.

JANSSEN AJH, LETTINGA G and DE KEIZER A (1999) Removal of hydrogen sulphide from wastewater and waste gases by biological conversion to elemental sulphur: Colloidal and interfacial aspects of biologically produced sulphur particles. *Colloids and Surfaces* **151** 389-397.

JARVIS AP and YOUNGER PL (2000) Broadening the scope of mine water environmental impact assessment – a UK perspective. *Environmental Impact Assessment Review* **20** 85-96.

JENKINS D, RICHARD MG and DAIGGER GT (1993) *Manual on the causes and control of activated sludge bulking and foaming*, Lewis Publishers, Chelsea.

JOHNSON DB (1995) Acidophilic microbial communities: Candidates for bioremediation of acidic mine effluents. *International Biodeterioration and Biodegradation.* Elsevier Science, 41-58.

JONES GJ and SIMON BM (1984) The presence and activity of *Desulfotomaculum* spp. in sulphate-limited freshwater sediments. *FEMS Microbiology Letters* **21** 47-50.

JORGENSEN BB (1982) Mineralization of organic matter in the sea bed: the role of sulfate reduction. *Nature* **296** 643-645.

JORGENSEN BB (1995) Die Mikrowelt der Meerebakterein. *Naturwissenschaften* **82** 269-278.

JORGENSEN BB and BAK F (1991) Pathways and microbiology of thiosulfate transformation and sulfate reduction in marine sediment (Kettegat, Denmark). *Applied and Environmental Microbiology* **57** 847-856.

JURETSCHKO S (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospora*-like bacteria as dominant populations. *Applied and Environmental Microbiology* **64** 3042-3051.

KALMBACH S, MANZ W and SZEWYK U (1997) Isolation of new bacterial species from drinking water biofilms and proof of their *in situ* dominance with highly specific 16S rRNA probes. *Applied and Environmental Microbiology* **63** 4164-4170.

KALYUZHNYI S (1997) Batch anaerobic digestion of glucose and its mathematical modelling. II “Description, verification and application of model. *Bioresource Technology* **59** 249-258.

KALYUZHNYI S and FEDOROVICH V (1998) Mathematical modelling of competition between sulphate reduction and methanogenesis in anaerobic reactors. *Bioresource Technology* **65** 227-242.

KAMPFER P, ERHART R, BEIMFOHR C, BOHRINGER J, WAGNER M and AMANN R (1996) Characterization of bacterial communities from activated sludge: culture dependent numerical identification versus in situ identification using group- and genus-specific RRNA targeted oligonucleotide probes. *Microbial Ecology* **32** 101-121.

KANE MD, POULSEN LK and STAHL DA (1993) Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridisation probes designed from environmentally derived 16S rRNA sequences. *Applied and Environmental Microbiology* **59** 682-686.

KARHADKAR PP, AUDIC JM, FAUP GM and KHANNA P (1987) Sulphide and sulphate inhibition of methanogenesis. *Water Research* **21** 1061-1066.

KARNER M and FUHRMAN JA (1997) Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiograph 13, 197-207.y, and nucleoid staining. *Applied and Environmental Microbiology* **63** 1208-1213.

KELLY MG (1988) *Mining and the freshwater environment*. Elsevier Applied Science, London.

KEMP PF, LEE S and LAROCHE J (1993) Estimating the growth rate of slowly growing marine bacteria from RNA content. *Applied and Environmental Microbiology* **59** 1594-2601.

KLIMANT I, KUHL M, GLUD RN and HOIST G (1997) Optical measurement of oxygen and temperature in microscale: strategies and biological applications. *Sensors and Actuators* **B38-39** 29-37.

KNOBEL A (1999) A mathematical model of a high sulphate wastewater, anaerobic treatment system, MSc dissertation, Department of Chemical Engineering, University of Cape Town, Cape Town, South Africa.

KNOBEL A and LEWIS AE (2001) A mathematical model of a high sulphate wastewater anaerobic treatment system, *Water Research* **36** 257-265.

KOCH AL (1996) What size should a bacterium be? A question of scale. *Annual Reviews of Microbiology* **50** 317-348.

KONISHI Y, YOSHIDA N and ASAI S (1996) Desorption of hydrogen sulphide during batch growth of the sulphate-reducing bacterium *Desulfovibrio Desulphuricans*. *Biotechnology Progress* **12** 322-330.

KOPCZYNSKI ED, BATESON MM and WARD DM (1994) Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Applied and Environmental Microbiology* **60** 746-748.

KREKELER D, SIGALEVICH P, TESKE A, CYPIONKA H and COHEN Y (1997) A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov. *Archives of Microbiology* **167** 369-375.

KUGELMAN IJ and CHIN KK (1971) Toxicity, synergism and the antagonism in anaerobic waste treatment processes. In: Pohland FG (ed.), *Anaerobic Biological Treatment Processes*, **105**, American Chemical Society Advances in Chemistry Series, 55-90.

KUHL M and JORGENSEN BB (1992) Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. *Applied and Environmental Microbiology* **58** 1164-1174.

KUHL M and REVSBECH NP (1998) Microsensors for the study of interfacial biogeochemical processes. In: Boudreau BP and Jorgensen BB (eds), *The benthic boundary leyer*. Oxford University Press.

KUHN A, KELSALL G and CHANAM (1983) A review of the oxidation of aqueous sulphide solutions. *Journal of Chemical Technology and Biotechnology*, 33

LACOUR S, VAN HILLE RP, PETERSON K and LEWIS AE (2005) Comparison of simulators for process and aqueous chemistry modelling. *AIChE Journal* **51** 2358-2368.

LANOIL BD and GIOVANNONI SJ (1997) Identification of bacterial cells by chromosomal painting. *Applied and Environmental Microbiology* **63** 1118-1123.

LARSEN KH, KJAER T and REVSBECH NP (1997) A micro-scale NO_3- Biosensor for environmental application. *Analytical Chemistr.* **69** 3527-3521

LAWRENCE AWM and MCCARTY PL (1965) The role of sulfide in preventing heavy metal toxicity in anaerobic treatment. *Journal of the Water Poluution Control Federation* **37** 392-406.

LAWRENCE AW, MCCARTY PL and GUERUN FJA (1966) The effects of sulfides on anaerobic treatment. *International Journal of Air and Water Pollution* **10** 207-221.

LEBARON P, CATALA P, FAJON C, JOUX F, BAUDART J and BERNARD L (1997) A new, sensitive, whole-cell hybridisation technique for detection of bacteria involving a biotinylated oligonucleotide probe targeting rRNA and tyramide signal amplification. *Applied and Environmental Microbiology* **63** 3274-3278.

LECLERC M, DELBES C, MOLETTA R and GODON J (2001) Single strand conformation polymorphism monitoring of 16S rDNA Archaea during start-up of an anaerobic digester. *FEMS Microbiology. Ecology* **34** 213-220.

LEE N, NIELSEN PH, ANDREASEN KH, JURETSCHKO S, NIELSEN JL, SCHLEIFER KH and WAGNER M (1999) Combination of fluorescence *in situ* hybridisation abd microautoradiography- a new tool for structure-function analyses in microbial ecology. *Applied and Environmental Microbiology* **65** 1289-1297.

LENS PNL, DE BEER D, CRONENBERG C, OTTENGRAF S and VERSTRAETE W (1995) The use of microsensors to determine population distribution in UASB aggregates. *Water Science and Technology* **31** 273-80.

LENS PNL, DE BEER D, CRONENBERG CCH, HOUWEN FP, OTEENGRAF SPP and VERSTRAETE WH (1993) Heterogeneous distribution of microbial activity in methanogenic aggregates: pH and glucose microprofiles. *Applied and Environmental Microbiology* **59** 3803-3815.

LETTINGA G (1995) Anaerobic digestion and wastewater treatment systems. *Antonie Leeuwenhoek* **67** 3-28.

LI J.-H, PURDY KJ, TAKII S and HAYASHI H (1999) Seasonal changes in ribosomal RNA of sulphate-reducing-bacteria and sulphate reducing activity in a freshwater lake sediment. *FEMS Microbiology. Ecology* **28** 31-39.

LIESACK W, WEYLAND H and STACKENBRANDT E (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microbial Ecology* **21** 191-198.

LIU W-T, LINNING KD, NAKAMURA K, MINO T, MATSUO T and FORNEY LJ (2000) Microbial community changes in biological phosphate-removal systems on altering sludge phosphorus content. *Microbiology.* **146** 1099-1107.

LIU WT, MARSH TL, CHENG H and FORNEY LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **63** 4516-4522.

LOY A, HORN M and WAGNER M (2003) probeBase- an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Research* **31** 514-516.

LUDWIG W, STRUNK O, KLUGBAUER S, KLUGBAUER N, WEIZENEGGER M, NEUMAIER J, BACHLEITNER M and SCHLEIFER K-H (1998) Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19** 554-568.

MACLEOD FA, GUIOT SR and COSTERTON JW (1990) Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. *Applied and Environmental Microbiology* **56** 1598-1607.

MAIDAK BL, OLSEN GJ, LARSEN N, OVERBEEK R, MCCAUGHEY MJ and WOESE CR (1997) The RDP (ribosomal database project). *Nucleic Acids Research* **25** 109-110.

MAILLACHERUVU KY, PARKIN GF, PENG CY, KUO W, OONGE ZI and LEBDUSCHKA V (1993) Sulphide toxicity in anaerobic systems fed sulphate and various organics. *Water Environment Research* **65** 100-109.

MAILLACHERUVU KY and PARKIN GF (1996) Kinetics of growth, Substrate utilisation and sulphide toxicity for propionate, acetate, and hydrogen utilises in anaerobic systems. *Water Environment Research* **68**, 1099-1106.

MAMASHELA M (2002) Biological Sulphide Oxidation to Elemental Sulfur using Mixed Culture. MSc thesis, Department of Chemical Engineering, University of Cape Town, Cape Town, South Africa.

MANZ W, AMANN R, LUDWIG W, WAGNER M and SCHLEIFER KH (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Systematic and Applied Microbiology* **15** 593-600.

MANZ W, WAGNER M, AMANN R and SCHLEIFER K-H (1994) In situ characterization of the microbial consortia active in two waste water treatment plants. *Water Research* **28** 1715-1723.

MANZ W, EISENBRECHER M, NEU TR and SZEWZYK U (1998) Abundance and spatial organization of Gram-negative sulphate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiology Ecology* **25** 43-61.

MAREE JP and STRYDOM WF (1985) Biological sulphate removal from a packed bed reactor. *Water Research* **19** 1101-1106.

MAREE JP, GERBER A and HILL E (1987) An integrated process for biological treatment of sulphate-containing industrial effluents. *Journal of Water Pollution Control Federation*. **12** 1069-1074.

MAREE JP and HILL E (1989) Biological removal of sulphate from industrial effluents and concomitant production of sulphur. *Water Science and Technology* **21** 265-276.

MAREE JP, HULSE G, DODS D and SCHUTTE CE (1991) Pilot plant studies on biological sulphate removal from industrial effluent. *Water Science and Technology*. **23** 1293-1300.

MAREE JP, GREBEN HA and M DE BEER (2004) Treatment of acid and sulphate-rich effluents in an integrated biological/chemical process. *Water SA* **30** 183-189.

MARSDEN DD (1986) The current limited impact of Witwatersrand gold mine residues on water pollution in the Vaal River system. *Journal of the South African Institute of Mining and Metallurgy* **86** 481-504.

MCCARTNEY DM and OLESZKIEWICZ JA (1991) Sulphide inhibition of anaerobic degradation of lactate and acetate. *Water Research* **25** 203-209.

MCCARTNEY DM. and OLESZKIEWICZ JA (1993) Competition between methanogens and sulphate reducers: effect of COD: Sulphate ratio and acclimation. *Water Environment Research* **65** 655-664.

MCCARTY PL (1964) *Anaerobic waste treatment fundamentals*. *Public Works*.

MEYERS H and MONTGOMERY DC (1995) *Response surface methodology: Process and product optimization using designed experiments*. Wiley, New York.

MINZ D, FISHBAIN S, GREEN SJ, MUYZER G, COHEN Y, RITTMANN BE and STAHL DA (1999) Unexpected population distribution in a microbial mat community: ulfate-redusing bacteria localised to the highly oxic chemocline in contrast to a eukaryotic preference for anoxia. *Applied and Environmental Microbiology* **65** 4659-4665.

MINZ D, FLAX JL, GREEN SJ, MUYZER G, COHEN Y, WAGNER M, RITTMANN BE and STAHL DA (1999) Diversity of sulfate-reducing bacteria in oxic and anoxic regions of microbial mat characterised by comparative analysis of dissimilatory sulfite reductase genes. *Applied and Environmental Microbiology* **65** 4666-4671.

MOBARRY BK, WAGNER M, URBAIN V, RITTMAN B and STAHL D (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology* **62** 2156-2162.

MOLWANTWA JB, WHITTINGTON-JONES KJ and PD ROSE (2004) An investigation into the mechanism underlying enhanced hydrolysis of complex carbon in a biosulphidogenic recycling sludge bed reactor (RSBR) *Water SA* **30** 150-154.

MOOSA S (2000) A kinetic study on anaerobic sulphate reduction- Effect of sulphate and temperature. PhD Thesis, University of Cape Town.

MOOSA S, NEMATI M and HARRISON STL (2002) A kinetic study on anaerobic sulphate reduction, Part I: Effect of sulphate concentration. *Chemical Engineering Science* **57** 2773–2780.

MOOSA S; NEMATI M. and HARRISON STL (2004) A kinetic study on anaerobic reduction of sulphate. Part II: Incorporation of temperature effects in the kinetic model, Chemical Engineering Science, (in press).

MORGAN B, HEARNE G, LAHAV A AND LOEWENTHAL RE (2005) A one-step ambient temperature ferrite process for treatment of acid mine drainage waters, WRC CONTRACT NO. K5/1244/0/1

MOSEY FE (1983) Mathematical modelling of the anaerobic digeston process: regulatory mechanisms for the formation of short-chain volatile acids from glucose. *Water Science Technology* **15** 209-232.

MOSEY FE, SWANWICK JD and HUGHES DA (1971) Factors affecting the availability of heavy metals to inhibit anaerobic digestion. *Journal of the Water Pollution Control Federation* **6** 668-680.

MULDER A (1984) The effects of high sulphate concentrations on the methane fermentation of wastewater. *Progress in Industrial Microbiology* **20** 133-143.

NAICKER K, CUKROWSKA E and MCCARTHY TS (2003) Acid mine drainage arising from gold mining activity in Johannesburg, South Africa and environs. *Environmental Pollution* **122** 29-40.

NATIONAL RESEARCH COUNCIL. (1996) Use of Reclaimed Water and Sludge in Food Crop Production. National Academy Press, Washington D.C. [online] available from: http://books.nap.edu/books/0309054796/html/R1.html#pagetop [Accessed 20 October 2004].

NIELSEN AT, LIU W-T, FILIPE C, GRADY L, MOLIN S and STAHL DA (1999) Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. *Applied and Environmental Microbiology* **65** 1251-1258.

NIELSEN PH, AQUINO DE MURO M. and NIELSEN JL (2000) Studies on the *in situ* physiology of *Thiothrix* spp. in activated sludge. *Environmental Microbiology* **2** 389-398.

O' FLAHERTY V, LENS P, LEAHY B and COLLERAN E (1997) Long-term competition between sulphate-reducing and methane-producing bacteria during full-scale anaerobic treatment of citric acid production wastewater. *Water Research* **32** 815-825.

O' FLAHERTY V, MAHONY T, O'KENNEDY R and COLLERAN M (1998) Effect of pH on growth kinetics and sulphidde toxicity thresholds of a range of methanogenic, syntrophic and sulphate-reducing bacteria. *Process Biochemistry* **33** 1-15.

O' FLAHERTY V and COLLERAN E (1998) Effect of sulphate addition on volatile fatty acid and ethanol degradation in an anaerobic hybrid reactor. I: process disturbance and remediation. *Bioresource Technology* **68** 101-107.

O' FLAHERTY V and COLLERAN E (2000) Sulphur problems in anaerobic digestion. In: Lens PNL and Hulshoff Pol L (eds), *Environmental Technologies to Treat Sulphur Pollution, Principles and Engineering.* IWA Publishing, London.

OKABE S, NIELSON PH and CHARACKLIS WG (1992) Factors affecting microbial sulphate reduction by *Desulfovibrio Desulphuricans* in continuous culture: Limiting nutrients and sulphide concentration. *Biotechnology and Bioengineering* **40** 725-734.

OKABE S, NIELSON PH, JONES WL and CHARACKLIS WG (1995) Sulphide product inhibition of *Desulfovibrio desulphuricans* in batch and continuous cultures. *Water Research* **29** 571-578.

OKABE S, ITOH T, SATOH H and WATANABE Y (1999) Analyses of spatial distribution of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. *Applied and Environmental Microbiology* **65** 5107-5116.

OLI SYSTEMS, INC. (2000) OLI Systems Aqueous Speciation Software. OLI Systems, Inc., Morris Plains, New Jersey, USA.

OMIL F, LENS P, HULSHOFF POL L and LETTINGA G (1998) Long-term competition between sulphate reducing methanogenic bacteria in UASB reactors treating volatile fatty acids. *Biotechnology and Bioengineering* **57** 676-685.

O' ROURKE JT (1968) Kinetics of anaerobic treatment at reduced temperatures, PhD thesis, Stanford University, Stanford, California

OUDE ELFERINK SJWH, BOSCHKER HTS and STAMS AJM (1998a) Identification of sulfate reducers and *Syntrophobacter* sp. in anaerobic granular sludge by fatty-acid biomarkers and 16S rRNA probing. *Geomicrobiology* **15** 3-17.

OUDE ELFERINK SJWH, VORSTMAN WJC, SOPJES A and STAMS AJM (1998b) Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating paper mill wastewater. *FEMS Microbiology Ecology* **27** 185-194.

OUVERNEY C and FUHRMAN JA (1997) Increase in fluorescence intensity of 16S rRNA *in situ* Hybridisation in natural samples treated with chloramphenicol. *Applied and Environmental Microbiology* **63** 2735-2740.

PARKIN GF and OWEN WF (1986) Fundamentals of anaerobic digestion of wastewater sludges. *Journal of Environmental Engineering* **112** 867-920.

PATTRICK RAD, MOSSELMANS JFW, CHARNOCK JM, ENGLAND KER, HELZ GR, GARNER CD and VAUGHN DJ (1997) The structure of amorphous copper sulphide precipitates: An X-ray absorption study. *Geochimica et Cosmochimica Acta* **61** 2023-2036.

PLATEN H, TEMMAS A and SCHINK B (1990) Anaerobic degradation of acetone by *Desulfococcus biacutus* sp. Nov. *Archives of Microbiology* **154** 355-361.

PORTER KG and FEIG YS (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* **25** 943-948.

POSTGATE JR (1984) *The Sulphate-Reducing Bacteria*, Cambridge University Press, UK.

POULSEN LK, BALLARD G and STAHL DA (1993) Use of rRNA fluorescence *in situ* hybridisation for measuring the activity of single cells in young and established biofilms. *Applied and Environmental Microbiology* **59** 1354-1360.

PULLES W, HOWIE D, OTTO D and EASTON J (1995) A Manual on Mine Water Treatment and Management Practices in South Africa. *Water Research Commission*, Report No. TT 80/96.

PULLES W, VAN NIEKERK A, WOOD A, BATCHELOR A, DILL S, DU PLESSIS P, HOWIE D and CASEY T (2001) Pilot-scale development of integrated passive water treatment systems for mine effluent streams. *Water Research Commision Report 700/1/01*.

PULLES, W., ROSE, P., COETSER, L. and HEATH, R. (2003). Development of integrated passive water treatment systems for the treatment of mine waters. *Proceedings of the 6th International Conference on Acid Rock Drainage* 801-808.

PURDY KJ (1997) The use of the 16S rRNA-targeted oligonucleotide probes to study the ecology of sulphate-reducing bacteria. Ph.D. Thesis, University of Essex, Colchester, Essex.

PURDY KJ, NEDWELL DB, EMBLEY TM and TAKII S (1997) Use of 16S rRNA-targeted oligonucleotide probes to investigate the occurrence and selection on sulfate-reducing bacteria in response to nutrient addition to sediment slurry microcosms from a Japanese estuary. *FEMS Microbiology Ecol*ogy **24** 221-234.

PURDY KJ, NEDWELL DB, EMBLEY TM and TAKII S (2001) Use of the 16S rRNA-targeted oligonucleotide probes to investigate the distribution of sulphate-reducing bacteria in eustarine sediments. *FEMS Microbiology Ecology* **36** 165-168.

PURDY KJ, MUNSON MA, CRESSWELL-MAYNARD T, NEDWELL DB and EMBLEY TM (2003) Use of the 16S rRNA-targeted oligonucleotide probes to investigate function and phylogeny of sulphate-reducing bacteria and methanogenic archaea a UK estuary. *FEMS Microbiology Ecology* **44** 361-371.

RABUS R, FUKUI M, WILKES H and WIDDEL F (1996) Degradative capacities and 16S rRNA-targeted whole cell hybridisation of sulphate-reducing bacteria in an anaerobic enrichment culture utilising alkylbenzenes from crude oil. *Applied and Environmental Microbiology* **62** 3605-3613.

RAMSING NB, KUHL M and JORGENSEN BB (1993) Distribution of sulphate-reducing bacteria, O_2 and H_2S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. *Applied and Environmental Microbiology* **59** 3840-3846.

RASKIN L, POULSEN LK, NOGUERA DR, RITTMANN BE and STAHL DA (1994) Quantification of Methanogenic Groups in Anaerobic Biological Reactors using Oligonucleotide Probe Hybridizations. *Applied and Environmental Microbiology* **60** 1241-1248.

RASKIN L, RITTMANN BE and STAHL DA (1996) Competition and coexistence of sulphate-reducing and methanogenic populations in anaerobic biofilms. *Applied and. Environmental Microbiology* **62** 3847-3857.

RAVENSCHLAG K, SAHM K, KNOBLAUCH C, JORGENSEN BB and AMANN R (2000) Community structure, cellular rRNA content and activity of sulphate-reducing bacteria in marine Arctic sediments. *Applied and Environmental Microbiology* **66** 3592-3602.

REIN NB, FOURIE HJ, GUNTHER P, NKOSI F AND SCHULTZ CE (2005) Sustainable treatment of acid mine drainage at Anglo Coal's Landau Colliery, Witbank South Africa using Paques Thiopaq® technology. *Proceedings of the 16th International Biohydrometallurgy Symposium* 455-461.

REIS MAM, ALMEIDA JS, LEMOS PC and CARRONDO MJT (1992) Effect of hydrogen sulphide on growth of sulphate reducing bacteria. *Biotechnology and Bioengineering* **40** 593-600.

REVSBECH NP and JORGENSEN BB (1986) Microelectrodes: their use in microbial ecology. *Advances in Microbial Ecology* **9** 293-352.

RISATTI JB, CAPMAN WC and STAHL DA (1994) Community structure of a microbial mat: the phylogenetic dimension. *Proceedings of the National Academy of Sciences of the United States of America* **91** 10173-10177.

RISTOW N (1999) The modelling of a falling sludge bed reactor using AQUASIM, Masters Thesis, Department of Chemical Engineering, University of Cape Town, Cape Town, South Africa.

RISTOW N, SOTEMANN SW, LOEWENTHAL RE, WENTZEL MC and EKAMA GA (2004) Hydrolysis of Primary Sewage Sludge under Methanogenic, Acidogenic and Sulfate-reducing Conditions. *Water Research Commission Report* 1216/1/04.

ROONEY-VARGA JN, DEVERUEX R, EVANS RS and HINES ME (1997) Seasonal changes in the relative abundance of uncultivated sulfate-reducing bacteria in a salt marsh sediment and in rhizosphere of *Spartina alterniflora*. *Applied and Environmental Microbiology* **63** 3895-3901.

ROSE PD, BOSHOFF GA, and MOLIPANE NP (2002) Salinity, Sanitation and Sustainability Vol 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 3: Mine Drainage Wastewaters - The ASPAM Model (Report 6), *Water Research Commission Report* TT 192/02, Technol. 21 265-276.

ROSSELLO-MORA RA, WAGNER M, AMANN R and SCHLEIFER K.-H (1995) The abundance of *Zoogloea ramigera* in sewage treatment plants. *Applied and Environmental Microbiology* **61** 702-707.

SAHM K, MACGREGOR BJ, JORGENSEN BB and STAHL DA (1999) Sulfate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridisation in a coastal marine sediment. *Environmental Microbiology* **1** 65-74.

SANTEGOEDS CM, DAMGAARD LR, HESSELINK G, ZOPFI J, LENS P, MUYZER G and DE BEER D (1999) Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. *Applied and Environmental Microbiology* **65** 4618-4629.

SANTEGOEDS CM, FERDELMAN TG, MUYZER G and DE BEER D (1998) Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. *Applied and Environmental Microbiology* **64** 3731-3739.

SANTOS S, MACHADO R, CORREIA MJN and CARVALHO JR (2004) Treatment of acid mining waters. *Minerals Engineering* **17** 225-232.

SCHAECHTER M, MAALOE O and KJELDGAARD NO (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. *Journal of General Microbiology* **19** 592-606.

SCHMID M, TWACHTMANN U, KLEIN M, STROUS M, JURETSCHKO S, JETTEM MSM, METZGER JW, SCHLEIFER K-H and WAGNER M (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Systematic and Applied Microbiology* **23** 93-106.

SCHOEMAN JJ and STEYN A (2001) Investigation into alternative water treatment technologies for the treatment of underground mine water discharged by Grootvlei Propriety Mines Ltd into the Blesbokspruit in South Africa. *Desalination* **133** 13-30.

SCHONHUBER W, FUCHS B, JURETSCHKO S and AMANN R (1997) Improved sensitivity of whole-cell hybrisdization by the combination of horseradish peroxidase-labelled oligonucleotides and tyramide signal amplification. *Applied and Environmental Microbiology* **63** 3268-3273.

SCHRAMM A, LARSEN LH, REVSBECK NP, RAMSING NB AMANN R and SCHAEFFER K-H (1996) Structure and fucnction og nitrifying biofilms as determined by in situ hybridisation and the use of microelectrodes. *Applied and Environmental Microbiology* **62** 4641-4647.

SCOTT R (1995) Flooding of Central and East Rand gold mines: An investigation into controls over the inflow rate, water quality and the predicted impacts of flooded mines. *Water Research Commission Report* No. 486/1/95, 238pp.

SEKIGUCHI Y, KAMAGATE Y, NAKAMURA K, OHASHI A and HARADA H (1999) Fluorescence in situ hybridisation using 16S rRNA-targeted oligonucleotides reveals localisation of methanogens and selected uncultured bacteria in mesophilic and thermophile sludge granules. *Applied and Environmental Microbiology* **65**, 1280-1288.

SHEA D and HELZ GR (1988) The solubility of copper in sulphidic waters: Sulphide and polysulphide complexes in equilibrium with covellite. *Geochimica et Cosmochimica Acta* **52** 1815-1825.

SINCERO AP and SINCERO GA (1996) *Environmental Engineering: A Design Approach*, 1st edition, New Jersey, Prentice-Hall.

SINNOTT R.K. (2000) *Coulson and Richardson's Chemical Engineering*, 3rd Edition, Oxford, Butterworth-Heinemann.

SMITH RL and KLUG MJ (1981) Reduction of sulfur compounds in the sediments of a eutrophic lake basin. *Applied and Environmental Microbiology* **41** 1230-1237.

SNAIDR J, AMANN R, HUBER I, LUDWIG W and SCHLEIFER KH (1997) Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Applied and Environmental Microbiology* **63** 2884-2896.

SPEECE RE (1983) Anaerobic biotechnology for industrial wastewaters. *Environmental Science and Technology* **17** 416A-427A.

SPEECE RE (1996) *Anaerobic biotechnology for industrial waste waters*. Archae Press, USA.

SPEKSNIJDER AG (2001) *Applied and Environmental Microbiology* **67** 469-472.

STACKEBRANDT E and GOEDEL BM (1994) *International Journal of Systematic Bacteriology* **44** 846-849.

STAMATELOU K, LOKSHINA L and LYBERATOS G (2003) Performance of a glucose fed periodic anaerobic baffled reactor under increasing organic loading conditions: 2. Model prediction. *Bioresource Technology* **88** 137-142.

STRUNK O and LUDWIG W (2001) http://www.arb-home.de/.

STUCKI G, HANSELMANN KW and HURZELER RA (1992) Biological sulphuric acid transformation: reactor design and process optimization. *Biotechnology and Bioengineering* **41** 303-315.

STUMM W and MORGAN JJ (1996) *Aquatic Chemistry*. Third edition. John Wiley and Sons, New York.

SUBLETTE KL (1990) Microbial treatment of sour gases for the removal and oxidation of hydrogen sulphide. *Gas Separation and Purification* **4** 91-96.

SZEWZYK R and PFENNING N (1987) Complete oxidation of catechol by the strictly anaerobic sulphate-reducing *Desulfobacterium catecholicum* sp. Nov. *Archives of Microbiology* **147** 162-168.

SZWERINSKI H, GAISER S and BARDTKE D (1985) *Applied Microbiology and Biotechnology* **21** 125-128.

TESKE A, WAWER C, MUYZER G and RAMSING NB (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Applied and Environmental Microbiology* **62** 1405-1415.

TIJERO J, GUARDIOLA E, MIRANDA F and CORTIJO M (1991) Effect of copper, nickel and zinc on an anaerobic digestion system. *Journal of Environmental Scientific Health* **A26: 6** 779-811.

TIPPER JC (1979) *Paleobiology* **5** 423-434.

TOLKER-NIELSEN T, HOLMSTROM K and MILIN S (1997) Visualization of specific gene expression in individual *Salmonella typhimurium* cells by *in situ* PCR. *Applied and Environmental Microbiology* **63** 4196-4203.

TRIANGLE SOLVENTS (2004) Triangle Solvents –Price list", Gauteng, South Africa [online] available from: http://www.trianglesolvent.co.za/ [Accessed 26 June 2004].

TRIMMER M, NEDWELL DB and PURDY KJ (1997) Process measurement and phylogenetic analysis of the sulphate-reducing-bacterial communities of two contrasting benthic sites in the upper eustary of the Great Ouse, Norfolk, UK. *FEMS Microbiology Ecology* **24** 333-342.

TSUKAMOTO TK and MILLER GC (1999) Methanol as a carbon source for microbiological treatment of acid mine drainage. Water Research **33** 1365-1370.

TURTON R, BAILIE RC, WHITING WB and SHAEIWITZ JA (1998) *Analysis, synthesis, and design of chemical processes*, 1st edition, New Jersey, Prentice Hall.

UNITED STATES SUGAR CORPORATION (2004) Molasses Composition, Molasses and Liquid Feeds Division, Clewiston, Florida [online] available from: http://www.suga-lik.com/molasses/composition.html [Accessed on 3 July 2004].

VAN DE PEER Y, JANSEN J, DE RIJK P and DE WACHTER R (1997) Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research* **25** 111-116.

VAN HILLE RP (2001) Biological generation of reactive alkaline species and their application in a sustainable bioprocess for the remediation of acid and metal contaminated wastewaters. PhD thesis, Rhodes University, Grahamstown.

VERSTRAETE W, DE BEER D, PENA M, LETTINGA G and LENS P (1996) Anaerobic bioprocessing of waste. *World Journal of Microbiology and Biotechnology* **12** 221-238.

VISSER A, VAN DE ZEE F, BEEKMAN I, STAMS AJM and LETTINGA G (1993) Anaerobic degradation of volatile fatty acids at different sulphate concentrations. *Applied Microbiology and Biotechnology* **40** 549-556.

VISSER A (1995) The anaerobic treatment of sulphate containing wastewater. Ph.D. thesis. Wageningen Agricultural University, Wageningen, The Netherlands.

VU JCV, NIEDZ PR and YELENOSKY G (1995) Activities of sucrose metabolism enzymes in glycerol-grown suspension cultures of sweet orange (*Citrus sinensis* L. Osbeck). *Environmental and Experimental Botany* **35** 455-459.

WAGNER M, AMANN R, LEMMER H and SCHLEIFER KH (1993) Probing activated sludge with oligonucleotides specific for pretobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Applied and Environmental Microbiology* **59** 1520-1525.

WAGNER M, ARMUS B, HARTMANN A, HUTZLER P and AMANN R (1994) In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy. *Journal of Microscopy* **176** 181-187.

WAGNER M, RATH G, AMANN R, KOOPS HP and SCHLEIFER KH (1995) In situ identification of ammonia-oxidizing bacteria. *Systematic and Applied Microbiology* **18** 251-264.

WAGNER M, ROGER AL, FLAX JL, BRUSSEAU GA and STAHL DA (1998) Phylogeny of dissimilatory sulfite reductase supports an early origin of sulfate respiration. *Journal of Bacteriology* **180** 2975-2982.

WALLNER G, FUCHS B, BEISKER W, SPRING S and AMANN R (1997) Flow cytometric sorting of microorganisms for moledcular analysis. *Applied and Environmental Microbiology* **63** 4223-4331.

WASHINGTON STATE DEPARTMENT OF HEALTH (2001) Hydrogen Sulphide – Fact Sheet", [online]. Environmental Health Programs, Office of Environmental Health Assessments, Washington. Available from: http://www.doh.wa.gov/ehp/oehas/hydrogen%20sulphide.doc [Accessed 10 December 2004].

WAWER C and MUYZER G (1995) Genetic diversity of *Desulfovibrio* sp. in environmental samples analysed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. *Applied and Environmental Microbiology*.**61** 2203-2210.

WEAST RC and ASTLE MJ (eds) (1988) *CRS Handbook of Chemistry and Physics*. CRC Press Incorporated, Boca Raton, Floridda, USA, First Student Edition.

WHITE C and GADD GM (1996) Mixed sulphate-reducing bacterial cultures for bioprecipitation of toxic metals: factorial and response-surface analysis of the effects of dilution rate, sulphate and substrate concentration. *Microbiology* **142** 2197-2205.

WHITE C and GADD GM (2000) Copper accumulation by sulphate-reducing bacterial biofilms. *FEMS Microbiological Letters* **183** 313-318.

WIDDEL F and BAK F (1992) Gram negative mesophilic sulphate-reducing bacteria. In: Balows A., Truper HG, Dworkin M, Harder W and Schleifer K-H (eds), *The Procaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Identification, Application*, Springer Verlag, New York.

WIDDEL F (1988) Microbiology and ecology of sulphate- and sulphur-reducing bacteria. In: Zehnder AJB (ed.), *Biology of anaerobic microorganisms*. John Wiley and Sons, Inc., New York.

WINFREY MR and ZEIKUS JG (1977) Effect of sulphate on carbon and electron flow during microbial methanogenesis in freshwater sediments. *Applied and Environmental Microbiology* **33** 275-281.

WHITTINGTON-JONES KJ, CORBETT CJ and ROSE PD (2002) Salinity, Sanitation and Sustainability Vol 4. The Rhodes BioSURE® Process. Part 2: Enhanced Hydrolysis of Organic Carbon Substrates - Development of the Recycling Sludge Bed Reactor (Report 10) *Water Research Commission Report* Number: TT 196/02.

WOESE CR, KANDLER O and WHEELIS ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America* **87** 4576-4579.

WOOD SC, YOUNGER PL and ROBINS NS (1999) Long-term changes in the quality of polluted water discharges from abandoned underground coal workings in Scotland. *Quarterly Journal of Engineering Geology* **32** 69-79.

YOUNGER PL (1997) The longevity of minewater pollution: a basis for decision making. *The Science of the Total Environment* **194/195** 457-466.

YOUNGER PL (1998) Coalfield abandonment: geochemical processes and hydrochemical products. In: Nicholson K. (ed.) *Energy and the Environment: Geochemistry of fossil, nuclear and renewable resources*. MacGregor Science, pp 1-29.

YOUNGER PL (2000) Predicting temporal changes in total iron concentrations in groundwaters flowing from abandoned deep mines: a first approximation. *Journal of Contaminant Hydrology* **44** 47-69.

YOUNGER PL, BANWART SA and HEDIN RS (2002) *Mine Water: Hydrology, Pollution, Remediation*. Kluwer Academic Publishers, Dordrecht, The Netherlands.

ZEHDNER JB, INGVORSEN K and MARTI T (1982) Microbiology of methane bacteria. In: Hughes DE (ed.) *Anaerobic Digestion,* Elsevier, Amsterdam.

ZEHNDER JB (1988) Environmental and technological aspects. *Biology of Anaerobic Microorganisms*, Wiley Publications, 794-831.

ZEIKUS JG and HENNING DL (1975) *Methanobacterium arboriphilicum* sp.nov. an obligate anaerobe isolated from wetwood of living trees. *Antonie van Leeuwenhoek* **41** 543-552.

ZINDER SH (1984) Microbiology of anaerobic conversion of organic wastes to methane: Recent developments. *ASM News* **50** 294-298.

ZOETERMEYER RJ, VAN DEN HEUVAL JC and COHEN A (1982a) pH influence on acidogenic dissimilation of glucose in an anaerobic digestor. *Water Research* **16** 303-311.

ZOETERMEYER RJ, MATTHIJSEN AJCM, COHEN A and BOELHOUWER C (1982b) Product inhibition in the acid forming stage of the anaerobic digestion process. *Water Research* **16** 633-638.

APPENDIX I

A1.1 TECHNICAL OUTPUTS

The technical outputs of this research have contributed to 4 journal papers published, a further 3 in press, 3 submitted for publication and 1 in preparation (given in Table A1.1), 7 international conference proceedings and 8 national conference presentations (given in Tables A1.2 and A1.3) over the period 2001 to 2004.

Table A1.1 : Journal Papers published or 'In Press': 2001 – 2005

Knobel, A.K and Lewis, A.E. (2001). A mathematical model of a high sulphate wastewater anaerobic treatment system, *Water Research*, 36, 257-265.
Guillard, D., and Lewis, A.E. (2001). Nickel Carbonate precipitation in a fluidised bed reactor: *Industrial and Engineering Chemistry Research,* No 23, Vol 40*,* 5564-5569
Guillard, D., and Lewis, A.E. (2002). Optimisation of Nickel Hydroxycarbonate Precipitation Using a Laboratory Pellet Reactor: *Industrial and Engineering Chemistry Research,* No 13, Vol 41, 3110 – 3114.
Moosa S, Nemati M and Harrison STL (2002). A kinetic study on anaerobic reduction of sulphate, Part I: Effect of sulphate concentration. *Chemical Engineering Science*, 57, 2773
Moosa S, Nemati M and Harrison S T L (2005). A kinetic study on anaerobic reduction of sulphate. Part II: Incorporation of temperature effects in the kinetic model. *Chemical Engineering Science,* 60, 3517-3524
Lacour, S., van Hille, R. and Lewis, A.E.. Comparison of simulators for process and aqueous chemistry modelling (in press)
Petersen, K, Lacour, S. and Lewis, A.E. Copper sulphide precipitation in a fluidised bed reactor (*Chemical Engineering Science,* in press)
Icgen B and Harrison S T L. Use of 16s rRNA-targeted oligonucleotide probes to investigate community structure of anaerobic sulphate bacteria in a continuous bioreactor. (Submitted to *FEMS Microbial Ecology*)
Van Hille, R.P., Lewis, A.E. and Duncan, J.R. Removal and recovery of copper from aqueous solution by non-viable biomass of *Azolla filiculoides,* (submitted to *Water Research*)
Van Hille, R.P., Storey A., Foster, T. and Lewis A.E. Heavy metal precipitation by sulphide and bicarbonate: evaluating methods to predict anaerobic digester overflow performance (submitted to *Minerals Engineering*)
Gopal H and Harrison S.T.L. Evaluation of three carbon sources for the biological treatment of acid mine drainage through process modelling. (In prep for *Minerals Engineering*)
Moosa S and Harrison S T L. The effect of the concentration and speciation of the soluble sulphide product of biological sulphate reduction on process kinetics. (In prep for *Hydrometallurgy*)

Table A1.2: Refereed Conference Proceedings: 2001 – 2005

Harrison S.T.L., Nemati M, Moosa S (2001) A kinetic study on anaerobic sulphate reduction – effect of temperature. Proceedings of International Biohydrometallurgy Symposium (ed. V S T Ciminelli, O Garcio), Part B, pp417

Lewis, A.E., Petersen, K. and Lacour, S. (2002). Copper removal from acid mine drainage using a pellet reactor, 15th Industrial Crystallisation Conference, Sorrento, Italy, 15-18th September 2002

Moosa, S, Nemati, M and Harrison S T L (2002). A kinetic study on anaerobic sulphate reduction – effect of temperature. Bio-& Hydrometallurgy 2002, 13-15 March 2002, Cape Town, South Africa

Swartbooi, A., Lacour, S. and Lewis, A. (2003). An investigation into the precipitation of nickel and cobalt as sulphides, XXII International Minerals Processing Conference, Cape Town, South Africa, 28 Sept – 3 Oct 2003.

Lewis, A.E and van Hille, R. (2003). Metal removal: crystallising some issues, XXII International Minerals Processing Conference, Cape Town, South Africa, 28 Sept – 3 Oct 2003.

Petersen, K., Lewis, A.E., Lacour, S. and van Hille, R. (2003). Copper sulphide precipitation within a fluidised bed reactor, XXII International Minerals Processing Conference, Cape Town, South Africa, 28 Sept – 3 Oct 2003.

Van Hille, R.P., Foster, T., Storey A., Duncan, J. and Lewis A.E. Heavy metal precipitation by sulphide and bicarbonate: evaluating methods to predict anaerobic digester overflow performance. Proceedings of Mine Water 2004: Process Policy and Progress (ed. Jarvis A.P., Dudgeon B.A. and Younger P.L.). Newcastle upon Tyne, 19-23 Sept 2004.

Moosa S and Harrison S.T.L. (2005). Product Inhibition by Sulphide Species on BiologicalSulphate Reduction for the Treatment of Acid Mine Drainage. Proceedings of 16th International Biohydrometallurgy Symposium (IBS2005). In press.

Table A1.3: National Conference Presentations: 2001 – 2004

Petersen, K., and Lewis, A.E. (2001). Removal of metals from acid mine drainage in a fluidised bed reactor. Mineral Processing 2001, Cape Town, 2-3 August 2001
Moosa S, Ball H, Schroeder M and Harrison S T L (2002). Applicable carbon sources for the biological treatment of high sulphate mining effluents: a case study. Minerals Processing 2002, August 2002, Cape Town.
Swartbooi, A., Lacour, S. and Lewis, A. (2002). An investigation into the precipitation of nickel and cobalt as sulphides in a fluidised bed reactor, Mineral Processing 2002, Cape Town, 1-2 August 2002.
Petersen, K., Lewis, A.E., Lacour, S. and van Hille, R., (2002). Copper sulphide precipitation using a fluidised bed reactor, Mineral Processing 2002, Cape Town, 1-2 August 2002.
Van Hille, R., Foster, T., Storey, A and Lewis, A.E. (2003). Heavy metal precipitation by sulphide and bicarbonate: Evaluating methods to predict anaerobic digester overflow performance, South African Chemical Engineering Congress, Sun City, 3 - 5 September 2003
Swartbooi, A., van Hille, R., and Lewis, A.E. (2003). Precipitation of nickel(ii) and cobalt(ii) using hydrogen sulphide gas, South African Chemical Engineering Congress, Sun City, 3 - 5 September 2003
Moosa S, Nemati M, and Harrison S. T. L. (2003). Biological sulphate reduction for treatment of AMD: a kinetic comparison of complete and incomplete oxidisers using acetate and ethanol as carbon sources. Cape Biotech 2003, Cape Town, November 2003.
Icgen B, Moosa S and Harrison S T L (2003). Use of 16S rRNA-targeted oligonucleotide probes to investigate the community structure of anaerobic sulphate-reducing bacteria in continuous bioreactor. Cape Biotech 2003, Cape Town, November 2003.
Icgen B, Moosa S and Harrison S T L (2004). FISH used to understand the population shifts in sulphate reducing consortia upon sulphide loadings. SASM & SASG National Meeting, Stellenbosch, 5 – 7 April 2004.

A1.2 HUMAN RESOURCES

The following personnel have been involved with this project over the period 2001 to 2004:

Senior researchers:	Dr Shehnaaz Moosa (post doc in 2001)
	Dr Rob van Hille (post doc in 2001-2)
	Associate Professor Alison Lewis
	Professor Sue Harrison
Post doctoral researchers:	Dr Shehnaaz Moosa (post doc in 2001)
	Dr Rob van Hille (post doc in 2001-2)
	Dr Stella Lacour (2002)

Dr Bulent Icgen (2003 -2004)

Research assistants:

G Berend:
Tech in-service trainee (ChemEng): July ’00 – June ‘01

K Ntshabele:
Tech in-service trainee (Biotechnol.): July ’01 – Dec’02

T Jacobs:
Tech in-service trainee (Chem Eng): July ’01 – June ’02
Research assistant (part-time BTech: till present)

Post graduate students:

Student	Research Topic	Degree	Supervisor	Date Completed
H Gopal	Selection of carbon sources for the treatment of acid mine drainage by biological sulphate reduction in South Africa	MSc	STLH, SM	2005
M Mamashela	Kinetic studies on biological sulphide oxidation	MSc	STLH	2002
J. Moon	Immobilised cell culture for bacterial sulphate reduction	PhD	STLH	Ongoing
Karen Petersen	Copper sulphide precipitation for treatment of acid mine drainage	MSc	AEL	2002
Vinnie Pillay	Predictive model for metal sulphates in solutions using EXCEL and GPROMS	MSc	AEL	2004
Ashton Swartbooi	Copper and nickel precipitation as sulphides in a fluidized bed reactor	MSc	AEL	Ongoing
M Hove	Metal removal from acid mine drainage	PhD	AEL	Ongoing

Honours level students:

Students	Research Topic	Supervisor	Year
Schroeder and Ball	Applicable carbon sources for the biological treatment of high sulphate mining effluents: a case study	STLH, SM	2001
Storey and Foster	Mixed metal precipitation for treatment of acid mine drainage	AEL	2002

A1.3 CAPACITY BUILDING

The Department of Chemical Engineering has a comprehensive programme for the training and education of engineers at both the undergraduate and postgraduate level. The department has an undergraduate student body of some 300 students, graduating on average 45-50 students per year of whom two thirds are Black and one third women. The postgraduate student body has some 100 students of which 45% are Black and 32% women.

Within this student group, the WRC project has given opportunity for the training of five MSc and two PhD students over its three year duration. Of these, five students were Black and three are woman. Further it has provided Honours level students with opportunity for exposure to research and has provided in service training for three technikon diplomas (two in chemical engineering and one in biotechnology).

The project has provided postdoctoral opportunity for four researchers where cross-disciplinary research has been key. These researchers have included a molecular biologist, microbiologist and two chemical engineers. Through continued involvement as senior researchers, the project is affording opportunity Dr van Hille and Dr Moosa to gain experience as senior researchers.

APPENDIX II

MOLECULAR TOOLS FOR MICROBIAL POPULATION ANALYSIS: REVIEW OF THE LITERATURE

A variety of tools are reported in the literature in which a molecular biological approach is used for analysis of the diversity of the microbial population present. These are reviewed here to provide context for the selection of the tool FISH in this study.

A2.1. FLUORESCENT ANTIBODIES

Identification and quantification of bacteria in wastewater treatment plants is offered by the use of fluorescent antibodies (FA). Production of these antibodies requires the prior isolation of the target organism, restricting the method to culturable micro-organisms. Once available, the FA-technique can be used to identify and quantify defined microorganisms within activated sludge or biofilms. For example *Sphaerotilus natans*, *Acinobacter* sp. (Cloete and Steyn, 1987), and *Thiotrix* sp. were detected by this technique (Brigmon et al., 1995). The specificity of antibodies cannot be adjusted and is usually below the species level. This offers advantage if specific strains should be monitored. On the other hand, the high serological diversity of many microbial genera and species (Belser, 1979; Wagner et al., 1995) requires the simultaneous use of a large number of FA for their detection, rendering the method cumbersome. Further, the large FA molecules do not penetrate through dense extracellular polymeric substances of flocs and biofilms easily and high background fluorescence may be caused by non-specific binding of the FA to detritus (Szwerinski et al., 1985; Wagner et al., 1995) and filament sheaths. The expression of the epitopes detected on the cell surface can vary with changing environmental conditions, causing false-negative results. Despite these limitations, the FA technique has the unique advantage that detection does not impair cell viability; hence antibodies can be used to enrich the target cells via flow cytometry, coated microtiter plates, or magnetic beads for subsequent cultivation.

A2.2. 16S rDNA AND rRNA BASED METHODS

A2.2.1. 16S rDNA and rRNA sequence analysis

The comparative sequence analysis of environmentally retrieved 16S rDNA sequences has become the standard for cultivation-independent assessment of bacterial diversity in natural and engineered systems (Amann et al., 1995). Current 16S rDNA databases contain more than 20 000 entries (Strunk and Ludwig, 2001). These rDNA or rRNA sequences can be obtained from environmental or medical samples without cultivation. This direct retrieval is facilitated by PCR exploiting highly conserved primer binding sites on the 16S and 23S rRNA genes. To facilitate this, commercially available 16S rRNA sequences have increased rapidly in the last decade (Maidak et al., 1997; van de Peer et al., 1997). Based on these sequence collections, rDNA or rRNA-targeted oligonucleotide probes can be designed in a directed way. These probes may be targeted to signature sites of the rRNA molecules characteristic for defined taxonomic entities such as species, genera, families, orders, or even domains, since the rRNA molecules also have conserved signatures that separate the three lines of descent: the archaea, bacteria and eucarya (Woese et al., 1990).

A2.2.2. Modern Techniques for Population Analysis: non-quantitative 16S rDNA library analysis

The availability of 16S rDNA databases provides a high-resolution framework for the assignment of those sequences obtained in 16S rDNA libraries from environmental diversity surveys (Hugenholtz et al., 1998). These libraries are developed through DNA extraction, subsequent PCR amplification of (a fragment of) the 16S rDNA gene using primers targeting regions conserved in the bacterial domain, cloning and sequencing. The sequences obtained are analysed together with adequate reference sequences to infer their phylogenetic affiliation. A meaningful phylogenetic analysis requires the use of almost full-length 16S rDNA sequences and of different treeing methods applied to different data sets (Ludwig et al., 1998).

Due to development of pre-manufactured kits and automated sequencers, the analysis of relatively high clone numbers per library has become possible. It is important to determine whether the number of analysed clones represents the diversity in the established library sufficiently well; however, this is rarely reported in published studies. For this purpose the clones should be grouped into operational taxonomic units (OTUs) according to their 16S rDNA similarities. However, organisms with highly similar or even identical 16S rDNA

sequence may nevertheless represent two different species (Stackebrandt and Goebel, 1994); hence the OTU concept leads to underestimation of the actual species richness. Following assignment of clones to OTUs, rarefaction analyses (Heck et al., 1975; Tipper, 1979) or coverage estimates (Giovannoni et al., 1995) should be performed to determine whether the analysed clone number represents a sufficient sample size.

The refraction analyses and coverage estimates show that most studies do not harvest the diversity of their 16S rDNA libraries adequately. The number of identical or highly similar clone sequences increases with the number of sequenced clones and thus extensive clone sequencing creates highly redundant information. Therefore, screening of the 16S rDNA clones with fingerprinting techniques for selection of different clones for sequence analyses is recommended.

Even if the complete diversity of an environmental 16S rDNA clone library is harvested; the species inventory obtained may not represent the naturally occurring diversity i.e. not all and sometimes even not all numerically important bacterial populations of an environmental sample will be found in a respective 16S rDNA library. This may be caused by inefficient DNA extraction (Juretschko et al., 1998), inadequate coverage of the selected PCR primers, kinetic and stochastic biases introduced by the PCR amplification, and cloning biases. For example the high coverage but low overall OTU number obtained in the 16S rDNA library analysis of activated sludge from a municipal high load wastewater treatment basin (Snaidr et al., 1997) does not necessarily reflect low species richness but was more likely caused by the omission of a dedicated DNA extraction protocol prior to PCR amplification. For establishing a more representative 16S rDNA library, one may use different DNA extraction techniques and combine the isolated nucleic acids prior to PCR amplification, more than one primer set for amplification, and different vectors for cloning. Regarding the PCR conditions best suited for a general diversity survey, extremely stringent annealing conditions should be avoided in order to allow binding of the primers to organisms with nucleotide exchanges in the target position. This procedure may lead to the amplification of undesired PCR products and the necessity to purify the expected PCR product by agarose gel electrophoresis and band excision prior to cloning. Furthermore, the PCR cycle number has a profound influence on the composition of the PCR product if complex template mixtures are used. Highly abundant and less abundant organisms are more likely to be represented in comparable numbers in the PCR product if high cycle numbers are used (an effect caused by the template reannealing at high concentrations). In contrast, the 16S rDNA composition of PCR products generated with low cycle numbers more accurately represents the composition of the 16S rDNA sequences in the isolated nucleic acids.

The 16S rDNA approach can create artificial diversity. Small sequence differences between multiple rRNA operons of a single bacterium, the so-called micro-heterogeneities (Dryden and Kaplan, 1990), might be interpreted after amplification and cloning as microdiversity. Furthermore, the formation of chimeric sequences via *in vitro* recombinations (Liesack et al., 1991; Kopczynski et al., 1994) and the introduction of point mutations via the thermostable DNA polymerase (Arnheim and Erlich, 1992; Spesksnijder et al., 2001) lead to the retrieval of sequences which differ from their natural counterparts. While chimeric sequences composed of distantly related sequence fragments can be recognised easily by standard software tools (Larsen) or comparative phylogenetic analyses of different sequence regions (Juretschko et al., 1998), it is difficult to identify chimera between closely related organisms. Polymerase-induced mutations can only be detected with a certain probability in highly conserved sequence regions by database comparison.

In essence, the 16S rDNA approach described above is an essential tool for microbial diversity analyses in wastewater. However, due to the numerous biases inherent to this approach, quantitative data on the microbial community composition can only be obtained if it is combined with quantitative dot blot or *in situ* hybridisation techniques and must be interpreted with caution.

A2.2.3. Non-quantitative 16S rDNA fingerprinting techniques

16S rDNA-based fingerprinting techniques supplement the 16S rDNA approach. The common principle of these methods is to separate PCR products of the same length but different sequence to visualise the diversity within the amplificate by a banding pattern. Many of these techniques were developed to analyse mutations in medical research and were later on adapted to environmental microbiology. The most frequently applied fingerprinting technique in wastewater microbiology is the denaturing gradient gel electrophoresis (DGGE) (Wawer and Muyzel, 1995; Nielsen et al., 1999; Liu et al., 2000). Terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997), gel retardation (Schmid et al., 2000), and single strand conformation polymorphism (SSCP) (Leclerc et al., 2001; Dabert et al., 2001) have been used successfully. High sample numbers can be processed through fingerprinting techniques in a relatively short time to gain an overview on the dynamics of complex microbial communities in wastewater. DGGE and gel retardation bands of interest can be excised, cloned and sequenced for subsequent identification. DGGE offers a higher resolution compared to gel retardation but, in contrast to the latter method, is limited to fragments smaller than 500 nucleotides which are not well-suited for phylogenetic analyses. The organism represented by a T-RFLP or SSCP band can only be identified if a 16S rDNA

clone library is established, the clones are sequenced for identification and used as reference in the respective fingerprinting protocol. In general, the fingerprinting techniques are affected by the same DNA-extraction and PCR amplification biases as the 16S rDNA approach and thus can not provide quantitative data.

A2.2.4. Quantitative 16S rRNA fluorescent in situ hybridisation (FISH)

In situ identification of individual microbial cells with fluorescently labelled, rRNA-targeted oligonucleotide probes, the so-called phylogenetic stains (DeLong et al., 1989), is based on the high cellular content of usually more than 1000 ribosomes, and consequently as many 16S and 23S rRNA molecules. The FISH method involves application of oligonucleotide probes to permeabilised whole microbial cells. Probes can be designed to specifically target narrow to broad phylogenetic groups (from species to domain) by virtue of variable evolutionary conservation within the 16S rRNA molecule. The major steps in probe design are identifying short regions (usually 15-25 nucleotides in length) in a sequence alignment unique to the target group of interest, centralising mismatches to nontarget organisms (where possible), and modifying the sequence to meet probe design criteria such as minimum melting temperature. The probes enter the cells and specifically hybridise to their complementary target sequence in the ribosomes. If no target sequence is present in the ribosomes of the cells, the probes are unable to hybridise and the unbound probe is removed by a subsequent wash step. Hence only specifically targeted cells retain the probes under appropriate stringency conditions in the hybridisation and wash steps. Probes are typically labelled at the 5' end with fluorochrome reporters such as fluorescein and sulfoindocyanine (Cy3, Cy5) dyes. Microbial cells containing hybridised probes can be directly observed under epifluorescence microscopy owing to the natural amplification of the fluorescent signal by large numbers of ribosomes in any given target cell. An advantage is that multiple probes with varying target specificity can be used in the same preparation providing they are labelled with clearly distinguishable fluorochromes.

The method was first applied using radioactive reporters, which provided only limited microscopic resolution of cells and required an extra step (visualisation by microautoradiography). The first demonstration of FISH method in its modern form was by DeLong and co-workers (1989), using a simple artificial microbial consortium. Subsequently, FISH has been applied in a variety of natural and artificial ecosystems confirming the value of the method (Amann et al., 1995). However, there are also number of limitations associated with the method such as poor cell permeability, ribosome accessibility

and content, and sample autofluorescence. FISH probes have been designed mainly to target 16S rRNAs but 23S rRNA targets are also used (Amann et al., 1995).

There are several interesting technical developments in the area of FISH to increase sensitivity of *in situ* identification of small environmental bacterial cells. Tyramide System Amplification (TSA) combined with horseradish peroxidase labelled oligonucleotides (Schohuber et al., 1997) resulted in an increase of fluorescence of at least one order of magnitude. Similar results were obtained with an indirect approach in which biotin-labeled probes mediated binding of streptavidin-horseradish peroxidase conjugates that subsequently catalysed immobilisation of fluorescein-labelled tyramide (Lebaron et al., 1997). Even though TSA yields very bright fluorescent signals, it should be noted that FISH of whole fixed cells relies on penetration of the probe molecules across the cell periphery to intracellular target molecules. This is more easily achieved with the much smaller fluorescently labelled oligonucleotides than with biotinylated probes, and, consequently, in aqueous samples more cell could be detected with Cy3 (carbocyanine dye)-labelled oligonucleotides (Glockner et al., 1996) than with TSA-based methods (Schohuber et al., 1997). Treatment of aqueous samples with chloramphenicol, an inhibitor of protein synthesis and RNA degradation, was reported to further increase the percentage of cells detectable with a general 16S rRNA-targeted, fluorescent oligonucleotide probe (Ouverney and Fuhrman et al., 1997).

A2.2.5. Chromosomal Painting

Lanoil and Giovannoni (1997) have demonstrated that identification of bacterial cells can also be achieved by chromosomal painting, a method originally developed to identify genetic material on eukaryotic chromosomes. They prepared probes from purified genomic DNA by nick-translation, which resulted in a statistical mixture of fragments with an average length of 50-200 nucleotides. After removal of cross-hybridising fragments even two closely related serotypes of *Salmonella choleraesuis* could be distinguished. By preparing the probes from operons encoding metabolically important functions, they could be used to analyse for defined activities as well as identification purposes (Lanoil and Giovannoni, 1997). *In situ* reverse transcription was shown by Chen et al. (1997) to bear potential for characterisation of genetic diversity and activity of bacteria. This technique relies on the initiation of reverse transcription of rRNA or mRNA at specific primers and the incorporation of labelled nucleotides in the resulting cDNA. Potential disadvantages of this technique are similar to those encountered for *in situ* PCR, a method described by the same group (Hodson et al., 1995). Cells need to be permeabilised for the fairly large polymerase molecules to gain

access and transcription initiation from non-specifically bound primers or internal priming sites may result in background signals. The future will show whether these methods are robust enough to be applied to the *in situ* detection of specific genes and gene products in complex environmental or medical samples.

A2.2.6. Quantitative 16S rRNA –based dot blot

In the dot blot, total RNA is extracted from the environment and is immobilised on a membrane together with dilution series of RNA of reference species. Reference rRNA of uncultured microorganisms can be obtained by *in vitro* transcription of the respective cloned 16S rDNA sequences. Subsequently, the membrane is hybridised with a radioactively labelled probe and after a stringent wash step, the amount of target rRNA is quantified with a Phospho-Imager. Results are expressed as nanograms of target rRNA per weight or volume of the environmental sample. Alternatively, the membrane can be re-hybridised with a bacterial or universal probe and the amount of population-specific rRNA detected with the specific probe is expressed as the fraction of the total (bacterial) rRNA (Raskin et al., 1994). Quantitative dot blot hybridisation measures the abundance of the target population via its rRNA content of a microbial cell. However, as this varies dependent on cellular activity, physiological history (Flardh et al., 1992) and environmental conditions, the data obtained can not be translated to cell numbers, giving a lumped indication of metabolic activity. Furthermore, the method may be biased by varying efficiency of RNA extraction from different microbial populations and degradation of the rRNA (which might affect different probe target sites in a different way) during the procedure. Quantitative dot blot analyses have been used in wastewater microbiology (Rosselo-Mora et al., 1995; Mobarry et al., 1996; de los Reyes et al., 1997, 1998) but are more widely applied on environments like soil and microbial mats (Risatti et al., 1994; Minz et al., 1999) which, due to their high background fluorescence, are not well-suited for FISH.

A2.3. Conclusions

Several studies have achieved *in situ* identification of bacteria not cultured to date based on 16SrRNA sequences directly retrieved from samples as a different as *acanthamoebae* (Amann et al., 1997), the epibiotic microflora of the hydrothermal vent annelid *Alvinella pompejana* (Cary et al., 1997), activated sludge (Snaidr et al., 1997), and soil (Ludwig et al., 1997). Taking into account the vast undiscovered microbial diversity, it is interesting that whole fixed cells, sorted in a flow cytometer on the basis of parameters such as light scatter,

DNA content or probe-conferred fluorescence, could be used for subsequent retrieval of almost full length 16S rRNA sequences (Wallner et al., 1997). Using this technique, defined fractions of the total population can be selected for molecular identification. As soon as 16S or 23S rRNA sequences are available, specific probes can be designed rapidly. With freely available software packages like ARB, probe target sites can be selected and tested against all other available rRNA sequences (Woese et al., 1990; Van de Peer et al., 1997). Successful *in situ* identification also requires permeabilisation of the target cells prior to hybridisation. In the case of the filamentous bacterium *Microthrix parvicella* (a frequent cause of activated sludge bulking and foaming), permeabilisation has proven difficult and ultimately required enzymatic treatment of the Gram-positive cell wall (Erhart et al., 1997).

New group-specific probes have been developed that enlarge the set of group-specific, rRNA-targeted oligonucleotide probes available for a rapid classification of single cells by FISH (e.g. for Gram-positive bacteria linked with activated-sludge foaming (De los Reyes et al., 1997) and for thermophilic bacteria present at deep-sea hydrothermal vent sites (Harmsen et al., 1997a,b)). It is also notable that, on comparing traditional isolation of bacteria with FISH, Hess et al. (1997) demonstrated that hydrocarbon-degrading *Azoarcus* sp. strains isolated from a diesel fuel contaminated laboratory aquifer made up only 1-2% of all bacteria present in this system. Kalmbach et al. (1997) used FISH to identify those strains that are the major constituents in drinking water biofilms from the 234 strains originally isolated from these communities. These two studies clearly show that rDNA and rRNA molecular methods and cultivation-based methods of microbial population analysis are not competing but complementary techniques.

The choice of molecular method used will depend on the application environment and the degree of quantification required. In the application of anaerobic SRB cultures for active treatment of AMD and related effluents, FISH has potential to provide simultaneous quantification of key microbial species or genera.

APPENDIX III

SIMULATION MODEL
FOR ASSESSING FEASIBILITY OF CARBON SOURCES

The stoichiometry and rate constants used in the analysis framework for evaluating carbon sources and electron donors for BSR, detailed in Section 9.3, are given here. Further detail is available in Gopal (2005).

Stoichiometry and rate constants used in the model

Table A3-1: Stoichiometry of Hydrolysis reactions used in the model

$C_5H_7O_2N + H_2O \rightarrow$	$C_5H_9O_3N$
$C_6H_{10}O_5 + H_20 \rightarrow$	$C_6H_{12}O_6$
$C_{51}H_{98}O_6 + 3H_20 \rightarrow$	$CH_2OHCHOHCH_2OH + 3CH_3(CH_2)_{14}COOH$

Table A3-2: Stoichiometry of Fermentation reactions used in the model

Amino Acid Fermentation		
$C_5H_9O_3N + 3H_2O$	$\rightarrow$	$2CH_3COOH + CO_2 + 2H_2 + NH_3$
$C_5H_9O_3N + 3H_2O$	$\rightarrow$	$CH_3CH_2COOH + 2CO_2 + 3H_2 + NH_3$
$C_5H_9O_3N$	$\rightarrow$	$C_5H_7O_2N + H_2O$
Glucose Fermentation		
$3C_6H_{12}O_6 + 2H_2O$	$\rightarrow$	$2CH_3COOH + 2CH_3CHOHCOOH + CH_3CH_2CH_2COOH + 6H_2 + 4CO_2$
$C_6H_{12}O_6 + NH_3$	$\rightarrow$	$C_5H_7O_2N + CO_2 + 2H_2O + 2H_2$
Lactate Fermentation		
$CH_3CHOHCOOH$	$\rightarrow$	$0.5CH_3COOH + 0.5CH_3CH_2COOH + 1.33CO_2$
$5CH_3CHOHCOOH + 3NH_3$	$\rightarrow$	$3C_5H_7O_2N + 9H_2O$
Glycerol Fermentation		
$2CH_2OHCHOHCH_2OH$	$\rightarrow$	$3CH_3COOH + 2H_2$
$CH_2OHCHOHCH_2OH + NH_3$	$\rightarrow$	$C_5H_7O_2N + CO_2 + H_2O + 5H_2$

Table A3-3: Stoichiometry of Long Chain Fatty Acid Beta Oxidation used in the model

$CH_3(CH_2)_{14}COOH + 14H_2O$	$\rightarrow$	$8CH_3COOH + 14H_2$
$5CH_3(CH_2)_{14}COOH + 16NH_3 + 22H_2O$	$\rightarrow$	$16C_5H_7O_2N + 70H_2$

Table A3-4: Stoichiometry of Acetogenesis reactions used in the model

Butyrate Acetogenesis		
$CH_3CH_2CH_2COOH + 2H_2O$	$\rightarrow$	$2CH_3COOH + 2H_2$
$5CH_3CH_2CH_2COOH + 4NH_3$	$\rightarrow$	$4C_5H_7O_2N + 2H_2O + 10H_2$
Propionate Acetogenesis		
$CH_3CH_2COOH + 2H_2O$	$\rightarrow$	$CH_3COOH + CO_2 + 3H_2$
$5CH_3CH_2COOH + 3NH_3$	$\rightarrow$	$3C_5H_7O_2N + 4H_2O + 5H_2$

Table A3-5: Stoichiometry of methanogenesis reactions used in the model

H2 Methanogenesis		
$4H_2 + CO_2$	→	$CH_4 + 2H_2O$
$5CO_2 + 10H_2 + NH_3$	→	$C_5H_7O_2N + 8H_2O$
AcH Methanogenesis		
CH_3COOH	→	$CH_4 + CO_2$
$5CH_3COOH + 2NH_3$	→	$2\ C_5H_7O_2N + 6H_2O$

Table A3-6: Stoichiometry of Sulphate reduction equations used in the model

H2 sulphate reduction		
$4H_2 + H_2SO_4$	→	$H_2S + 4H_2O$
$5CO_2 + 10H_2 + NH_3$	→	$C_5H_7O_2N + 8H_2O$
AcH Sulphate Reduction		
$CH_3COOH + H_2SO_4$	→	$H_2S + 2CO_2 + 2H_2O$
$5CH_3COOH + 2NH_3$	→	$2\ C_5H_7O_2N + 6H_2O$
LaH Sulphate Reduction		
$2CH_3CHOHCOOH + H_2SO_4$	→	$2CH_3COOH + H_2S + 2CO_2 + 2H_2O$
$5CH_3CHOHCOOH + 3NH_3$	→	$3C_5H_7O_2N + 9H_2O$
PrH Sulphate Reduction		
$CH_3CH_2COOH + 0.75H_2SO_4$	→	$0.75H_2S + CH_3COOH + CO_2 + H_2O$
$CH_3CH_2COOH + 3NH_3$	→	$3C_5H_7O_2N + 4H_2O + 5H_2$
BuH Sulphate Reduction		
$2CH_3CH_2CH_2COOH + H_2SO_4$	→	$4CH_3COOH + H_2S$
$5CH_3CH_2CH_2COOH + 3NH_3$	→	$4\ C_5H_7O_2N + 2H_2O + 8.5H_2$
EOH Sulphate reduction		
$C_2H_5OH + H_2SO_4$	→	$H_2S + 2CO_2 + 2H_2 + H_2O$
$5C_2H_5OH + 2NH_3$	→	$2\ C_5H_7O_2N + 5H_2 + H_2O$

Table A3-7: Stoichiometry of acid base reactions

$$HS^- + H^+ \Leftrightarrow H_2S$$
$$S^{2-} + H^+ \Leftrightarrow HS^-$$
$$H_2CO_3 \Leftrightarrow CO_2 + H_2O$$
$$HCO_3^- + H^+ \Leftrightarrow H_2CO_3$$
$$CO_3^{2-} + H^+ \Leftrightarrow HCO_3^-$$
$$HSO_4^- + H^+ \Leftrightarrow H_2SO_4$$
$$SO_4^{2-} + H^+ \Leftrightarrow HSO_4^-$$
$$NH_3 + H^+ \Leftrightarrow NH_4^+$$
$$CH_3COO^- + H^+ \Leftrightarrow CH_3COOH$$
$$CH_3CH_2COO^- + H^+ \Leftrightarrow CH_3CH_2COOH$$
$$CH_3CH_2CH_2COO^- + H^+ \Leftrightarrow CH_3CH_2CH_2COOH$$
$$CH3CHOHCOO^- + H^+ \Leftrightarrow CH3CHOHCOOH$$

Table A3-8: Rate constants used for hydrolysis reactions

Rate constant	Value	Unit	Source
$K_{protein}$	0.09	d-1	O' Rourke, 1968
$k_{carbohydrates}$	0.29	d-1	O' Rourke, 1968
K_{lipids}	0.09	d-1	O' Rourke, 1968

Table A3-9: Rate constants used for simulation model

Reaction	μ_{max}	Y	K_s	$K_{I,H2S}$	$K_{I,VFA}$	K_{SO4}
Units	d^{-1}	mmole/mmole	mmole/l	mmole/l	mmole/l	mmole/l
Amino acid fermentation	1.5 [1]	0.57 [1]	0.153 [1]	3.12 [1]	10 [1]	-
Glucose fermentation	5.124 [2]	0.112 [2]	0.082 [2]	17.19 [1]	10 [3]	-
Lactate fermentation	2.552 [2]	0.1 [2]	1.11 [2]	3.12 [1]	10 [3]	-
Glycerol fermentation	10 [1]	0.4 [1]	0.25 [1]	3.12 [1]	10 [1]	-
Long chain fatty acid beta oxidation	1.2 [4]	0.674 [4]	0.19 [4]	3.12 [1]	10 [1]	-
Butyrate acetogenesis	0.264 [5]	0.04 [5]	1.1 [5]	0.811 [1]	3 [3]	-
Propionate acetogenesis	0.36 [6]	0.03 [6]	0.5 [6]	0.83 [7]	30 [3]	-
Hydrogen sulphate reduction	5 [8]	0.021 [8]	0.0015 [8]	4.65 [7]	100 [1]	0.0093 [8]
Acetate sulphate reduction	0.51 [8]	0.023 [8]	0.375 [8]	4.75 [8]	10 [1]	0.2 [8]
Lacate sulphate reduction	2.5 [9]	0.02 [9]	0.0488 [9]	7.83 [10]	10 [1]	0.00877 [9]
Propionate sulphate reduction	0.81 [8]	0.03 [8]	2.56 [8]	8.89 [8]	10 [1]	0.077 [8]
Butyrate sulphate reduction	0.41 [11]	0.04 [7]	0.309 [7]	15.6 [1]	10 [1]	0.17 [1]
Ethanol sulphate reduction	0.8 [12]	0.02 [12]	0.124 [12]	5.6 [12]	10 [13]	0.124 [12]
Hydrogen methanogenesis	1 [8]	0.002 [8]	0.008125 [8]	20.71 [7]	3 [3]	-
Acetate methanogenesis	0.36 [5]	0.0127 [3]	0.875 [8]	3.65 [7]	10 [3]	-

1 - Knobel, A. and Lewis, A. (2002)
2 - Stamatelatou et al. (2003)
3 - Costello et al. (1991)
4 - Gujer and Zehnder (1983)
5 - Kalyuzhnyi (1997)
6 - Lawerence and McCarty (1969)
7 - Maillacheruvu and Parkin (1996)
8 - Kalyuzhni anf Fedorovich (1998)
9 - Traore (1982)
10 - Okabe (1995)
11 - Schauder (1986)
12 - Erasmus (2000)
13 - Value assumed