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BIOWASTE AS ENERGY SOURCE FOR BIOLOGICAL SULPHATE REMOVAL

By

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Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Water Resouce Management)

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DECLARATION

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

Signature:

Date:

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SUMMARY

Due to mining operations, polluted mine waters are continuously produced. The characteristics of these polluted waters, often referred to as Acid Mine Drainage (AMD), are high concentrations of acidity (low pH), salinity (mainly in the form of sulphate) and metals (e.g. iron, manganese, magnesium, calcium and sodium). From a water management perspective, the treatment of mine effluents is a necessity since water is a scarce commodity in South Africa, due to increasing demands on water resources. Globally, as well as in South Africa, studies are focussed on finding the best possible AMD treatment technologies. Neutralisation of AMD, using limestone and in some instances a combination of limestone and lime can not reduce the sulphate concentration to values < 1500 mg/ℓ, while the stipulation for the sulphate (SO_4^{2-}) concentration is set at 500 mg/ℓ by the Department of Water Affairs and Forestry (South African water quality guidelines).

The biological treatment technology can remove sulphate to concentrations of < 200 mg/ℓ. The disadvantage of the biological treatment process is the need for a carbon and energy source, which is most often not present in AMD and thus needs to be added, resulting in increased operational costs. In current systems ethanol is the preferred electron donor, however, its price is related to the oil price and thus has the tendency to increase. Investigations into identifying a cheaper carbon and energy source are therefore critical. Since grass is often grown around mining operations, it

was thought that further investigations in the use of the degradation products of grass-cellulose were feasible.
It was hypothesized that natural occurring cellulose degrading microorganisms from ruminants (cattle, sheep) could be utilised to hydrolyse and ferment grass cellulose to polymers, monomers, volatile fatty acids and other intermediates, which could be used by the sulphate reducing bacteria (SRB) as the carbon and energy sources for biological sulphate removal. The study presented here shows that the degradation products of cellulose could be used as the carbon and energy source for the biological sulphate removal in mine and other industrial effluents.

Initially, batch operated reactors were used, while later a two and three stage continuous reactor system for a combined fermentation and sulphate removal process were operated. It was shown that cellulose degrading microorganisms produced short chain volatile fatty acids (VFA), such as butyric-, propionic- and acetic acids and other intermediates from grass-cellulose. Sulphate reduction was obtained when these VFAs were subsequently used for biological sulphate removal. For all studies the grass cuttings were collected from the CSIR garden service and stored at 4 ℃ before use. No moisture was observed on the grass cuttings.

The sulphate removal rate, using the VFA produced as the carbon and energy source, was slightly higher than when using sugar as the control carbon source. When the amount of grass cuttings per litre feed water were increased as substrate to the reactors, a direct relationship between grass concentrations and sulphate removal was observed, since the fastest sulphate removal occurred in the reactor containing the highest amount of grass. A residual VFA concentration was observed for the highest concentration grass cuttings (90 gram gass per litre SO_4 rich feed water). This result indicated a positive correlation between grass addition and the subsequent sulphate reduction.

Using microbes from rumen fluid for cellulose fermentation and SRB as the sulphate removers in one reactor, sulphate removal was achieved, even after the addition of extra sulphate loads. In batch experiments it was observed that grass-cellulose was initially faster degraded by SRB, but the cellulose fermentation bacteria from the rumen produced higher propionic acid concentrations, a preferred carbon source for SRB.

After the different batch tests were conducted, the technology was tested in a continuous mode. A continuously fed biological sulphate removal reactor, containing grass cuttings, bacteria obtained from a bovine rumen and SRB was used. This reactor system was fed synthetically prepared sulphate rich water as well as mine water. Sulphate reduction (average of 86% removal efficiency), feeding synthetic sulphate rich water was observed during an experimental period of 77 days, adding fresh grass cuttings (150 g) four times to the reactor. When pre-treated mine water was used as feed water, the highest percentage sulphate removal was 78%. When the feed rate to the reactor was doubled (from 15 to 30 ℓ/d), without increasing the amount of grass cuttings added, the percentage sulphate removal decreased to 55%. These results showed a clear relationship between grass addition and sulphate reduction. When operating a two and three stage reactor system, the results showed that the highest sulphate removal occurred in the first reactor. Thus the fermentation process and sulphate removal was already achieved in a one stage reactor, which made the second and third stage superfluous.

A process description using mass balances was developed on the basis of the results obtained when the first reactor received diluted mine water as feed water. Factors, such as the COD concentration utilised for cell growth were based on theoretical based assumptions. The outcome of the calculations showed that in order to remove 1.5 g/ℓ/d sulphate treating 2000 m^3 mine water per day, a total surface area of 1.1 km^2 is needed to cultivate enough grass under irrigation, using (partly-treated) mine water, to sustain continuous sulphate reduction for one year. Although the described process offers promises for the biological sulphate removal process, it must be kept in mind that the the reactor was operated at 37 ℃ for optimal performance of the rumen associated bacteria. Heating of mine water to elevated temperatures is not cost-effective. Future research should focus on adapting the anaerobic fermentation consortium, originating from rumen fluid and grass cuttings to ambient temperatures in order to make the process competitive. It is envisaged that further development of the technology may result in a viable process, comparable to other South African developed sulphate removal treatment systems.

“ WATER”

“When I was a child, it was hard to get water. We walked for long distances to find water. We fetched water from a water hole. We had to wake up early in the morning to make sure that we were at least first or second at the water hole, otherwise the water hole would be empty. If we were too late and the water hole was already empty, we used to cook and drink run-off rainwater from the roof, although it was rusted. We used to catch run-off rain water from the roof in buckets and drums. If the water hole and the buckets and the drums were empty, we had to walk for even a longer distance to get water from another river and we had to ask permission from those people. We used to wash in the river, which was dangerous, especially for the boys, who loved swimming. We used to get very itchy from the river and it caused bilharzia”

Esther Ntombi Kaba. “*Water and when I was little*”. (translated from isiZulu). Hydropolitics in the Developing World – *A Southern African Perspective* (A. Turton and R. Henwood, eds.), AWIRU, Pretoria, 2002, 269 pp.

Quoted from p.113.

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"I can do what you can't do, and you can do what I can't do; together we can do great things."
- Mother Teresa of Calcutta -

In order to write a PhD thesis, extensive research has to be conducted, for which funding is required. I was in the fortunate position to obtain a BioPAD project, together with the University of Stellenbosch. BioPAD was initiated by the Department of Science and Technology to promote research in Biotechnology in South Africa. Our project received just under R 3 million in funding, while the total duration of the project was 2 years. The research focus was to investigate the production of degradation products from cellulosic material, to function as suitable carbon sources for the biological sulphate reduction. Mining is an important contributor to the economy, but results in vast volumes of polluted mine water, which needs to be treated, prior to discharge into rivers and dams. A great deal of research is focussed globally on the treatment of mine water.

The CSIR team concentrated on the operation of batch and continuous reactors, while at the University of Stellenbosch, Professor Alf Botha and Dr. Lydia Joubert and their teams, focussed on the microbiology of the degradation and fermentation of plant biomass. Without the input and support of this team, the CSIR team would not have been able to deliver the same kind of output. I learned the meaning of true collaboration through this BioPAD project. Together we managed to keep on brief, budget and time and delivered good science in the process, part of which is captured in this thesis.

I would like to acknowledge BioPAD, the CSIR, the Stellenbosch team as well as Thrip (for funding postgraduate students) for invaluable financial support. Special thanks go to Richard Eijsberg, who developed the technological description (Chapter 6) of the process and to Ellenore Steyn for analytical assistance. Thanks to the encouragement of my husband Dr. Jan Meint Greben, my colleague Dr. Jannie Maree and ex- Programme Manager, Dr. Johan de Beer, I felt encouraged to tackle this challenging task.

Last but certainly not least, thanks to my promoter, Professor Fanus Venter, who spent many hours going throught the different versions of this thesis.

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LIST OF ABBREVIATIONS

AMD	Acid Mine Drainage
AB	Acetogenic Bacteria
AF	Anaerobic Filter
AMB	Acetate Utilizing Methanogenic Bacteria
ASRB	Acetate Utilizing SRB
ATP	Adenosine Triphosphate
CH_4	Methane
CO_2	Carbon Dioxide
COD	Chemical Oxygen demand
DWAF	Department of Water Affairs and Forestry
EPS	Extracellular Polymeric Substances
FB	Fluidized Bed Reactor
GC	Grass cuttings
HSRB	Hydrogen Utilizing SRB
HMB	Hydrogen Utilizing Methanogenic Bacteria
HRT	Hydraulic Retention Time
IMPI	Integrated Managed Passive Treatment Process technology
MB	Methanogenic Bacteria
MPB	Methane Producing Bacteria
SRB	SRB
SO_4	Sulphate
RB	Rumen Bacteria
RR	Reduction Rate
SEM	Scanning Electron Microscopic
UASB	Upflow Anaerobic Sludge Bed
VSS	Volatile Suspended Solids
WSP	Waste Stabilisation Pond
WW	Waste water

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1 Water demand

Due to the limited annual rainfall, South Africa is considered a semi arid country. For that reason, water has been identified as the country's most limiting natural resource. Due to both the rapidly growing population and its upliftment, the total water demand for agriculture, domestic use, industrialisation and mining has increased rapidly. Estimates of the current patterns of use and anticipated future uses of South Africa's water resources, based on existing patterns of water exploitation, indicate that the demands for water in each sector of the economy will increase. It is estimated that this increase will amount to 111% for the mining industry by the year 2030 (Basson *et al*. 1997). Water demand in several regions of the country has already exceeded the available supplies. These demands are being met by progressively larger water transfers from those catchments where demands have not exceeded supplies and "excess" water is still available (Ashton & Haasbroek, 2000). Consequently the water allocation priorities must be aligned with national development objectives and hence should place greater emphasis on ensuring that scarce water resources are used in such a way that maximum long-term benefits for the country as a whole can be derived (Basson *et al.* 1997; Muller, 2000; Ashton & Haasbroek, 2000). Improved water management in the mining industry could be achieved by pollution prevention, e.g contamination of clean water with pollutants caused by mining operations should at all times be avoided (Pulles, 2006). This can be achieved by preventing the transport of the generated contaminants to the water resource.

Water management efforts should not only be directed at source level, but should focus equally strongly on the re-use of industrial effluent waters. For this reason the treatment and re-use of industrial and mining effluents has become not only a priority but a necessity. The re-use of industrial effluent waters may furthermore have economical benefits. Jovanovic *et al*. (1998, 2002) investigated the use of partially treated mine water for irrigation. Greben *et al.* (2003) showed that treated mine water from a nickel and copper mine in Botswana could potentially be used for the irrigation of citrus crops. It can be envisaged that this form of agriculture will result in additional job creation and consequently in some degree of poverty alleviation, showing that treated mine water, used for irrigation as opposed to non treated mine water, which is stored in decommissioned mines, can benefit the country and its people.

1.1.2 Origin of AMD

The scarcity of water is exacerbated by pollution of the surface- and ground- water resources due to industrial activities such as mining. By its very nature and scale, mining has a marked and visual impact on the environment. Mining is implicated as a significant contributor to water pollution, the prime reason being, that most of the geological formations that are mined contain pyrites which oxidize to form sulphuric acid when exposed to air and water. Due to the weathering of pyrite, sulphate as soluble ferrous irons are released. Metal sulphides other than pyrite will also release soluble ions, such as zinc, copper, lead, nickel and cadmium. The combination of auto-oxidation and microbial sulphur and iron oxidation produces large volumes of sulphuric acid, which is highly corrosive and when discharged into river systems can cause major environmental problems, one of them being the high toxicity level towards aquatic biota. This polluted, often acidic and sulphate rich water is referred to as Acid Mine Drainage (AMD). Sulphate needs to be removed from mining effluents to avoid salination of surface water. The removal of sulphate also reduces the risk of scaling as well as the possibility of biocorrosion of pipes and mining equipment. The present recommended sulphate discharge concentration imposed by the Department of Water Affairs and Forestry (DWAF) is at a concentration lower than 500 mg/ℓ (South African water quality guidelines).

1.1.3 Environmental impact due to coal mining activities in South Africa

While a mine is operational, the act of mining, i.e. sinking of shafts or open pits and the excavation of ore, can have a significant impact on the natural water environment, as mining activities inevitably disrupt pre-existing hydrological pathways (Younger & Wolkersdorfer, 2004). In excess of 200 Mℓ/day of mining effluent is discharged annually into the water bodies of the Gauteng region, which approximately accounts to sulphate loads of 73 000 tonnes/annum, while this contribution is estimated to be 12 000 tonnes/annum in Mpumalanga. Mine water in the Upper Olifants River Catchment in Mpumalanga (upstream of Loskop Dam) is at times discharged, resulting in local acidification and regional salination of surface water resources. Although mine water in the Olifants River Catchment currently amounts to only 4.6% of the total water usage, it contributes 78.4% of the sulphate load. Mine water in the catchment of the Witbank Dam and Middelburg Dam is rich in calcium, magnesium and sulphate and is acidic. When the pH is below 5.5, water can be toxic to plant and fish life and corrosive to pipelines and equipment.

1.1.4 Approaches for the treatment of AMD

Because of the variety of mine waters encountered in nature and because of the familiarity of the mining sector with the physical and chemical processes, necessary to separate metals and water, there is a wide range of conventional treatment methods for mine waters (Younger *et al*. 2002). Mine waters can be treated *chemically* applying lime and limestone neutralization technologies, however the residual sulphate in the form of gypsum ($CaSO_4$) is dependent on the solubility of gypsum, which is about 1500 mg/ℓ as sulphate (SO_4). For removal of sulphate to below this concentration, the *biological* sulphate reduction technology can be applied. In order to achieve biological sulphate reduction, anaerobic conditions, favoured by the SRB and the presence of suitable carbon and energy sources, have to be adhered to. Successful sulphate reduction is typically associated with a pH increase due to the production of sulphide and alkalinity. Therefore, the biological sulphate reduction technology is particularly beneficial to industries experiencing AMD problems, as it results in removal of sulphate, in an increase in the pH of the treated water and often in metal removal. The latter occurs as a result of the formation of sulphides, followed by metal precipitation as metal-sulphides. To avoid incurring high additional treatment costs, the idea of an integrated treatment system was conceived, in which initially the high sulphate load is treated chemically with limestone until the sulphate concentration is reduced to approximately 1500 mg/ℓ. The remaining sulphate concentration can then be treated biologically, with the advantage that less carbon and energy source is required than in the case of a full biological treatment at sulphate concentrations of e.g. 2500 mg/ℓ (Maree *et al.* 2004)

1.1.5 Biological sulphate removal technology

In the presence of sulphate, the SRB utilize organic products as the carbon and energy source, providing electrons, while sulphate is used as the terminal electron acceptor with hydrogen sulphide (H_2S), CO_2, H_2O or HCO_3^- and in some cases acetic acid as the end products (Greben *et al*. 2002). When sugars are used as carbon sources, intermediate products, such as volatile fatty acids (VFA's), e.g., butyrate and propionic acid, as well as ethanol are formed. In a well functioning bioreactor, these products will be subjected to acetogenesis, performed by the acetogenic bacteria (AB), to produce acetic acid. Good results for sulphate removal have recently been obtained using ethanol (De Smul *et al*. 1997, Greben *et al*. 2000a), sucrose (Maree *et al*. 1986; Greben *et al*. 2000b) as well as methanol, both at thermophilic (Weijma *et al*. 1999) and at ambient temperatures (Tsukamoto & Miller, 1999).

1.1.6 Bio-waste Products

Inexpensive but complex carbon sources such as saw dust and sewage sludge (Butlin *et al.* 1949, 1960; Knivett, 1960; Sadana & Morey, 1962; Tuttle *et al.* 1969; Conradie & Grütz, 1973) have also been evaluated. Although good sulphate removal was obtained using these different carbon sources, long retention times of 5-10 days were required. Maree and Strydom (1985) treated mine water with pulp mill effluent and sewage as energy sources.

Recently, the use of other easily available organic waste, in the form of cow manure, grasses, hay, corn stalks etc. has come to the forefront. The study of Coetser *et al.* (2000) evaluated several complex as well as simpler carbon sources for potential use in passive biological removal treatment systems to treat AMD. They found that Kikuyu grass cuttings, silage and hay, together with propionic-butyric- and lactic acids were the preferred carbon sources to give the most effective sulphate reduction, while in their investigation, acetic acid, pyruvate and ethanol did not result in effective sulphate reduction. Studies, executed by Dill *et al.* (2001) showed that when using hay as the carbon and energy source, a 99% SO_4 removal efficiency was obtained, while this was 97.8% when using Kikuyu grass.

1.2 STUDY OBJECTIVES

The objective of the study was to find an appropriate and cost effective treatment method for AMD using alternative and possible cheaper carbon and energy sources for the biological sulphate removal process. The potential use of a bio-waste product is attractive to the biological sulphate removal technology, as the challenge is to develop technologies that economically produce simple sugars and/or fatty acids from complex polymers such as cellulose/lignin. This approach emphasizes the utilisation of a bio-waste product, such as grass cuttings, rather than its treatment thus shifting the process from reducing the potential for pollution to productive utilisation.

It is hypothesized that anaerobic cellulose degrading microorganisms originating from rumen fluid can produce energy sources in the form of VFA and other intermediates for SRB in the biological sulphate removal process, when contacted with grass cuttings.

1.3 RESEARCH QUESTIONS

1.3.1 Cellulose degradation, VFA production, sustainable sulphate reduction

To achieve the set objectives, the main research questions are focussed on the fermentation of grass-cellulose and on the use of these fermentation products as substrates in the biological sulphate removal. Can it be proven that the proposed technology is feasible to obtain a sustained removal of sulphate from mine water? With the aim to answer this hypothesis, the following research questions were stipulated.

- Can VFA be produced from cellulose in grass cuttings, using naturally occurring micro organisms and can biological sulphate removal be obtained using the formed fermentation products?
- Will a larger amount of grass cuttings (and thus an increased cellulose concentration) affect the VFA concentration and the sulphate reduction rate?
- When using the same amount of grass cuttings, which fermentation products are generated using 1) SRB for fermentation and 2) utilising rumen as the inoculum and in what concentrations will they be present? Can the generated VFA be used for sulphate removal?
- What are the VFA production and sulphate reduction rates when utilising rumen microbes as the inoculum in reactors containing grass cuttings in combination with 1) sulphate, 2) no sulphate and 3) tryptone?
- What is the sulphate removal rate using a two and three stage reactor system containing rumen organisms to produce the carbon and energy source for the biological sulphate removal through fermentation of cellulose in grass cuttings, when feeding 1) artifical feed water and 2) pretreated mine effluent?
- Can a process description of the reactor be developed using mass balances based on the operational results of the combined fermentation and sulphate removal reactor.
- How does the sulphate removal technology described compare to other processes in the market place

The study portrayed in this thesis aimed to find a technology to treat AMD using biowaste products to allow the treated water to, be re-used in the coal processing plant, be used for irrigation or be re-charged to rivers from where some of it originated.

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CHAPTER 2: LITERATURE REVIEW

2.1 SULPHUR CYCLE

The sulphur cycle (Figure 2.1) is, like the carbon and nitrogen cycles, an essential process in nature. However, due to human activities, the cycle can be easily disturbed, both on a local and on a global scale (Kuenen & Robertson, 1992). One of the major environmental pollutants in the sulphur cycle is the formation of SO_2 and other sulphur compounds by the burning of fossil fuels, due to global industrialization. The other major environmental contributor to the sulphur pollution is the formation of SO_4 as a consequence of mining operations. The sulphur cycle consists of several steps, including an oxidative and a reductive component, which in a natural ecosystem should be in balance. On the reductive side, sulphate and sulphur function as electron acceptors in the metabolic pathways, used by a wide range of anaerobic bacteria. On the oxidative side of the cycle, reduced sulphur compounds serve as electron donors for anaerobic phototrophic bacteria, which gain their energy from (sun)light or provide growth energy for the colourless sulphur bacteria. From an industrial management perspective, the best way to manipulate the sulphur cycle is to produce sulphur, which being insoluble, can be easily recovered.

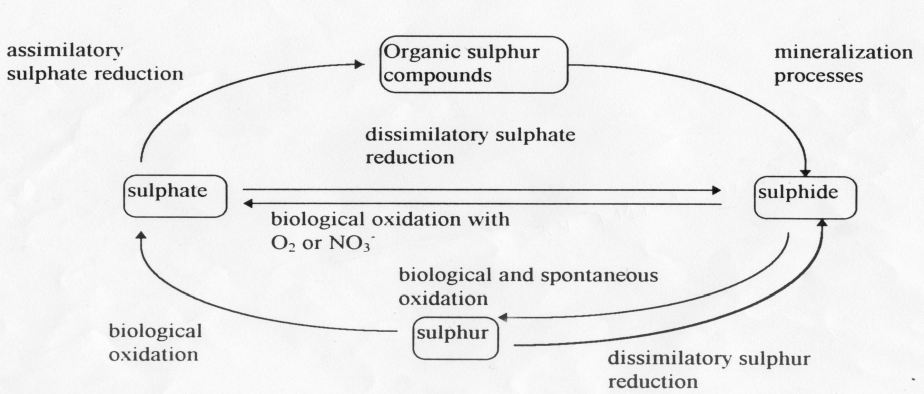

Figure 2.1: The Biological Sulphur Cycle (Pfenning & Widdel, 1982)

2.2 IMPACT OF MINING AND MINE EFFLUENTS ON THE ENVIRONMENT

Mining almost always impacts on the natural water environment. These impacts can be beneficial as some mine waters are of good enough quality that they can be used for public supply (Banks *et al*. 1996). The potential magnitude of environmental impacts associated with excess mine water discharging from old mine workings can

be established through the evaluation of excess mine water production, the geochemical properties of mine water, the safe environmental level to which the rising mine water can be allowed to increase before impacting on the groundwater and surface water resources, as well as probable surface decant points if the old workings were allowed to fill and decant. Dewatering in mining operations is essential for the safety of the mine workers. The consequences of dewatering of mines can include surface or groundwater pollution if the mine water is of poor quality and is discharged to the natural environment without prior treatment.

Underground mining tends to have less conspicuous impacts on surface water than an open pit, surface mining. But all types of mining have the potential to directly disrupt the ground water flow, which can affect surface waters that are in hydraulic continuity with affected groundwater systems (Booth 2000). However, the impact on the natural water environment arising from the act of mining itself tends to be relatively localized and limited when compared to other mining related impacts, such as those associated with dewatering and seepage of contaminated leachate from waste rock piles and tailings dams (Younger & Wolkersdorfer, 2004). Waste products from both mining and mineral processing operations are often contained in large heaps or in tailings dams. Seepage of contaminated leachate from waste rock piles and tailing dams is a significant cause of surface and ground water pollution in many mining areas. This kind of water pollution often occurs when the mine is in operation and without remediation can persist long after mine closure (Younger & Wolkersdorfer, 2004). This is the case in the operation of the South African mining industry, which inherited the legacy of the past regarding contaminated mine water.

The metals and salts containing mine effluents can deplete the oxygen in the receiving waters, which can have strong impacts on the survival of invertebrates and fish present in these receiving water bodies. Oxidation of Fe^{2+} to Fe^{3+} is a particular problem in the affected streams due to the precipitation of voluminous orange/red rusty coatings of ferric hydroxides/oxyhydroxides, called “yellow buoy” in the USA and “ochre” in the United Kingdom. The formation of these iron hydroxides/oxyhydroxides can have detrimental effects on the aquatic biota.

The pH of the mine water is usually acidic and can be as low as 2. When the pH is maintained below 6.5 for an extended period, it can result in decreased reproduction and growth of fish and aquatic invertebrates (Ikuta & Kitamura, 1995).

A significant cause of surface water pollution is contaminated leachate from waste rock piles and tailing dams in most mining districts. Younger (1997) states that re-vegetated waste rock piles can continue to release acidic leachates over several decades from shallow water table systems perched within the spoil. Drainage of leachate through the unlined bases of old tailingss dams is also known to produce polluted surface and ground water (Manzano *et al*. 1999; Johnson, 2000).

2.3 ACID MINE DRAINAGE (AMD)

The formation of AMD is primarily a function of the geology, hydrology and mining activities. It is formed due to complex geo-chemical and microbial reactions, which occur when water and oxygen come into contact with pyrite in the coal seam. Bacterial oxidation of sulphide minerals is the major factor in the formation of acid mine drainage, a common environmental problem in coal mining regions. When pyrite is first exposed during mining operations, it is slowly oxidised according to reaction 2.1:

$$FeS_2 + 3\,½\,O_2 + H_2O \rightarrow Fe^{2+} + 2\,SO_4^{2-} + 2\,H^+ \qquad (2.1)$$

This reaction depicts the oxidation of pyrite by oxygen, when sulphur is oxidized to sulphate and ferrous iron is released. As can be seen by the reaction (2.1), 2 moles of acidity are formed for each mole of pyrite. The ferrous iron formed is converted to ferric iron due to the biological oxidation of ferrous (Fe^{2+}) to ferric ions (Fe^{3+}), which can react with more pyrite according to reaction 2.2:

$$FeS_2 + 14\,Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2\,SO_4^{2-} + 16H^+ \qquad (2.2)$$

When more Fe^{2+} ions are formed, the bacterial oxidation to Fe^{3+} continues, thus initiating a cycle referred to as the *propagation cycle*. The breakdown of pyrite leads ultimately to the formation of Fe^{2+} and SO_4^{2-} ions, resulting in acidic water, with a pH as low as 2. Furthermore, pyrite, occurring in coal discard heaps can be oxidized with similar results as for the mine water effluents. The run-off from coal mining discards often causes contamination of ground waters (Madigan *et al.* 1997; Younger *et al.* 2002). Under undisturbed conditions, the coal is not exposed to air, water or bacteria.

2.4 ACID MINE DRAINAGE TREATMENT TECHNOLOGIES

Before 1980, the only proven technologies for mine water treatment were the active treatment methods. By active treatment is meant conventional waste water engineering applied to mine waters (Younger *et al*. 2002). Therefore, in most cases the mine effluents can be treated following the design of infrastructure for similar unit processes in ordinary waste water treatment plants.

2.4.1 Physical and Chemical Technologies

Due to salination by AMD and the associated scaling and biocorrosion problems, as well as increased environmental awareness among the general population, methods are being investigated to remove the high sulphate concentration of AMD. Physical (reverse osmosis, electrodialysis and ion exchange) and chemical methods (precipitation with barium salts and limestone neutralisation followed by lime precipitation, for Mg removal) have been tested and applied.

2.4.1.1 The Barium removal technology

Kun (1972) studied sulphate removal using barium carbonate ($BaCO_3$) producing barium sulphate ($BaSO_4$). Volman (1984) and Maree *et al*. (1989) demonstrated that $BaSO_4$ could be reduced efficiently and economically with coal under thermic conditions to produce barium sulphide (BaS). The BaS can then be re-used for the sulphate removal process. For certain mine waters this technology can be applied and the benefit of the technology is that the chemicals required for the technology can be retrieved and re-used, which results in substantial savings on running/operation costs.

2.4.1.2 The limestone neutralisation and precipitation technology

It was demonstrated that limestone ($CaCO_3$) instead of lime ($Ca(OH)_2$) can be utilized for neutralization of acid water, resulting in a 50% saving in operating costs (Maree *et al*. 2003). The other advantages of the use of limestone are that limestone is safer to handle than lime and that the pH after neutralisation cannot exceed a pH of 8. The limestone neutralisation technology consists of the following stages: the $CaCO_3$ handling and dosing, $CaCO_3$-neutralization and gypsum crystallization to achieve neutralised water and partial sulphate removal.

2.4.2 Biological treatment

The biological sulphate reduction technology is particularly beneficial to mining industries experiencing acid mine drainage problems, as it results in removal of

sulphate, in a pH increase of the treated water and often in metal removal. The SRB utilize organic products as the carbon and energy source, providing electrons, while sulphate is used as the terminal electron acceptor. The products of biological sulphate removal are sulphide and alkalinity. Sulphide production often results in metal-sulphides precipitation, e.g FeS, since most AMDs contain high concentrations of iron. Due to the production of alkalinity, the pH of the treated water often increases to neutral values.

Biological treatment of AMD can be applied after neutralisation and partial sulphate removal, which is advantageous for two reasons:

a) It is cheaper to use limestone than a carbon and energy source
b) For biological treatment a neutral pH is more favourable for the SRB

There are two options for the biological treatment, namely the passive and active treatment technologies, both of which will be discussed as both treatment systems have applications in South Africa.

2.4.2.1 Passive treatment

Passive treatment requires little maintenance and can find its application in rural mining areas, however, it can only treat relative small volumes of mining effluents (Pulles, 2000). “Passive treatment is the deliberate improvement of water quality using only naturally-available energy sources (e.g. gravity, microbial metabolic energy, photosynthesis), in systems which require infrequent (albeit regular) maintenance in order to operate effectively over the entire system design life” (Younger *et al.* 2002). Thus passive treatment technologies use natural materials to promote naturally occurring chemical and biological processes. Particular contaminant removal processes are optimized by manipulating the environmental conditions to obtain a cost effective technology. For this purpose, locally sourced materials, such as carbonate rocks and organic substrates, are utilised (Younger *et al.* 2002).

The advantage of a passive treatment system is that it can be used for more than 10 years with minimal requirement for operator intervention and costly maintenance. The ecological advantage is that they include constructed wetlands, which provide wildlife habitat and can have substantial values of social and ecological values (Hawke & José, 1996, Younger 1998). The plant-microbe associations in wetlands can serve both as the reactor and as source of carbon for the sulphate reduction and

water quality improvement (Batchelor *et al.* 1998). A wide range of electron donors, such as manure, spent mushroom compost, peat, sawdust and woodchips have been used. The natural occurring vegetation or specifically planted vegetation can be used as a continuous source of reduced carbon (Johnson, 2000). Passive treatment systems occur in North America as well as in Europe (UK and Spain) and it is the technology of choice for long-term use, wherever the hydrogeochemical prognosis is favourable and land space is available. These systems are usually applied in situations where mining was stopped many years ago and where no funds are available for costly high-tech solutions for the treatment of the remaining acid mine waters. In this kind of situation, where acid mine water needs to be treated, a relatively cheap passive treatment system can be operated with low maintenance and little supervision.

With the above mentioned conditions in mind, a novel South African passive treatment system has been developed, called the Integrated Managed Passive Treatment Process Technology (IMPI). This development is the result of many years of collaborative research between water professionals, research institutions and mining companies (Heath, 2002). The IMPI technology focussed both on the microbiology as well as on the chemical engineering of the processes by fundamentally investigating the breakdown and use of lignocellulose material, observing the sulphate reduction followed by the sulphide production as well as the reactor hydraulics (Heath, 2002). The IMPI technology can treat one Mℓ per day of mine water at a relatively low capital cost of R3 million to remove one ton of sulphate per day at an operating cost of R 0.60 per m^3 (Heath, 2002).

2.4.2.2 Active treatment

The emphasis of the investigation described in this thesis will be on active biological sulphate reduction technology. A major advantage of the active technology is the increased rate of reaction, which in turn allows for larger volumes of effluents to be treated. Sulphate-rich effluents can be treated biologically when SRB and organic matter are present. In the presence of sulphate, but also of sulphite (SO_3^{2-}) and thiosulphate ($S_2O_3^{2-}$), SRB are able to use several intermediate products of the anaerobic mineralization process. Besides the direct methanogenic substrates, such as hydrogen, formate, acetate, methanol and pyruvate (Bock *et al.* 1997), they can also use propionate, butyrate, higher and branched fatty acids, lactate, ethanol and higher alcohols, fumarate, succinate, malate and aromatic compounds (Colleran *et al.* 1995). In sulphidogenic breakdown of VFA, two oxidation patterns can be

distinguished. Some SRB are able to completely oxidize VFA to CO_2 and sulphide as end-products, whereas other SRB can only carry out an incomplete oxidation of VFA with acetate and sulphide as end-products. The carbon sources most commonly used are listed in Table 2.1 (Maree, 1988). Some of these substrates will be discussed in greater detail in section 2.11.1.

Table 2.1. Organic substrates, most commonly used for biological sulphate removal

Acetate	Ethanol	Glycerol	Pyruvate
Alanine	Formate	Lactate	Siccinate
Butyrate	Fructose	Malate	Sucrose
Citrate	Glucose	Propionate	Tartrate

Although the biological sulphate removal is an attractive option, worldwide not many active full scale operations are in operation, however several demo-scale plants have been constructed. Research is still being conducted to find the optimal reactor configuration (UASB, EESB, Completely Mixed), to maintain the biomass and to identify a cost effective carbon and energy source, as discussed in this thesis.

2.5 REACTOR DESIGN

Due to the development of improved reactor configuration, anaerobic, as opposed to the traditionally aerobic, treatment of wastewater was established as a feasible option. As the biological sulphate removal also occurs under anaerobic conditions, similar reactor configurations as typically used for the anaerobic COD removal, can be used for biological removal of high sulphate concentrations. A biological sulphate reduction process was developed at the CSIR, Pretoria, South Africa (Maree & Strydom, 1985; Maree *et al.* 1986). This three-stage process (anaerobic - aerobic – anaerobic) when treating mining effluents, employed up-flow, packed bed reactors for anaerobic treatment, followed by an activated sludge system for aerobic treatment. Once the biological sulphate reduction process had been proven, efforts concentrated on obtaining the most efficient reactor design. Among these are the Upflow Anaerobic Sludge Bed (UASB) Reactor (Lettinga, *et al*. 1980), the Fluidized Bed (FB) Reactor (Iza, 1991) and the Anaerobic Filter (AF) (Young & McCarty, 1969). These reactors are based on sludge immobilization and sludge retention, so that high biomass concentrations can be maintained in the reactors and high organic loading rates can be applied. The advantage of sludge immobilization and the

formation of biofilms is that wash-out of only small particles of the biomass occurs. To avoid sludge loss due to wash-out, the addition of a clarifier with a sludge return cycle to the reactor can be considered. However, due to the surface area of the clarifier, which is in contact with the atmosphere, it can be assumed that a fair amount of air will be introduced into the anaerobic reactor. A reactor system based on this principle was introduced by Maree *et al.* (1997) as the single-stage, completely-mixed reactor configuration, which removed sulphate and sulphide simultaneously, due to air diffusion into the reactor system.

2.6 MICROORGANISMS IN THE ANAEROBIC BIOREACTOR

2.6.1 Sulphate Reducing Bacteria (SRB)

Ten genera of dissimilatory SRB are currently recognised and are placed in two broad physiological subgroups (Postgate, 1984, Rinzema & Lettinga, 1988, Madigan *et al.* 1997). The genera in group I, *Desulfovibrio, Desulfomonas, Desulfotomaculum,* and *Desulfobulbus* utilize lactate, pyruvate, ethanol, or certain fatty acids as carbon and energy sources, reducing sulphate to hydrogen sulphide. The genera in group II, *Desulfobacter, Desulfococcus, Desulfosarcina, and Desulfonema*, specialise in the oxidation of fatty acids, particularly acetic acid, reducing sulphate to sulphide. The SRB are all obligate anaerobes and strict anaerobic techniques must be used for their cultivation. SRB are widespread in aquatic and terrestrial environments that become anaerobic due to active decomposition processes. The best known genus is *Desulfovibrio*, which is common in aquatic habitats or water-logged soils containing abundant organic material and sufficient levels of sulphate. *Desulfotomaculum* consist of endore-spore forming rods primarily found in soil. *Desulfomonas* can be isolated from the mammalian intestine. Certain SRB, among which are the *Desulfosarcina, Desulfococcus* and certain species of *Desulfovibrio,* are unique in their ability to grow chemolithotrophically with H_2 as electron donor, sulphate as electron acceptor and CO_2 as sole carbon source (Autotrophic growth).

2.6.2 Acetogenic Bacteria (AB)

Acetate is an important intermediate degradation product in an anaerobic reactor and can be produced by both AB and homoacetogenic bacteria. Homoacetogenic bacteria are obligate anaerobes that utilize CO_2 as a terminal electron acceptor, producing acetate as the sole product of anaerobic respiration. Electrons for the reduction of CO_2 to acetate can be derived from H_2, a variety of C_1 compounds,

sugars, organic acids, alcohols, amino acids and certain nitrogen bases. Many homoacetogens can also reduce NO_3^- and $S_2O_3^{2-}$. However, CO_2 reduction is probably the major reduction of ecological significance (Madigan *et al.* 1997).

2.6.3 Methanogenic bacteria (MB)

The MB are CO_2 reducing bacteria, belonging to a major group of Archaea. They utilize H_2 as the electron donor, to produce CH_4 from CO_2 according to equation (2.3):

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad (2.3)$$

When growing on H_2 and CO_2, the MB are autotrophic, with CO_2 serving as both carbon source and electron acceptor. In addition to CO_2, some alcohols, formate, methanol and several methylamines can be converted to methane by certain MB species. The three classes of methanogenic substrates known are listed below:

1 The CO_2 substrates, CO_2, CO and formate ($HCOO^-$)
2 Methyl groups (CH_3OH) (through reduction)
3 Acetate: $CH_3\,COO^-$ (equation 2.4)

$$CH_3\,COO^- + 2H_2O \rightarrow CH_4 + HCO_3^- \qquad (2.4)$$

2.6.4 Cellulose degrading microorganisms

Cellulose degrading microorganisms are ubiquitous and are found in various environments including soils, sediments, compost heaps and the gut of vertebrate herbivores such as the ruminants (Coughlan and Mayer, 1992). They include protozoa, fungi and bacteria, aerobes and anaerobes, mesophiles and thermophiles. In the natural environment, cellulose is mainly oxidized by aerobic fungi and bacteria, producing CO_2 and water, while only 10% is converted by anaerobic microorganisms producing methane and carbon dioxide. The anaerobic digestion of cellulose utilising rumen fluid as the inocculum will be discussed in this thesis. The level of microorganisms in the rumen is as high as typically found in any other natural habitat. These bacteria are adapted to live in a slightly acidic environment between pH 5.5 and 7.0 at a preferred temperature of 39–40 ˚C. The steady supply of food and continuous removal of fermentation products and food residues maintain relatively constant conditions, in which an extremely dense population develops (Hungate, 1966). The diversity amongst rumen bacteria is striking, which may be due to the

complex feed intake by the ruminants. The feed typically contains carbohydrates, proteins, fats and numerous other organic compounds and minerals (Hungate, 1966).

Already in 1832, Karel Sprenger published that decomposition of plant materials in the rumen was known to give rise to volatile substances which, at that time, were assumed to consist of acetic and butyric acids. Hungate in 1966 writes "the ruminant and the rumen microbial population exist in an equally beneficial relationship in which many of the plant materials consumed by the mammalian host are digested and fermented by the rumen microbes to form chiefly carbon dioxide, methane and volatile acids." The rumen is a complex ecosystem where microorganisms thrive in symbiotic relationship that facilitates fibre digestion. Therefore, anaerobic degradation of plant material can be executed efficiently using the bacteria, fungi and protozoa occurring in the rumen as they produce cellulose fibre degrading enzymes (Lee *et al.* 2000). Cellulose degradation in anaerobic environments can be carried out by different *Clostridium* species, producing glucose and cellobiose, which are then fermented to lactate, acetate, ethanol, CO_2 and H_2. Ljungdahl and Eriksson, (1985) described the fermentation of sugars to produce carbon dioxide and hydrogen according to Equation (2.5)

$$C_6H_{12}O_6 + 6\ H_2O \rightarrow 6\ CO_2 + 12\ H_2 \qquad (2.5)$$

The hydrogen-utilizing bacteria assimilate hydrogen and use it for the reduction of CO_2 to acetate or methane, sulphate to H_2S or nitrate to ammonia or N_2. The end product of the degradation process depends on the nature of the hydrogen-utilizing bacterium in the second stage, which in our studies will be mainly the SRB, producing hydrogen-sulphide.

The anaerobic species of cellulose degraders comprise *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens* and *Fibrobacter succinogenes*, *Caldocellum saccharolyticum*, the *Clostridium* species, the *Erwinia* species and the *Ruminococcus* species. Ruminococci have been isolated from cattle and sheep rumen fluid in Africa, Europe and the USA (Hungate, 1966). Several species of the most primitive group of fungi (anaerobe Chytridomycete) are well known for their ability to degrade cellulose in the gastrointestinal tracts of ruminant animals (Carlile & Watkinson, 1997; Lynd *et al*. 2002).

Cellulose fibers are imbedded in a matrix of structural biopolymers, primarily hemicellulose and lignin, which comprise 20 - 35 and 5 - 30% of plant dry weight, respectively (Lynd *et al*. 1999). Many bacteria can grow on cellulose producing enzymes that catalyse the degradation of soluble derivates of cellulose. However few bacteria synthesise the complete enzyme system, which can totally hydrolyse the crystalline material found in nature (Coughlan and Mayer, 1992). Hemicellulose is a plant carbohydrate, which forms a large percentage of the forage consumed by ruminants. Its digestion is similar to that of cellulose and is almost completely digested in the rumen (McAnally, 1942). In contrast to cellulose that is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolysed by dilute acid or base, but nature provides an arsenal of hemicellulase enzymes for its hydrolysis (Marchessault & Sundararajan, 1993).

2.6.4.1 Acetivibrio cellulolyticus

This anaerobic bacterium attaches itself to cellulose and produces acetic acid, hydrogen, carbon dioxide and traces of propanol, butanol and ethanol. It was first isolated from municipal sewage sludge.

2.6.4.2 Bacteroides cellulosolvens (*Fibrobacter succinogenes)*

The genus of *Bacteroides* includes species of obligately anaerobic, mesophilic, non-sporeforming gram-negative rods (Holdeman *et al.* 1984). They form an important part of the cellulytic rumen flora (*Bacteroides succinogenes).*

2.6.4.3 Caldocellum saccharolyticum

These species are thermophilic, anaerobic, cellulytic bacteria (Reynolds *et al*. 1986). The best three isolated strains can hydrolyse cellulose and lignin cellulose, comparable to *Clostridium thermocellum.*

2.6.4.4 Clostridium species

Most clostridia are mesophilic, which includes *Clostridium cellobioparum*, which is isolated from rumen fluid.

2.6.4.5 Erwinia species

These species are responsible for the soft rot of crops, both in the field and in storage. The bacteria secrete hydrolytic enzymes, including pectinases, cellulases, proteases and nucleases into extracellular fluids.

2.6.4.6 Ruminococcus species

These species are after the Bacteroides (Fibrobacter) group, the most important cellulose-digesting of the rumen flora. These species of rumen origin ferment cellulose and various sugars, to produce acetate, formate, succinate, ethanol, hydrogen and carbon dioxide as major end products (Bryant, 1986).

2.7 PRODUCTS OF THE MICROBIAL ACTIVITY IN THE RUMEN

Most rumen species produce acetic acid as the final fermentation product. One forth of the species forms propionate e.g. *Selenomonas*, which is usually second in abundance (Hungate, 1966, Bergman 1990). Ruminal VFA production is closely related to ruminal pH (Russell & Dombrowski, 1980). Hydrogen is a major intermediate in organic matter degradation in the ruminal ecosystem (Hungate, 1966; Wolin & Miller, 1988). It is produced by fermentative microorganisms and can potentially be used by MB, SRB and the AB, to produce acetate. Interspecies hydrogen transfer between H_2-producing and H_2-utilizing microorganisms allows growth of fermentative and hydrolytic microorganisms (Morvan *et al*. 1996). In the rumen, H_2 is used by MB to reduce CO_2 and produce CH_4, while in the sulphidogenic bioreactor it is used by SRB for biological sulphate reduction.

2.8 COMPETITION FOR SUBSTRATE IN THE ANAEROBIC REACTOR

When considering the affinity of the SRB, the AB and the MB for substrates such as acetate, CO_2 and H_2, it is evident that these groups of bacteria may out-compete each other for their preferred substrate. In the sulphate reducing stage, a complete reduction of sulphate to sulphide is desired. Channelling of reducing equivalents towards the SRB is enhanced by the ability of the SRB to effectively compete with other anaerobic bacteria for the available organic substrate and the sensitivity of other bacteria for sulphide (Lens *et al.* 1998b). The anaerobic process can become very complex in the presence of sulphate, because SRB will compete with MB for compounds such as formate and hydrogen, and with AB for compounds such as propionate and butyrate (Colleran *et al.* 1995). Until recently, only limited investigations have been conducted on the likely outcome of the competition between SRB and MB. Once the factors, influencing the outcome of this competition are known and applied, they can avoid the risk of process failure. Moreover, practical engineering manipulations could force the bacteria to either go the sulphidogenic or the methanogenic route.

O'Flaherty *et al.* (1998) studied the population structure of biomass from a full-scale anaerobic reactor after 5 years of operation, with the purpose to obtain an improved understanding of long-term competition between SRB and other anaerobic microorganisms, such as the MB, the AB and other (synthropic) bacteria. The results showed that SRB carried out an incomplete oxidation of propionate to acetate. It was observed that the SRB and synthropic bacteria competed for butyrate and ethanol. However, in the case of hydrogen, the SRB out-competed the MB, which confirmed the results of other studies, which demonstrated that H_2 and CO_2 are primarily used by the SRB, provided that sufficient sulphate is available (Visser, 1995). It is thought that the SRB keep the hydrogen concentration below the threshold level required by the MB (Lovley, 1985). Oude Elferink *et al.* (1994) showed that the hydrogen utilizing SRB (HSRB) gain more energy from the consumption of molecular hydrogen, have a higher substrate affinity, growth rate and cell yield than the hydrogen utilizing methanogenic bacteria (HMB). These authors also suggested that in the presence of sulphate, compounds, such as alcohols, lactate, propionate and butyrate, may be oxidized directly by the SRB without the intermediate formation of hydrogen. They presented the following conclusions from their investigation:

- SRB will compete with MB for hydrogen, formate and acetate.
- In general, SRB have better growth kinetic properties than MB, since SRB have a higher growth rate than the MB and the conditions in a sulphidogenic reactor generally favour the SRB
- Reactor conditions, such as pH, temperature, sulphate and sulphide concentrations, can influence the microbiological processes in the bioreactor and can determine whether these processes will proceed via the sulphidogenic or the methanogenic pathway.

O'Flaherty *et al.* (1998) further showed that acetogenic bacteria also played a role in the utilisation of H_2 and CO_2 in their study of the microbial activity in an anaerobic reactor. It was shown that even after 5 years of reactor operation, the SRB failed to out-compete the acetate utilizing MB. In general, the findings of O'Flaherty *et al.* (1998) were a confirmation by those of Harada *et al.* in (1994). They showed that when the sulphate concentration in the bio-reactor increased from 30 to 100 to 600 mg SO_4/ℓ, the SRB utilized almost 5, 30 and 40-75% of the COD present. It was observed that propionate accumulated significantly when no or low levels of sulphate were present. Therefore, it can be deduced that SRB strongly contribute to the degradation of propionate to acetate. The study of Harada *et al.* (1994) indicated

furthermore that the activity of the HMB decreased with increasing sulphate concentrations. It can be assumed that the SRB contribute to the degradation of propionate to acetate using hydrogen. It was also shown that the SRB were poor competitors of MB for acetate. Only during long-term operation, the SRB started to out-compete the MB for acetate.

Omil *et al.* (1997) also studied the competition between acetate utilizing MB and SRB, operating two UASB reactors, at a reactor pH of 8. The UASB reactors received VFA mixture of acetate, propionate and butyrate (5:3:2), on the basis of COD and only acetate, respectively, at different COD: Sulphate ratios. It was found that in the precence of excess sulphate concentration (COD: Sulphate concentration ratio < 0.67), the SRB became predominant in relation to the MB, when the reactors were operated from 250 to 400 days.

2.9 CARBON AND ENERGY SOURCES FOR BIOLOGICAL SULPHATE REMOVAL

Since the 1970's the application of anaerobic wastewater treatment has increased dramatically. Some of the advantages of anaerobic treatment over aerobic treatment are the low energy input required and the low sludge yield. The main advantage is that the end product of the anaerobic degradation of organic matter is the production of methane gas (CH_4), a potential energy source. However, as already indicated, when sulphate forms part of the organic waste, the SRB will use the available organic matter as their carbon and energy source to reduce sulphate with hydrogen sulphide, partly as gas and partly dissolved in the treated water, as the end product. Due to this reason, many operators of anaerobic treatment plants consider sulphate rich effluents troublesome, as during anaerobic treatment of these wastewaters, the reactor will turn sulphidogenic rather than methanogenic. When treating AMD or other sulphate containing industrial effluents, which contain no or insufficient electron donor and carbon source for a complete sulphate reduction, addition of an appropriate electron donor is required. The selection of the electron donor depends on the costs of the added electron donor per unit reduced sulphate and on the pollution potential of the additive in the waste stream.

2.9.1 Traditional carbon and energy sources

2.9.1.1 Sucrose

The studies of Greben *et al*. (2000a and 2000b) showed that sucrose can be used as the carbon and energy source for the biological sulphate removal. When operating a single stage completely mixed biological sulphate removing reactor, using sucrose as the carbon and energy source, the experimental volumetric and specific sulphate reduction rates (maximum) were determined to be 10.4 g SO_4/(ℓ.d) and 0.79 g SO_4/(g VSS.d) respectively (Greben *et al*. 2000b). When sucrose is used as the carbon and energy source, the SRB can utilize sucrose and produce hydrogen (reaction 2.6), which can be utilized by the SRB, according to reaction (2.7). Some SRB species are unique in their ability to grow with H_2 as electron donor, sulphate as electron acceptor and CO_2 as sole carbon source.

$$C_{12}H_{22}O_{11} + 5\ H_2O + 4SO_4^{2-} \rightarrow 4CO_2 + 8H_2 + 4HS^- + 8HCO_3^- + 4H^+ \quad (2.6)$$

$$8H_2 + 2SO_4^{2-} + 2H^+ \rightarrow 2HS^- + 8H_2O \quad (2.7)$$

2.9.1.2 Ethanol

Ethanol in the presence of AB and SRB represents a substrate that can be oxidized to acetate, which then can be oxidized by the acetate utilizing SRB, such as *Desulfuromonas acetoxidans* and *Desulfobacter postgatei.* These microorganisms are often unable to metabolise lactate and pyruvate, but can oxidize ethanol completely to CO_2. The reactions involved are (2.8-2.12):

$$2C_2H_5OH + H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad (2.8)$$

The produced hydrogen can be used as the energy source by the SRB in the presence of sulphate:

$$2H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad (2.9)$$

$$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + H^+ + 2H_2O \quad (2.10)$$

$$2CH_3COO^- + 2SO_4^{2-} \rightarrow 4HCO_3^- + 2HS \quad (2.11)$$

$$2C_2H_5OH + 3SO_4^{2-} \rightarrow 3HS^- + 3HCO_3^- + 3H_2O + CO_2 \quad (2.12)$$

Ethanol has been identified as an intermediate during the degradation of organic matter in most anoxic ecosystems (Kaspar and Wuhrmann, 1978; Lovley *et al.* 1982; Schink *et al.* 1985). Szewzyk & Pfennig (1990) concentrated on the competition for ethanol by the SRB and other fermenting bacteria during their investigations. The

results in continuous culture showed that SRB are able to successfully compete with fermenting bacteria under low substrate concentrations. This confirms the important role of the SRB in the anaerobic degradation process. The study of Szewzyk & Pfennig (1990) showed that SRB compete successfully with the fermenting bacteria in the process of organic degradation.

De Smul *et al.* (1997) indicated that a sulphate reduction efficiency of 80-85% was obtained when the reactor pH was controlled above pH of 7.8, using ethanol as the energy source. They also found that in their reactors, the oxidation of ethanol proceeded mainly via acetate, but due to the higher reactor pH at 7.8, the ASRB out-competed the MB, confirming the findings of Visser (1995).

Greben *et al.* 2000b showed that ethanol could be used for the treatment of AMD in laboratory scale reactors obtaining a sulphate reduction rate of 6.8 g SO_4/ℓ.d, while Maree *et al.* (2004) described a sulphate removal rate of 12 g SO_4/ℓ.d operating a demonstration plant at Navigation Mine (Witbank, South Africa), using ethanol as the carbon and energy source to which sugar was added. Greben *et al.* (2002) indicated that adding 0.25 g/ℓ of sucrose to technical ethanol 96 % (1 mℓ ethanol/ℓ feed water) as the carbon and energy source resulted in a good sulphate reduction rate as well as in biomass growth.

2.9.1.3 Methanol

Braun & Stolp (1985) and Nanninga & Gottschall (1986) reported the use of methanol as electron donor for sulphate removal. Davidova and Stams (1996) researched the degradation of methanol in anaerobic sludge at temperatures over 60 °C. They found that a consortium of bacteria, obtained from anaerobic granular sludge could degrade methanol at 65 °C via sulphate reduction and acetogenesis. Tsukamoto and Miller (1999) proved sulphate reduction by using a combination of lactate and methanol as the substrate, followed by only methanol. Weijma (2000) described high sulphate removal rates of 4-7 g SO_4 (ℓ/d) at a HRT of 10 h when using methanol as the carbon and energy source operating under thermophilic conditions.

2.9.1.4 Hydrogen and Carbon dioxide mixture

Hydrogen gas is a clean and sustainable fuel, which can be considered an important alternative energy resource. Jules Verne, the well-known science fiction author, wrote as early as 1874 in his book “The mysterious Island” that hydrogen gas (H_2) is the “fuel” of the future. Hydrogen can be produced both chemically and biologically.

Verne (1874) indicated that it would be produced from a "plentiful" source, such as water. Water can be split by electrolysis into gaseous hydrogen and oxygen. To generate electricity for the electrolysis, other energy sources, such as coal combustion, have to be employed. Thus, in order to produce hydrogen chemically, another energy source needs to be provided, whereas for biologically produced hydrogen, fermented waste material can be used.

SRB can use hydrogen and CO_2 as energy source and carbon source, respectively, for the reduction of sulphate, which serves as the electron acceptor. The utilisation of hydrogen as energy source for biological sulphate removal has been reported by Du Preez *et al. (*1992) and van Houten, (1996). SRB have the advantage over MB, when H_2 is used as the energy source (Visser, 1995; Oude Elferink, 1998).

The study of Schutte & Maree (1989) reported on the autotrophic sulphate reduction using hydrogen. They operated both under batch and under continuous conditions, using the same upflow packed bed reactor for both experiments. The results showed that the sulphate removal efficiency was 91% at a hydraulic retention time (HRT) of 2.4 days. The need of the SRB for CO_2 was illustrated by omitting the CO_2, namely when the CO_2 flow to the reactor was stopped, the sulphate reduction ceased. When, however, the CO_2 flow was restored, the sulphate removal efficiency was brought back to previous levels. Schutte & Maree (1989) ascribed the dependence of SRB on CO_2 because in an anaerobic environment, synthrophic bacteria utilize carbon dioxide, forming intermediates such as lactate, ethanol and other carbon sources, which can then be used by SRB as source of carbon. The investigations of Van Houten (1996) confirmed this finding, showing that the hydrogen utilizing SRB (HSRB) are not autotrophic, so they do not assimilate CO_2 but are dependent on other anaerobes to produce acetate, which the SRB require as an additional carbon source. Acetate is formed by the homoacetogens, a group of obligate anaerobes, which utilize CO_2 as a terminal electron acceptor, producing acetate as the sole product of anaerobic respiration. Under H_2 limitating conditions, insufficient amounts of acetate become available for the HSRB, which may result in the predominance of HMB. It can also be assumed that under CO_2 limiting conditions, no acetate is produced, thus limiting or inhibiting the SRB respiration (Hulshoff-Pol *et al.* 1998).

When in the study, described by Schutte & Maree (1989), the hydrogen supply was stopped, the sulphate reduction ceased as well. This result led to the assumption that both growth and sulphate reduction seems to be strictly dependent on the

presence of an energy source (H_2). It can thus be concluded that when H_2 is available as the energy source and CO_2 as the electron acceptor for the homoacetogens, acetate will be produced, which the HSRB can use as carbon source for the reduction of sulphate present in e.g. the mine waste water.

Van Houten (1996) reported that the use of a mixture of H_2 and CO_2 (vol.:vol.as 80%: 20%), resulted in a sulphate reduction rate of 30 g SO_4/(ℓ.d) This rate was achieved within 10 days of operation at 30 °C using a gas-lift reactor, which provided good gas-mass transfer rates, with pumice as carrier material for the SRB. When examining the structure of the biomass, the results showed that the *Desulfovibrio* spp. and the *Acetobacterium* spp. were the most abundant microorganisms present. This confirmed the assumption that H_2, provided in the reactor, was consumed both by the SRB and the homoacetogens, which formed biofilms on the pumice particles.

2.9.1.5 Synthesis gas,

The studies of Du Preez *et al.* (1992), operating both under continuous as well as under batch conditions, showed that sulphate reduction was achieved using synthesis gas. This gas mixture can be generated from any material containing carbon and hydrogen and is readily available, as several industries dispose of it as a waste product. It originates from industrial sources such as steam and methane, through the oxidation of fuel oil and through coal gasification. The resultant mixture contains 29.7% hydrogen, 7.9% carbon dioxide, 59.1% carbon monoxide and 2.9% nitrogen gas and can be used as the energy and carbon source for SRB. When feeding this mixture, at 35 mℓ/min, to a 20 ℓ packed bed reactor, with pelletized ash as support medium, an average sulphate reduction of 2 g SO_4/day was achieved, feeding artificial SO_4 rich (1 350 mg SO_4/ℓ) feed water. When the SO_4 concentration in the feed increased to 2 000 mg SO_4/ℓ, the sulphate reduction was 3.3 g SO_4/day.

2.9.2 Complex organic products as alternative carbon and energy sources

2.9.2.1 Complex organic products

Probably the cheapest carbon and energy source to be used in the biological sulphate reduction technology is sewage and other types of industrial waste liquors. McKinney and Conway (1957) discussed sulphate as a possible terminal electron acceptor for anaerobic biological waste treatment and Pipes (1960) developed a process with potential practical application using activated sludge. Domka *et al.* (1977) surveyed a variety of municipal wastes, such as sewage, dairy waste and

sugar plants as carbon and energy sources for biological sulphate reduction (Postgate, 1984). Although sewage is a relative cheap product, the question in South Africa is whether enough sewage is available in the areas where AMD is produced. Recently, Rose (2000) and Joubert (2005) applied the use of primary sewage sludge as the carbon and energy source for the biological treatment of sulphate in AMD, operating the so-called Rhodes Biosure process. It is based on the hydrolysis of complex carbon sources in a novel Falling Sludge Bed Reactor, providing an easily accessible feed for SRB activity.

Algae can be considered a bio-waste product and have been also been used as a carbon and energy source. Boshoff *et al.* (1996) have investigated the anaerobic fermentation of waste-grown micro algae produced in waste stabilisation ponds and the linked production of sulphide by SRB. Waste Stabilisation Pond (WSP) technology involves the large-scale application of algal photosynthesis and the role of SRB in the anaerobic compartments of these systems. The study of Rose *et al.* (1996) also described the biological sulphate removal from a tannery effluent using dried *Spirulina spp.* as the organic substrate.

2.9.2.2 Production of Volatile Fatty Acids from complex organic material

Volatile Fatty Acids are products of the anaerobic digestion of complex organic material, forming methane as the final product of the process. The effective conversion of complex organic material into methane depends on the combined activity of a diverse microbial population consisting of various genera of obligate and facultative anaerobic bacteria. Koster (1988) showed that the activities of the mixed population present in an anaerobic digester can be summarised as seven distinct processes:

- Hydrolysis of suspended solids
- Fermentation of amino acids and sugars
- Anaerobic oxidation of long-chain fatty acids
- Anaerobic oxidation of intermediary products, mainly volatile fatty acids, such as butyric acid (C4), Propionic acid (C3) and Acetic acid (C2).
- Non-methanogenic conversions of acetate and hydrogen
- Acetoclastic and acetotropic methanonogenesis
- Hydrogenotrophic methanogenesis

These processes can be arranged as four distinct metabolic stages (McInerney *et al.* 1980) as depicted in Figure 2.2.

- *Hydrolysis*

During the hydrolysis process, complex, non-soluble organic compounds are solubilized by exoenzymes excreted by hydrolytic microorganisms. Basically, hydrolysis is the conversion of polymers into monomers.

- *Acidogenesis*

During the acidogenesis, soluble organic compounds, including the products of hydrolysis, are converted into organic acids, such as butyric, propionic and acetic acids.

- *Acetogenesis*

In the acetogenesis process, the products of the acidogensis are converted into acetic acid, hydrogen and carbon dioxide.

- *Methanogenesis*

In the methanogenesis process, methane is produced from acetic acid or from hydrogen and carbon dioxide. Methane can also be formed from other substrates, of which methanol and formic acid are the most important.

For purposes of using degradation products of organic waste as carbon and energy sources for biological sulphate reduction, the hydrolysis/fermentation processes are the most relevant. The anaerobic degradation of organic material in a methanogenic reactor will differ from that in a sulphidogenic reactor, due to the presence of sulphate and SRB. When sulphate is present in the wastewater, the SRB are able to couple the oxidation of organic compounds and hydrogen to sulphate reduction (Oude Elferink, 1998). Therefore, for the purpose of this study, the oxidation of organic compounds will be presented as occurring in a sulphidogenic reactor.

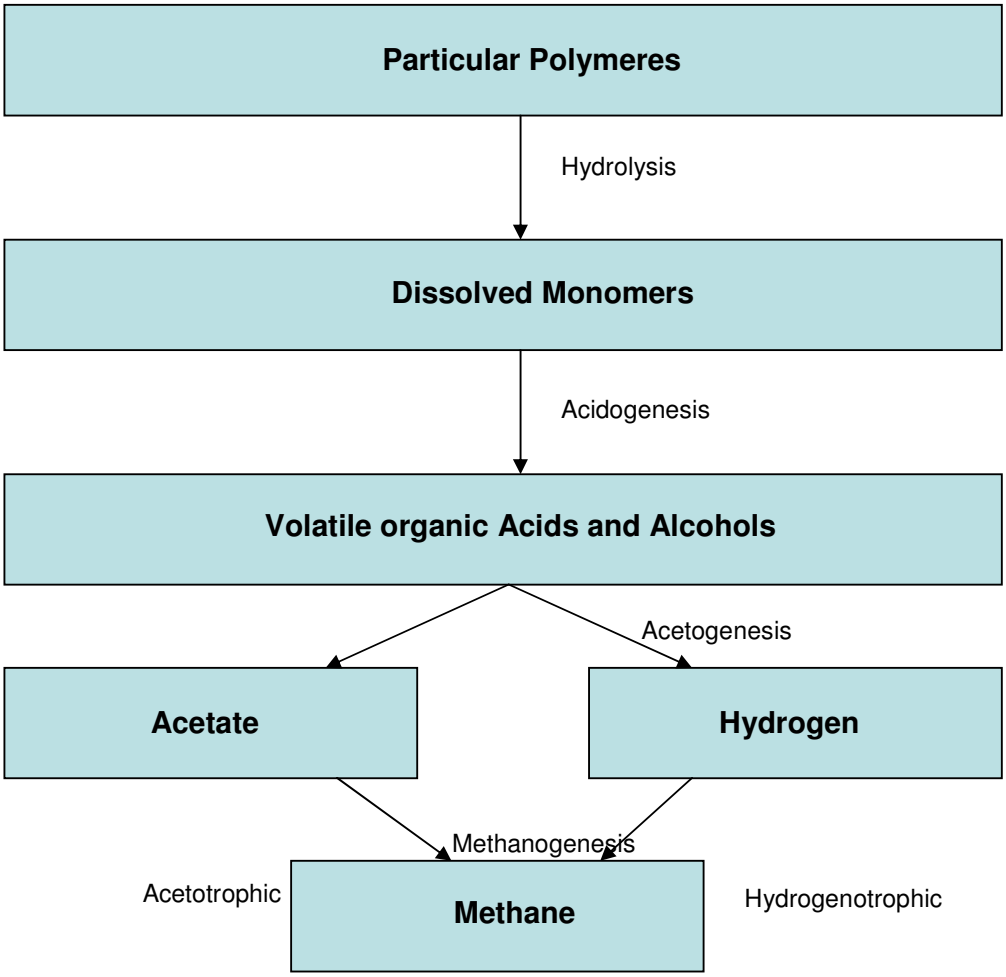

Figure 2.2 Metabolic stages and products in the anaerobic digestion of complex organic matter

Hydrolysis

In experiments on the fermentation of organic waste products to methane, the hydrolysis varied with the reaction time. Degradation percentages of 59% of the hemicellulose and 34% of the cellulose, respectively, were achieved when the reaction time was 15 days, whereas at 100 days reaction time, the degradation percentage increased to 96% and 76%, respectively (Lequerica *et al.* 1984).

The optimum pH for hydrolysis is different for various substrates. When degrading carbohydrates, the hydrolysis and acidogenesis processes proceed at maximum rates at pH 5.5-6.5 (Zoetemeyer *et al.* 1982; Zoetemeyer, 1982).

The rate and extent to which a substrate may be hydrolyzed is also influenced by the accessibility of the substrate by the exoenzymes (Hobson, 1982). This is especially true for the anaerobic digestion of fibrous materials, in which the cellulosic and hemicellullosic microfibrils are aggregated and embedded within the liquefied cell wall matrix. The crystallinity and surface area of the fibers are the most important features which determine the accessibility for exoenzymes (Fan *et al.* 1980). In some cases, physical pretreatment methods, such as heating or milling are applied. Alternatively, chemical treatment, comprising of scaling in NaOH is an option, whereas microbial pretreatment based on the capacity of "White Rot" fungi to degrade lignocellulose may be considered a more environmentally friendly option (Koster, 1988; Wicklow *et al.* 1980). In the hydrolysis step of the total degradation process, the particle size of the organic waste product influences the speed of the hydrolysis, due to the accessibility for the exoenzymes. In a fermentation reactor the methane production increased just over three times when the particle size was decreased from 20 to 1.3 mm (Hills and Nakano, 1984). In general, the slow rate of hydrolysis of organic waste products can be the limiting factor in the application of one-stage anaerobic digestion.

- *Fermentation process*

When defining the fermentation processes, one considers those processes that do not involve oxygen or nitrate as electron acceptors. Compared to aerobic processes, the anaerobic fermentation reactions result in smaller amounts of biomass attained per mole of substrate and the production of large amounts of fermentation products (Gottschalk, 1979). The fermentation products present after the degradation process depend on environmental conditions. When fermenting glucose in a separate acid producing reactor, the main products are butyric acid, acetic acid, hydrogen and carbon dioxide. However, when interrupting the feed supply for a period of 1–24 h, the fermentation pattern changed to an increased production of propionic and acetic acid. The study of Zoetemeyer *et al.* (1982) showed the influence of the pH on the fermentative bacteria. They showed that at pH values < 6 (pH=5.7), the main fermentation product of glucose is butyric acid, while the propionic acid concentration decreased. When the pH was increased, a successive change from butyric to lactic acid and subsequent change from lactic acid to acetic acid, ethanol and formic acid was observed. The product pattern of the fermentation also depends on the type of organic waste (Cohen, 1983). An increase in acetic, propionic and valeric acids was observed, when hydrolyzing and fermenting gelatine in a separate acid-producing reactor at pH values > 6.

Wolin (1976, 1979) showed the importance of hydrogen production and utilisation in the fermentation reactor. Removal of hydrogen, e.g. by the hydrogenotrophic bacteria, such as hydrogen consuming SRB (HSRB), can influence the kinds of products formed by the fermentative bacteria. When hydrogen is consumed by HSRB or HMB, the fermentative bacteria can produce further oxidized products than they would be able to at increased hydrogen levels, which supplies more energy per unit of substrate to the bacteria. This indicates that when HSRB are present, keeping the hydrogen partial pressure low, other organisms use the electrons generated in the fermentation process for hydrogen production rather than for the production of ethanol (Reddy *et al.* 1972). This observation may be significant when using the fermentation products of organic waste for the biological reduction of sulphate in the fermentation tank, when sulphate and SRB are present.

2.9.2.3 Anaerobic oxidation of Long-Chain Fatty Acids

The anaerobic degradation of long-chain fatty acids occurs by β-oxidation (Jeris & McCarty, 1965). When long chain fatty acids with an even number of carbons are oxidized, the fermentation products are acetate and hydrogen, but when acids with an uneven number of carbons are oxidized, the products are propionate and hydrogen. Anaerobic β-oxidation of long-chain fatty acids is thermodynamically unfavourable, unless the hydrogen partial pressure is maintained at a very low level (Hanaki *et al.* 1981). The affinity for hydrogen exhibited by HSRB is higher than that of HMB, and therefore the HMB are out-competed by HSRB in environments where a sufficient amount of sulphate is present (Robinson & Tiedje, 1984).

2.10 THE OXIDATION OF ORGANIC COMPOUNDS IN A SULPHIDOGENIC REACTOR

Compared to the MB, SRB are very diverse in terms of their metabolic capabilities. Acetate is the product, when oxidizing the C3 and C4 fatty acids, as is the case in the hydrolysis of the C3 and C4 fatty acids. The hydrolysis products as well as the oxidation products in the presence of sulphate of propionate and butyrate are acetate and hydrogen (Table 3.2). Both autotrophic and heterotrophic growth on hydrogen is possible. The hydrogen utilisation of the SRB will be discussed in a following section.

2.10.1 Fatty Acids in the sulphidogenic reactor

In 1928, Rubentschik as well as Baars isolated SRB capable of growing on fatty acids. Their work was in agreement with the observations of Hoppe-Seyler (1886) who had already documented the complete conversion of cellulose carbon, to carbon dioxide accompanied by sulphide production when sufficient sulphate was added. However, Postgate (1984) when reviewing the work done by Rubentschik and Baars referred to their findings as "historical errors". Widdel and Pfenning (1977) confirmed that SRB appear to have a large share in the mineralization of organic material. They isolated several new species of SRB capable of growing on fatty acids (Rinzema & Lettinga, 1988). Since then, there has been no doubt that the SRB are able to oxidize VFA and that the SRB can use all important intermediates in the anaerobic degradation of organic matter (Table 2.2).

Another important factor in the competition of SRB, MB and AB is the COD/SO_4 ratio in the fermentation reactor. This ratio determines which part of the organic material can be degraded via the sulphate reduction. The COD/SO_4 ratio in the sulphate removing reactor indicates the COD concentration versus the sulphate concentration in the reactor (mg/ℓ). The theoretical ratio value is 0.67, which indicates that, at that reactor ratio, all COD present will theoretically be used for the sulphate degradation. If the ratio is > 0.67, the MB and AB can participate in the degradation process as well. The propionate-oxidizing species of SRB (*Desulfobulbus propionicus*) can ferment lactate and ethanol in the absence of sulphate (Stams *et al.* 1984). Direct oxidation of hydrogen by the SRB and indirect hydrogen consumption by incomplete oxidation of propionate and higher fatty acids can be expected if sufficient sulphate is present. Under high sulphate concentrations, a sharp decrease in methanogenesis can be observed.

Both *Desulfobulbus propionicus* and acetogenic bacterial species grow on propionate. Visser (1995) showed that the propionate degrading AB are out-competed by the SRB due to the better growth kinetic property of the latter. He furthermore showed the crucial role of the SRB in the anaerobic degradation of butyrate and propionate in sulphate rich environments. When no sulphate is present the propionate concentration will increase in the reactor. The results of his study showed that the competition between the SRB and AB for propionate depends on the COD/SO_4 ratio. At COD/SO_4 ratios of about 10, the predominant route is a syntrophic oxidation of propionate by acetogens coupled to sulphate reduction by the

generated hydrogen. Under conditions of oversupply of sulphate (COD/SO_4 ratio of 0.5) the propionate is degraded mainly by direct oxidation by SRB.

Table 2.2. Acetogenic and methanogenic reactions, and sulphate-reducing reactions involved in the degradation of organic matter in methanogenic bioreactors and sulphate-reducing bioreactors, respectively.

Syntrophic Acetogenic reactions		
$Propionate + 3\ H_2O$	→	$Acetate^- + HCO_3^- + H^+ + 3\ H_2$
$Butyrate^- + 2\ H_2O$	→	$2\ Acetate\ H^+ + 2\ H_2$
$Lactate^- + 2\ H_2O$	→	$Acetate^- + HCO_3^- + H^+ + 2\ H_2$
$Ethanol + H_2O$	→	$Acetate^- + H^+ + 2\ H_2$
Methanogenic reactions		
$4\ H_2 + HCO_3^- + H^+$	→	$CH_4 + 3\ H_2O$
$Acetate^- + H_2O$	→	$CH_4 + HCO_3^-$
Sulphate-reducing reactions		
$4\ H_2 + SO_4^{2-} + H^+$	→	$HS^- + 4\ H_2O$
$Acetate^- + SO_4^{2-}$	→	$2\ HCO_3^- + HS^-$
$Propionate^- + ¾\ SO_4^{2-}$	→	$Acetate^- + HCO_3^- + ¾\ HS^- + ¼\ H^+$
$Butyrate^- + ½\ SO_4^{2-}$	→	$2\ Acetate^- + ½\ HS^- + ½\ H^+$
$Lactate^- + ½\ SO_4^{2-}$	→	$Acetate^- + HCO_3^- + ½\ HS^-\ ½\ H^+$
$Ethanol + ½\ SO_4^{2-}$	→	$Acetate^- + ½\ HS^- + ½\ H^+ + H_2O$

2.10.2 Competition for propionate and butyrate

As already indicated, in the anaerobic fermentation reactor in which a high sulphate concentration is present, the SRB will compete with the AB for butyrate and propionate. It is expected that for wastewater with an excess of sulphate, the SRB become predominant, because of their better growth kinetic properties. SRB grow much faster when sulphate is present than the synthrophic consortia (Oude Elferink, 1998). The C3 and C4 fatty acids are oxidized to acetate and hydrogen by the AB, followed by the hydrogen conversion via sulphate reduction. Harmsen *et al.* (1996) showed direct propionate oxidation by the SRB.

Other studies have shown that the SRB can be present (up to 15%) in the methanogenic sludge of the total biomass in an anaerobic fermentation reactor, even when no sulphate is present (Raskin *et al.* 1996). Under those conditions, the SRB grow similarly to the AB by oxidizing ethanol and lactate to acetate. Certain SRB can in the absence of sulphate, oxidize propionate in syntrophic association with hydrogen consuming anaerobes, while in the presence of sulphate they couple propionate to sulphate reduction. Growth of SRB on butyrate without the presence of sulphate has so far not been demonstrated (Oude Elferink, 1998).

2.10.3 Propionate utilisation treating sulphate rich effluent.

The study of Ghigliazza *et al.* (2000) concentrated on the biological treatment of gypsum-rich wastewater, using propionate as the organic carbon source. This carbon source was chosen as it is an important intermediate product, commonly found in anaerobic fermenting processes. The results of this study indicated that at a Feed: *Prop/*SO_4^{2-} ratio of 1.31, a 99.5% SO_4 removal at a HRT of 2 days could be achieved. This ratio could approach 1, after a longer acclimatization period. This finding agreed with others indicating that sulphate removal efficiency improves with time (Visser, 1995). While good propionate utilisation as well as efficient sulphate reduction was observed, the acetate concentration increased to constant levels as high as 1.2 g/ℓ. Utilisation of the produced acetate for further sulphate removal would be beneficial.

2.10.4 Acetate degradation

Acetate is the degradation product of the acetogenesis of the higher fatty acids (>C_2) and of the sulphidogenic activities of the propionate and butyrate utilizing SRB, mainly in the presence of sulphate. A specific ASRB (*Desulfotomaculum acetoxidans*) has been isolated from manure, rumen content and fresh water sediments contaminated with manure. This bacterium has a temperature optimum at 36 ℃ and does not grow at <10 ℃. This observation suggests that *D. acetoxidans* is primarily an intestinal microorganism, which most likely is present in digested sewage sludge, the most frequently used inoculum for anaerobic water treatment systems (Rinzema & Lettinga, 1988).

Acetate is the primary substrate for the MB, however, SRB interfere with methane production in the presence of sulphate. Anaerobic degradation of organic material is accomplished through a series of successive and parallel microbial processes. Besides methane, hydrogen sulphide (H_2S) can be an important end-product of this

mineralization process (Rinzema & Lettinga, 1988). It has been discussed that when oxidizing propionate and butyrate, acetate is the end product. Visser (1995) and Omil *et al.* (1997) have shown that acetate is the most recalcitrant VFA under sulphidogenic conditions.

The studies of Greben *et al.* (2000a, 2000b) have shown that the remaining COD in the anaerobic sulphate removing reactor using sucrose and ethanol consist of acetate. Lens *et al.* (1998a) indicated that the acetate removal capacity is the limiting factor of sulphidogenic VFA removal, using different reactor systems (UASB and staged USSB). They had envisaged that in the staged reactor, the presence of acetate would allow the development an ASRB population. They concluded that the period of 138 days may not have been sufficiently long to allow the ASRB to multiply, since ASRB have a low growth rate. Visser (1995) also showed that ASRB require a long period of time to become a dominant population under sulphidogenic conditions. The results of his study confirmed that after prolonged operation of the reactor, the ASRB were able to out-compete the AMB for acetate. It took 250 days in the one reactor and 400 days in the other to observe the acetate concentration used by the ASRB increased from 50 till 90%. The results of Visser's study furthermore indicated that at a reactor pH > 7.7, the ASRB became the predominant organisms. Moreover, the ASRB can out-compete the AMB at sufficiently high sulphate concentrations in the reactor. It seems that the competition is mainly determined by the kinetic growth properties of the bacteria. Another important conclusion was that, when seed sludge cultivated on a substrate with low sulphate levels, the ASRB will be absent or only present in low quantities, whereas the SRB become the predominant organisms in the sulphidogenic reactor. The non-preference for acetate is a nutritional characteristic of SRB (Oude Elfink, 1998) and even ASRB e.g. *Desulforhabdus amnigenus*, still prefer propionate and butyrate to acetate.

Visser (1995) showed in his study that regarding acetate utilisation in a sulphidogenic reactor, contradictory results have been reported. Several factors are known to influence acetate utilisation in the reactor, such as the acetate and sulphate concentrations, the type of seed sludge, as well as the effect of temperature and pH. Similar observations were made regarding the growth of the propionate degrading sulphate reducers, which will decrease under sulphate limitating conditions.

- *Alkalinity production*

Methanogenesis as well as sulphidogenesis is dependent on the digester/reactor pH which preferably should be in the range 6.7-7.4 for the methanogenic activity. SRB can tolerate a similar pH range, however, they prefer a pH as high as 8.0-8.5. Under balanced reactor conditions, the biochemical reactions tend to automatically maintain the pH within the required pH range. The acidogenic reactions in the reactor would result in a pH reduction due to the production of organic acids. However, this effect is counteracted by the concomitant formation of bicarbonate buffering ions. The most important buffering system in anaerobic digestion is the equilibrium between dissolved carbon dioxide and bicarbonate (reaction 2.13).

$$CO_2 + H_2O \rightarrow H_2CO \rightleftarrows H^+ + HCO_3^- \qquad (2.13)$$

- *Reactor temperature*

Anaerobic digestion can occur at two different temperature ranges, according to the two different groups of bacteria. The mesophilic MB grow and are active at temperatures up to 35-40 ℃, while the thermophilic MB operate at temperatures over 50 ℃, with an optimum between 55-75 ℃ . When the operating temperature is as low as 20-25 ℃, the mesophilic MB population usually predominates.

- *Reactor type*

O'Flaherty *et al.* (1998) attributed the competition between the ASRB and AMB to the reactor type, since biomass retention promotes dominance of AMB over ASRB. Omil *et al.* (1997) reported a selective washout of SRB from UASB reactors, operating at up-flow velocities of 4 and 6 m/h. Iza *et al.* (1986) attributed the dominance of MPB over SRB in anaerobic filters to the inferior attachment capacity of ASRB. Another consideration may be that only a small numbers of ASRB are present in the seed sludge and it may take a long time for SRB to eventually displace the AMB. However, O'Flaherty *et al.* (1998) showed that the AMB out-competed the ASRB even after 5 years under full-scale conditions, when treating a sulphate containing citric acid production wastewater at a COD/SO_4 ratio of 12 g COD/1.4 g SO_4^{2-}/ℓ, which ratio will favour the AMB.

2.11 BIOLOGICAL TREATMENT OF AMD: THE CHALLENGES

Mining contributes positively to the economy, but negatively to the environment, due to the production of contaminated effluents in the form of AMD, which should be treated so that it can either be discharged to a river system or re-used in the coal processing plant. Several treatment methods have been described, both in active

treatment plants as well as under passive conditions The main pollutants in AMD are the acidity and salinity and in some cases high metal concentrations. The most cost effective treatment option to remove the acidity is to apply the limestone neutralisation technology, which will result in treated water with a neutral pH and a partial sulphate reduction to ≈ 2000 mg/ℓ. In order to remove both the sulphate and the metals, the biological sulphate removal technology can be applied. The one product of biological sulphate removal is sulphide, which results in any heavy metals in the mine water being precipitated as metal-sulphides (MeS). The other product is alkalinity which assists in the pH increase of the treated water.

The most important factor for the biological sulphate removal technology is the need for a cost effective carbon and energy source (electron donor), while sulphate is the electron acceptor. Globally, many different carbon and energy sources have been described, varying from methanol, ethanol, sugar and gas mixtures, such as producer gas as well as a mixture of hydrogen and carbon dioxide. Recently, the emphasis has shifted to organic waste products, such as wheat straw, cow manure, mushroom compost and sewage sludge. These products all have cellulose in common. The advantage of the use of a bio-waste product is it can be used as energy source through the fermentation of cellulose to oligomers, monomers and ultimately volatile fatty acids, which then can be used as energy sources for biological sulphate removal.

2.12 CONCLUSIONS

The literature study has shown that the fermentation products from cellulose and hemicellulose, such as sugars, VFA, alcohols and hydrogen are favoured by SRB as carbon and energy sources. It has furthermore become evident that hydrogen as the final product of the degradation of organic product can be used by SRB in the reduction of sulphate and that the HSRB will out-compete the HMB for the utilisation of hydrogen in the presence of sulphate. SRB will select hydrogen, propionate, butyrate and acetate, in that order.

This information is important for the succesfull outcome of the study described in this thesis. The emphasis of the study therefore needs to be directed towards investigating which parameters are important for the production of VFA as well as the utilisation thereof for the biological sulphate reduction. The choice of the fermentative microbes to obtain the highest VFA production as well as the conditions under which

these microbes can be sustained will be investigated, as well as the use of the most applicable reactor system for maintaining a constant sulphate removal rate.

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CHAPTER 3

MICROBIAL CELLUOSE DEGRADATION FOR OPTIMAL VFA PRODUCTION AND BIOLOGICAL SULPHATE REDUCTION

3.1 INTRODUCTION

Elevated sulphate concentrations present in mine and other industrial effluents can be treated using biological sulphate removal technology. A disadvantage of this treatment method is the high cost of the carbon and energy sources. Therefore, cheaper alternatives have to be found and investigations into the use of the fermentation products of organic wastes have been initiated (Coetser *et al*. 2000; Rose, 2000; Dill *et al*. 2001). Waste utilisation, rather than treatment, reduces waste pollution and provides a source of energy (Sonakya *et al.* 2003). The hydrolisation of organic waste products, produce soluble intermediates due to the presence of exoenzymes e.g. cellulases, amylases, proteases and lipases (Sonakya *et al.* 2001). Anaerobic degradation of organic wastes in the presence of sulphate is a complex process since the SRB compete with MB for compounds such as acetate and hydrogen, whilst AB compete for compounds like propionate and butyrate (Oude Elferink, 1998). During biological sulphate removal, SRB utilize propionate and butyrate, of which some SRB oxidize these fatty acids completely to carbon dioxide, while others oxidize butyric acid (C4) and propionic acid (C3) to acetic acid (C2). SRB can also degrade the branched and long chain fatty acids to short chain volatile fatty acids (C4, C3 and C2). Since anaerobic hydrolysing/fermenting microbes can assist in the degradation of organic material, the investigations in this chapter focussed on detecting the naturally occurring microorganisms, which produce the highest concentration of VFA to be used for biological sulphate reduction.

The aim of the studies in this chapter was to investigate whether 1) VFA can be produced from a plant biomass using naturally occurring microorganisms and 2) the acids produced can function as the carbon and energy sources for biological sulphate removal.

The following microorganisms were sourced:

1) Natural occurring cellulose degrading microorganisms attached to grass cuttings
2) Microorganisms obtained from a municipal ww treatment Anaerobic Digester
3) SRB from a sulphidogenic demonstration reactor

3.2 MATERIALS AND METHODS

3.2.1 Plant biomass

Mainly Kikuyu grass cuttings (abbreviated as GC in the following text) was obtained from the CSIR, Pretoria Garden Service. After cutting, the GC were collected and kept at 4 ℃. GC used for the experiments described in this thesis came from the same stockpile from the cold room. The size of the GC was between1-2 cm. The weight of the grass in the thesis' text refers to air dried grass, due to storage at 4 ℃ (dry weight). Kikuyu grass *(Pennisetum clandestinum)* is a low growing, deep-rooted perennial with stolons and rhizomes, and forms a dense turf, which is very resistant to heavy grazing (Partridge, 2003). When the grass is degraded by cellulose fermenting organisms, nutrients, e.g. nitrate and phosphate are released. Generally, the average nitrate (NO_3^- N) concentration in the reactor was measured at ca. 20 mg/ℓ, while the phosphate (PO_4-P) concentration averaged 25 mg/ℓ in the grass degrading reactors.

3.2.2 Microbial biomass

For the cellulose degrading study, hydrolyzing microorganisms (essentially microorganisms occurring in and attached to decaying grass), as described in *3.2.3.1,* were used. A SRB mixture obtained from the CSIR-o-Sure demo plant (Navigation Mine, Witbank, South Africa) and an anaerobic sludge mixture obtained from the Daspoort Sewage Works, Pretoria, South Africa, were used for the following fermentation studies. The CSIR demo plant is a biological sulphate removing, one stage reactor system, which treated AMD at the Anglo Coal Navigation mine from 2000-2004 (Maree *et al*. 2004). The biomass from this sulphidogenic reactor was suitable seed sludge for the sulphate containing reactors in this study.

3.2.3 Experimental

All studies described in this chapter were executed using batch test conditions. The origin of the microbial populations used in the three studies is captured in Table 3.1.

3.2.3.1 Study 1: Hydrolysis study

The hydrolysis of cellulose in grass was investigated using two reactors (G1 and G2), which comprised two 5 ℓ plastic containers open at the top. G1 and G2 contained 30 g dry GC per ℓ tapwater (150 g GC/5 ℓ tapwater). G2 contained additional naturally occurring microorganisms obtained from a previous grass hydrolysis experiment. These were obtained by settling the contents of the reactor, mainly comprising the

partly degraded GC and microorganisms and discarding the supernatant from a previously used 5 ℓ reactor. The hydrolysis experiment was conducted over 42 days at room temperature. Fresh GC (30 g each time) were added to the reactors contents (5 ℓ) on days 1, 2, 6, 7, 22, 39 and 40. Samples for VFA analysis were taken daily.

Table 3.1. Overview of the three studies in this chapter

Study	Method	Microbial population	Study reactor	Control reactor
1	Hydrolysis	Natural occurring grass microbes	Hydrolysis biomass	No addition of biomass
2	Anaerobic degradation,	Digester sludge	See Table 3.2	
3	Anaerobic degradation,	SRB	See Table 3.3	

3.2.3.2 Anaerobic degradation/ SO_4 removal study

Three anaerobic reactors (F1, F2 and F3) for which the experimental conditions are given in Table 3.2 were operated at 35 °C to evaluate the VFA production using different cellulose degrading microbes. The supernatants of the three GC fermentation reactors (F1, F2 and F3) were used in sulphate removal batch tests, for which four stirred, anaerobically operated, glass bottles (volume 2 ℓ) with rubber stoppers, B1-B4, were used. Samples were taken from the bottoms of the four reactors, through an outlet fitted with a clamp. All reactors received 250 mℓ SRB biomass mixture (obtained from the CSIR sulphate removing demonstration plant in Witbank, (VSS was 10 g/ℓ), 2 mℓ/ℓ macro and micro nutrient mixture, as well as 2 ℓ of the supernatant from the fermentation reactors (F1, F2 and F3). The carbon sources for B1-B4 are given in Table 3.3. All four reactors received sulphate rich feed water ($MgSO_4$), so that the final SO_4 concentrations in B1-B4 were approximately 1 500 mg/ℓ SO_4. Reactors B1-B4 were operated at room temperature (25 °C).

Table 3.2 The experimental conditions in F1, F2 and F3

Reactor	Conditions
F1	30 g Grass Cuttings
F2	30 g GC + 100 mℓ Daspoort anaerobic sludge
F3	30 g GC+ 100 mℓ SRB mixture

Table 3.3 The carbon sources used in the different batch reactors

Reactor	B1	B2	B3	B4
Carbon Source	1 g sucrose/ℓ	Supernatant F1	Supernatant F2	Supernatant F3

3.2.4 Analytical

The sulphate, sulphide, alkalinity, COD, and pH were determined manually according to analytical procedures as described in Standard Methods (APHA, 1985). The analyses were all carried out on filtered samples, except for the COD analysis on feed water, the redox potential and the sulphide samples. Alkalinity was determined by titrating with 0.1N HCl to a pH of 4.3. Prior to the COD measurement, the sulphide in the samples from the reactors was removed by adding a few drops of 98% sulphuric acid and flushing the sample with nitrogen. The redox potential of the samples was calculated from the mV and stabilization temperature measured with a pH/redox meter (Metrohm 744) applying the following formula:

226 - (18 x temperature of reactor contents/25) = Value

Redox potential = Value + mV measurement of sample, where

226 is a Constant.

All VFA analyses were done using a gas chromatograph (Hewlett Packard. HP 5890 Series II) equipped with a flame ionisation detector (GC/FID), while the data analyses were done using the Chem Station (Hewlett Packard, software package). The column used was an HP-FFAP, 15 m x 0.530 nm, 1 micron. The N_2 flow rate was set at 1 mℓ/min. An outline of the GC/FID programme used is depicted in Table 3.4.

Table 3.4. The GC/FID programme for the detection of VFA

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

3.3 RESULTS AND DISCUSSION

3.3.1 VFA production from grass-hydrolysis by natural occurring microorganism on grass

The VFA concentrations (acetic, propionic and butyric acids) in the two hydrolysis reactors, G1 and G2, are given in Figures 4.1 to 4.3.

3.3.1.1 Acetic acid

The acetic acid concentrations in G1 and G2 were similar (Figure 3.1), showing that the addition of supplementary naturally occurring hydrolytic bacteria had no influence on the acetic acid production. Fresh GC were added on days 1, 2, 6, 7, 22, 39 and 40, after which no remarkable increase in the acetic acid concentration was observed. From day 22-39, no GC were added, which resulted in a decrease in the acetic acid concentration, which increased slightly on days 39 and 40 when fresh GC were added to the reactor.

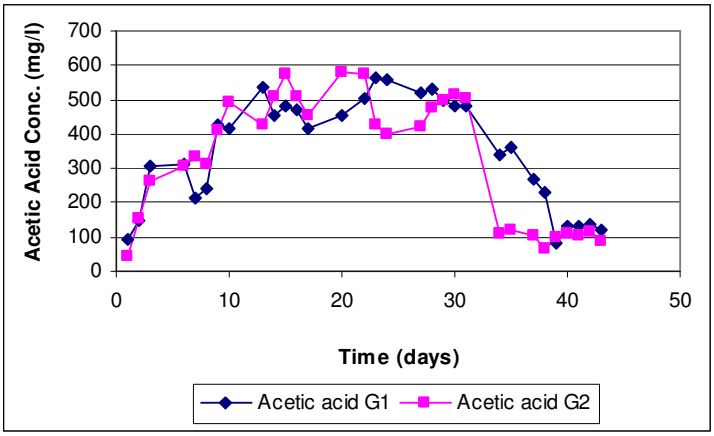

Figure 3.1. Acetic acid production in reactors G1 and G2.

3.3.1.2 Propionic acid

The propionic acid concentration in G1 increased from day 1-14 (Figure 3.2), whereafter it decreased to concentrations < 100 mg/ℓ up to day 22, on which day fresh GC were added to the reactor. The propionic acid concentration in G1 remained stable thereafter till day 31, after which it decreased. It increased again after day 34, to increase sharply on days 39 and 40, when fresh GC were added, indicating that the naturally grass occurring microbes degraded GC to propionic acid. The propionic acid in G2 followed a similar pattern as that in G1, except between day 28-38. The overall propionic acid concentration was higher in G2. This result

indicated that the addition of the natural cellulose degrading microorganisms resulted in a small increase in C3 acid concentration. The average propionic acid concentration in G1 was 129 mg/ℓ, while it was 193 mg/ℓ in G2, which was an improvement of 30%. This result indicated that the addition of hydrolytic microorganisms resulted in an increase of microorganisms. The results indicated that an increased microbial population proved beneficial for additional propionic acid production. Since SRB prefer propionic acid above acetic acid as the electron donor, this information is valuable as it showed that natural occurring microorganisms on grass can degrade to VFA , especially propionic acid.

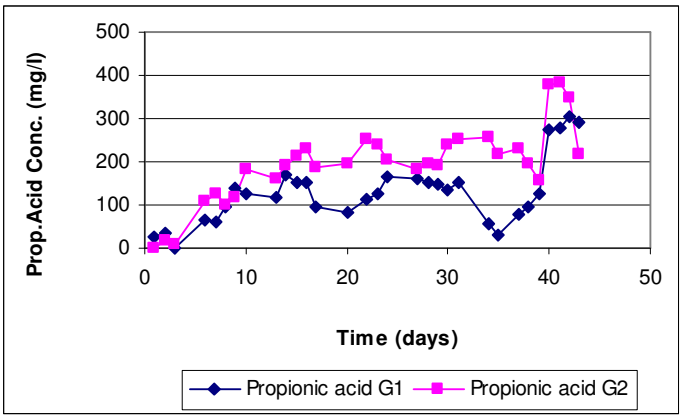

Figure 3.2. Propionic acid production in G1 and G2

3.3.1.3 Butyric acid

The results of the butyric acid production experiment are given in Figure 3.3. The butyric acid concentration in G2 was slightly higher than in G1, as was observed for propionic acid formation. The average butyric acid production in G1 during the total experimental period was 54 mg/ℓ, while it was 69 mg/ℓ in G2, which was 22% higher than in reactor G1. As in the case of the propionic acid production, the addition of the natural cellulose degrading microorganisms attached to grass appeared advantageous as it resulted in a slightly improved butyric acid production. It was noted, however, that only low concentrations of the acids were produced. The rate and extent to which a substrate is hydrolyzed is influenced by the accessibility to the substrate by the exoenzymes (Hobson, 1982). This is especially true for the anaerobic digestion of fibrous materials, in which the cellulosic and hemicellulosic microfibrils are aggregated and embedded within the liquefied cell wall matrix. The crystallinity and surface area of the fibres are the most important features which determine the accessibility for exoenzymes (Fan *et al.* 1980). In the hydrolysis step of the total degradation process, the particle size of the organic waste product influences the speed of the hydrolysis, due to the increased accessibility for the

exoenzymes. It seemed likely that when cellulose degrading microorganisms were adapted to the fermentation of the cellulose fibres in GC and subsequently added to the hydrolyzing reactors, it resulted in a small increase in C3 and C4 acid production (30 and 22%, respectively). This finding indicated that cellulose degrading microbes attached to grass naturally ferment cellulose to VFA, especially propionic and butyric acids, albeit in lower concentrations than the acetic acid concentration.

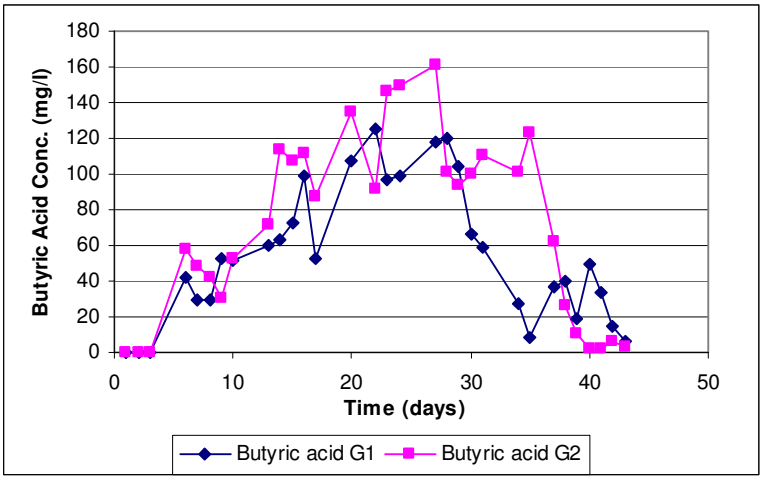

Figure 3.3. Butyric acid production in G1 and G2.

3.3.2 Effect of cellulose degrading anaerobic microorganisms on the VFA production, followed by SO_4 reduction

3.3.2.1 VFA production

The total VFA concentrations in F2 and the butyric acid concentrations in F1-F3 are given in Figures 3.4 and 3.5, respectively. The graphs in Figure 3.4 show the VFA production in F2 and those in Figure 3.5 show the butyric acid production in all three reactors during the first 80 hours of the experiment. It was observed from Figure 3.4 that the acetic acid concentration in F2 increased between 22-56 h and that it decreased thereafter, most likely due to methane production. The propionic acid concentration in F2 was the lowest. The graphs in Figure 3.5 show that the butyric acid production followed the same pattern in all three reactors during the first 50 h. After 72h, the butyric acid concentration was the highest in F2. This finding confirmed the result of Sonakya *et al.* (2003), who reported that the butyric acid concentration was higher than that of the C2 and C3 acids, when measured over the same period.

Zoetemeyer *et al.* (1982) showed that the degradation process depends on environmental conditions. When fermenting glucose in a separate, acid producing reactor, the main products were butyric acid, acetic acid, hydrogen and carbon dioxide. However, when interrupting the feed supply for a period of 1 – 24 h, the fermentation pattern changed to an increased production of propionic and acetic acid. They furthermore showed that at pH values < 6, the main fermentation product of glucose was butyric acid, and when the pH was increased, the product pattern changed, to lactic and acetic acid and to formic acid and ethanol. The product pattern of the fermentation also depends on the type of organic waste (Cohen, 1983). Wolin (1976, 1979) demonstrated the importance of hydrogen production and utilisation in the fermentation reactor. Removal of hydrogen, e.g. by the hydrogenotrophic bacteria such as hydrogen consuming SRB (HSRB), can influence the kinds of products formed by the fermentative bacteria. When hydrogen is consumed by the HSRB or HMB, the fermentative bacteria can produce further oxidized products than they otherwise would do at increased hydrogen levels, which supply more energy per unit of substrate to the bacteria. This finding indicates that when syntrophic bacteria, such as HSRB, are present in the reactor system, keeping the hydrogen partial pressure low, other organisms use the electrons generated in the fermentation process, for hydrogen production rather than for the production of ethanol (Reddy *et al.* 1972). This observation is of importance when using the fermentation products of cellulose for the biological reduction of sulphate in the fermentation tank, when sulphate and SRB are present, since SRB use hydrogen as energy source for sulphate reduction. Harmsen (1996) showed that SRB can participate in the degradation of organic material, to produce propionic acid, even when no sulphate is present.

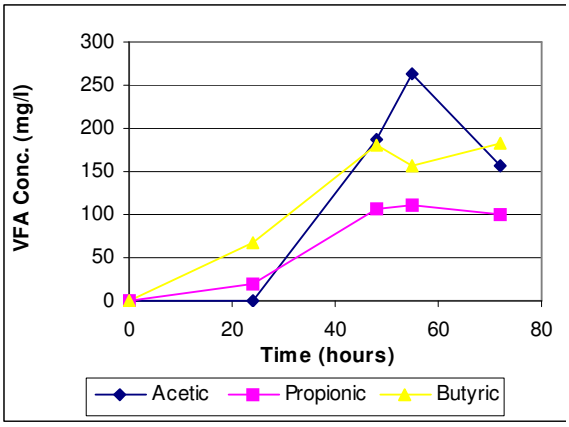

Figure 3.4 The VFA production in F2

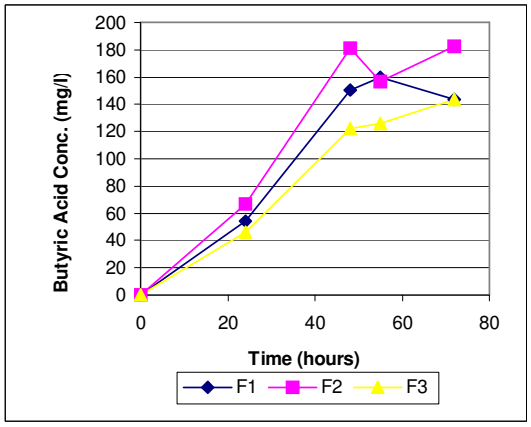

Figure 3.5 Butyric acid production in F1, F2 and F3

3.3.2.2 Sulphate reduction and sulphide production

The sulphate removal and sulphide production using sucrose (control) and the VFAs produced (from reactors F1-F3) are given in Figures 3.6 and 3.7. The results showed that the sulphate reduction rates in all four reactors B1-B4 were similar. However, when linear regression was applied to the four graphs, the concentrations of SO_4 removed were 279, 287, 266 and 349 mg SO_4/d for B1-B4, respectively. These results indicated that during the degradation process in the fermentation reactors (F1-F3), the most favourable fermentation products for sulphate removal were produced in F3, the reactor containing GC and the SRB biomass mixture. The sulphate reduction using sucrose (two step reaction) is given in equation 3.1 and for propionate and butyrate in 3.2 and 3.3, respectively:

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H_2 \quad (3.1)$$

$$8H_2 + 2SO_4^{2-} + 2H^+ \rightarrow 2HS^- + 8H_2O$$

$$C_{12}H_{22}O_{11} + 2SO_4^{2-} + 2H^+ \rightarrow 4CH_3COOH + 4CO_2 + 2HS^- + 3H_2O + H_2O$$

$$CH_3CH_2COO^- + \tfrac{3}{4}\, SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + \tfrac{3}{4}\, HS^- + \tfrac{1}{4}\, H^+ \quad (3.2)$$

$$CH_3CH_2CH_2COO^- + \tfrac{1}{2}\, SO_4^{2-} \rightarrow 2\, CH_3COO^- + \tfrac{1}{2}\, HS^- + \tfrac{1}{2}\, H^+ \quad (3.3)$$

Equations 3.1-3.3 show that acetate is the degradation product of the biological sulphate reduction of most carbon and energy sources. It is considered the most recalcitrant VFA (Lens *et al.* 1998; Vallero *et al.* 2003) and the rate limiting factor (Visser *et al.* 1993) in a sulphidogenic reactor and usually represents a final COD concentration of 200-500 mg/ℓ (Greben *et al.* 2000).

The highest sulphide concentration was obtained in the control reactor B1, followed by reactor B4, containing the carbon and energy source produced in F3.

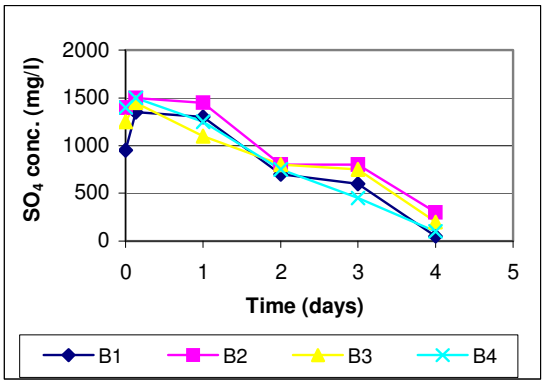

Figure 3.6 Sulphate reduction in B1-B4

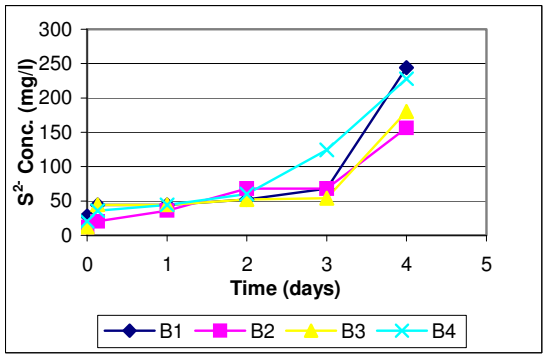

Figure 3.7 Sulphide production in B1-B4

3.3.2.3 VFA utilisation in reactors B1 to B4.

The VFA concentrations in B1 to B4 are given in Figures 3.8 to 3.11

3.3.2.3.1 Reactor B1

The graphs in Figure 3.8 showed that on day 0, acetic, propionic, butyric and valeric acids were present in B1, but that on days 1-3, most of the acids had been utilized, except for acetic acid. When glucose is fermented by the SRB, the final product is H_2, with many intermediate products, such as organic acids. Butyric and propionic acids can be used by SRB as the carbon and energy source. O'Flaherty *et al.* (1998) studied the population structure of biomass from a full-scale anaerobic reactor after 5 years of operation. The results showed that the SRB incompletely oxidised propionate to acetate. The SRB produce four moles of acetate from four moles of propionate for the reduction of three moles of sulphate. It was observed that the SRB and MB competed for butyrate and ethanol. It was suggested that in the presence of sulphate, compounds, such as alcohols, lactate, propionate and butyrate, may be oxidized directly by the SRB (Oude Elfering, 1998). The acetic and propionic acid concentrations had increased in the reactor by day 4 (Figure 3.8). Figure 3.6 showed that the SO_4 concentration decreased to <100 mg/ℓ, indicating that no further COD (VFA) was required by SRB in B1, thus the residual COD (1 394 mg/ℓ) in the reactor consisted mainly of acetic acid and propionic acids (Figure 3.8), higher fatty acids, alcohols and other organic matter.

3.3.2.3.2 Reactor B2

Reactor B2 contained the supernatant from F1, the reactor to which no additional microorganisms had been added. The graphs in Figure 3.9 showed that the C3, C4 and C5 acids were utilized by the SRB, producing the C2 acid as was also shown for reactor B1.

3.3.2.3.3 Reactor B3

Reactor B3 was operated on the supernatant from F2 (GC + Daspoort anaerobic biomass). The results for the VFA concentrations in B3 are given in Figure 3.10, while the graphs in Figure 3.11 show the VFA concentration in B4. The graphs in Figure 3.10 show that on day 2, a high concentration of propionic acid was present in B3, which decreased to zero on day 4. Usually, when propionic acid is utilised, the acetic acid concentration increased. The low acetic acid concentration in B3 may indicate that the acetic acid was utilised by the MB present in the anaerobic sludge obtained from Daspoort. A marginal increase in the butyric acid concentration from days 2-4 could be seen. It seems that when the propionic acid concentration decreased, the butyric acid slowly increased. This finding may indicate that when SRB used propionic acid, the butyric acid increased in the reactor.

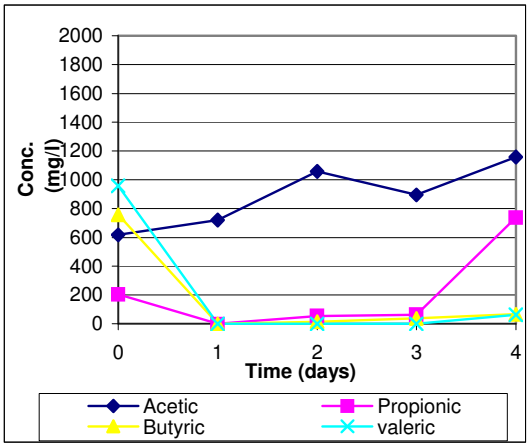

Figure 3.8 The VFA concentration in B1

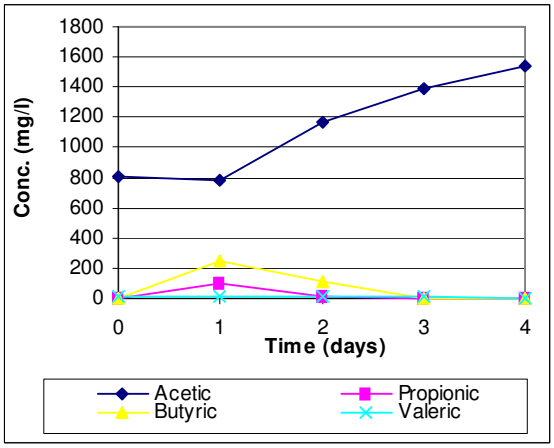

Figure 3.9 The VFA concentration in B2

3.3.2.3.4 Reactor B4

From Figure 3.11, it was seen that initially propionic acid, acetic acid and valeric acids were present in the reactor at concentrations ca. 1000, 700 and 450 mg/ℓ, respectively, which decreased to practically zero on day 1 and subsequently all

available VFA were used up during the experimental period. As soon as the butyric acid was produced (days 1-2), it was utilized (days 2-3). The graphs in Figure 3.11 furthermore showed that the acetic acid concentration decreased from a concentration of > 500 mg/ℓ to values < 100 mg/ℓ. This finding did not correspond with the result obtained from reactors B1 and B2, where an overall increase in the acetic acid concentration was observed. Acetic acid utilisation was observed in B3 as well. The acetic acid degradation in B3 and B4 can possibly be ascribed to the fact that during the fermentation process, bacteria from the SRB mixture were present in the fermentation tank F3, while the supernatant of F2 contained the sludge from the Daspoort anaerobic digester. The MB (present in the Daspoort sludge) most likely degraded the acetate to methane gas in B3, while the SRB in B4 may have used the acetate for further sulphate removal in the absence of the C3 and C4 acids. No gas analyses were conducted to substantiate this theory.

No acetic acid reduction was observed in reactor B1 (glucose reactor) and B2 (supernatant F1: containing only GC) to which no additional bacteria had been supplemented. Omil *et al*. (1996) reported that from a thermodynamic and kinetic point of view, the SRB out-compete the MB on hydrogen, acetic, propionic and butyric acids. Hydrogen is completely consumed by SRB, while propionic and butyric acids are used faster by SRB than by syntrophic consortia. For acetate, the sulphidogenic acetate conversion has been found predominant in marine and freshwater sediments and in mixed reactors, as was the case in this study. The SRB will use hydrogen, propionic- and butyric- and acetic acid as electron donors in that order. Thus, it can be assumed that when no other substrates are available, SRB will utilise acetate. ASRB are slow growers, but when no other substrate is available, SRB will use acetate. Generally, several factors can affect the acetate utilisation by SRB or MB, such as type of substrate and inoculums used (as in this study), reactor pH and temperature and immobilisation properties (Oude Elferink, 1998).

Omil *et al.* (1996) showed that an acetate rich mixture favoured the MB, even when the reactor was started up with sulphidogenic sludge, while a propionate and butyrate rich mixture promoted the SRB. This finding indicated that even when sulphate adapted sludge is used, as in the case of F3, MB may still be present, which can explain the utilisation of the acetate in B4.

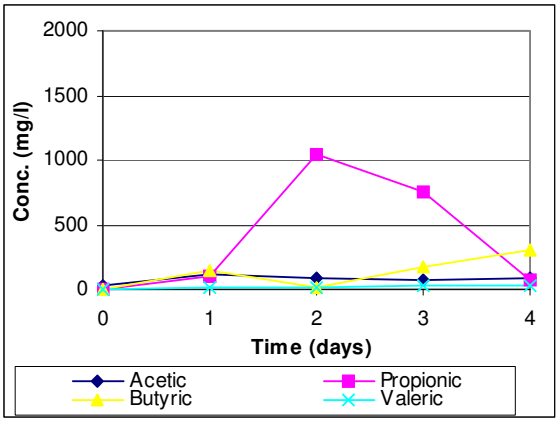

Figure 3.10 VFA concentrations in B3

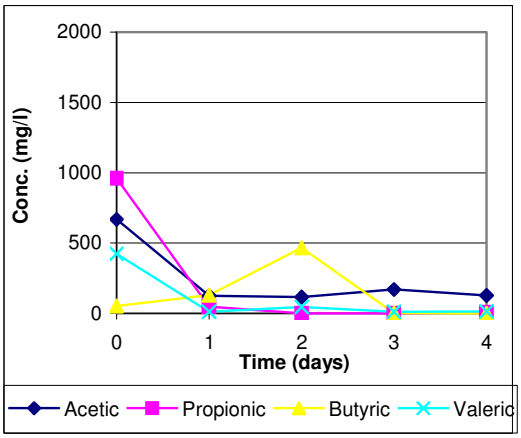

Figure 3.11 VFA concentrations in B4

3.4 CONCLUSIONS

It was concluded from these studies that VFA production from GC was observed. The results showed that VFA production occurred in a hydrolysis tank, using naturally occurring grass-degrading microorganisms. After adding extra hydrolysing bacteria during the hydrolysis process, the C2 and C3 acid concentrations increased by 30 and 22%, respectively. When using cellulose-degrading microorganisms from anaerobic digester sludge and sulphate adapted biomass, acetic acid was produced in the highest concentration (almost 600 mg/ℓ) as opposed to propionic acid at an average of 250 mg/ℓ and butyric acid at about 160 mg/ℓ.

Using the SRB biomass mixture in the fermentation process resulted in butyric acid production at a higher concentration and at a faster rate than when the bacteria from the anaerobic digester were used. This finding showed that SRB can produce VFA, even when no sulphate is present. When using the supernatant from the fermentation tanks, containing the VFA produced, for the removal of sulphate in a second reactor, sulphate reduction was observed over a period of 4 days. A slightly better sulphate reduction (349 mg SO_4/d) was obtained using the supernatant from the fermentation reactor containing GC and the SRB biomass mixture than when using sucrose in the control reactor as the carbon and energy source (279 mg SO_4/d). The VFA concentrations in the four sulphate reducing reactors showed that in the control (sucrose) reactor and in the reactor receiving the supernatant of the fermentation tank without additional biomass, the acetic acid concentration increased, indicating that the acetic acid was not utilized for the biological sulphate removal process. No residual acetic acid was measured in the reactors which received the supernatant

from the reactors to which the anaerobic biomass mixtures had been added. These results appeared to indicate that the MB used the available acetate to produce methane or, alternatively, other bacteria (e.g. AB) used the acetate to produce propionate thus providing the SRB with more substrate for subsequent sulphate reduction. It is possible that in absence of other substrates, acetate is used by the SRB as carbon and energy source.

Although all described microorganisms produced VFA, which could be used for subsequent sulphate reduction, it was observed that the concentrations especially of propionic and butyric acids were relatively low. The focus in the following chapter was therefore on the comparison between using SRB and rumen fluid microbes, natural cellulose degrading microorganisms, for VFA production.

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CHAPTER 4

THE EFFECT OF INCREASED GRASS CONCENTRATION ON THE VFA PRODUCTION AND SUBSEQUENT SULPHATE REDUCTION USING SRB AND RUMEN FLUID AS FERMENTATION INOCULA

4.1 INTRODUCTION

Cellulose is the major constituent of plant biomass, forming an important component in the carbon cycle. The formation of cellulose can be ascribed to photosynthesis and the CO_2 supply in the atmosphere (0.036%). The carbon cycle is closed as a result of the cellulose utilizing microorganisms present in soil and the guts of animals (Lynd *et al*. 2002). Plant biomass is a sustainable source of energy when cellulose is utilised during anaerobic degradation, resulting in the production of VFA and other degradation products. This process involves many species of bacteria, such as the AB and the MB. The SRB also play a role in the degradation of the complex polymers in the presence of sulphate (Oude Elferink, 1998). Greben and Baloyi (2004) showed that the anaerobic degradation of plant biomass (grass) to VFA could be enhanced when sulphate-adapted biomass was added to the fermentation process, even when no sulphate was present. This outcome indicated that the SRB participated in the degradation of the polymers and monomers to produce VFA. The utilisation of propionic acid in the absence of sulphate was shown by Harmsen (1996).

Fermentation of cellulose also occurs in the rumen of the ruminants. These are herbivorous mammals that possess a special organ, the rumen, within which the digestion of cellulose and other plant polysaccharides occurs through the activity of special microbial communities (Barnes and Keller, 2003, 2004). The energy containing components (carbohydrates, such as cellulose) in the ruminant feed are, during the fermentation process, converted into microbial cells and compounds such as CO_2, CH_4, and acetic, propionic and butyric acids, of which the acids are utilized by the host. The rumen is inhabited by between 10^{10}-10^{11} bacteria and 10^6 protozoa per mℓ rumen fluid. The rumen houses a complex ecosystem where microorganisms live in symbiotic relationships that facilitate fibre digestion. Therefore, it seemed likely that anaerobic degradation of plant material may be executed more efficiently using the bacteria, fungi and protozoa occurring in the rumen (Lee *et al*. 2000). Recent work published by Sonakya *et al*. 2003 demonstrated this concept with the use of digested cattle feed for the production of VFA from grass cuttings.

The results in the previous chapter showed that VFA production was possible from the cellulosic components of plant biomass (GC) using different inocula. However, the results also indicated that the VFA production was low when using the SRB as fermentation microorganisms. In order to achieve higher VFA concentrations it was decided to use grass-cellulose fermenting organisms, obtained from rumen fluid. It also appeared likely that higher grass concentrations could result in improved VFA production. The following studies were conducted with the aim of producing high concentrations of VFA, which subsequently would result in enhanced sulphate removal rates.

The first objective of the study was to investigate whether an increase in GC concentration would result in an increased SO_4 removal rate.
The second objective was to compare the production and utilisation of VFA obtained from grass cuttings (GC) when bacterial communities obtained from
1) Biological sulphate removal systems and
2) Rumen fluid of sheep alone and combined with SRB
were added to the fermentation reactors, containing GC, tap- and SO_4 rich water.
The third objective was to investigate the conditions under which the rumen bacteria would produce VFA consistently for sustained biological sulphate reduction.

4.2 MATERIALS AND METHODS

In order to achieve the above mentioned objectives, three different studies were conducted. Study 1 investigated whether the highest grass concentration would result in the highest VFA production and thus in the highest sulphate removal rate, using an SRB community as the added fermentative microorganisms. During Study 2, the VFA production and utilisation was compared, using microorganisms originating from the rumen (RB) of sheep as well as a combination of RB and SRB as the cellulose degraders. During Study 3, different reactor conditions affecting the VFA production and subsequent sulphate reduction were investigated, using RB as the sole cellulose degrading microorganisms. In the first reactor sulphate rich water was used to which RB were added. In the second reactor tap water and RB were mixed, while tryptone was added to the third reactor, since tryptone can stimulate propionic acid production using RB (personal communication, Professor P.J. Weimer, Dairy Forage Research Center and Department of Bacteriology, Madison, Wisconsin, USA).

4.2.1 Study 1

4.2.1.1 Experimental

This study was carried out under anaerobic conditions (closed to the environment, Dissolved Oxygen concentration of 0 mg/ ℓ) in three 2.5 ℓ Perspex batch reactors: T30, T60 and T90. To each reactor 250 mℓ mixture of SRB (VSS: 10 g/ℓ) obtained from the CSIR Demo plant in Witbank, was added as the fermentation and sulphate removing inocula. All three reactors contained sulphate rich water, made up of Na_2SO_4 (Merck), to which macro and micro nutrients (1mℓ/ℓ) were added (Table 4.4). When the SO_4 concentration in the reactors approached zero, fresh SO_4 solution was added to the reactor (indicated by arrows in the figure), to monitor further SO_4 removal. Different GC concentrations: 30, 60 and 90 g/ℓ in sulphate rich water were used. No fresh GC was added during the experimental period of 42 days. This investigation was conducted at room temperature (25 ˚C). The pH of the reactors was maintained at 7.0-7.5. The experimental conditions are given in Table 4.1.

Table 4.1. Experimental conditions for Study 1

Parameter	**Reactors**		
	T30	**T60**	**T90**
GC (g/ℓ)	30	60	90
SO_4 concentration (mg/ℓ)	1600	1700	1600

4.2.2 Study 2

4.2.2.1 Experimental

Four 500 mℓ batch reactors (R1-R4) were used as the fermentation reactors. R2 and R3 contained 25 mℓ rumen fluid, obtained from fistulated sheep at the University of Pretoria, while R1 and R4 contained a mixture of SRB and RB (12.5 mℓ each). When collecting the rumen fluid from the University, it was transported in a closed vessel, placed in a bucket of warm water (body temperature) and stored in an incubator (37-39 ℃) upon arrival at the CSIR laboratories. It has to be taken into account that shifts in microbial composition of the rumen fluid will occur continuously, due to the "foreign" conditions in the storage vessel as well as in the bio-reactors.

R1 and R2 contained tap water, while sulphate rich water was used in R3 and R4. All four reactors (Vol.: 450 ml) contained 30 g GC (≈ 60 g GC/ℓ). The experimental conditions of Study 2 are given in Table 4.2. The reactors were shaken in an incubator at 39 °C. The pH in the reactors was maintained between 6.6-6.9, to ensure the optimum conditions for the rumen microorganisms. The experimental period was 53 days.

Table 4.2. Experimental conditions of Study 2

Reactors	SO_4 rich water	Tap water	RB	SRB
R1		X	12.5 mℓ	12.5 mℓ
R2		X	25 mℓ	
R3	X		25 mℓ	
R4	X		12.5 mℓ	12.5 mℓ

4.2.3 Study 3

4.2.3.1 Experimental

Three batch reactors (Vol. 2.5 ℓ) were used: L1, L2 and L3. The experimental details are given in Table 4.3. The duration of Study 3 was 25 days.

Table 4.3. Experimental conditions of Study 3

Reactor	Contents
L1	1500 mg/ℓ SO_4 + 30 g/ℓ GC + 250 mℓ RB + nutrients (Table 4.4)
L2	Tap water + 30 g/ℓ GC + 250 mℓ RB + nutrients
L3	Tap water + 30 g/ℓ GC + 250 mℓ RB + 2.5 g tryptone + nutrients

Table 4.4. The chemical composition of the nutrient solution

MACRO NUTRIENTS	MICRO NUTRIENTS
6.5% N ,2.7% P, 13.0% K, 7.0% CA, 2.2% MG , 7.5 % S	0.15% FE, 0.024% MN, 0.024% B, 0.005% ZN, 0.002% CU , 0.001% MO

4.2.4 Analytical

The same analytical procedures as described in Chapter 3 (3.2.4) were followed.

4.3 RESULTS AND DISCUSSION

4.3.1 Study 1. The use of SRB as fermentative and SO_4 removing bacteria

4.3.1.1. Sulphate reduction

Reactor T30

The graphs in Figure 4.1 showed that from days 1-8 sulphate removal occurred and that the sulphate concentration was < 50 mg/ℓ after 8 days. During this period, the propionic and butyric acid concentrations in the reactor were too low to be measured, which indicated that as soon as the VFA were produced they were utilised by the SRB. Generally, when propionic acid is utilised by SRB as the carbon and energy source, acetic acid is produced. The results in Figure 4.1 confirmed the production of acetic acid up to day 15. When the sulphate concentration decreased to < 50 mg/ℓ, fresh sulphate was added to the reactor (Day 8: *arrow*). From days 9-13 the sulphate reduction continued. However, during the following period, the sulphate concentration in the reactor increased, for which no explanation can be given. The decrease in propionate concentration coincided with cessatation of sulphate removal. The graphs in Figure 4.1 showed that no propionic acid was available in the reactor and only a small amount of butyric acid, which most likely resulted in no further sulphate reduction.

Reactor T60

Similarly, as in T30, sulphate removal could be observed during the first 8 days of operation in reactor T60. During that period, the butyric and propionic acid concentrations were very low, while the acetic acid concentration (oxidation product of the propionate utilisation) increased. On day 8, fresh sulphate was added (arrow), which was initially reduced during days 9-14 to 500 mg/ℓ, whereafter sulphate reduction ceased. No further sulphate reduction was ascribed to the low butyric and propionic acids concentrations in the reactor.

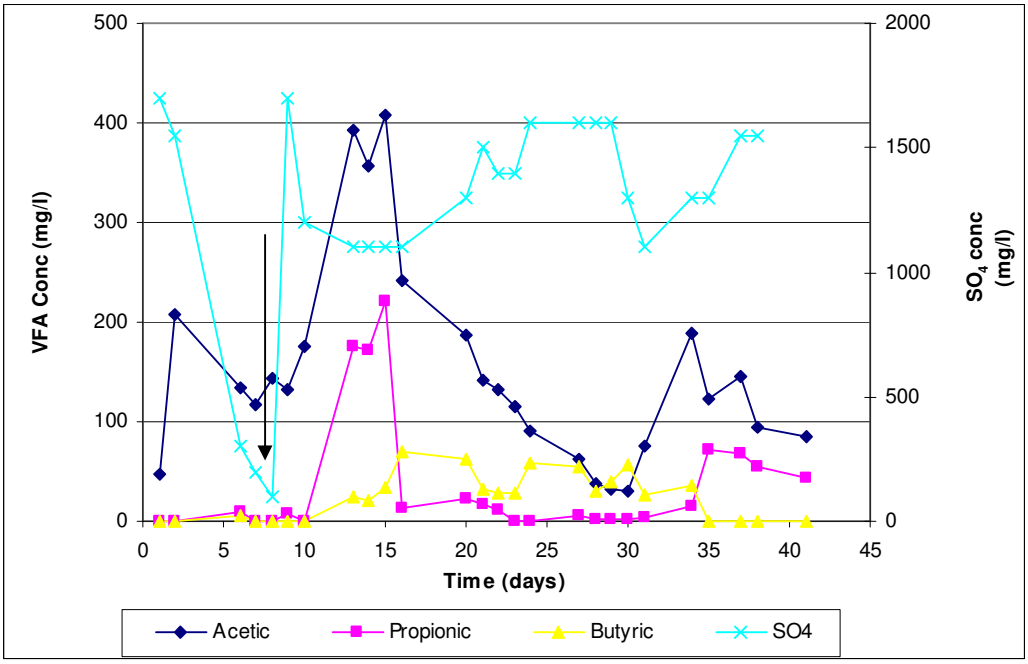

Figure 4.1. The SO_4 reduction and VFA pattern in T30.

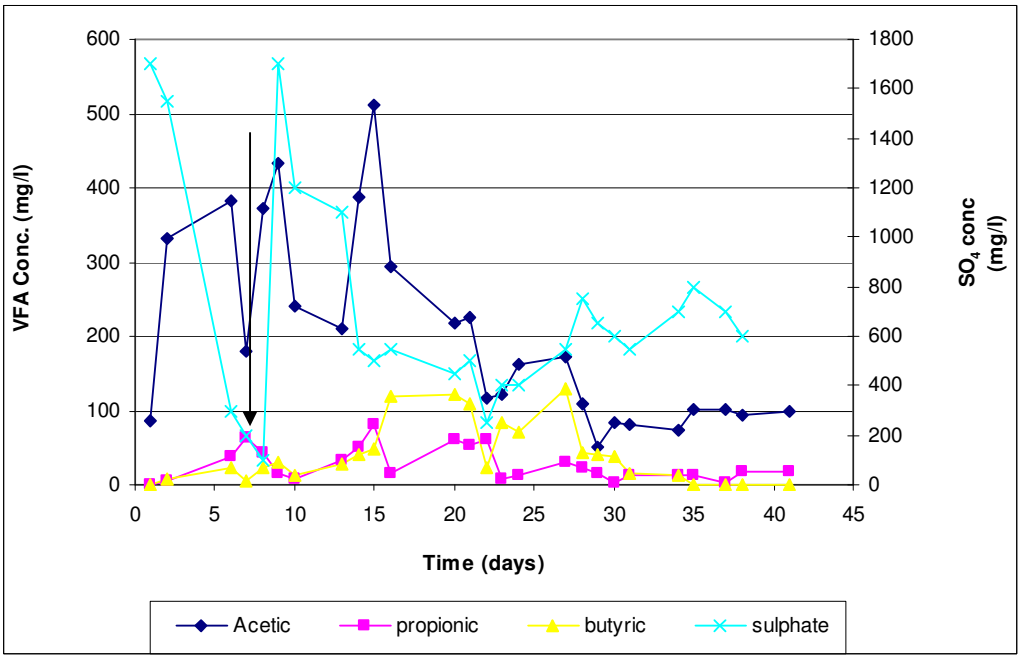

Figure 4.2. The SO_4 reduction and VFA pattern in T60.

When evaluating the VFA concentrations and extents of sulphate reduction in T30 and T60, it was evident that the sulphate reduction could be maintained over a longer period in T60 due to the higher concentrations of the C3 and C4 acids. Furthermore, it was noted that the acetic acid concentration in T60 was higher than in T30. This

result indicated that when sufficient C3 and C4 acids were present in the reactor to reduce the available sulphate load, no acetic acid was used by the SRB. It is interesting to note that as soon as fresh sulphate solution was added to the reactor, the VFA, as well as the SO_4 concentration decreased, showing that the SRB indeed utilised the available VFA produced from the GC for sulphate reduction. After day 30, the sulphate reduction stopped, due to the low propionic and butyric acid concentrations.

Reactor T90

The graphs in Figure 4.3 showed that sulphate reduction in T90 occurred during the first few days of operation and that the VFA production was higher than its utilisation. When fresh sulphate was added to the reactor (Day 8, *arrow*), the sulphate concentration decreased, but not as rapidly as during the first week. This was ascribed to the lower propionic acid concentration in the reactor (from day 16 onwards). The acetic acid concentration decreased when both the sulphate and propionic acid concentrations decreased in the reactor. On day 22 (*arrow*), a fresh supply of sulphate was added to the reactor, which was reduced during the subsequent period (up to day 34), coinciding with a very low concentration of propionic acid in the reactor. During days 22-34 the butyric acid and the acetic acid concentrations decreased in the reactor, which may indicate that the SRB used these substrates for their respiration because of the low propionic acid concentration. In some instances the homoacetogenic bacteria, which normally produce acetate, using H_2 and CO_2, can also produce butyric acid from 2 molecules of acetic acid. It can be assumed that symbiotic interactions between the different microorganisms occurred in the reactor, when SRB require a carbon source to reduce the available sulphate.

Comparing the experimental results obtained from operating T30, T60 and T90, it was observed that when the initial concentration of GC was 30 g/ℓ, all VFA produced were utilized by the SRB, including acetic acid. When, however, the GC concentration was increased to 60 g/ℓ, the sulphate reduction was faster, but not all VFA produced were utilised. When 90 g/ℓ GC was added to the reactor, the sulphate reduction was initially faster and more consistent. It seemed that a shift in the utilisation of the different acids occurred. Initially sufficient C3 and C4 acids were present. After the propionic acid had been utilised, the SRB started utilising the butyric and even the acetic acid. From the graphs in Figures 4.1-4.3, it was noted that the reactor containing the highest concentration of GC (90 g/ℓ) formed the highest concentration of acids and the sulphate removal rate was the highest, e.g.

1500 mg/ℓ sulphate being removed within 5 days in T90, while not all VFA were utilised. This result showed that the SRB utilised the VFA selectively, i.e. the C3 before the C4 acid. This observation is in agreement the findings of Harmsen (1996) and Harada (1994). When enough GC were added to the fermentation reactor, adequate amounts of VFA were produced to reduce the available sulphate in a short time.

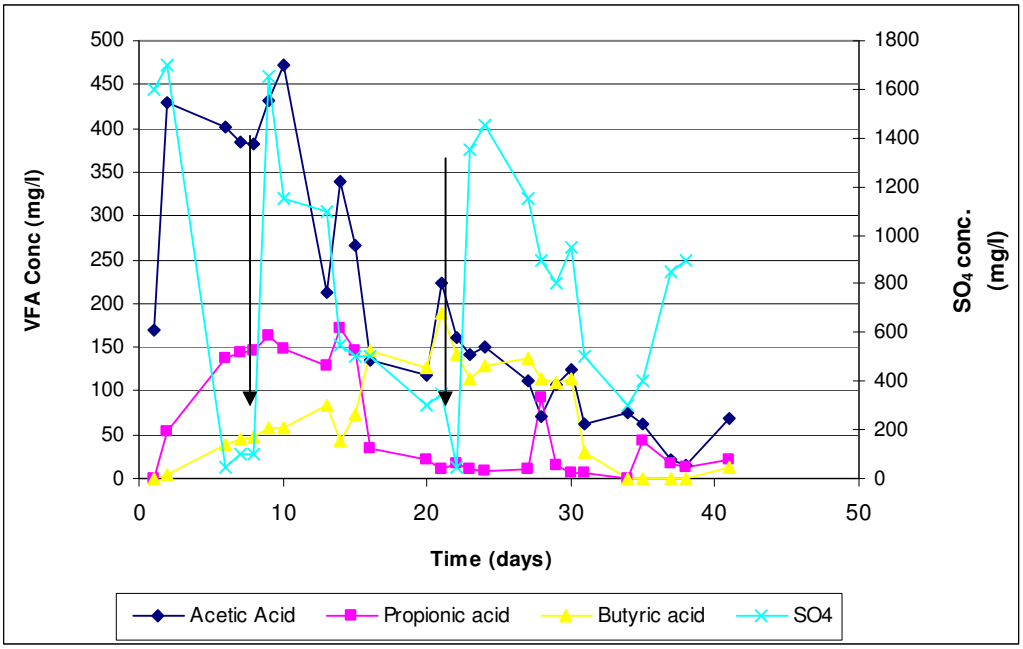

Figure 4.3. The SO_4 reduction and VFA pattern in T90.

4.3.1.2 VFA concentration

The C2, C3 and C4 acid concentrations in T30, T60 and T90, respectively, are given in Figures 4.4-4.6.

4.3.1.2.1 Acetic acid

The acetic acid (C2) production in Reactors T30, T60 and T90 followed a similar pattern: the C2 acid concentration increased during the first two weeks (up to day 15) of the experimental period, whereafter it decreased. This increase in the acetic acid concentration correlated with the sulphate reduction. When the SRB utilised the propionic and butyric acids to reduce the sulphate, acetic acid was produced. The average C2 concentrations from day 1-15 were 214, 312 and 329 mg/ℓ, in T30, T60 and T90, respectively. These results indicated that when the GC concentration was doubled from 30 to 60 g/ℓ, the average acetic concentration did not increase by the same factor. When the GC concentration was increased to 90 g/ℓ, the acid production

hardly increased any further. This irregular acetic acid production/utilisation from an increased concentration of GC can possibly be ascribed to the utilisation of acetic acid by the SRB in the T30 reactor and/or by another microbial population, such as the MB, producing methane gas. Gas production was not measured, though gas production was observed in the reactor during the experimental period.

4.3.1.2.2 Propionic acid

The C3 acid concentration in T30 was low during the first few days of operation, which was in agreement with the sulphate reduction during the first 8 days. After day 10, the propionic acid in reactor T30 increased untill day 15, to decrease thereafter at the same rapid rate. Sulphate reduction was observed in T30 between days 1-8 and days 9-15. The sulphate concentration stabilised after day 15, which can possibly be ascribed to the low propionic acid concentration in T30. No further C3 acid production was observed, except after day 35, despite no fresh GC having been added. However, during that time, butyric acid production decreased and the increase of the C3 acid was ascribed to the possible degradation of butyric acid by microorganisms other than SRB. The propionic acid concentration in T60 was stable at about 50 mg/ℓ for several days, whereafter it decreased. The propionic acid concentration correlated with the sulphate reduction in T60. It was noted that the highest C3 concentration occurred in the reactor together with the highest GC concentration (T90) and that, especially during the first 15 days, a high propionic acid concentration was noticed, even though sulphate was being reduced concomitantly. Barnes and Keller (2003) indicated that an increase in the propionic acid concentration is related to overloading of the reactor. They noted that build-up of cellulose resulted in a significant change in fermentation stoichiometry pattern. The higher concentration of grass-cellulose, when 90 g/ℓ GC were added to the reactor, may have resulted in the high propionic acid concentration from day 0-15 in T90.

The sulphate reduction was of such nature that on days 8 and 22 a fresh sulphate solution was added to the reactor, which was reduced throughout the duration of the experiment. The highest propionic acid concentration was measured at ca. 150 mg/ℓ in T90 from days 6-15, during which time sulphate reduction was observed.

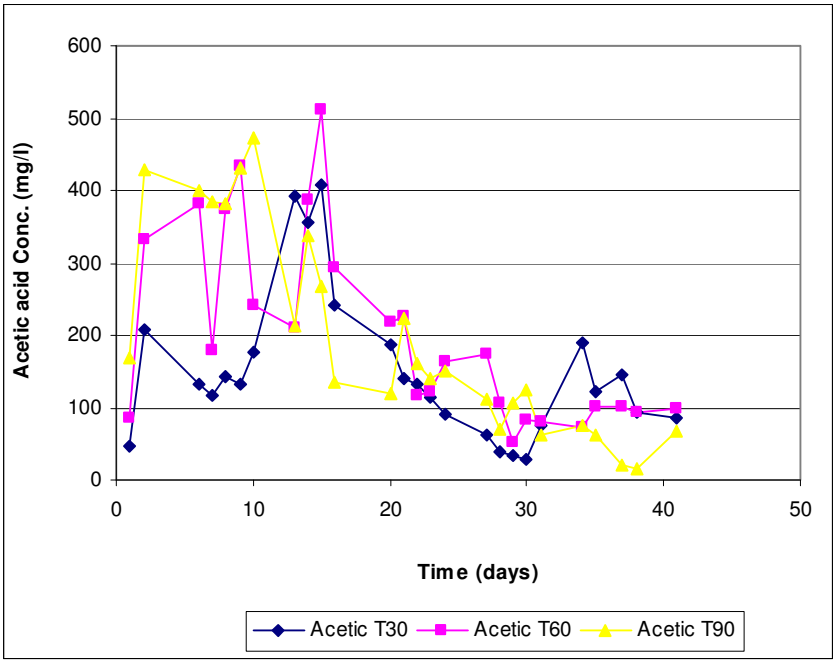

Figure 4.4. Acetic acid concentration in T30, T60 and T90.

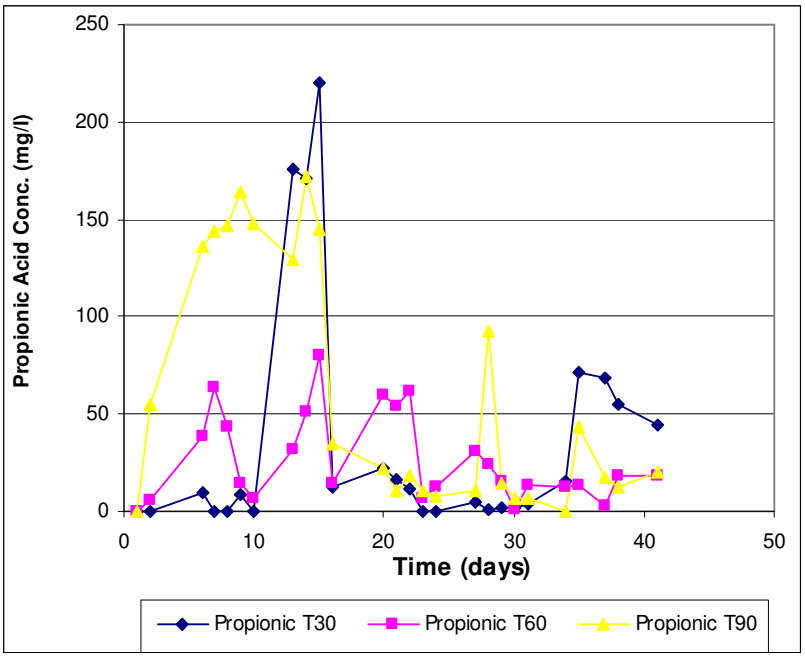

Figure 4.5. Propionic acid concentration in T30, T60 and T90.

4.3.1.2.3 Butyric acid

The butyric acid production in Reactors T30, T60 and T90 is depicted in Figure 4.6. The highest butyric acid concentration was observed during days 10-35 in all three reactors. The average values of the butyric acid concentrations in the three reactors during the full experimental period of 41 days, was 23 mg/ℓ in T30, 39 mg/ℓ in T60

and it was 70 mg/ℓ in T90. These results seemed to indicate that, unlike the acetic acid and propionic acid concentrations, the butyric acid concentration was proportional to the GC concentration. The higher GC concentration yielded the highest butyric acid concentration measured in the reactor. Generally, during the anaerobic degradation process, the SRB utilise the propionic acid, while the Acetogenic Bacteria (AB) use the C4 acid, to produce C2 acid, which in turn is used by the MB to produce methane. This pattern, however, is interrupted when sulphate and SRB are present in the bioreactor (Harmsen, 1996; Oude Elferink, 1998). SRB utilise hydrogen as soon as it is produced by the hydrogen producing microorganisms. This implies that hydrogen is not available to the MB.

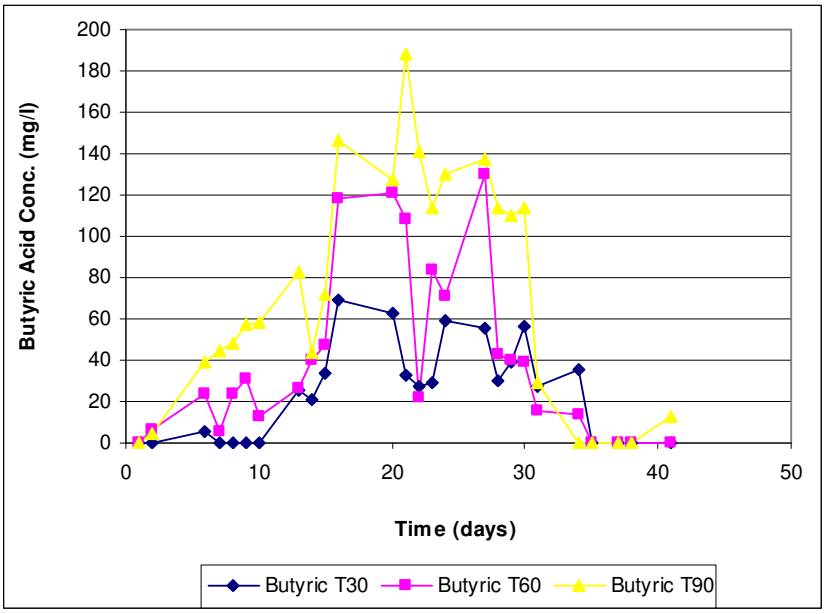

Figure 4.6. Butyric acid concentration in T30, T60 and T90.

4.3.2 Study 2. The use of RB and SRB as fermentative and SO_4 removing microorganisms

4.3.2.1 Sulphate reduction

While in Study 1, the fermentation capabilities of SRB were studied, in this study it was investigated whether RB can ferment cellulose to substrates usable by SRB in the biological sulphate removal process. The sulphate reduction results of Study 2 are given in Figure 4.7. Complete sulphate removal occurred in R3 and R4 during the first 12 days of the experimental period. The microorganisms in R4 consisted of a mixture of SRB and RB which were expected to afford better sulphate reduction than in R3, due to the presence of the SRB mixture. However, the net sulphate reduction

rates in R3 and R4 were similar. Fresh sulphate was added to the reactors on day 14 (*arrow*). These sulphate concentrations decreased at the same rate up to day 32 and day 35, for R3 and R4, respectively. Thereafter the sulphate concentrations reached steady state. No fresh GC were added during this period.

From the results obtained from R3, it was observed that sulphate reducers were present in the rumen consortia. This observation bears out the findings of Matteuzzi, (1964) and Cummings *et al.* (1995), who found that a fairly high count of sulphate reducers is present in rumen fluid. Postgate and Campbell (1965) found a bacterium in rumen fluid that reduced sulphate to sulphide, which they named *Desulfotomaculum ruminis,* while Huisingh *et al.* (1974) isolated *Desulfovibrio* spp. from the rumen fluid of sheep. Several bacteria are able to derive sulphur as nutrient from sulphate (Prescott, 1961) since sulphate is as effective as any form of Sulphur (Block *et al*. 1951)

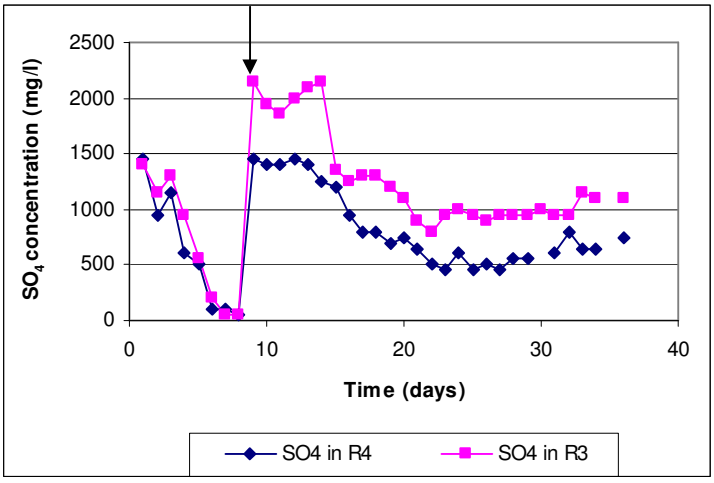

Figure 4.7. The sulphate removal in reactors R3 and R4.

4.3.2.2 VFA concentration

Propionic acid

The propionic acid concentration in reactors R1 and R2 (control reactors, containing no SO_4, Figure 4.8) increased from day 0-15, till values between 500-600 mg/ℓ. This result showed that using a rumen consortium to ferment GC resulted in an improved propionic acid concentration in the reactors, compared to the propionic acid concentration in the fermentation reactors using SRB as the degradation bacteria (Study 1). The reactor containing only rumen fluid produced a higher propionic concentration than the reactor containing the mixture of SRB and rumen, indicating

that the rumen consortia are better cellulose degraders. The principle products of the rumen microbial activity are fatty acids from the fibre and grain part of the food (grass, hay), which the cow uses as energy sources and amino acids from the protein-rich food components such as bean meal or good quality hay. The interaction between the cellulose fermentation and the rumen bacteria was already acknowledged by Hungate (1966). Since the rumen is a highly cellulytic ecosystem with a complex microbial population of bacteria, archaea, protozoa and fungi, rumen research has expanded during the last few decades (Barnes and Keller, 2003). Many researchers are investigating cellulose degradation of plant biomass to generate biogas, while the study in this thesis focussed on the use of the produced VFA and hydrogen for biological sulphate removal.

The propionic acid concentration in R3 and R4 was noticeable lower than in the control reactors (R1 and R2). This result showed the correlation between the propionic acid concentration and the biological sulphate reduction in R3 and R4, which agreed with the SO_4 reduction (day 1-12, Figure 4.7). After day 12, the propionic acid concentration in R3 increased till ca. 200 mg/ℓ, while SO_4 removal occurred concurrently. The SO_4 removal ceased on ca. day 25, which coincided with a propionic acid concentration <100 mg/ℓ. A similar pattern was observed in R4, although the propionic acid in that reactor was lower. This may possibly be ascribed to the difference in microbial origin: R3 contained only rumen fluid and R4 a mixture of rumen fluid and SRB. When comparing the propionic concentrations in R3 and R4, with that in reactors R1 and R2, it can be seen that when no sulphate is present, the propionic acid concentration in the reactors is substantial higher.

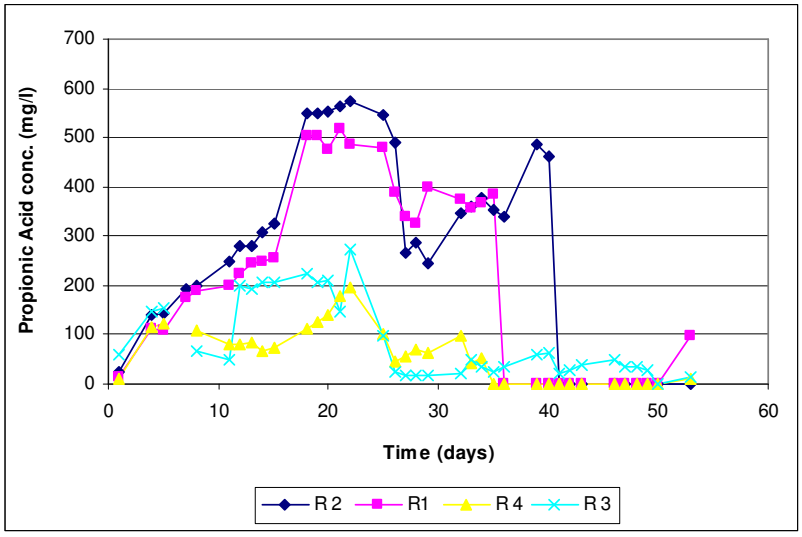

Figure 4.8. The propionic acid concentration in reactors R1-R4

Acetic acid

The acetic acid concentrations in R3 and R4 were higher than in R1 and R2 (Figure 4.9). This result can be expected because the utilisation of propionic acid for the biological sulphate reduction resulted in the formation of acetic acid. The higher acetic acid concentrations corresponded with the data illustrating the sulphate reduction.

The acetic acid concentration in reactor R1 is higher than in R2. This can possibly be ascribed to the fact that in reactor R1 a mixture of micro organisms (SRB and rumen inocculum) is present and that in reactor R2 only the rumen microbes are responsible for the VFA production. This finding seems to indicate that the SRB (in the organisms mixture in R1) favour the acetic acid production, whereas the rumen microbes seem to favour the propionic acid production (Figure 4.8: R2, containing the rumen organisms). The highest acetic acid concentration was measured in R3 at 500 mg/ℓ. The acetic acid concentration increased from day 5 to day15, where after it decreased (days 15 to 25), at which time the propionic acid concentration was the highest. This result confirms the correlation between the propionic and acetic acid concentrations in the reactors. In order to reduce 3 mol of sulphate, 4 mol of C3 acid are used and 4 mol of C2 acid are produced. This observation is in agreement with the acetic acid concentration in the sulphidogenic reactors, where the acetic acid concentration is higher than in the control reactors. This finding indicated that the acetic acid was not ued for the sulphate removal.

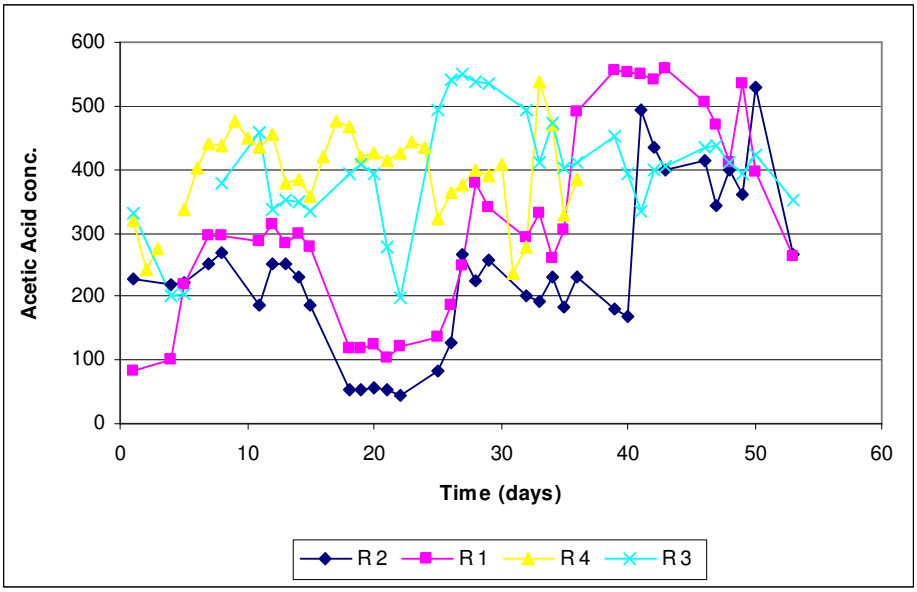

Figure 4.9. The acetic acid concentration in reactors R1-R4.

4.3.2.3 Butyric acid

The butyric acid concentration is presented in Figure 4.10, which shows that in the reactor containing the GC and the rumen microbes (R2), the butyric acid concentration is higher than in the reactor containing a SRB and rumen microbes mixture (R1). The average butyric acid produced in R1 from start to day 15 is 74 mg/ℓ, while in R2 this is 165 mg/ℓ, which is an increase of 55%. This result shows that the rumen microbes can ferment the GC more effectively to butyric acid than the SRB combined with the rumen microbes. After day 15, no further butyric acid was observed in both reactors. The butyric acid concentration in R3 and R4 increased after day 34, which coincided with no further SO_4 removal and with a low propionic acid concentration. This result seems to indicate that the conditions in the reactors favoured the butyric acid production (lower pH) but at the same time that the SRB did not utilise the produced butyric acid for further sulphate removal. The remaining SO_4 concentration in R3 was about 1000 mg/ℓ, while this was on average 500 mg/ℓ in R4. It might be that the increased acetic acid concentration of 400 and 500 mg/ℓ in R4 and R3, respectively, was the rate limiting factor in the reactors.

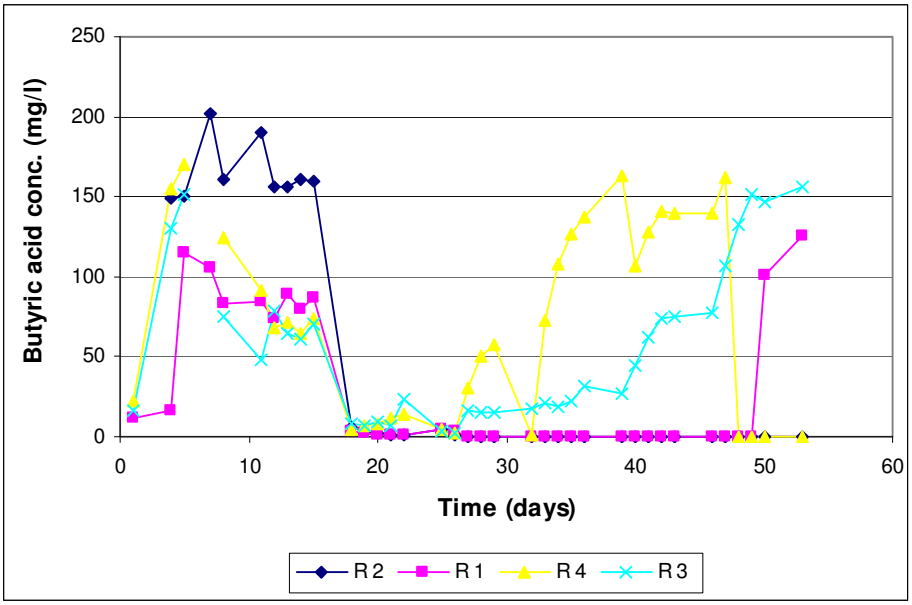

Figure 4.10. The butyric acid concentrations in R1, R2, R3 and R4.

4.3.2.4 Theoretical COD used/Sulphate reduced ratio

When SO_4 is reduced, using the VFA as the carbon and energy sources, equations 1, 2 and 3 can be applied:

Butyrate

$$CH_3CH_2CH_2COO^- + 2{,}5\,SO_4^{2-} + 0{,}25\,H_2O \rightarrow 4HCO_3^- + 2{,}5\,HS^- + 0{,}75\,H^+ + 0{,}25\,OH^- \quad (4.1)$$

Propionate

$$CH_3CH_2COO^- + 1{,}75\,SO_4^{2-} + 0{,}25\,H_2O \rightarrow 3HCO_3^- + 1{,}75\,HS^- + 0{,}5H^+ + 0{,}25\,OH^- \quad (4.2)$$

Acetate

$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad (4.3)$$

Equations 4.1 to 4.3 show that 1 mol butyrate is needed to reduce 2.5 mol sulphate, that 1 mol propionate is needed to reduce 1.75 mol sulphate and that 1 mol acetate can theoretically reduce 1 mol sulphate, assuming that total sulphate removal is obtained and that no residual VFA is present. Further correlations and calculations between the theoretical COD used and sulphate removed ratios are given in Table 1(Appendix A). A total of 9.5 g/ℓ propionic acid over 40 days was produced in R2, containing 30 g GC, 450 ml tap water and 25 ml of rumen inoculum mixture. Theoretically this amount of propionic acid can remove 21.6 g sulphate. When more GC were added to the reactors, higher VFA concentrations were produced, reducing higher concentrations of sulphate. However, when more VFA and other intermediates are produced the reactor COD/SO_4 ratio will increase, resulting in competition of the MB as well as in a high residual COD concentration in the reactor effluent.

3.2.5 Study 3

The results of Study 2 indicated that the rumen microbial population could ferment grass-cellulose to degradation products, which functioned as carbon and energy sources for SRB reducing sulphate in the feed water of the reactors. In order to understand the conditions under which rumen microbes can produce increased VFA concentrations, Study 3 was conducted. Three reactors were operated under different conditions: with and without the addition of sulphate and with and without the addition of tryptone, a protein rich nutrient, which can enhance propionic acid production rather than C2 or C4 acids from the degradation of cellulose and hemicellulose (personal communication from Paul Weimer, rumen specialist, University of Winconsin, USA).

4.3.3.1 Sulphate reduction

The sulphate removal in L1 is presented in Figure 4.11. The sulphate removal graph shows that initially, from days 0-11, the sulphate concentration decreased from 1250 to 800 mg/ℓ, while by day 14, the sulphate had been reduced to 40 mg/ℓ. Fresh 5.5 g Na_2SO_4 was added to L1 (*Arrows* in Figure 4.11) on days 14-18 (inclusive). This newly added SO_4 solution was removed within 16-18 h. These results indicated a good sulphate removal, which was confirmed by the sulphide concentration in L1, which was 600 mg/ℓ on day 25. Since the high sulphide concentration can inhibit the rumen (fermentation) microorganisms, the experiment was stopped.

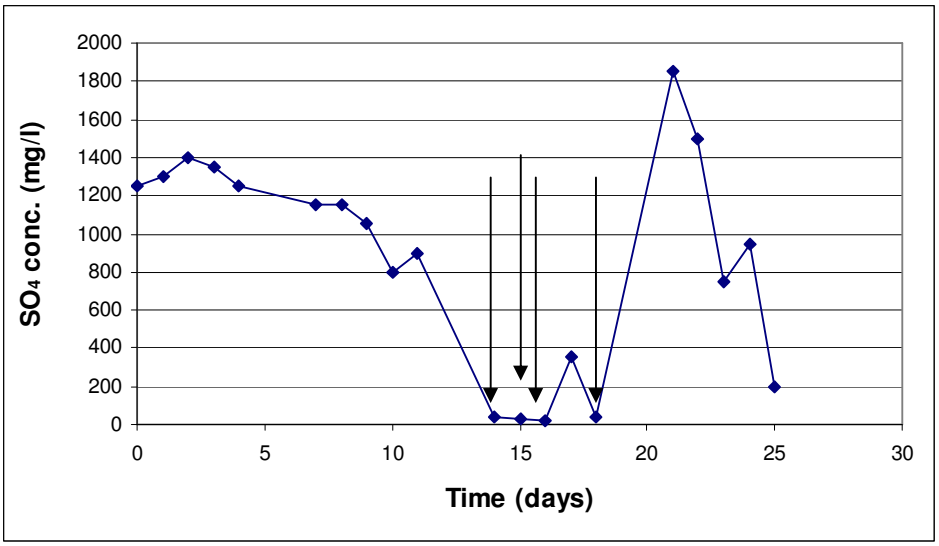

Figure 4.11. The biological sulphate reduction in reactor L1.)

(Arrows indidcate the addition of fresh SO_4: as soon as SO_4 was added it was reduced when next sample was analysed 16-24 hours later, see text

When no metals are present, sulphide accumulation can result in a severe inhibition of the biological sulphate removal process and in some cases may even cause total process failure. Many studies have been dedicated to the effect of sulphide toxicity on the biological sulphate reduction efficiency. In general, these studies demonstrated that, under mesophilic conditions, both granular and suspended sludges are more tolerant of H_2S inhibition at a pH of around 8. At neutral pH values, free H_2S, which is more toxic than HS^-, accounts for 50% of total dissolved sulphide, whereas at pH 8 it is only around 10% (Lens & Hulshoff Pol, 2000). Speece (1996) listed the sulphide toxicity levels investigated by different researchers, which showed that 100-150 mg/ℓ sulphide is toxic for lactate and glucose utilizing SRB in a continuously stirred tank reactor (CSTR). Moreover, 60-75 mg/ℓ sulphide was not tolerated by acetate and propionate utilizing SRB (in a CSTR), while Parkin *et al*.

(1990) reported that when the sulphide concentration was 60-70 mg/ℓ, in an acetate and propionate-fed chemostat, it resulted in process failure. Since the pH in this study operating L1 was > 6.5, the sulphide is not in its toxic form. Greben *et al*. (2004, 2005) have shown that a high sulphide concentration in a sulphidogenic reactor may be beneficial for sulphate reduction when using ethanol as the carbon and energy source. Eloff *et al*. (2004) showed that when sulphide was added to the sulphate rich influent feeding a biological sulphate removal reactor, the sulphate reduction improved compared to feeding sulphide-free influent.

4.3.3.2 VFA production and utilisation

Propionic acid

The propionic acid concentration in L1 was the lowest of the three reactors (Figure 4.12). This was ascribed to the C3 acid utilisation for sulphate removal in L1 (Figure 4.11). Whenever the sulphate concentration decreased to < 100 mg/ℓ, fresh sulphate solution was added to the reactor. The sulphate removing microorgnisms utilised the propionic acid in L1 and resulted in the C3 acid concentration in that reactor being the lowest. The highest propionic acid concentration (Figure 4.12) occurred in reactor L3. Tryptone (1g) was added to L3 daily. This is used as a source of nitrogen (amino acids) and nutrients in many culture media. Tryptone, as well as peptone as amino acid polymers are an excellent choice for bacterial fermentation and most likely will stimulate the rumen microbes to ferment the cellulose in the GC more efficiently. However, the additional costs when adding tryptone to the fermentation reactor have to be taken into account as 1 g tryptone currently costs R 0.50/g. Moreover, tryptone will add to the COD concentration in the reactor and thus to the residual COD concentration in the reactor effluent.

Acetic acid

The utilisation of propionic acid in L1 resulted in the reduction of sulphate and in the production of acetic acid (Figure 4.13). Thus the highest acetic acid concentration was observed in reactor L1. It increased with time up to a concentration of ca. 800 mg/ℓ, which according to Hill *et al*. (1987) can result in process failure. In the reactor which contained the rumen population and tap water, the highest acetic acid concentration obtained was almost 400 mg/ℓ, while it was 450 mg/ℓ in the reactor containing tryptone. These results indicated that the tryptone addition resulted in a 50 mg/ℓ increase in the acetic acid concentration. Moreover, due to the biological

sulphate reduction in L1, the acetic acid concentration increased with about 200-300 mg/ℓ.

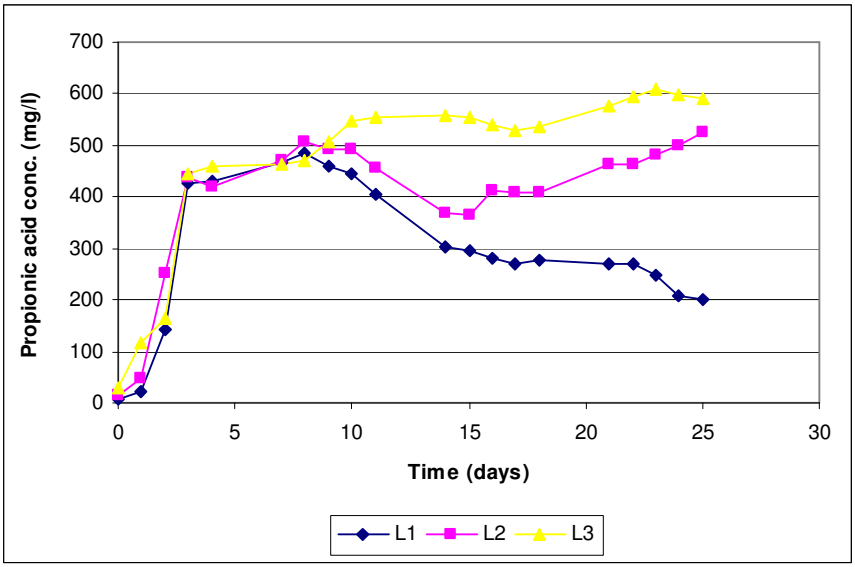

Figure 4.12. Propionic acid concentrations in reactors L1, L2 and L3.

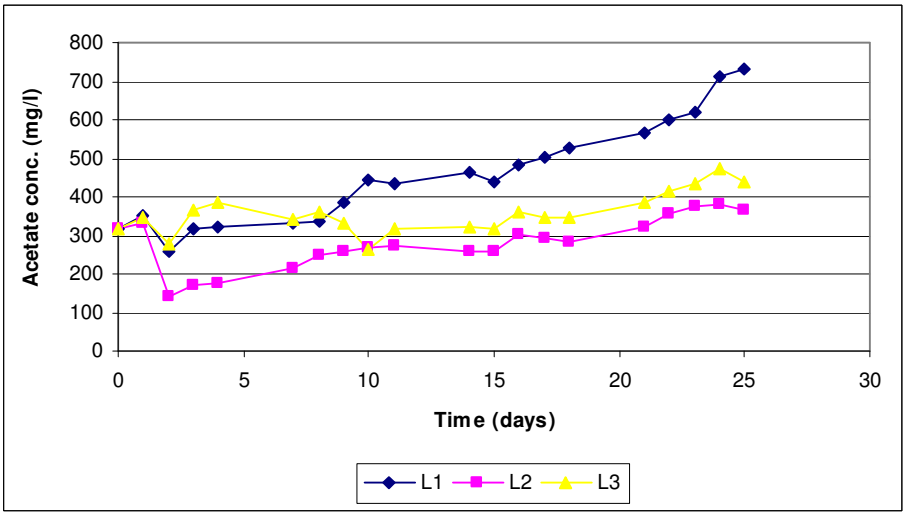

Figure 4.13. Acetic acid concentration in reactors L1, L2 and L3.

Butyrate

The butyrate concentrations in L1-L3 are given in Figure 4.14, which showed that the butyric acid concentration was zero in the three reactors during the first 14 days of operation. This lag in butyric acid production may have been due to an increased C3 production. Thereafter, the butyric acid concentration in L1 was low, as it was being utilised for the sulphate reduction. In L2 and L3 the butyric acid concentration increased due to cellulose degradation. The butyric acid concentration in L2 (control) was higher than in the tryptone reactor (L3). This result seemed to indicate that the

propionate concentration, which is the preferred energy source for the SRB, rather than butyric acid concentration increased when tryptone was added to the fermentation reactor.

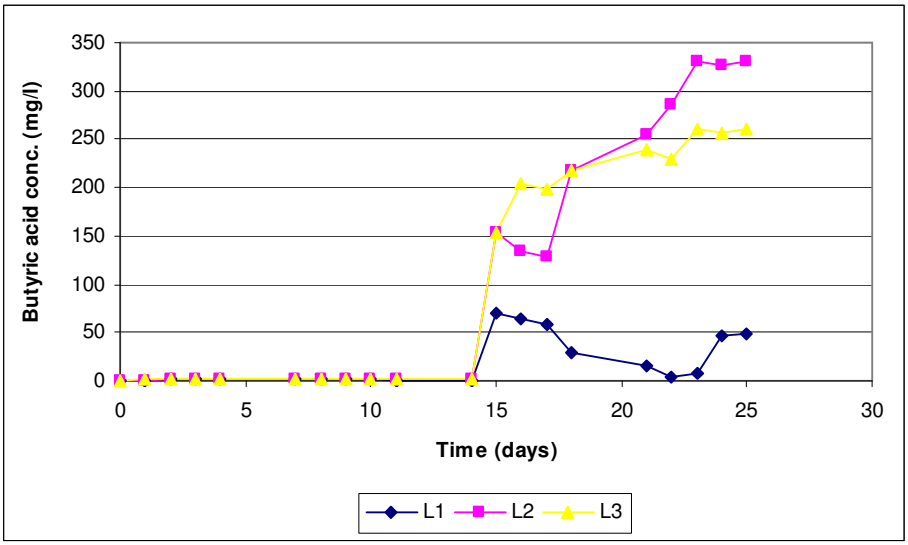

Figure 4.14. The butyric acid concentrations in reactors L1, L2 and L3

4.3.3.3 Sulphate removed/VFA utilised

The high peaks in the graph in Figure 4.11 indicated the sulphate concentration after fresh sodium sulphate was added to the reactor. The sulphate removal, as depicted in Figure 4.11, corresponded to the utilisation of the C3 and C4 acids. An estimate of the total sulphate removal from day 0-21 was calculated, during which period no fresh GC was added to the reactor. During that period ca. 9 g sulphate was removed, while 75 g GC was present in the reactor. Thus it can be deduced that, in order to reduce 1 g sulphate, 8 g GC is needed. The $SO_{4\ removed}$/$grass_{used}$ ratio will again be addressed in subsequent chapters

4.4 CONCLUSIONS

The results from Studies 1, 2 and 3 showed that sulphate reduction was obtained in the sulphate containing reactors in all three studies. It was also shown that when fresh sulphate was added to the reactors, continued sulphate removal occurred. In Study 1, the sulphate reduction was dependant on the concentration of GC: the higher the amount of grass added to the reactor, the faster the sulphate removal. In Study 2, sulphate removal was obtained in both sulphate rich reactors. In Study 3, total sulphate removal was achieved after 14 days, using the rumen fluid

microorganisms. In all three studies it was observed that mainly propionic and butyric acids were used as the carbon and energy sources for the SRB, producing acetic acid.

VFA production in T30, T60 and T90 (Study 1) resulted in removal of sulphate. It was shown that SRB utilised maily propionate. However, the results obtained from reactor T30 seemed to indicate that acetic acid was used during the period that the C3 or C4 acid concentrations were insufficient. It was furthermore hypothesized that the C2 acid was used by other microorganisms to produce the C3 and C4 acids for further sulphate reduction. Sulphate reduction occurred in the reactors T30 and T60 during the first 8 days of operation**,** while in T90, full SO_4 reduction took place during the first 5 days. Thus a higher GC concentration resulted in increased SO_4 removal.

Sulphate reduction was observed in the reactor containing SO_4 rich water and only rumen fluid microorganisms. This finding indicated that sulphate reducers form part of the rumen consortium. The results of Study 2 formed the basis for Study 3 as it demonstrated that the rumen inocula could ferment the grass cuttings to produce VFA and that the VFA produced could be utilised for biological sulphate removal. When comparing the sulphate reduction in reactors T30 (study 1) and in L1 (study 3), containing the same amount of grass cuttings and biomass mixture, it was seen that the sulphate reduction in T30 was higher than in L1. However, the rumen microbes produced more C3 and C4 acids. The improved sulphate reduction in T30 over that seen in L1 was ascribed to the sulphate adapted biomass. When tryptone was added to L3, the propionic acid concentration increased while the butyric acid concentration did not.

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CHAPTER 5

VFA PRODUCTION AND CONTINUOUS BIOLOGICAL SULPHATE REMOVAL OPERATING A TWO AND THREE STAGE REACTOR SYSTEM FEEDING SYNTHETIC FEED WATER AND DILUTED ACID MINE WATER

5.1 INTRODUCTION

Biological sulphate reduction is a competitive alternative to other sulphate removal technologies utilized for the full scale treatment of mine and other industrial effluents (Colleran *et al*. 1995). The major disadvantage of applying the biological process for the treatment of sulphate and acid rich effluents is the operational costs associated with the carbon and energy source. In order to save on operational costs, the biological sulphate removal process can be coupled to limestone and lime neutralisation of acidic mine water (Maree, 2003a; Geldenhuys *et al*. 2002; Maree *et al*. 2003b). When the sulphate rich water is firstly treated with limestone, the sulphate concentration is reduced to 2300 mg/ℓ, and after lime treatment the sulphate concentration is further reduced to 1500 mg/ℓ, the solubility level of gypsum. After lime treatment, the pH of the treated water is between 11and 12. This pH level can be reduced to pH 7.5 by bleeding CO_2 gas, generated in the limestone treatment phase, through the lime-treated high pH water. After contact with CO_2, the partially treated water with a near neutral pH is fed to the biological treatment system to further lower the remaining sulphate to concentrations below 200 mg/ℓ. This integrated chemical-biological treatment of acid and sulphate rich mine effluents saves considerably on operational costs compared to treating the total mine water stream biologically (Maree *et al*. 2004).

The utilization of a range of low molecular weight substrates for biological sulphate removal such as lactate (Laanbroek *et al*. 1983), ethanol (Greben *et al*. 2000; De Smul *et al*. 1997) and hydrogen (Du Preez *et al*. 1992, Van Houten, 1996; Eloff *et al.* 2004) has been demonstrated. A possible way of treating mine water more cost effectively is by using a cellulose-containing bio-waste product (e.g. plant biomass) as the carbon and energy source. When cellulose, a polysaccharide, is fermented, polymers, oligomers, monomers (sugars), fatty acids and other fermentation products are formed, which can be utilized as energy sources by the SRB to remove sulphate from industrial waste waters, such as mine effluents.

It was shown in Chapter 4 that the fermentation of a bio-waste product containing cellulose can effectively be mediated by microorganisms originating from rumen fluid

obtained from ruminants. The products generated in this fermentation process are, amongst others, fatty acids, such as butyric acid, propionic acid and acetatic acid. When adding tryptone to the fermentation reactor, the rumen microbes utilise this protein based, nutrient supplement to produce more propionic acid than butyric acid. The SRB can utilise the C4 and C3 acids in the sulphate reducing process, producing C2 acid, which is the rate limiting factor in the sulphidogenic reactor (Visser *et al*. 1993) and may lead to failures of anaerobic wastewater treatment systems. The oxidation of propionic acid in the sulphate reducing process is more beneficial than that of butyric acid as only one mol of acetate is formed by oxidizing propionate, whereas butyric acid oxidation results in two moles of acetic acid.

The results in Chapter 4 showed that the degradation of GC produced sufficient organic products to sustain biological SO_4 removal, in laboratory batch test reactors. The objectives of the studies in this chapter were to investigate whether continuous sulphate reduction can be achieved using GC as carbon and energy source, operating the process in a continuous mode. The study comprised of two parts: Part 1 describes the feeding of synthetic influent, into a two and a three stage reactor system, while in Part 2, pre-treated AMD from a closed mine in the Witbank area was used as feed water to the same reactor system.

5.2 MATERIAL AND METHODS

5.2.1 Study 1a. Two stage reactor system

In Study 1a, a two stage reactor system, comprising a fermentation reactor (FR) and a sulphate reducing reactor (SR), was operated and fed continuously with synthetic sulphate rich water. The effluent from FR was fed to SR, which also received synthetic feed water, the objective being that the cellulose degradation products formed in FR should function as the carbon and energy sources in SR.

5.2.1.1 Feed water

The feed water for FR and SR had a SO_4 concentration of ≈ 2500 mg/ℓ, (Na_2SO_4, Crest Chemicals, Johannesburg). Macronutrients consisting of (w/w): 6.5% N, 2.7% P, 13.0% K, 7.0% Ca, 2.2% Mg and 7.5 % S) and micronutrients (0.15% Fe, 0.024% Mn, 0.024% B, 0.005% Zn, 0.002% Cu and 0.001% Mo were made up in a stock solution (1g/5ℓ) of which 1mℓ/ℓ was added to the feed water (Hydroponic nutrient powder, Kompel, Chemicult). Both reactors received feed water at a rate of 5 ℓ/d.

5.2.1.2 Carbon and energy source

The fermentation products of the cellulose containing GC, produced in FR, served as the carbon and energy source for sulphate removal in FR and SR.

5.2.1.3 Reactor system

A two stage reactor system was operated, consisting of a fermentation reactor (FR) and a sulphate removal (SR) as depicted in Figure 5.1.

5.2.1.3.1 Fermentation reactor (FR)

For the fermentation reactor (FR), a 20 ℓ reactor was used, operated at 39 °C using a water jacket surrounding the reactor, which was connected to a waterbath set at the required temperature. FR was operated as a "hybrid" reactor. The lower part of the reactor contained ceramic rings as packing material for biofilm formation. Biomass (250 mℓ), obtained from the sulphate removal CSIR Demonstratoin Plant Navigation Colliery (Witbank) was added to promote SRB growth on the ceramic rings. The upper part of the reactor contained 1000 g GC at the start of Study 1a. The GC were replenished on a weekly basis, added to the top of the reactor. Due to its light weight, cut grass floats on water and therefore stayed at the upper part of the reactor. Initially 250 mℓ rumen fluid, (VSS of 10.6 g/ℓ) obtained from fistulated sheep (University of Pretoria, South Africa) was added to the GC. The pH of the reactor was maintained between 6.6-6.9, to accommodate the optimum conditions for rumen microorganisms. The SRB prefer a reactor pH of 7.5, but can function at pH 7. The feed water (5ℓ/d) entered FR at the top (HRT = 4 days), to allow it to make contact with the grass cuttings. A recycle stream (360 ℓ/d) was fitted from the lower GC part of the reactor to the top, with the rationale that the polymers and monomers produced could further be fermented to the substrates required for the SRB in the bottom part of the reactor. The effluent left FR at the bottom, from where it entered SR. The FR effluent contained partly removed sulphate, alkalinity, sulphide, as well as residual COD concentration, obtained from the fermentation process in FR.

5.2.1.3.2 Sulphate removal reactor (SR)

For the biological sulphate reduction a packed bed reactor (SR) was used (5 ℓ). The active volume was 2 ℓ, due to the fact that the reactor was packed with a geotextile blanket (material product used in road construction) for SRB biofilm formation. Geotextile is a coarse material, ideally suited for biofilm formation, due to the many threads per cm^2 of fibrous cloth to which the bacteria can attach. SR was inoculated with 150 mℓ biomass already adapted to sulphate removal and 100 mℓ anaerobic

sludge, obtained from Daspoort Sewage Works, Pretoria. The Anaerobic sludge was added with the rational to introduce a wider diversity of SRB, since the Demo plant SRB were adapted to ethanol as the carbon and energy source. SR received two feed streams, each of 5 ℓ/d: the effluent of FR and SO_4 rich synthetic feed water, which resulted in a HRT of ½ d. The reactor was operated at room temperature (22-25 ˚C). A recycle stream (20 ℓ/d) was installed between the upper and lower parts to improve mixing inside the reactor.

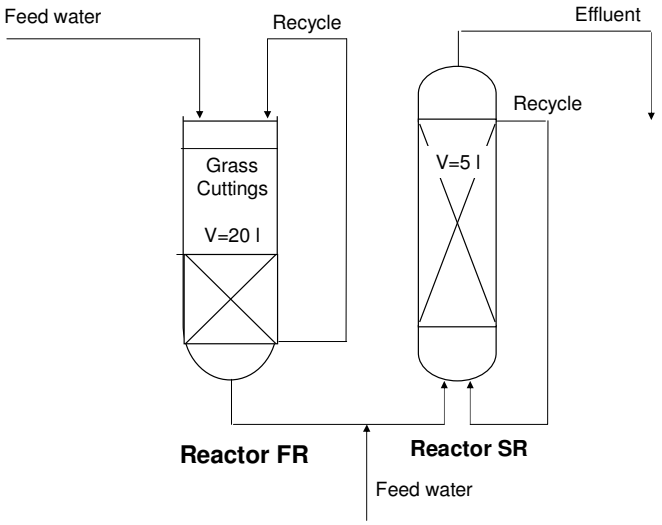

Figure 5.1 Schematical representation of the two stage reactor system.

5.2.1.4 Analytical

For all described experiments, daily samples were taken from feed and treated water from the reactors and analysed on the same day. The same analytical procedures as described in Chapter 3 (3.2.4) were followed.

5.2.2 Study 1b. Three stage reactor system

During Study 1b, a third reactor was added to the reactor system as described in *5.2.2.2* (Figure 5.3). The aim of Study 1b was to investigate whether further sulphate removal occurred by the acetate consuming SRB (ASRB), using the acetate produced in the other two reactors, as the carbon source, which would possibly be more beneficial than removing the residual COD (acetate) concentration in an additional aerobic reactor, prior to discharge in receiving water bodies.

5.2.2.1 Feed water

The feed water used in Study 1b was identical to the feed water in Study 1a.

5.2.2.2 Reactor system

The same procedure as for Study 1a was followed, except that the two stage reactor system was changed to a three stage reactor system, comprising FR and SR as well as a third reactor, the Acetate Sulphate Reactor (ASR, Figure 5.2). ASR was exactly the same as SR, also with a volume of 5 ℓ and was similarly packed with strips of geotextile (road construction material, see 5.2.1.3.2). The feed rate of the synthetic SO_4 rich feed water to ASR was 5 ℓ/d, while ASR also received the effluent of SR at a feed rate of 15 ℓ/d, resulting in a HRT of 1/3 day.

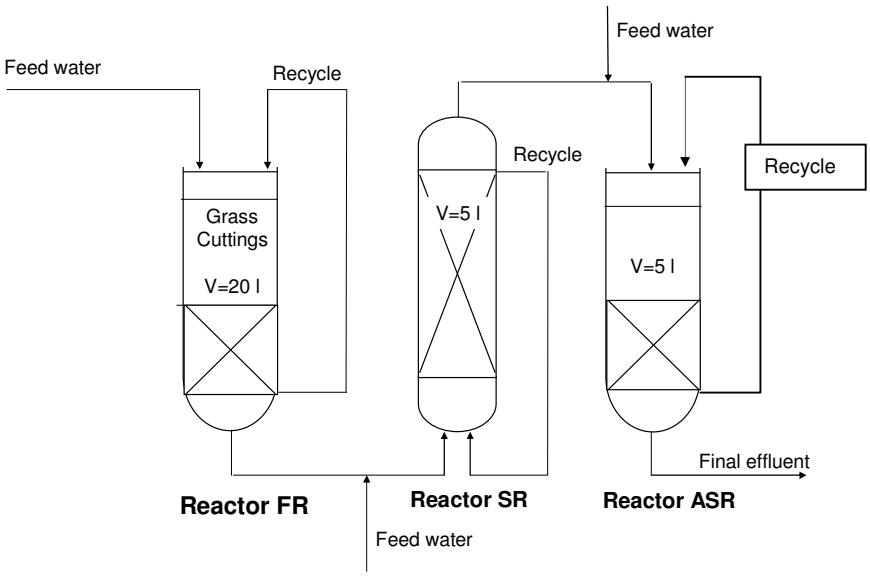

Figure 5.2 Schematic representation of the three stage reactor system

5.2.2.3 Biomass

ASR received the same biomass (SRB and anaerobic digester sludge from Daspoort, see 5.2.1.3.2) as FR and SR in Study 1a as well as 100 mℓ acetate utilising SRB (ASRB). These microorganisms were prepared on Postgate medium, with acetate as the carbon and energy source, isolated from anaerobic digester sludge, obtained from the Pretoria Sewage Works.

5.2.2.4 Carbon and Energy Source

As in Study 1a, grass cuttings were added to FR to be fermented by the rumen microorganisms to degradation products, providing SRB with the electron donor. In this study, 150 g grass cuttings were added on days 1, 32, 46 and 62.

5.2.2.5 Analytical

Daily samples were taken of feed water and treated water from the reactors. The same analytical procedures as described in Chapter 3 (3.2.4) were followed.

5.2.3 Study 2a. Effect of AMD on activity of rumen microorganisms

This study was conducted to investigate whether the rumen inoculum were affected by diluted AMD as feed water, before it was decided to feed this diluted AMD to FR in the following study. To reach the objective, a short batch reactor test was conducted, operating two reactors. The first reactor received diluted AMD as feed water (SO_4 concentration ≈ 1300 mg/ℓ), while the second reactor was fed with synthetic feed water (SO_4 concentration ≈ 950 mg/ℓ). All other conditions in the two reactors were the same.

5.2.3.1 Feed water

AMD effluent (chemical composition Table 5.1), which originated from a closed coal mine in the Witbank (South Africa) area, was used as feed water for reactor R1. The mine effluent was diluted prior to use with sulphide rich effluent from the biological sulphate removing reactor SR (ratio 1:1) so that the sulphide and the produced alkalinity present in the effluent could precipitate the metals and increase the pH of the AMD, respectively. The SO_4 concentration of the diluted feed water was approximately 1500 mg/ℓ. Artificial SO_4 rich water (SO_4 ≈1500 mg/ℓ) was used as feed water for reactor R2. Metal removal due to pre-treatment of AMD is presented in Table 5.2.

5.2.3.2 Reactors

Two completely mixed Perspex batch reactors, R1 and R2 (Vol: 2.5 ℓ), were operated under anaerobic conditions at 37 °C by means of a water jacket surrounding the reactor.

5.2.3.3 Biomass

Both R1 and R2 were seeded with100 mℓ rumen bacteria (VSS: 10g/ℓ), 100 mℓ SRB (VSS: 9.7 g/ ℓ), 50 g GC, 1 mℓ/ℓ macro nutrients (as described for Study 1a: 5.2.1.1).

5.2.3.4 Experimental

The pH in R1 and R2 was controlled (by pH controller) between pH 6.6 -6.9, which is the required pH for the rumen bacteria. In total 8.3 mℓ/ℓ of 0.1 N HCl was used to lower the increased pH due to biological sulphate reduction. The total experimental period was 10 days.

Table 5.1. Chemical composition of AMD obtained from Witbank South Mine

Parameter	Units (mg/ℓ, except for pH)
Ph	2.5
Acidity	1 200
SO_4	2 600
Cu	0.75
Total Fe	76
Pb	0.25
Mg	77
Mn	9.3
Ni	0.61
Zn	4.0
Na	19
K	7

Table 5.2. The metal concentration of AMD before and after dilution with sulphide rich effluent

Metal (mg/ℓ)	AMD	Diluted AMD
Al	11.7	5
Cu	0.75	0.04
Fe	41	0.2
Mn	9.3	5.7
Zn	4.0	0.27
Ni	0.61	0.24

5.2.4 Study 2b. Operating the two stage reactor system using pre-treated AMD as feed water

5.2.4.1 Feed water

Mine seepage water from a closed coal mine situated in the Witbank area functioned as feed water for the two stage reactor system, as described under 5.2.1.3 (Figure 5.1). The chemical composition of this mine water is presented in Table 5.1. Only FR received feed water in this study at a feed rate of initially 15 ℓ/d and later at 30 ℓ/d, while the effluent from FR was the only feed water to SR. The pH of this mine water of 2.5 was too low and the acidity concentrations too high (1 200 mg/ℓ) to use untreated AMD as feed water to the hybrid reactor FR (Table 5.1). This AMD therefore required treatment prior to feeding it to the reactor system. The biological pre-treatment method was used as described in 5.2.3.1 (Greben *et al*. 2000, 2003).

5.2.4.2 Carbon and energy source

The fermentation products of the cellulose present in grass cuttings (GC), formed in FR, served as the carbon and energy source for both FR and SR.

5.2.4.3 Reactor system (FR and SR)

The two stage reactor system FR and SR was used (Figure 5.1). In this study, feed water only entered FR, while SR received the effluent of FR as the only feed water.

5.2.4.4 Analytical

The same analytical procedures as described in Section 3.2.4 were followed.

5.2.4.5 Experimental

The same procedure as in Study 1a was followed. The feed rate was 15 ℓ/d from day 1-37 and 30 ℓ/d from day 38-78. Grass cuttings were added according the following pattern: 25 g on day 2, 100 g on days 6, 12 and 34 and 1000 g on day 27. From day 41-60 it was 20 g/d and from day 61-78, it was 40 g/d. There were three experimental periods: d 1-37, d 41-49 and d 50-78.

5.3 RESULTS AND DISCUSSION

5.3.1 Study 1a

5.3.1.1 Sulphate removal

The chemical compositions, based on the averages of the daily analyses during the total experimental periods of the treated waters of FR and SR, is given in Table 5.3. The effluent of FR was the feed stream to SR at a rate of 5 ℓ/d, while SR also received sulphate-rich feed water at 5 ℓ/d, thus the total feed-rate to SR was 10 ℓ/d. The average SO_4 load to FR was 1.7 g/ℓ, while the average SO_4 removal in FR was 1.6 g/ℓ (Table 5.4) and the average SO_4 removal in SR was 1.1 g/ℓ. Thus the combined SO_4 removal in FR and SR amounted to 2.7 g/ℓ. The data in Table 5.4 furthermore showed that the total average removal was 19 g/d SO_4 over the two reactors during the experimental period (Table 5.4).

Table 5.3. The chemical composition of the treated water from FR and SR

Parameter (mg/ℓ)	**Treated FR (Feed SR)**	**Treated SR**
SO_4	895	597
pH (Value)	7.31	7.49
COD	1417	1228
Sulphide	294	295
Alkalinity	1818	1894
C2	530	977
C3	248	98
C4	123	-
S^{2-}/SO_4	0.18	0.13
Alk/SO_4	1.13	0.68

Table 5.4. The SO_4 removal pattern in FR and SR

Reactor	SO_4 Removed(mg/ℓ)	Qfeed (ℓ/d)	SO_4 removed (g/d)	SO_4 RR* gSO_4(ℓ.d)
FR	1 614	5	8	0.8
SR	1 105	10	11	2.2

***RR=reduction/removal rate**

The sulphate concentrations in the feed- and treated water and the reactor COD concentrations in FR and SR are presented in Figures 5.3 and 5.4, respectively. The graphs in Figure 5.3 showed that initially the COD concentration in the treated water of FR is >1000 mg/ℓ, which resulted in a SO_4 concentration generally < 500 mg/ℓ in the effluent. This result showed that when the residual COD concentration is high, a sustained SO_4 removal can be achieved. When the COD concentration decreased on days 32-60 (to concentrations <1000 mg/ℓ) the SO_4 concentration in the effluent increased to values >1000 mg/ℓ, respectively. The reversed pattern was observed from day 60-70: high COD concentrations in the effluent, resulting in a lower SO_4 concentration. These results clearly indicated the relationship between a high residual COD concentration and high SO_4 removal rate.

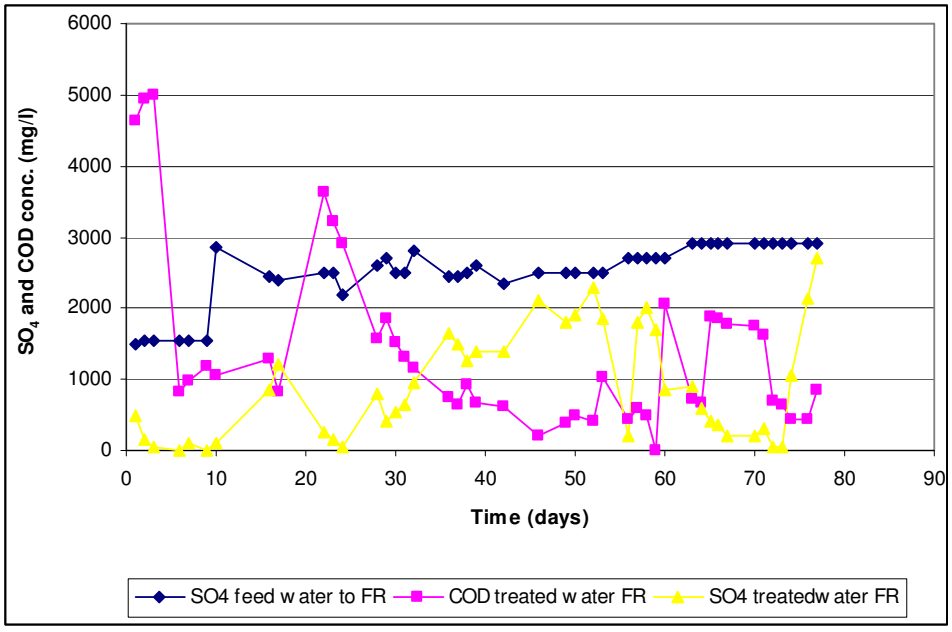

Figure 5.3. The SO_4 and COD concentrations relating to operation FR

A similar pattern can be noted for SR (Figure 5.4), where high COD concentrations (± 5000 and 4000 mg/ℓ) resulted in low SO_4 concentrations in the treated water. When the experiment was conceived, it was hypothesized that the fermentation process would occur in FR, followed by SO_4 removal in SR, utilizing the fermentation products from FR for biological SO_4 removal in SR. However, as shown in the results of study 1a, SO_4 removal already occurred in FR.

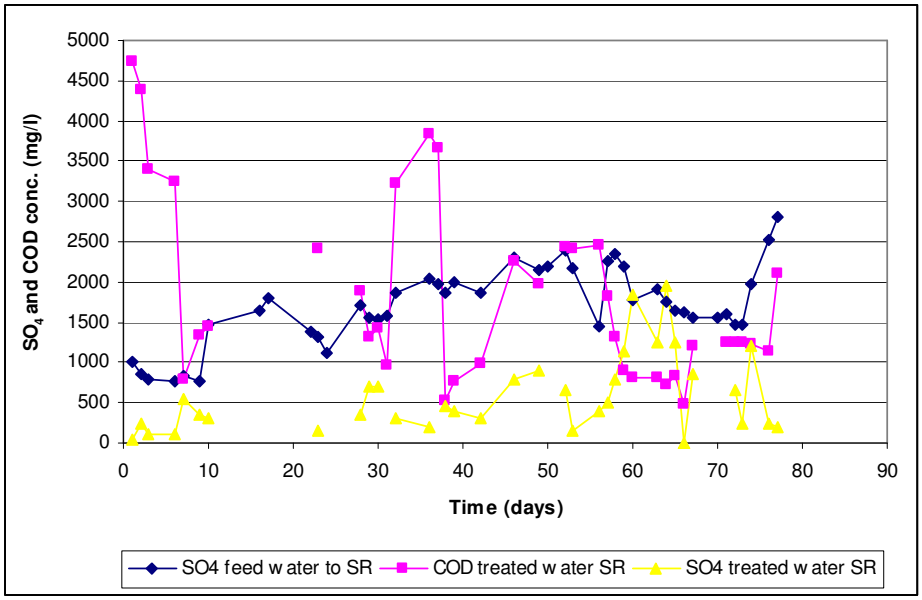

Figure 5.4. The SO_4 and COD concentrations relating to SR

5.3.1.2 VFA utilisation

The results in Table 5.4 showed that the C4 and C3 acids were utilised, producing C2 acid, since the C2 acid concentration increased with 447 mg/ℓ, while the C3 acid concentration decreased by 150 mg/ℓ and the butyric acid concentration by 123 mg/ℓ. From these and the sulphate removal results (total removed 2688 mg/ℓ SO_4) it became evident that the SRB, in addition to VFA, utilised other intermediate products from the fermentation process, e.g. hydrogen and alcohols. Hydrogen is a major intermediate in the degradation of organic matter and is used by SRB as soon as it is produced by fibrolytic and fermentative microorganisms (Hungate, 1966; Wolin and Miller, 1988). Interspecies hydrogen transfer between H_2-producing and H_2-utilising microorganisms allows growth and activities of the fermentative and sulphate reducing microorganisms. During the total experimental period of 60 days, 95 g sulphate was removed, while 447 g acetate was produced (based on the daily results). Acetate production can be ascribed to the weekly addition of 1000 g of grass cuttings. When utilising the C3 and C4 acids, the amount of acetate produced can

be the rate limiting factor and potentially cause reactor failure. Hill *et al*. (1987) proposed that an acetic acid concentration > 800 mg/ℓ or a C2:C3 ratio > 4:1 can have an adverse effect on the reactor processes. It was therefore decided to add less grass cuttings to FR during Study 1b.

5.3.1.3 Sulphide production (Table 5.3)

During the biological sulphate removal process, sulphide is produced according to reactions 5.1-5.2. This can be biologically oxidised to elemental sulphur in the presence of oxygen (reaction 5.3):

$$\text{Propionate}^- + \tfrac{3}{4}\ SO_4^{2-} \rightarrow \text{Acetate}^- + HCO_3^- + \mathbf{\tfrac{3}{4}\ HS^-} + \tfrac{1}{4}\ H^+ \quad (5.1)$$

$$\text{Butyrate}^- + \tfrac{1}{2}\ SO_4^{2-} \rightarrow 2\ \text{Acetate}^- + \mathbf{\tfrac{1}{2}\ HS^-} + \tfrac{1}{2}\ H^+ \quad (5.2)$$

$$H_2S + \tfrac{1}{2}\ O_2 \rightarrow H_2O + S^\circ \quad (5.3)$$

From these reactions, for every mole of SO_4 (96 g), one mole of sulphide is formed (32 g), which results in the experimental S^{2-}/SO_4 ratio of 0.33. Although small amounts of air could diffuse into the reactor, the Dissolved Oxygen (DO) concentration was always measured at zero, while the redox potential was on average -190 mV. Dilling and Cypionka (1990) described the aerobic respiration of SRB. They found that cultures of *Desulfovibrio desulfuricans* (strain CSN) reduced 5 mM O_2 with H_2 as electron donor. Aerobic respiration was not coupled with growth, but resulted in ATP formation. Besides H_2, organic electron donors, such as formate, lactate, ethanol and pyruvate, as well as inorganic sulphur compounds, e.g. H_2S, thiosulphate, sulfite, were utilised for aerobic respiration. Sulphite and thiosulphate were completely oxidized to sulphate.

The sulphide production in FR was 294 mg/ℓ, while the SO_4 removal was 1614 mg/ℓ. Therefore the S^{2-}/SO_4 ratio in FR was 0.18, which is lower than the theoretical value of 0.33, showing that part of the S^{2-} formed was not accounted for. This was explained by the biological oxidation of sulphide to sulphur (reaction 6.3) as well as by the fact that part of the sulphide escaped in the gaseous form, due to the lower reactor pH. Weast (1981) described that the pK_a value of the dissociation equilibrium of H_2S is 7.04 at 18 ℃. Above pH 8.0-9.0 virtually all dissolved sulphide is present in its ionised form, while at neutral pH values 20 to 50% of the dissolved sulphide is present as H_2S, depending on the reactor temperature (O'Flaherty & Colleran, 2000).

Furthermore, part of the sulphate is reduced to intermediate products, such as thiosulphate and sulphite, which are not analysed in the sulphide analysis, during the biological sulphate removal process. The increase in reactor pH after sulphate reduction (due to alkalinity production) is therefore beneficial in lowering the sulphide toxicity. In most cases it is advisable to keep the pH of the sulphidogenic reactor between 7.5 and 8.5. However, this higher reactor pH is not advisable when rumen bacteria are present in the same reactor, since they prefer a pH of 6.6-6.9.

The sulphide rich effluent from FR entered SR, which also received sulphate-rich feed water. The sulphide load to SR was 147 mg/ℓ. The sulphide concentration in the treated water of SR was 295 mg/ℓ, thus sulphide produced in SR was 148 mg/ℓ. The SO_4 removal in FR was 1105 mg/ℓ, thus the S^{2-}/SO_4 ratio in FR was 0.13, which is lower than the theoretical ratio of 0.33. The lower ratio was partly explained by the sulphur formation at the top of the reactor. Air diffusion was possible at the top of the reactor, resulting in sulphide oxidation according to reaction (5.3).

5.3.1.4 Alkalinity production

The average alkalinity production in FR was 1818 mg/ℓ $CaCO_3$, while the sulphate reduction was 1614 mg/ℓ, resulting in an Alkalinity/SO_4 ratio of 1.13, which is higher than the theoretical ratio of 1.04. This was ascribed to pH correction in FR using $NaHCO_3$, as initially the fermentation of the grass resulted in high VFA concentrations and thus in the reactor pH decrease. The alkalinity concentration entering SR was diluted by the feed stream into SR, which resulted in an average alkalinity concentration of 909 mg/ℓ. The treated water from SR contained an average alkalinity concentration of 1669 mg/ℓ, which implied an average alkalinity production of 760 mg/ℓ in SR. The average sulphate removal in SR was 1105 mg/ℓ, thus the Alkalinity/SO_4 ratio was 0.69, which is << the theoretical value of 1.04. The lower experimental value (0.69) was ascribed to addition of 0.1N HCl to correct for the increased reactor pH (values > 7), as a result of the biological sulphate removal in FR. The reactor pH in FR needed to be maintained between 6.6 and 6.9 to accommodate the rumen microorganisms.

5.3.1.5 Reactor pH

The average pH of the FR treated water was 7.31, while the average pH of the SR treated water was 7.39. The reactor pH increased due to sulphate removal, followed by alkalinity production in FR and SR.

5.3.2 Study 1b

A three stage reactor system was used (Figure 5.2). All three reactors received synthetic feed water at 5 ℓ/d. In addition SR received the effluent of FR (total feed rate 10 ℓ/d) and ASR received the effluent of SR (total feed rate 15 ℓ/d), thus FR had a HRT of 4 days, SR of ½ day and ASR of ⅓ day. Study 1b was divided into four periods, during which 150 g grass was added to FR on days 1, 32, 46 and 62. The duration of the four periods was 19, 15, 15 and 14 days, respectively. The monitoring of the reactor system started on day 14.

5.3.2.1 Sulphate removal in FR, SR and ASR

The chemical composition of the average results of the feed and treated water from reactors FR, SR and ASR during the four periods are given in Table 5.5. The highest sulphate removal took place in FR, followed by SR and ASR (Table 5.6). Sulphate removal was followed by sulphide production as deduced from the data in Tables 5.5 and 5.6. The $S^{2-}_{produced}/SO_{4\ removed}$ ratios were 0.19, 0.21, 0.19 and 0.20 during periods 1-4 in FR. The graphs in Figures 5.5-5.8 show the relationships between the available COD concentrations and the sulphate reduction in the three stage reactor system (FR, SR and ASR). The SO_4 removal pattern in FR was initially irregular, however, it improved after day 35. During the periods that the COD concentration was lower than 1000 mg/ℓ, the sulphate reduction was less efficient (≈ day 45). Fresh GC (150 g per addition) was added on days 32, 46 and 62 (arrows). It was observed from Figure 5.5 that after each grass addition, the COD concentration increased, while it decreased during the periods of sulphate reduction.

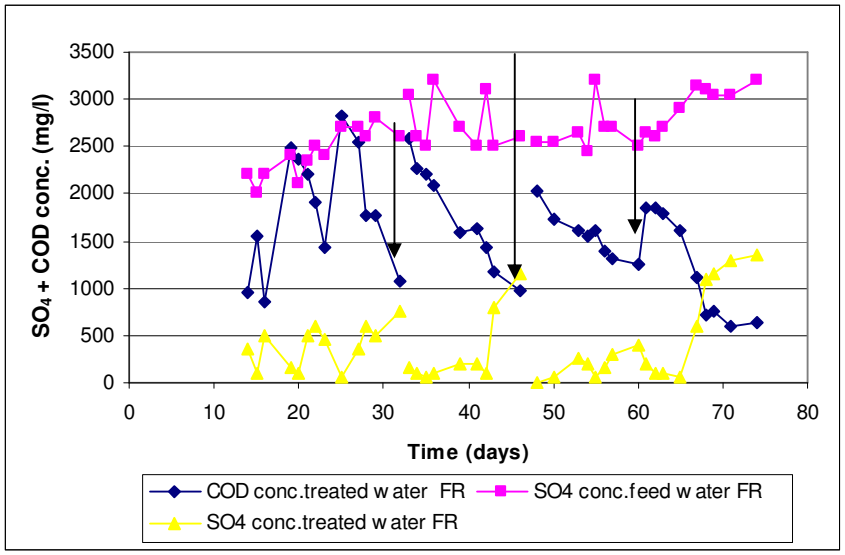

Figure 5.5. Sulphate removal and COD concentration in FR

A similar relationship between the COD and SO_4 concentration pattern was observed in Figure 5.6. When the COD concentration was high, the SO_4 concentration was low and on days when the COD concentration decreased to values < 1000 mg/ℓ, the SO_4 concentration in the reactor increased. When the COD concentration was lower than 500 mg/ℓ, the sulphate reduction seemed to come to a halt. This can be clearly seen from the graphs in Figure 5.6, after day 56. Sulphate was most efficiently removed in FR (volume: 20 ℓ), in which the HRT was 2 days for the fermentation process and 2 days for the sulphate removal process (total HRT: 4 d). The SO_4 removal in SR (volume: 5 ℓ) was not as efficient as in FR, which was ascribed to the addition of fresh sulphate feed water, to the lower COD concentration entering SR and to the shorter HRT of ½ day.

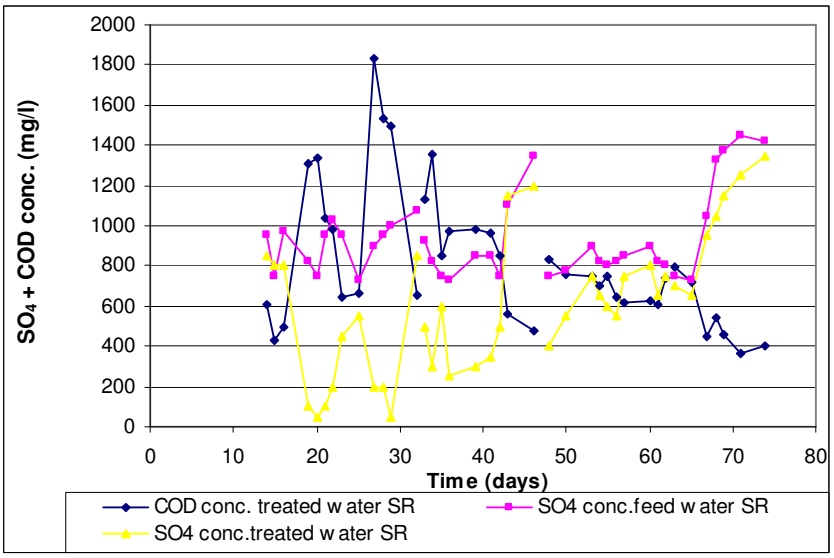

Figure 5.6. Sulphate removal and COD concentration in SR

Figure 5.7 showed that the SO_4 removal was the least efficient in ASR (volume: 5 ℓ), (HRT: ⅓ d) since the COD concentration was the lowest in ASR compared to FR and SR. The overall sulphate removal was the highest in FR, followed by SR, while it was the lowest in ASR (Table 5.4).

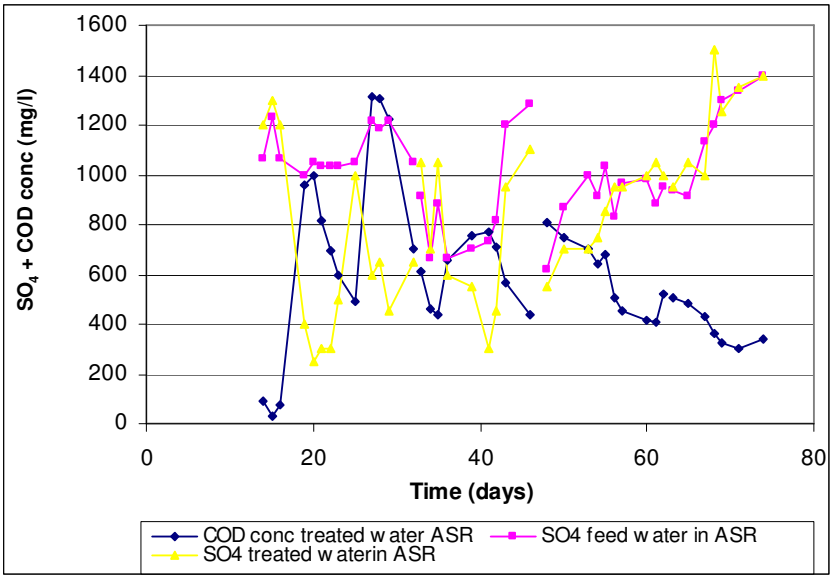

Figure 5.7. Sulphate removal and COD concentration in ASR

There were four experimental periods during study1b, determined by adding fresh GC (150 g) on days 1, 32, 46 and 62. The monitoring of the reactor system started on day 14. The chemical compositions of the feed and treated water from FR, SR and ASR during the four periods are given in Table 5.5, indicating that the highest sulphate removal took place in FR, followed SR and ASR. Sulphate removal was followed by sulphide production as deduced from the data in Table 5.5. The $S^{2-}_{produced}/SO_{4removed}$ ratios were 0.19, 0.21, 0.19 and 0.20 during periods1-4 in FR. Although these ratios were lower than the theoretical value of 0.33, it was noted that the ratios throughout the four experimental periods were stable. The sulphate removal values that occurred in the three stage reactor system, during the four experimental periods, are presented in Table 5.6. The results showed that during each period a total of 435, 245, 223 and 190 g SO_4 was removed using 150 g grass per period. It was noted from this data that the amount of sulphate removed decreased over periods 1-4.

The results from FR (Table 5.6) indicated that the average SO_4 removal in FR was similar at 176, 175 and 172 g over a period of 14 and 15 days during periods 2, 3 and 4. The higher total sulphate removal of 194 g in the first period can be ascribed to a longer period of 19 days.

It was calculated from the total sulphate removal over the four periods that from 1 g grass, in the first period, 3 g sulphate was removed; in the second period it was 1.6 g, while it was 1.5 g and 1.3 g in the third and fourth period, respectively. This

removal yield was relatively high compared to the results obtained from the batch tests as described in Chapter 4, which showed that in order to remove 1 g SO_4, 8 g grass was needed. The decrease in the sulphate removal during the four periods was ascribed to the fact that as no further GC was added, cellulose became depleted. The sulphate removal efficiency in FR during the four periods was 84, 91, 88 and 80%, respectively, while in SR the SO_4 removal decreased from 36 to 22 to 12 and to 9%, respectively, while little additional SO_4 removal was observed in ASR. The results in FR compared well to the experiments where commercial propionate was used as the carbon and energy source, since during that study, the percentage sulphate removal was 78% (Greben *et al*. 2004). The low sulphate removal in the third reactor prompted the decision not to continue with the three stage reactor design. When taking into account the SO_4 removal efficiency in FR and SR, the question also arose whether a second reactor (SR) adds sufficient value to the reactor system to warrant its use. This observation will be discussed later.

Table 5.5. The chemical compositions of the feed and treated water during the four periods in FR, SR and ASR.

Period 1						
Parameter	**Feed FR**	**Treated FR**	**Feed SR**	**Treated SR**	**Feed ASR**	**Treated ASR**
COD (mg/ℓ)		1724		922		649
pH (value)	7.15	7.23	7.21	7.46	7.30	7.66
SO_4 (mg/ℓ)	2367	383	910	490	1095	747
S^{2-} (mg/ℓ)		386		352		290
Redox Potential (mV)		-173		-168		-143
Period 2						
COD (mg/ℓ)		1965		928		604
pH (value)	7.20	7.26	7.39	7.48		7.55
SO_4 (mg/ℓ)	2761	244	864	544	859	750
S^{2-} (mg/ℓ)		522		459		364
Redox Potential (mV)		-174		-163		-152
Period 3						
COD (mg/ℓ)		1519		633		583
pH (value)	7.30	7.45	7.43	7.63	6.57	7.70
SO_4 (mg/ℓ)	2650	315	903	730	968	870
S^{2-} mg/ℓ)		446		302		225
Redox Potential (mV)		-171		-158		-147
Period 4						
COD (mg/ℓ)		1276		590		416
pH (value)	7.33	7.46	7.36	7.52	7.34	7.86
SO_4 (mg/ℓ)	2895	600	1040	905	1087	1095
S^{2-} (mg/ℓ)		467		239		159
Redox Potential (mV)		-154		-143		-138

Table 5.6. The sulphate removing data in the three reactor system

Period 1				
Parameter	*FR*	*SR*	*ASR*	*TotalSO_4 (g) removed over each period*
Av SO4 removal (g/ℓ)	2.04	0.51	0.42	
Av SO4 removal (g/d)	10.21	6.10	6.27	
Av SO_4 removed during period 1 (g)	194	122	119	435
% SO_4 removal efficiency	84	36	n.a	
Period 2				
Av SO4 removal (g/ℓ)	2.52	0.33	0.11	
Av SO4 removal (g/d)	12.58	3.25	1.64	
Av SO_4 removed during period 2 (g)	176	45.5	23	245
% SO_4 removal efficiency	91	22	n.a	
Period 3				
Av SO4 removal (g/ℓ)	2.33	0.17	0.10	
Av SO4 removal (g/d)	11.67	1.73	1.48	
Av SO_4 removed during period 3 (g)	175	26	22	223
% SO_4 removal effieciency	88	12	n.a.	
Period 4				
Av SO4 removal (g/ℓ)	2.29	0.14	No removal	
Av SO4 removal (g/d)	11.49	1.35	No removal	
Av SO_4 removed during period 4 (g)	172	20	No removal	190
% SO_4 removal efficiency	80	9	n.a.	

5.3.2.2 COD profile in reactors FR, SR and ASR

The graphs in Figure 5.8 show that the highest COD utilisation occurred in FR (difference in COD concentrations between FR and SR), which is in agreement with the sulphate reduction in FR. The residual COD concentration of FR was available for further SO_4 removal in SR, while the COD concentration in the effluent of SR was required for further SO_4 removal in ASR. This latter reactor was coupled to the reactor system, because during Study 1a, 1000 g GC/week were added to FR, which resulted in high concentrations of acetate in the final effluent. Due to the fact that less grass was added to the reactor system during this investigative period, ASR was no longer needed. The residual COD concentration in the final effluent was most likely in the form of recalcitrant COD (e.g lignin), as the VFA profile in Table 5.7 showed that most VFA was utilised, except for small concentrations of acetate.

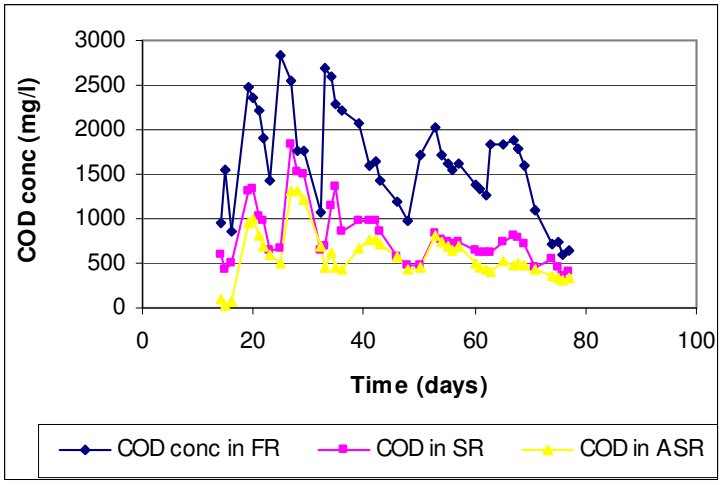

Figure 5.8. COD profile in the three reactor system

Anaerobic digestion is mediated by a complex system of various microbial populations and pathways. Several cultures coexist that derive energy from degradation of various substrates (Ahring 2003). The results of this study showed that the fermentation products required for biological sulphate reduction were obtained from the fermentation reactor (FR), which received grass cuttings on a regular basis.

5.3.2.3 VFA profile in FR, SR and ASR

The data in Table 5.7 indicated that the C3 and C4 VFAs were absent in FR, SR and ASR, whereas acetic acid was present in all reactors. During Periods 1-4, the C2 acid concentration was 649 mg/ℓ, 449 mg/ℓ, 88 mg/ℓ and 27 mg/ℓ, respectively. Thus either less butyric and propionic acids were utilised, thus less acetic acid was

produced or due to the low butyric and propionic acid concentrations, the acetic acid was utilised for sulphate reduction in ASR. The ASRB will use propionate and butyric acid, when available and only use acetic acid, when no other carbon source is obtainable. Acetate utilisation was reported in the previous chapters, when insufficient C3 and C4 acids are available for SO_4 reduction.

Table 5.7. The VFA profile in the three stage reactor system over the four experimental periods

Period 1			
Parameter (mg/ℓ)	FR	SR	ASR
Acetate	649	227	73
Propionate	16	0	0
Butyrate	3	0	0
Period 2			
Acetate	449	126	0
Propionate	3	0	0
Butyrate	1	0	0
Period 3			
Acetate	88	150	79
Propionate	0	0	0
Butyrate	0	0	0
Period 4			
Acetate	27	121	63
Propionate	2	0	0
Butyrate	0	0	0

The finding that the VFA concentrations in the three reactors decreased over the consecutive experimental periods agreed with the correspondingly decreasing COD concentrations in the reactors during the same periods. The COD and VFA results indicated that most of the available COD was utilised in FR followed by SR.

5.3.3 Study 2a

5.3.3.1 Sulphate removal

In this study the results of using pre-treated AMD and synthetic SO_4 rich water as feed water, respectively, operating batch test reactors, are shown in Figure 5.9. It was observed that the sulphate concentration in R1 decreased from 1350 mg/ℓ to 150 mg/ℓ over a period of 10 days. Initially, the sulphate removal in R2 was faster than in R1, however, the overall sulphate reductions in R1 and R2 were similar. The results, as presented in Figure 5.9, seemed to indicate that feeding diluted AMD had no adverse effects on the rumen microorganisms, as in both cases adequate COD was present for the biological sulphate reduction to take place (Figure 5.10).

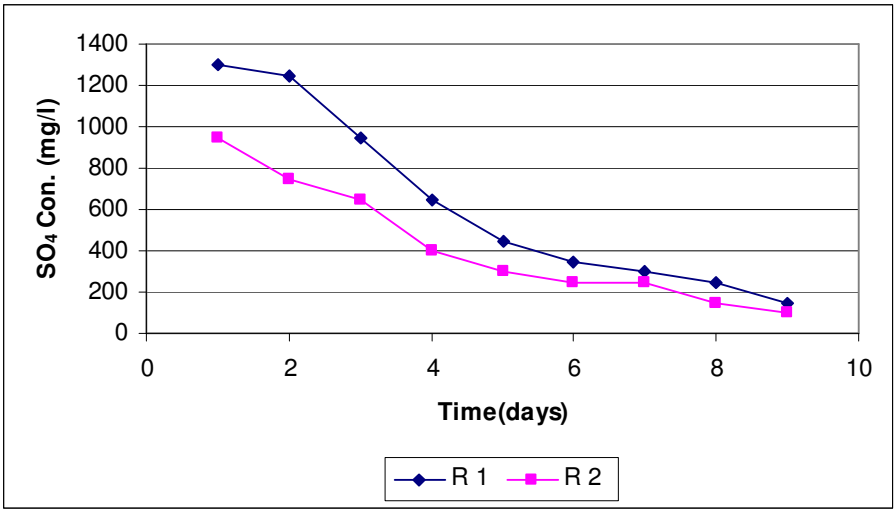

Figure 5.9. The SO_4 removal patterns in R1 and R2
R1 received pretreated AMD
R2 received synthetic sulphate-rich water

5.3.3.2 COD/Sulphate

The results in Figure 5.10 show the COD concentrations in R1 and R2. From the start of the experiments, the COD concentrations in both R1 and R2 increased to more than 5000 mg/ℓ COD, which demonstrated the good cellulose fermentation potential of the rumen bacteria. The available COD concentrations in both reactors were used for the sulphate reduction and, as was observed for the sulphate removal

pattern (Figure 5.9), the COD concentrations in both reactors were similar. These results indicated that pre-treated AMD does not affect the rumen microbes in the cellulose degrading process. It was concluded from Study 2a, that Study 2b could be executed.

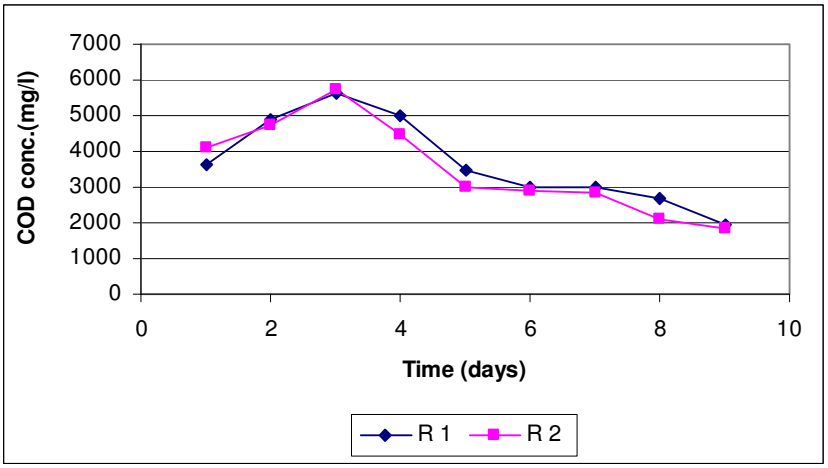

Figure 5.10. The COD concentration in R1 and R2
R1 received pretreated AMD
R2 received synthetic sulphate-rich water

5.3.4 Study 2b

5.3.4.1 Sulphate removal

The sulphate removal profile is illustrated in Figure 5.11. The graphs showed that the sulphate concentration of the treated water in both reactors was very similar at between 500 and 1500 mg/ℓ up to day 29. On day 29 the reactor received 1 kg grass and 1 ℓ rumen bacteria mixture (VSS of 10 g/ℓ) in order to improve the cellulose degradation and to generate a higher COD concentration to improve the biological sulphate removal in both reactors. The addition of fresh GC and the rumen inoculum resulted in a sulphate concentration of 400 mg/ℓ in the treated water on the next day, while it increased again to 1900 mg/ℓ on day 34 to decrease again to 350 mg/ℓ on day 35. From day 40-60, 20 g GC/d were added, which, after the feed rate was doubled on day 50, was increased to 40 g/d from day 61-78. The results indicated that after day 75, the sulphate reduction was <500 mg/ℓ for several consecutive days. On day 50, the feed-rate was increased to 30 ℓ/d, which resulted in an average sulphate load of 70.5 g/d. During this period, different amounts of GC were added. From d 50-62, it was 20 g/d and from d 62-78, it was doubled to 40 g/d, while on d 75, 500 g GC were added. This increased grass addition immediately resulted in improved sulphate reduction as seen in Figure 5.11, which again showed the relationship between GC addition and sulphate removal.

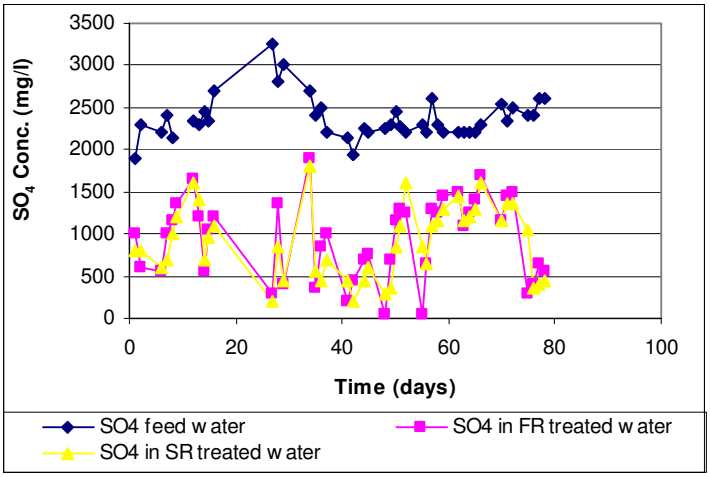

Figure 5.11. SO_4 concentration in feed and treated water in FR and SR reactors

The chemical composition of the feed and the treated water of FR and SR during three experimental periods is presented in Table 5.8. The data is based on the analyses of daily sample taking, which is averaged and presented in Table 5.8. During the first two periods, when the feed-rate was 15 ℓ/d, the highest sulphate reduction occurred in FR, followed by additional SO_4 reduction in SR. This resulted in an increased alkalinity and sulphide concentration and in a further decrease in the redox potential. These different results indicated preferred reactor conditions for sustained sulphate reduction and a further decrease in COD concentration. The highest sulphate reduction was obtained during Period 2, when the feed-rate was 15 ℓ/d and 20 g/d GC were added daily over 8 days. During this total experimental period 160 g GC were added while 205 g SO_4 was removed in FR and 215 g in FR and SR combined. These results indicated that 1 g GC resulted in a total of 1.34 g SO_4 removal. During the first period, the SO_4 reduction in FR was 1472 mg/ℓ, while this was 1708 mg/ℓ during the second period. For SR an additional SO_4 removal of 74 mg/ℓ (5.0%) and 83 mg/ℓ (4.9%), respectively, was noted. During period 3, the removal in FR was 1284 mg/ℓ, while no further reduction took place in SR. The sulphate removal during period 3 was less efficient, because the residual COD concentration at 480 mg/ℓ was too low for sustained SO_4 removal. The low COD was ascribed to the increased sulphate load on day 50, when the feed rate was doubled while initially the amount of GC stayed the same, but was doubled on day 62. From the sulphate reduction data in SR, it was concluded that an additional sulphate removal reactor is unwarranted as the additional capital costs are unjustifiable in order to increase the total sulphate reduction by only 5%. The higher sulphate reduction during Period 2 was ascribed to the daily GC addition of 20 g/d.

5.3.4.1.1 Grass added/sulphate removal ratio

From the batch tests results as described in Chapter 4 it was indicated that 8 g GC were needed to remove 1 g SO_4. From the studies using synthetic, sulphate-rich feed water, the sulphate removal was 3, 1.6, 1.5 and 1.3 g from 1 g GC, over 4 experimental periods, respectively. The decrease in sulphate removal over the four periods was ascribed to the lower COD concentration obtained from the 1000 g GC. From the results as obtained from the study feeding pre-treated AMD, it was shown that the sulphate removal using 1 g GC was 1.34 g in Period 2. This result compared favourably with that obtained using synthetic, sulphate-rich feed water. Most results obtained from the different studies demonstrated that regular addition of GC was essential in order to obtain a high COD concentration in the reactor and hence a sustained sulphate removal, as will be shown in the following paragraph.

5.3.4.2 COD concentration

Sulphate removal is dependant on the COD concentration in the reactor as was shown in the previous chapters. Fresh GC and 1 ℓ rumen fluid were added on d 27, which resulted in a rapid COD increase (Figure 5.12). However, the available COD concentration was utilised instantly and thereafter the COD concentrations in both FR and SR were too low for continued sulphate reduction. Only on d 62 (first day of adding 40 g/d GC) and on d 75 (adding 500 g GC, once off) were COD concentrations increases observed. This resulted in immediate sulphate reduction as shown in Figure 5.11. These results once again showed the relationship between a high COD and low SO_4 concentration in the bioreactor. The COD concentration in the reactor is dependant on the addition of GC and on the cellulose degraders (rumen microorganisms) providing the substrates for the SRB to carry out sulphate reduction. The COD concentration in FR during Period 3 was 480 mg/ℓ, (Table 5.8) while the SO_4 concentration was 1068 mg/ℓ, which resulted in a COD/SO_4 ratio of 0.45 in FR, which was too low to sustain sulphate reduction. The theoretical feed COD/SO_4 ratio is 0.67, in which case the available COD is used for the biological sulphate removal. Ideally the feed water COD/SO_4 ratio should be approximately 1, providing enough COD to sustain sulphate removal and cell growth (Rinzema & Lettinga, 1988).

5.3.4.3 $Sulphide_{produced}/Sulphate_{removed}$ ratio

The sulphide concentrations were 396, 504 and 355 mg/ℓ, respectively, which resulted in S^{2-}/SO_4 ratios of 0.27, 0.30 and 0.28. These ratios were similar to the theoretical ratio of 0.33. The somewhat lower values were ascribed to the metal-sulphide precipitation in the reactor, especially of FeS (Table 5.9).

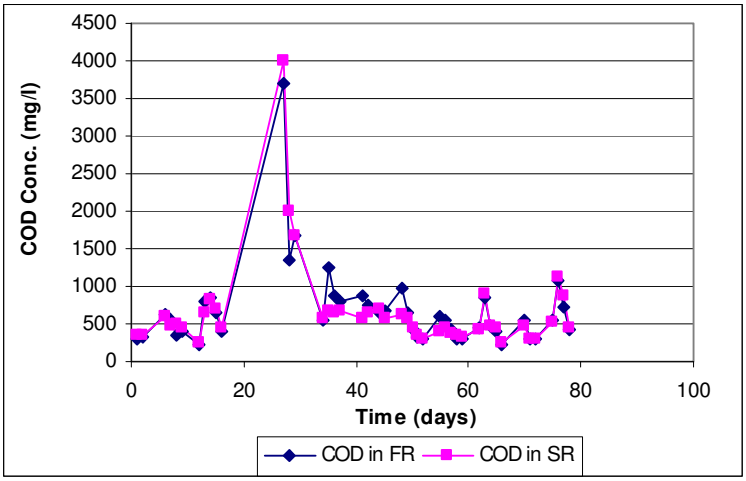

Figure 5.12. COD concentration in the FR and SR reactors

Table 5.8. The chemical composition of the feed and treated water in FR and SR

Parameter	Period 1 (d1-37)	Period 2 (d41-49)	Period 3 (d50-78)
Feed-rate (ℓ/d)	15	15	30
pH Feed (Value)	6.58	7.19	7.12
pH FR (Value)	7.68	7.65	7.42
pH SR (Value)	7.71	7.70	7.45
SO_4 Feed (mg/ℓ)	2489	2183	2352
SO_4 FR (mg/ℓ)	1017	475	1068
SO_4 SR(mg/ℓ)	943	392	1070
Alk Feed (mg/ℓ)	189	518	328
Alk FR (mg/ℓ)	1709	2208	1543
Alk SR (mg/ℓ)	1819	2482	1659
COD FR (mg/ℓ)	922	757	480
COD SR (mg/ℓ)	847	615	478
S^{2-} FR (mg/ℓ)	396	504	355
S^{2-} SR (mg/ℓ)	397	520	345
S^{2-}/ SO_4 ratio	0.27	0.30	0.28
Redox pot. FR (mV)	-175	-192	-183
Redox pot. SR (mV)	-176	-194	-183
VSS in effluent FR(mg/ℓ)	68	50	46

5.3.4.4 Metal removal

It was shown in Table 5.1 that the raw AMD from the mine in Witbank, South Africa, contained several metals. In order to remove the metals prior to feeding this AMD to the reactor, the AMD was pre-treated with the effluent from SR. This effluent contained high concentrations of sulphide: 397, 520 and 345 mg/ℓ, respectively in the three experimental periods (Table 5.8). The data in Table 5.9 show the metal concentrations in AMD, in pre-treated AMD and treated AMD, in both FR and SR. Most metals were removed to a large extent after the pre-treatment and those not completely removed were mostly precipitated during the biological sulphate removal process. All metal concentrations were < 0.10 mg/ℓ, except for iron and manganese. The precipitation of MnS is pH related and occurs at pH > 7.5. At the time of the metal analyses, the average pH in FR and SR were approximately 7.42 and 7.45, respectively (Period 3).

Table 5.9. Metal concentrations in AMD, in pre-treated AMD and in treated AMD

Metal (mg/ℓ)	AMD	Pre treated AMD	FR out	SR out
Aluminium	122	11	<0.07	<0.07
Copper	0.75	<0.04	<0.04	<0.04
Iron	76	2.2	0.22	0.14
Lead	0.25	<0.07	<0.07	<0.07
Manganese	9.3	6.8	3.8	3.0
Zinc	1.7	<0.07	<0.07	<0.07

5.4 CONCLUSIONS

The results of this study confirmed those presented in the previous chapters, namely that the fermentation products of grass cuttings can serve as carbon and energy sources for continuous, biological sulphate removal. Conducting the different studies using synthetic feed water led to the use of two and three stage continuous reactor systems, comprising a hybrid reactor (FR), followed by (two) packed bed reactor(s) SR and ASR, repectively. It was shown that the VFA produced, except acetic acid, were utilised for the biological sulphate removing process, although the sulphate removal rate was not dependant solely on the VFA concentration. Other intermediate products were utilised for sulphate reduction as higher sulphate removals were obtained than were expected from the available VFA concentrations. The addition of a large amount of grass (1000 g/week) resulted in a high COD_{used}/$Sulphate_{removed}$ ratio and in a high residual COD (acetate) concentration. When 150 g GC per two weeks were added, a continuous sulphate reduction was obtained. It was noted that

sustained sulphate reduction was dependant on a continuous COD production. The highest and stablest sulphate removal efficiency was achieved in FR in which the fermentation and sulphate reduction occurred simultaneously at a HRT of 4 days.

The results of the two stage reactor, to which 1000 g GC/week were added, showed a total SO_4 removal rate of 3.0 g SO_4 (ℓ.d). Under these conditions, it was found that the residual COD and acetate concentrations in the treated water were high at 1428 and 977 mg/ℓ, respectively. When operating a three stage reactor system, to which 150 g GC/2 weeks was added, the results showed a stable sulphate removal efficiency in the first reactor, during the four experimental periods (84, 91, 88 and 80%), respectively. Sulphate reduction was obtained in the second sulphate removal reactor, but the percentage removal decreased with time (36, 22, 12 and 9%, respectively over the four periods). This was most likely due to a shortage of readily available substrate (COD/VFA). The total sulphate removal in the third reactor was low and its use added no value to the sulphate removal process. It was shown that 1 g grass could remove 3, 1.6, 1.5 and 1.3 g sulphate over the four experimental periods, respectively.

When pre-treated AMD was used as feed water for the two stage reactor system at a feed rate of 15 and 30 ℓ/d, most of the sulphate was removed in the first reactor. The highest sulphate removal was obtained when GC (20 g) were added daily and when the feed rate was 15 ℓ/d. When the feed rate was doubled and the GC kept constant, a lower SO_4 removal resulted. Both sulphide and alkalinity were produced, the reactor pH increased and the redox potential in the reactor was at -194 mV, when the highest sulphate removal rate was obtained. Since most sulphate was removed in the first reactor with low additional concentrations of sulphate removed in the second and third reactor it was concluded to only operate a one stage (hybrid) reactor system. In order to take this technology to pilot plant scale, the fermentation and removal process in a one stage reactor needs to be well understood.

A process description based on practical results from these studies as well as on theoretical values, was developed to simulate the treatment of AMD. This is described in the following Chapter (6). Only the one stage reactor was used for the calculations in Chapter 6.

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CHAPTER 6

PROCESS DESCRIPTION OF THE GRASS-CELLULOSE FERMENTATION AND BIOLOGICAL SULPHATE REMOVAL TECHNOLOGY APPLYING MASS BALANCE EQUATIONS

6.1 INTRODUCTION

Mine effluents have to comply with the standards set by the Department of Water Affairs and Forestry (DWAF), when discharged into river systems. This governing body specifies that the sulphate concentration in AMD discharged should not exceed 500 mg/ℓ in most areas of South Africa. In addition, metals and acidity should be removed before the water is released to public water bodies. The main components after biological treatment usually consist of sulphide, alkalinity as well as residual COD and SO_4. The pH of treated water should be 7 to 8.

In this chapter attention was given to the design of an anaerobic bioreactor for the removal of sulphate, (heavy) metals and the elevation of the pH in AMD. The description of the process was based on treating AMD of a specific quality (obtained from a closed coal mine located in the Witbank area, South Africa) and on the results obtained in Chapter 5 where pre-treated AMD was used as the feed water for the process. The volume of this to be treated AMD was 2 000 m^3/d with a sulphate concentration of 2 kg/m^3 and a pH of 2.5 (data provided by mine management).

The suite of mass balance equations incorporated all streams in and out of the reactor system and took the chemical and biochemical reactions into account that occurred in the system. The stoichiometric equations and growth kinetics for the rumen bacteria and the SRB were mainly based on theoretical values. All important parameters, such as sulphate concentration and flow-rates were taken into account for the process description. It was envisaged that such analysis of the process could provide the understanding of the biological processes of the fermentation and sulphate reduction in the one stage reactor system, when treating mine effluents.

6.2 PROCESS DESCRIPTION

For the reduction of sulphate to sulphide 8 electrons, equivalent to 0.67 g of COD per g of sulphate, are required (equation 6.1)

$$8[H] + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \qquad (6.1)$$

This reaction generates approximately 1 mol of ATP (Schlegel, 1993). SRB have a preference for hydrogen, propionate, butyrate and acetate in that order. The results of the previous chapter showed that sulphate reduction on hydrogen, propionate and butyrate proceeded well, while small concentrations of acetate was generally detected in the reactor effluent, which agreed with the findings of Visser (1995). Hydrogen produced by rumen bacteria was immediately used by SRB, thus the SRB kept the dissolved hydrogen concentration low and consequently, rumen bacteria were not inhibited by the production of hydrogen (Visser, 1995). The energy/carbon source is oxidized when sulphate is reduced to sulphide, which produces carbon dioxide and water. When higher carbon sources are oxidised acetate is produced. Some SRB can subsequently oxidise acetate to carbon dioxide and water. Considering substrate affinity and growth rates, the reduction reactions for hydrogen and the various VFA's are presented in equations 6.2 - 6.5:

$$Hydrogen:$$
$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \qquad (6.2)$$
$$Butyrate:$$
$$2C_3H_7COO^- + SO_4^{2-} \rightarrow 4CH_3COO^- + HS^- + H^+ \qquad (6.3)$$
$$\text{Pr}opionate:$$
$$1\frac{1}{3}C_2H_5COO^- + SO_4^{2-} \rightarrow 1\frac{1}{3}CH_3COO^- + 1\frac{1}{3}HCO_3^- + HS^- + \frac{1}{3}H^+ \qquad (6.4)$$
$$Acetate:$$
$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \qquad (6.5)$$

The degradation of GC by rumen microorganisms and the subsequent sulphate reduction by SRB, using VFAs and hydrogen as carbon source are presented in Figure 6.1.

Like most other anaerobic bacteria, SRB have low growth rates, which are in the range of 0.55/day for the acetate oxidizing SRB up to about 2.5/day for hydrogen and propionate utilizing SRB (Visser, 1995). Other anaerobic bacteria, e.g. MB and AB have growth rates in the same range as SRB. These groups of bacteria are in constant competition for the available substrate when growing under anaerobic conditions. If a reactor is operated for a long period, consequently either SRB or MB/AB will dominate. This is dependent on kinetic parameters such as growth rate and substrate affinity of the bacteria groups. Visser (1995) showed that SRB can out-compete MB and AB for VFAs (except acetate) and hydrogen as substrates.

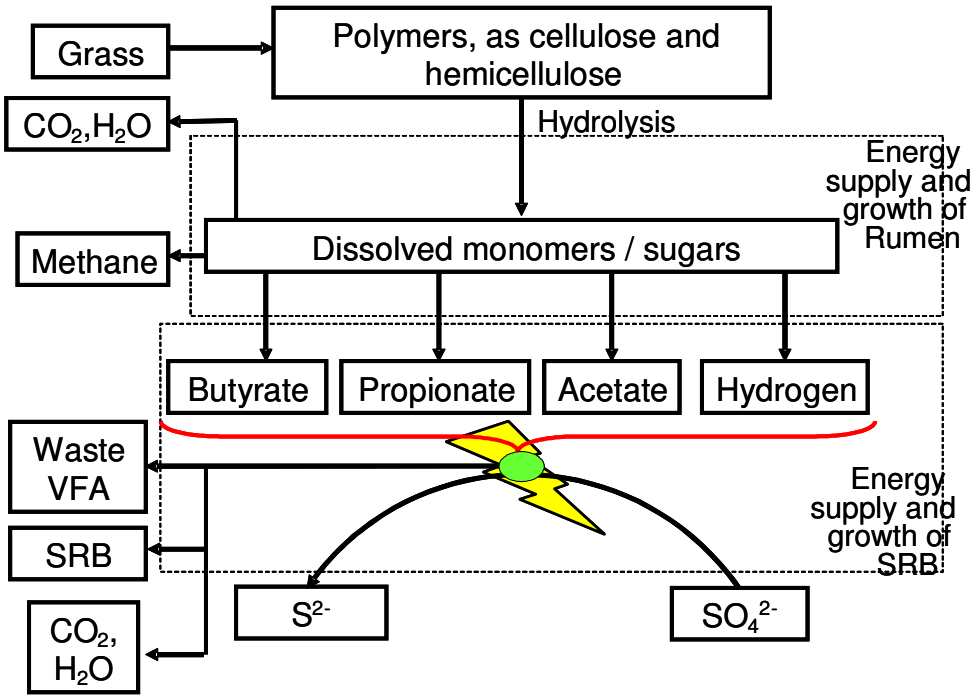

Figure 6.1. Grass (cellulose) degradation by rumen bacteria and subsequent biological sulphate reduction by SRB.

6.2.1 Metal Removal

The sulphide produced during biological sulphate reduction precipitated metals present in AMD to insoluble metal-sulphides, prior to feeding AMD to the reactor. Thus in full scale operation, part of the reactor effluent can be mixed with the incoming AMD. The metal-sulphides formed precipitated and were removed in a settler (Figure 6.2). The alkalinity concentration of the treated water neutralised the acidity in the AMD, thus the pH of the AMD increased, prior to its entering the reactor. The ratio, AMD: treated water was dependent on the acidity of the AMD and the alkalinity in the treated water. It was noted that the recycling stream also included the recycling of other substances such as the non-reduced sulphate in the treated water, which, however, was of no significance to the results presented.

6.2.2 Waste streams

6.2.2.1 Metal sulphides

Metals such as iron, copper, zinc and manganese precipitated with sulphide at the operating reactor pH (Janssen and Warmoeskerken, 1997). The precipitated metal-sulphides settled prior to the diluted AMD entering the reactor, thus preventing microbial toxicity. In full scale operation, the metal sulphides sludges can be stored in

the tailings dams, depending on the metal concentration. When these concentrations are relatively low, the metal sludge can be used as fertiliser on land.

6.2.2.2 Sludges

Bacterial sludges (rumen microorganisms and SRB) were produced in the bio-reactor and these need to be removed. Generally, waste sludge can be used as fertilizer.

6.2.2.3 Waste VFA or COD

The fermentation products generated by rumen bacteria, which were not used by SRB for the sulphate reduction, represented a certain residual COD concentration, which should comply with effluent standards on COD discharge. If the COD effluent concentration was higher than 100 mg/ℓ, aerobic treatment needs to follow the anaerobic stage to digest the residual COD. The biomass produced after COD degradation can be separated in a clarifier and disposed of together with the other sludges.

6.2.2.4 Gases

When a reactor is optimized for sulphate reduction the amount of the methane produced will be very low. Furthermore, most of the sulphide produced did not escape as hydrogen sulphide gas, but remained dissolved as HS^- since the reactor pH was higher than 6.5.

6.2.2.5 Sulphide

The sulphide produced can be converted chemically to elemental sulphur using a Fe^{3+} solution (Maree *et al*. 2004). The elemental sulphur can be sold for the production of sulphuric acid. There is a market for sulphur in South Africa and in other African countries. At present, South Africa is a net importer of sulphur.

6.2.3 Process Flow diagram

The combined description of the biological processes in the reactor assisted to understand the degradation and the sulphate removal process and how these two processes were dependant on each other. In the previous chapters, it was observed that the degradation of grass supplied the SRB with a carbon and energy source to reduce sulphate to sulphide. The sulphide produced precipitated the metals, present in AMD, while the alkalinity produced was beneficial to increase the pH of the treated AMD.

The objective of this chapter was to construct a process flow diagram of the proposed biological sulphate removal process (Figure 6.2) to understand both the chemical and biological processes involved. As shown in Chapter 5, the incoming AMD stream was mixed with the treated effluent from the biological sulphate removing bio-reactor (1:1) in a mixing tank. Next the pre-treated AMD stream entered the biological sulphate removing bio-reactor. To this reactor, GC were added regularly with the aim to degrade the cellulosic component, using a rumen inocula as fermenters, to VFA and hydrogen. These degradation products were used by SRB as carbon and energy sources for biological sulphate removal to sulphide. During this syntrophic degradation and utilisation of fermentation products, rumen microorganisms and SRB used carbon for growth. The effluent stream of the reactor contained residual sulphate and COD concentrations, which consisted mainly of un-degraded grass (lignin). It also contained sulphide, alkalinity and some of the washed out biomass (dead cells and debris). It was predicted that the gas phase contained carbon dioxide and only small quantities of methane since most of the hydrogen produced was utilised by the SRB.

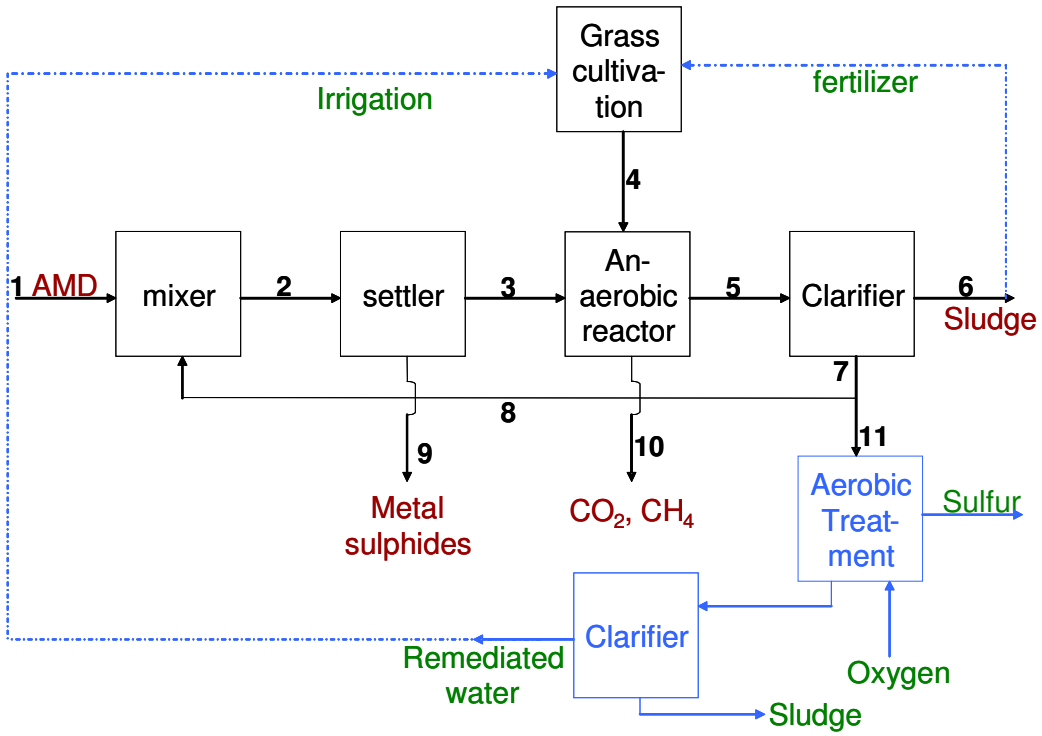

Figure 6.2. Process Flow- diagram of the proposed AMD treatment.
(Blue streams and reactors are outside the scope of this design).

In the proposed treatment system the grass would be cultivated on-site and irrigated with partly treated AMD and fertilized with waste sludge. Part of the effluent stream would be recycled to the front of the reactor system where it would be mixed with the feed stream. The other part of the effluent could be used for the irrigation of grass or could be discharged to rivers and dams, if the effluent standards of DWAF were adhered to. The predicted compositions of the separate streams in the process are presented in Table 6.1.

Table 6.1. Key components of each stream in the biological treatment

Stream*	Origin	Key components
1	AMD	Sulphate, Acidity and Metals
2	Mixed AMD+ effl (1:1)	Metal Sulphides, Sulphate and Sulphide
3	Supernatant of 2	Sulphate and Sulphide
4	Dry GC	Water, Cellulose, Hemicellulose, Lignin
5	Reactor effluent + sludge waste	Residual COD(acetate), Non-degraded grass, Biomass, Sulphate, Sulphide, Alkalinity
6	Sludge waste	Biomass
7	Overflow clarifier	Residual COD(acetate), Non-degraded grass,Sulphate,Sulphide, Alkalinity
8	Recycle stream	Non-used VFA, Non-degraded grass, Sulphate, Sulphide, Alkalinity
9	Metal waste	Metal Sulphides
10	Exhaust gas	Carbon Dioxide, Methane, Hydrogen-sulphide
11	Treated water	Residual COD(acetate), Non-degraded grass, Sulphate, Sulphide, Alkalinity

*Stream numbers are given in Figure 6.2.

6.2.4 Mass Balances

The mass balances were based on the assumption that the entire process was treated as in steady state. Firstly, the flow rates of the streams (1-11) in the process (Table 6.1) were taken into account. Secondly, the overall process balances for sulphur and sulphate were calculated and subsequently the mass balances over each process unit were reviewed. The grass cultivation was also analysed in the

mass balances and the surface area for grass cultivation was calculated. Thereafter, the stream tables of the entire process were presented.

6.2.4.1 Stream balance

The volume flow rates of each stream were determined. The following volume balance over the total system holds:

$$0 = feed\ flowrate + flow\ entering\ with\ grass - effluent\ flowrate - flow\ in\ sludge \quad (6.6)$$

The amount of water associated with the GC was negligible in comparison to the amount of water present in the feed flow. This is also true for the amount of water removed with the sludge, since very little sludge was produced. Consequently the feed flow rate is equal to the effluent flow rate. A ratio of 1:1 of the AMD and the sulphide/alkalinity rich effluent from the reactor was experimentally determined to give the required increase in pH value in the pre-treated AMD used as feed water. This complied with the amount of sulphide required for metal precipitation. The following balance applied for the mixing tank:

$$0 = feed\ flow + recycle\ flow - flow\ to\ settler \quad (6.7)$$

Since the feed flow was equal to the effluent flow, the sulphide/alkalinity rich recycle stream had the same volume as the feed flow. The flow to the settler is thus twice the feed flow rate. All water flow rates throughout the process were given in m^3/y (Table 6.2).

Table 6.2. Flow rates of the various process streams

Stream no*	Flowrate [m^3/y]	Stream no	Flowrate [m^3/y]	Stream no	Flowrate [m^3/y]
1	730 000	5	1 460 000	9	0
2	1 460 000	6	0	10	0
3	1 460 000	7	1 460 000	11	730 000
4	0	8	730 000		

** Stream numbers are presented in Figure 6.2.*

6.2.4.2 Overall balances for sulphate and sulphide

Sulphate entered the biological reactor system with the feed stream and was used by the SRB as electron acceptor to reduce sulphate to sulphide. In addition, sulphate is assimilatively taken up by SRB, which however formed a negligible part of the total sulphate concentration. The following balance, considering sulphate, of the total system in steady state holds:

$$0 = sulphate\,in - sulphate\,out + sulphate\ produced - sulphate\,consumed \qquad (6.8)$$

Sulphate is not produced in the process, thus the balance can be converted to:

$$0 = \phi_{v,in} \cdot C_{SO_4^{2-}} - \phi_{v,out} \cdot C_{SO_4^{2-},out} - sulphate\,consumed \qquad (6.9)$$

ϕ is flow rate
C is concentration

The maximum allowed effluent sulphate concentration is 0.5 kg/m^3 based on the DWAF requirements. The inflow and the outflow of the total system were equal and was 730 000 m^3/y. From the balance it follows that 1 095 000 kg/y of sulphate is reduced to sulphide.

A similar balance for sulphide as for sulphate can be expressed:

$$0 = sulphide\,in - sulphide\,out + sulphide\ produced - sulphide\,consumed \qquad (6.10)$$

Sulphide was produced through the conversion of sulphate. For every mole of sulphate reduced one mole of sulphide was produced. Sulphide was however also consumed for the precipitation of metals. The balance can be reduced to the following equation:

$$0 = -\phi_{v,out} \cdot C_{S^{2-},out} + \frac{32}{96} \cdot sulphate\,produced - sulphide\,in\,metal\,precipitation \qquad (6.11)$$

For the reactor it meant that the total amount of sulphide produced equaled 365 000 kg/y. The amount of sulphide required for metal precipitation equaled 142 248 kg/y (calculated from the metal concentration, present in the AMD, which will be discussed later). As a consequence 222 753 kg/y sulphide will leave the system via stream 11 at a concentration of 0.3 kg/m^3.

6.2.4.3 Mixer settler

The composition of stream 2 can be calculated. A balance of the mixing tank for sulphate is the following:

$$0 = \phi_{v,AMD} \cdot C_{SO_4^{2-},AMD} + \phi_{v,\mathrm{Re}c} \cdot C_{SO_4^{2-},\mathrm{Re}c} - \phi_{v,Mix} \cdot C_{SO_4^{2-},Mix} \quad (6.12)$$

It follows that the concentration of sulphate leaving the mixing tank is 1.25 kg/m^3. The concentration of sulphide leaving the mixing tank and settler can be calculated from a similar equation as follows:

$$0 = \phi_{v,AMD} \cdot C_{S^{2-},AMD} + \phi_{v,\mathrm{Re}c} \cdot C_{S^{2-},\mathrm{Re}c} - \phi_{v,Mix} \cdot C_{S^{2-},Mix} - sulphide\,in\,precipitation \quad (6.13)$$

Since there is no sulphide in the AMD feed, the sulphide concentration after the mixer and settler becomes 0.05 kg/m^3.

The metal concentrations in the incoming AMD are given in Table 6.3. The pH value of > 6.5 in the mixer will ensure the precipitation of all metals as well as manganese, except potassium and sodium. The precipitated metal sulphides will be removed in the settler. The metals precipitated as metal-sulphides and the concentrations thereof are presented in Table 6.3. The amount of sulphide needed to precipitate all metals can be calculated. This equals 142 248 kg/y. The amount of sulphide in the recycle stream equals 0.3 kg/m^3 X 730 000 m^3/y = 219 000 m^3/y, the recycle ratio is thus sufficient to deliver enough sulphide to precipitate all metals present in the AMD.

The minimum recycle ratio, only to precipitate the metals, can be calculated from the recycled sulphide mass-flow according to:

$$\phi_{mass,S^{2-},\mathrm{Re}c} = \phi_{v,\mathrm{Re}c} \cdot C_{S^{2-},\mathrm{Re}c} \quad (6.14)$$

The concentration of the sulphide (stream 11) equals 0.3 kg/m^3. The mass flow rate is the minimal amount of sulphide required for the precipitation of all the metals. This gives a minimum required recycle flow rate of 474 160 m^3/y, or a recycle ratio of 0.65:1.

Table 6.3. Amounts of removed metal sulphides

Metal sulphide	Metal sulfide effluent via stream 9 (kg/y)
CuS	44
Fe_2S_3	103 165
MnS	10 743
ZnS	2 175

6.2.4.4. The reactor

For the reactor the following influent and effluent streams can be conceptualised and will be discussed in the following sections.

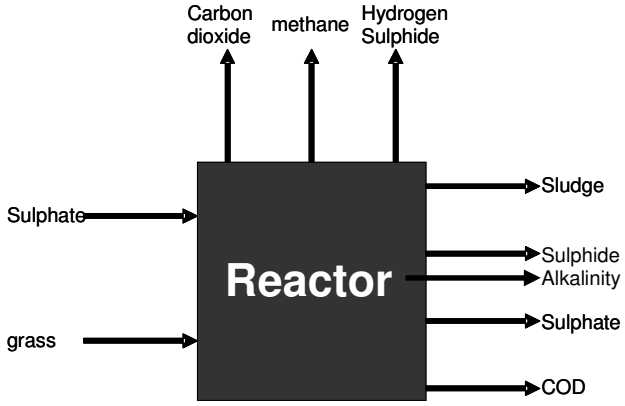

Figure 6.3. The reactor showing all in- and out-going streams.

The sulphate concentrations entering the reactor as well as the sulphide concentrations leaving the reactor were discussed in the previous sections, when the total mass balances were considered. The sulphate concentration entering the reactor is 1.25 kg/m^3 while the sulphide concentration entering the reactor is 0.05 kg/m^3. The effluent concentrations of sulphate and sulphide were 0.5 kg/m^3 and 0.3 kg/m^3, respectively. It was assumed that the hydrogen sulphide formed was dissolved (HS^- form), due to the relatively high reactor pH (pH>6.5). The mass balances in the reactor are derived by means of the following description:

- Firstly, the amount of waste was determined. This waste was presented in the form of COD of which the constituents were described in a later section

- Secondly, the growth of SRB was described, by determining the amount of COD (in the form of VFA) required for growth as well as for energy supply of SRB. The energy supply and growth are linked through microbial growth/yield relations i.e. the Yield ATP (Y_{ATP}) concept (ATP: Adenosine triphosphate).
- Thirdly, the VFA production (by rumen microorganisms) necessary for sulphate reduction was calculated. Subsequently the amount of grass/cellulose was calculated for growth, VFA production and for total sulphate reduction.

To estimate the amount of unutilised VFA, the theoretical values were compared with the experimental data and were used accordingly. In order to calculate biomass or sludge production, yields of bacteria growing on the substrates were required and thus theoretical ATP yields (for every mol of ATP produced 1 gram of biomass was produced) were used. The amount of ATP generated in SRB is 1 mol of ATP per mol sulphate reduced (Schlegel, 1993). Since 1 095 000 kg/y of sulphate was reduced an amount of 11 406 kmol of ATP was produced. Bauchop and Eldsen (1960) measured the amount of ATP required for the production of 1 g of biomass for several organisms. Their results showed an average yield of 10.5 g cells/mol ATP. This gives a biomass production of SRB of 120 000 kg/y. The SRB biomass is formed from COD, mainly consisting of acetate, butyrate and propionate obtained from the fermentation of cellulose by the rumen bacteria.

Biomass growth relations were calculated from the amount of each of the individual substrates (COD) needed for biomass production, namely 1 C-mol of biomass can on average be represented as $CH_{1.8}O_{0.5}N_{0.2}$ (Heijnen and Roels, 1979). Furthermore it was assumed that the N-source derived from grass can be represented as ammonium and that the nitrogen from grass is abundant. The following growth relations can then be derived:

$$a\,Substrate + b\,NH_4^+ + c\,H_2O + d\,HCO_3^- + e\,H^+ + 1CH_{1.8}O_{0.5}N_{0.2} = 0 \qquad (6.15)$$

For each equation there are 5 unknowns and 5 balances, namely 4 elemental balances and one charge balance. Solving these balances gives the following growth relations for the production of 1 C-mol of biomass with butyrate, propionate and acetate as substrates:

$$Acetate:$$
$$0.525\,CH_3COO^- + 0.2\,NH_4^+ + 0.275\,H^+ \rightarrow 0.4\,H_2O + 0.05\,HCO_3^- + CH_{1.8}O_{0.5}N_{0.2}$$
$$\text{Pr}opionate:$$
$$0.3\,C_2H_5COO^- + 0.2\,NH_4^+ + 0.1\,HCO_3^- + 0.2\,H^+ \rightarrow 0.4\,H_2O + CH_{1.8}O_{0.5}N_{0.2}$$
$$Butyrate:$$
$$0.21\,C_3H_7COO^- + 0.2\,NH_4^+ + 0.16\,HCO_3^- + 0.17\,H^+ \rightarrow 0.4\,H_2O + CH_{1.8}O_{0.5}N_{0.2}$$

(6.16-6.18)

The energy for growth of the SRB was provided by the oxidation of COD and hydrogen, produced by the rumen bacteria, with the simultaneous reduction of sulphate to sulphide. For butyrate, propionate, acetate and hydrogen this occurred according to the following reactions:

$$Hydrogen:$$
$$4\,H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\,H_2O$$
$$Butyrate:$$
$$2\,C_3H_7COO^- + SO_4^{2-} \rightarrow 4\,CH_3COO^- + HS^- + H^+$$
$$\text{Pr}opionate:$$
$$1\frac{1}{3}\,C_2H_5COO^- + SO_4^{2-} \rightarrow 1\frac{1}{3}\,CH_3COO^- + 1\frac{1}{3}\,HCO_3^- + HS^- + \frac{1}{3}\,H^+$$
$$Acetate:$$
$$CH_3COO^- + SO_4^{2-} \rightarrow 2\,HCO_3^- + HS^-$$

(6.19-6.22)

The growth relations and energy production reactions were linked through the ATP yield. Total growth relations can be derived which incorporated energy production and growth:

$$Acetate:$$

$$2.87\,CH_3COO^- + 0.2\,NH_4^+ + 0.28\,H^+ + 2.34\,SO_4^{2-} \rightarrow$$
$$0.4\,H_2O + 4.74\,HCO_3^- + 2.34\,HS^- + CH_{1.8}O_{0.5}N_{0.2}$$

$$\text{Pr}opionate:$$

$$3.42\,C_2H_5COO^- + 0.2\,NH_4^+ + 2.34\,SO_4^{2-} \rightarrow$$
$$0.4\,H_2O + 3.02\,HCO_3^- + 0.58\,H^+ + 2.34\,HS^- + 3.12\,CH_3COO^- + CH_{1.8}O_{0.5}N_{0.2}$$

$$Butyrate:$$

$$4.90\,C_3H_7COO^- + 0.2\,NH_4^+ + 0.16\,HCO_3^- + 2.34\,SO_4^{2-} \rightarrow$$
$$0.4\,H_2O + 2.17\,H^+ + 2.34\,HS^- + 9.36\,CH_3COO^- + CH_{1.8}O_{0.5}N_{0.2}$$

(6.23-6.25)

The SRB used hydrogen only as the energy source and needed carbon sources for cell growth, such as acetate or pyruvate, which were the main intermediates in biomass growth and energy reactions (Schlegel, 1993). Hydrogen was used by the acetogenic bacteria to produce acetate from H_2 and CO_2. The following growth relation with acetate and hydrogen were derived:

$$Hydrogen:$$

$$0.525\,CH_3COO^- + 0.2\,NH_4^+ + 2.62\,H^+ + 9.37\,H_2 + 2.34\,SO_4^{2-} \rightarrow$$
$$9.77\,H_2O + 0.05\,HCO_3^- + 2.34\,HS^- + CH_{1.8}O_{0.5}N_{0.2} \qquad 6.26$$

In order to establish the cell growth obtained from the utilization of the different VFA and hydrogen, it is necessary to know the distribution of the VFA and hydrogen produced by the rumen bacteria to calculate the total biomass yield on the total VFA. The hydrogen produced by the rumen bacteria inside the rumen is immediately used by MB, which produce methane from carbon dioxide and hydrogen. In the presence of SRB and sulphate, the MB were outcompeted by the SRB (Visser, 1995). In an optimized sulphate reducing reactor the hydrogen produced by rumen microbes was used by the SRB. The production of the various gases and VFAs by rumen bacteria occurred through the degradation of cellulose and hemicellulose in grass, which consisted mainly of hexoses (monomers, such as glucose). Hungate (1966) gives the following reaction for the degradation of hexoses by rumen bacteria:

$$58\,Hexose \rightarrow 62\,HAc + 22\,H\,\text{Pr} + 16\,HBut + 66\frac{1}{2}CO_2 + 33\frac{1}{2}CH_4 + 27\,H_2O \qquad 6.27$$

This equation was derived from the stoichiometric relationships in microbial pathways and energy conservation laws and agreed fairly well with experimental results (Hungate, 1966). The formation of methane was ruled out since sulphate and SRB were present in the culture. Experimental results showed that the average amount of carbon dioxide produced in the experimental reactor was about 84 %. Methane is produced according to:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (6.28)$$

Due to the presence of SRB and sulphate in the bioreactor, 4 mols of hydrogen were gained since instead of methane production, the hydrogen was utilised by the SRB as the energy source. The overall reaction for the degradation of cellulose:

$$58\,Hexose + 28\,H_2O \rightarrow 62\,HAc + 22\,H\,\mathrm{Pr} + 16\,HBut + 88\,CO_2 + 6\,CH_4 + 110\,H_2 \quad (6.29)$$

For each of the substrates (VFA and hydrogen) produced an amount of biomass was produced. On average, 30 % of the COD produced was not used in the reactor, based on experimental data. The COD values for 1 mol each of butyrate, propionate and acetate are 160, 112 and 64 g of oxygen, respectively. The VFA produced from cellulose is distributed in COD amounts as presented in Table 6.4 (Hungate, 1966).

Table 6.4. Distribution of the produced VFA represented as COD

VFA	Percentage COD
Butyrate	29
Propionate	27
Acetate	44

Visser (1995) reported that hydrogen was immediately used by the HSRB, which implied that rumen bacteria and SRB cultures lived in syntrophy: as soon as the hydrogen was produced, the SRB utilised it in the presence of SO_4. Furthermore a preference for propionate and butyrate before acetate was determined experimentally as the preferred substrate for SRB. Considering the, on average highest growth rate of SRB on propionate, this will be assumed to be the preferred substrate (Visser, 1995). It was assumed that the SRB initially consumed the hydrogen produced for energy in combination with acetate as the carbon source. Secondly the SRB used propionic acid followed by butyric acid. During the consumption of the C3 and C4 acids, acetate was formed which was the substrate used last in the sequence. Part of the waste COD/VFA consisted almost entirely of acetate, which was experimentally confirmed (Chapters 3-5).

The total growth and the cellulose conversion equations provided the yield of biomass on cellulose (or hexose). The usage of hydrogen, propionate and butyrate will give the following equations:

$$58\,Hexose + 4.28\,NH_4^+ + 50.16\,SO_4^{2-} \rightarrow$$
$$106.47\,CH_3COO^- + 50.16\,HS^- + 108.63\,H_2O + 1.43\,HCO_3^- + 21.43\,CH_{1.8}O_{0.5}N_{0.2}$$
$$+ 106.06\,CO_2 + 6\,CH_4 \qquad (6.30)$$

Note, that in the reaction representing the degradation of cellulose, acetate, butyrate and propionate are represented as un-dissociated acids, while in the above equation they were dissociated. Furthermore, the acid produced reacted with the produced carbonate according to:

$$H^+ + HCO_3^- \rightarrow H_2O + CO_2 \qquad (6.31)$$

Only the acetate produced by the rumen bacteria and SRB had to be incorporated into the process description. The total VFA concentration, expressed as COD, was produced by the rumen bacteria in degrading 58 hexose units to 8992 g O_2. A COD waste of 30 % was equivalent to 2698 g O_2. Thus an amount of 4116 g COD (as acetate) was used for energy production and growth. This equaled 64 mol of acetate. Thus, the total equation for the growth of SRB on VFA produced from cellulose by rumen bacteria, with a 30 % waste of COD becomes:

$$58\,Hexose + 8.77\,NH_4^+ + 102.61\,SO_4^{2-} \rightarrow 42.15\,CH_3COO^- + 102.61\,HS^- + 173.31\,H_2O + 51.94\,HCO_3^- + 43.85\,CH_{1.8}O_{0.5}N_{0.2} + 161.78\,CO_2 + 6\,CH_4 \quad (6.32)$$

According to Hungate (1966) the molar mass of hexoses in making up cellulose is reported to be 162 g/mol (dehydrated hexose). The yield from biomass on cellulose is thus 0.11 g biomass/g of cellulose, including loss of COD or acetate. Without loss the yield calculated would be 0.15 g biomass/g cellulose.

6.2.4.5 GC requirements based on the process description calculations

The cellulose or hexose was related to grass, in order to calculate the amount of grass required to sustain the total removal of sulphate. Sonakya (2003) reported the composition of fresh grass as presented in Table 6.5.

Table 6.5 Composition of fresh grass (Sonakya *et al.* 2003)

Compound	Percentage w/w
Water	51.84
Cellulose	14.00
Hemicelluse	28.30
Lignin	5.40
Ash	0.46

Only the cellulose and hemicellulose were digested by the rumen bacteria (Kalia *et al*. 2000). It is assumed that hemicellulose consisted of only hexoses as well. Thus 88 % m/w of the grass dry matter consisted of degradable hexoses. From the

equations derived above the amount of grass required for the reduction of sulphate and the growth of SRB was calculated:

For the reduction of 1 095 000 kg/y of sulphate and growth of SRB, 1.0 million kg of cellulose was needed, thus 2.5 million kg of fresh grass was required. Based on dry mass, an amount of 1.2 million kg of dry grass was needed for the reduction of sulphate and growth of SRB. The growth of rumen bacteria should however also be incorporated. This was also be done by means of ATP yield.

Bergen (1977) reported that rumen bacteria gain 2 mol of ATP for every mol of acetate produced and 3 mol of ATP for every mol of propionate or butyrate produced. The degradation of 1 mol of hexose (cellulose) thus provided 4.1 mols of ATP. The ATP yield of rumen bacteria was 16.5 g biomass/mol of ATP (Baldwin *et al*. 1970). The VFA and hydrogen produced for the reduction of 1.095 million kg of sulphate thus delivered 26 million mols of ATP to the rumen bacteria which resulted in 436 000 kg of rumen biomass produced per year. The hexoses in grass was represented as glucose minus water (hence the molecular weight of 162 g/mol). The following growth relation for rumen follows:

$$0.175\,C_6H_{10}O_5 + 0.2\,NH_4^+ \rightarrow 0.275\,H_2O + 0.05\,CO_2 + 0.2\,H^+ + CH_{1.8}O_{0.5}N_{0.2} \quad (6.33)$$

The yield of rumen-biomass on cellulose, without growth requirements was thus 0.87 g biomass/hexose. This implied that the amount of cellulose needed for the biomass growth of the rumen bacteria is 502 000 kg/y. The total amount of grass required for rumen bacterial growth is therefore 1.18 million kg/y of fresh grass or 572 000 kg/y, dry mass. The total amount of sludge produced is equivalent to the amounts of SRB, the amount of rumen bacteria and other debris produced. This provided a total sludge production of 556 000 kg/y.

The amount of grass required was for the reduction of sulphate and for the growth of the SRB and the rumen microbes. Thus the total amount of grass required was 3.65 million kg/y of fresh grass or 1.76 million kg/y dry mass (DM). This provided a removal efficiency of 0.62 g SO_4^{2-} removed per g of dry grass. The work of Mappledoram (1998) indicated that in an arid area of South Africa the yield of Kikuyu grass varied from 6-12 tonne DM per hectare, with a mean of 10.8 tonne DM/ha. In order to achieve this yield, the grass needed to be fertilised with 300 kg N/ha. In addition, phosphate and potassium were added on an annual basis to maintain a P

and K concentration in the soil of 20 and 120 mg/ℓ, respectively. When grass was grown under irrigation, the yield was higher at 15.3 tonnes DM/ha (Harris, 1990). Since the Kikuyu grass grown at the mine will be irrigated regularly with the mine water, the yield of 15.3 tonne DM/ha will be used for further calculations. In order to grow enough grass to provide a yield of 15.3 DM/ha, a surface area of 131 ha of grass land is needed, which translated to an area of e.g.1.14X1.14 km.

6.3 CONCLUSIONS

The calculations presented in this chapter provided a good understanding of the full technology description. It showed the implication of the various streams entering and leaving the reactor and the processes occurring in the reactor, e.g the COD utilization for the sulphate reduction as well as for the growth of the SRB and the rumen microbial population. From the technological process description it could be calculated that in order to treat AMD from a particular mine in the Witbank area, 15.3 tonnes DM/ha of grass cuttings was needed, which equated to a surface area of 1.14 x1.14 km of grassland. The presented calculations provided the basis for AMD treatment which contained 2000 mg/ℓ sulphate and metals (mainly 76 mg/ℓ iron, 10 mg/ℓ manganese and 2 mg/ℓ zinc) at a flow of 2 000 m^3/d. In the process description it was assumed that the treated water will have a sulphate concentration of 500 mg/ℓ and that all heavy metals will be removed. The residual metals such as calcium, magnesium and sodium can be removed with alternative treatment methods such as the desalination technology. Alternatively, the treated water can be used for irrigation or dust suppression at the mining site.

From the information in this and the previous chapters, it is evident that sulphate rich AMD can be treated biologically, using the degradation products of grass-cellulose as the carbon and energy sources. From the technology description, the required amount of grass was calculated for the operation of the process. For this technology to be economically feasible the costs associated with the cultivation and harvesting of the grass at the mining sites have to be taken inot account. The advantage of the biological sulphate removal technology is that the sulphate in AMD can be reduced to concentrations of 500 (and lower) mg/ℓ, that sulphide is produced to precipitate the metals in the mine effluent and that the alkalinity formed can increase the pH of the AMD.

From the above mentioned calculations, this system was compared to another biological sulphate removal process, which also operated on a waste product, e.g. primary sewage sludge as the carbon and energy source. This so called Rhodes BioSure plant, developed at Rhodes University (Rose, 2000), was commissioned recently at Grootvlei Gold Mine, with the aim to treat 10 Mℓ/day mine water, at a cost of R15 million. Grootvlei Mine is close to ERWAT's Ancor Waste water treatment works. The polluted mine water is piped by gravity to the Ancor sewage works into the BioSure plant. The treated water, after sulphide removal, is then directed to the Ancor sewage works for COD removal. Although the Rhodes Biosure Plant is an elegant biological sulphate removal technology, it has to be taken into account that not many mines are close enough to sewage plants to make this technology generally feasible. Another, similar plant is under consideration, but in that case the primary sewage sludge has to be transported by road to the mine, which is expensive and a potential health hazard. The BioSure Plant has taken 10 years to develop, by many scientists from different universities, mainly funded by the Water Research Commission (WRC) and Innovation Fund (IF).

At Anglo Coal's Navigation colliery, a biological pilot plant is currently in operation, which treats 3 Mℓ/d of AMD, with a sulphate concentration of 2.5 g/ℓ. This plant, constructed by Paques, The Netherlands, uses waste ethanol as the carbon and energy source, making it more cost effective than using technical grade ethanol. The sulphide produced is biologically oxidised to sulphur, using redox potential measurement to regulate the oxygen supply. The sulphur produced is contaminated with biomass, for which there is currently no application, except as soil improver. However, when this produced sulphur-sludge needs to be transported from the mining sites to areas where it is to be apllied to the soil, additional costs are incurred for transportation, placing the technology at an economic disadvantage. Although this technology is full-proof, the main disadvantage is the rising price of ethanol coupled to the oil price, which also affects the price of waste ethanol.

When comparing the biological sulphate removal technologies to the chemical sulphate reduction process, in which the mine water is neutralised either with lime or limestone or a combination of the two, to also remove the metals such as iron and manganese, the sulphate concentration can only be reduced to ≈ 1500 mg/ℓ, the solubility of gypsum ($CaSO_4.2H_2O$). Thus, although the water is neutralised, the sulphate concentration in some areas may still be a concern depending on the waste load allocation requirements of DWAF. After the water is neutralised, the biological

sulphate removal technology can form a second stage. The overall proces would then be considered an integrated mine water treatment system. Another chemical process with which to treat mine water is the barium process, in which BaS is added to the mine water, precipitating $BaSO_4$ and resulting in sulphate values lower than 200 mg/ℓ. The $BaSO_4$ can be roasted at ≈ 1500 ˚C to regenerate BaS, for re-use in the process. The diasadvantage of this technology is the heating of chemicals to a high temperature, which is costly in energy usage.

The neutralisation technology, which to recently employed lime, and which was replaced by limestone, took approximately 20 years to develop to the implementation stage. Since the early 2000s, several full-scale plants were constructed, of which the first one in Empangeni on the northern KZN coast. This plant is a combination of limestone and lime treatment. Two limestone plants have been erected in Witbank at the Navigation and Kromdraai Collieries. Further full scale implementation continues, both in South Africa (Northern Cape) as well as abroad (Botswana, Australia). Although this technology has found market acceptance, research is still continuing, aiming to improve on the challlenges of sludge recycle and settlement, to render the technology even more cost effective. It was shown that when limestone is replaced by lime, the operational costs were cut by approximately 40-50%.

At present, a physical treatment plant is being built by Anglo Coal, where the mine effluent from two Anglo mines and one Ingwe mine will be treated with Reverse Osmosis (RO) membrane technology. This will result in potable water and in a brine, containing metals and other salt residuals. This plant will treat 120 Mℓ/day underground mine water at a cost of R 300-million. Part of the costs incurred will be recovered by the sale of drinking water to the Emalahleni (Witbank) Municipality, which has a shortage of potable water. This treatment of mine water is very attractive from a water management point of view, since useless polluted water can be rendered potable for a most urgent need, such as for human consumption.

The passive treatment system, as developed by PHD Consulting over the past 10-15 years in conjunction with the Inovation Fund (IF) and the Water research Committee (WRC), is receiving recognition. A demonstration-scale, passive treatment ponding sytem will be constructed at one of the BHPBilliton mines, treating 200 m^3/day of AMD. The principle of this passive treatment system was explained in the literature review (Chapter 2).

Different treatment technologies are being implemented at different mines. Although the chemical compositions of most mine waters differ and mines are located in dissimilar areas, for most mine effluents, there is a treatment solution that has been developed in South Africa. This indicates that even though the AMD treatment research competition might initially seem to be superfluous, it has resulted in several, sound, treatment technologies, most of which have found applications.

The technology described in this thesis may, after further investigations, competes well with established technologies. To date (May 2007) only 3 years of research has been invested compared to 10-20 years with the above mentioned technologies. When comparing with other mentioned biological treatment methods, the advantage of using cellulose as the carbon source is that grass can in principle be grown anywhere, using sunlight as the energy source. Although primary sewage sludge will always be available, it may not always be available near a mining site, while plant biomass is sustainable, when partly treated mine water can be used for irrigation during the dry Souyh African winter months. The main observation of this study was the relationship between high COD concentration, and sulphate removal. Thus an increased sulphate load will result in an increased need for grass which may require a large reactor for the one stage operation. The other observation made from the study is that rumen microbes require increased temperatures for the fermentation of cellulose. Further research should be directed to investigating whether the more robust cellulose degraders from the rumen inoculum can adapt to ambient temperature.

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CHAPTER 7

CONCLUDING REMARKS ON BIOLOGICAL MINE WATER TREATMENT TECHNOLOGY USING THE DEGRADATION PRODUCTS OF CELLULOSE

7.1 INTRODUCTION

To comply to sound water management, pollution prevention of effluents is the key parameter. Mining as well as other industries produce large volumes of polluted waste water, which should be kept separate from clean water to prevent pollution of these clean water sources. In the situation that good housekeeping of water management is adhered to, water treatment can be applied. The studies presented in the previous chapters showed that the biological sulphate removal technology can be used to remove the salinity and acidity of mine water.

In order to list the results of this study and how these results can possibly be used for future mine water treatment, a summary table is presented, reviewing the aims and results from Chapters 3-6 (Table 7.1). The information summarised in Table 7.1 shows that the investigations described in the different chapters all have one common objective: the production and utilisation of VFA for biological sulphate removal. The outcomes of each study was dicussed separately in the following sections

7.1.1 Mine effluents as feed water for the biological reactor

The characteristics of mine effluents are high acidity (low pH), high sulphate and high metal concentrations. The high sulphate concentration in AMD can be treated biologically using SRB, an electron donor and sulphate as the electron acceptor, operating a sulphidogenic bioreactor. The results of the studies in the different chapters showed that biological sulphate removal was obtained when using either artificial, sulphate-rich, feed water or pretreated AMD, obtained from a closed mine as feed water. In order to increase the pH of the AMD prior to feeding it to the reactor, one part of mine water was mixed with one part of the effluent of the biological reactor. This mixing had several advantages, namely the neutralisation of the AMD acidity by the alkalinity present in the reactor efluent, the metals in the AMD were precipitated as metal sulphides by the sulphide present and the sulphate concentration of the AMD was diluted.

Table 7.1. Summary of aims and results of this study

Chapter	Aim	Results
3	1. VFA production from GC using microorganisms from: - Natural grass degradation - Anaerobic digester consortium (ADC) - SO_4 adapted SRB consortium 2. Can VFA produced by cellulose degraders be used for SO_4 removal	1. VFAs were produced: Increased C3 and C4 acid production (30 and 22%, respectively), when more natural grass degraders were added to reactor ADC produced VFA, resulting in SO_4 removal Highest C2, followed by C3 and C4 acids 2. Highest SO_4 removal using VFA produced by cellulose degrading SRB
4	1. VFA production using SRB versus RB as fermentation organisms 2. Will highest concentration GC result in highest SO_4 removal?	1. SRB and RB both produced VFA: RB produced higher concentration of propionic acid, especially when adding tryptone to reactor 2. Highest conc. of GC resulted in highest SO_4 removal, using C3 and C4 acids, producing C2 acid.
5	Operation of 2 stage reactor system using • Artificial Feed water • AMD as feed water Operation of 3 stage reactor system using Artificial Feed water	An average 86% SO_4 removal was achieved in FR and 20% in SR, using artificial feed water at HRT of 4 d and ½ d, in FR and SR, respectively 78% SO_4 removal was obtained using pre-treated AMD as feed water, at HRT of 2 d in FR. No noticeable SO_4 removal in SR Av. 86% SO_4 removal in FR, 20% in SR and negliable SO_4 removal in ASR at 4 d, 1/2 d and 1/3 d HRT, resptively. The results of this chapter showed that the technology is feasible as long as grass is added in relation to sulphate load
6	Technological description of technology, based on obtained and theoretical data	Technological description was developed: Technological description provided overview of processes and was used to indicate amount of grass needed for SO_4 reduction from specific AMD, removing SO_4 to DWAF standards

7.1.2 Sulphate removal efficiency

The results from the initial feasibility (batch operated) studies, investigating whether cellulose degradation products could serve as carbon and energy sources for the biological sulphate removal process, indicated that the set objectives were attainable. The results obtained from the initial batch tests formed the basis for the subsequent studies.

7.1.3 Reactor System

The results described in Chapter 5 showed that a 2 stage reactor system was suitable for the purpose of the treatment of synthetic feed water, when high concentrations of grass-cellulose were added to the first reactor. However, it was observed that not only the GC were fermented in the first reactor, but that the degradation products were already utilized for biological sulphate removal in the first reactor. The highest sulphate removal efficiency was achieved in the first reactor at 86% while an additional 20% removal was obtained in SR. When a third reactor was added, the results indicated that hardly any additional sulphate was removed in the third stage, which was ascribed to a low residual COD concentration in that reactor. Most of the COD concentration was already utilised in the first and second reactors. When diluted AMD was used as feed water, operating the two stage reactor system, it was again observed that little additional sulphate was removed in the second stage and that actually the highest sulphate removal was again achieved in the first reactor. This finding was un-expected, since the fermentation of organic matter usually requires a low pH (4-6), while the biological SO_4 removal occurs at a preferred pH of 7.5. The first reactor contained GC, rumen consortia, SRB and packing material (ceramic rings) for SRB biofilm formation.

The VFA and other intermediates produced by the rumen microorganisms were thus mainly utilised by the SRB in the first reactor. It was postulated that the SRB kept the hydrogen partial pressure low, there by stimulating the degrading bacteria to produce more hydrogen. This microbial interaction showed potential syntrophic and symbiotic interactions between the different microorganisms in the reactor.

The three stage system added no value to the technology as no additional sulphate removal was observed in the third stage. Since the highest sulphate removal was observed in FR, containing the immobilised SRB, GC and rumen fluid in one reactor, future work will concentrate on a one stage reactor system.

7.1.4 The use of rumen inoculum for the fermentation of cellulose

The rumen associated bacteria degraded the grass-cellulose to VFA and other degradation products, e.g. hydrogen, for SRB to use as the electron donor for biological sulphate removal. It was observed that the rumen bacteria produced mainly C2, followed by C3 and C4 acids. The obtained results showed the interactions among the different groups of microorganisms. It can be expected that the symbiotic relationship between groups of bacteria and other microorganisms in the reactor is similar to processes occurring in natural environments. When a good understanding of the biological processes occurring during fermentation and sulphate removal is acquired, this knowledge can be applied to harness and enhance the activities in a bioreactor. Understanding part of the complex processes in the bioreactors can enable fine-tuning of the technology.

The information obtained during the studies described in this thesis showed that cellulose was degradable by rumen microorganisms outside the ruminant, which provided some understanding of natural occurring biological processes now taking place in a created environment, such as the bioreactors. Microbes change and mutate continuously due to environmental conditions (e.g. chemicals) and therefore it is difficult to predict the exact metabolisms and mechanisms taking place in the described reactors. A better understanding of the processes can be attained by applying molecular techniques to the microbial populations in the reactor, such as for instance the terminal restriction fragment length polymorphism (t-RFLP) procedure. This is a tool for a rapid fingerprinting method, studying diversity, structure and dynamics of microbial communities.

The use of molecular studies/tools would enable the researchers to investigate the changes in the composition of the rumen fluid microorganisms. It can be well inmagined that when the rumen fluid is extracted from the fistualted animal and placed in a container, the population in the rumen fluid will change, since certain microbes cannot live outside the rumen of the ruminant. When the rumen fluid is transported from Pretoria University to the CSIR (under the required anaerobic and temperature conditions), further changes in the microbial population are anticipated. This transformation process will proceed and thus the composition of the rumen fluid will undergo changes continuously. When, in this thesis was referred to rumen consortia, it must be understood, that different compositions of the rumen microorganisms were used in the reactors. Furthermore, when GC were added to the

reactor other natural occurring microbes entered the reactor as well. The “un-known” consortium of rumen microorganisms was thus “contaminated” with other natural cellulose occurring microorganisms. Therefore, the rumen fluid microorganisms described for the different studies in the thesis most likely comprised a certain robust consortium of rumen microorganisms, mixed with anaerobic soil/grass microbes, responsible for cellulose degradation in the reactors. Although applying molecular techniques to the microbial populations in the reactors did not form part of this thesis, it is proposed that applying this tool will be incorporated in future research regarding the degradation of cellulose by rumen and grass obtained microorganisms.

7.1.5 The use of VFA and other fermentation products from biowaste product as energy sources for biological sulphate removal

SRB utilised the degradation products of cellulose as substrates for the biological sulphate removal. The preferred products were hydrogen, propionic acid and butyric acid in that order. Acetic acid is the product of propionate and butyrate degradation when the C3 and C4 acids were oxidized as electron donors for the biological sulphate reduction. The results showed, that in some instances, acetate was utilised in the sulphate removal process. It was speculated that other groups of bacteria (e.g. homoacetogenic bacteria) produced butyrate from 2 mols of acetate so that butyrate was available for the SRB. It was furthermore hypothesized that as soon as the RB produced VFA and hydrogen, these products were utilized in the sulphate removal process, which explained why the highest sulphate removal was always achieved in the first reactor.

7.1.6 Process description for the bioreactors

A process description was compiled on the basis of the chemical composition of the mine water, the volume of mine water to be treated and the results obtained from FR, when diluted mine water was used as feed water. In order to create a representative account of the biological sulphate removal technology using cellulose degradation products as the carbon and energy sources, several parameters were considered, such as the target sulphate concentration required in the treated water, the metal concentration in the AMD and the amount of sulphide used for the precipitation of the metals as well as the residual COD concentration in the treated water. Other factors included were the expected growth-rates of the sulphate reducing and rumen biomass, since part of the available COD in the reactor was used for sustained cell growth. The cellulose concentration needed had to be translated into the amount of

GC required. This necessitated an estimate of the cultivated areas to grow grass for the process to be sustainable. The technological process description was developed on the basis of treating a known mine water with a specific chemical composition.

7.1.7 The sociological and economic implications

In the previous section the amount of feed grass required by the technology was calculated on the basis of the outcomes of the continuous studies in Chpater 5. If the technology described in this thesis is feasible and be brought into operation at a coal mine, it would result in social and economic advantages. The reactor system would be erected near a dam containing mine effluent. Mining companies grow grass on the premesis surrounding the mining operations. The cultivation and harvesting of grass would provide employement for labourers, who would cut and mill the GC and make it available to the biological plant. The grass can be irrigated with mine water and fertilised with the waste sludge produced in the reactor(s), depending on the concentration of heavy metals. The provision of jobs is crucial for the social upliftment of communities and the project could develop in an SMME.

The technology described is likely to be a more economical option compared to other treatment technologies. An overview of the total process is presented in Figure 7.1. The principle of the technology presented in this thesis could also find application in the passive treatment of mine water, which would result in the treatment of smaller volumes. The process, however, is aimed at active treatment. The volume of mine water treated is dependant on the amount of grass that can be grown in the immediate vicinity of the mine.

7.1.8 Limitations of the presentated study

During the investigations described in the different chapters, limitations of the technology presented themselves. As already indicated the rumen fluid microbial population was mixed with natural occurring microorganisms attached to GC. This implied that the reactors comprised mixtures of microbes that most likely were never the same, although it can be hypothesized that a consortium of robust microorganisms populated the reactors. The main aim of this study concentrated on the possible use of a potential bio-waste product rather than adding that product to landfill or other disposal facilities. For this purpose GC were degraded and the degradation products were tested for a possible more cost effective mine water treatment option. Although the GC used for the investigations were collected from a stockpile of GC, stored in the cold room, differences in the composition of GC might have occurred. It is unavoidable that small parts of leaves sometimes entered the

reactors. When this study will result in a pilot scale unit near a mining operation, it can be envisaged that the added GC will most likely also change in compostion.

Since the outcome of a study could not be predicted, the results of a certain investigation were often the reason from diverting from an original study plan to further explore the reason why promising results from the initial study were obtained. During all investigations, the practical implications of applying the technology to mine effluents had to be kept in mind. A one stage reactor system is a more cost effective way of operation than a two or three stage reactor system.

The presented study is a good example of the application of environmental microbiology to the treatment of polluted waste water.

7.1.9 Recommendations for future studies

Heating mine effluents (2Mℓ/d) to 37-39 ℃ is not feasible, due to high costs. Therefore, future studies will concentrate operating at decreasing temperatures and even at ambient temperature. It is enviseaged that certain microbes from the rumen fluid can adapt to ambient temperature, since certain researches obtain degradation of cellulosic material using mature cow manure. During future studies molecular tools will be applied to microbial populations in the reactors.

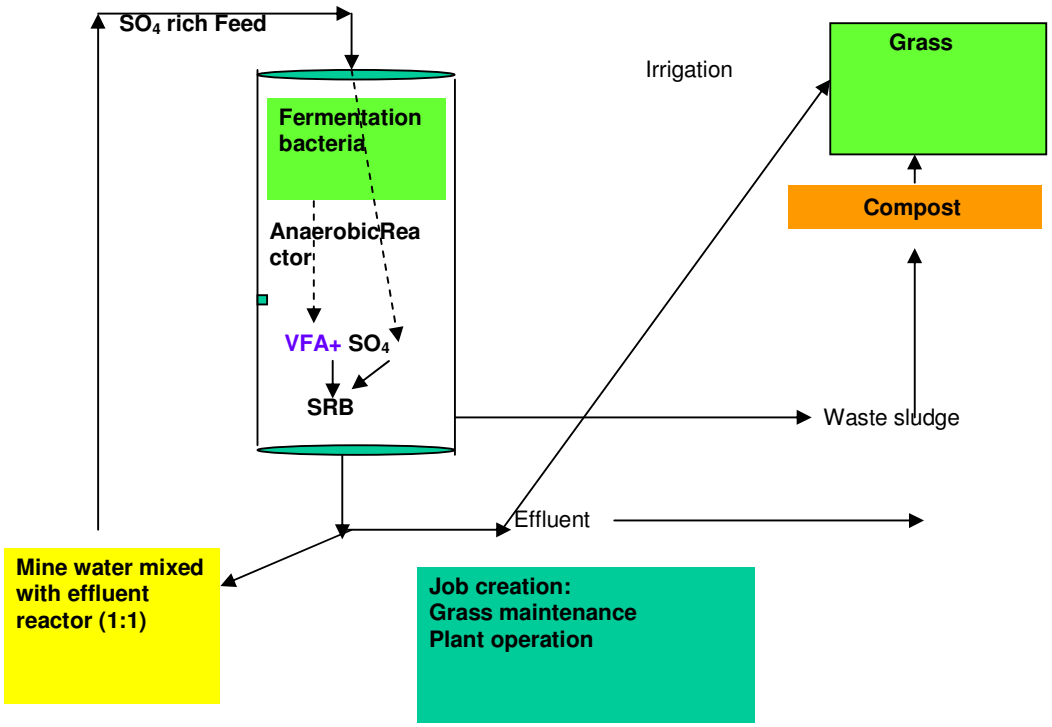

Figure 7.1. Grass cultivation and utilization for biological sulphate removal

7.2 CONCLUSIONS OF RESEARCH STUDY

The following conclusions were made from the studies as reported in this thesis:

- Naturally occurring microorganisms attached to grass produced acetic acid- (maximum of 600 mg/ℓ) propionic acid- (400 mg/ℓ) and butyric acid (160 mg/ℓ)
- Sludges, originating from an anaerobic digester (AD) and from a sulphidogenic pilot scale reactor (SRB) resulted in VFA production, mainly butyrate and acetate. SRB assisted in the degration of polymers and monomers.
- When the produced VFAs (by AD and SRB sludges) were used in a sulphate removal reactor, sulphate reduction was obtained
- The sulphate removal rate using the VFA produced by SRB in the biological sulphate removal process was slightly higher than when using sucrose as the carbon source in the control reactor.
- Grass-cellulose was fermented to VFAs, which subsequently were used in the biological sulphate removing reactor as electron donor.
- Rumen bacteria fermented grass-cellulose in higher propionic acid concentrations than SRB
- Adding tryptone to the reactor resulted in increased concentration of propionic acid compared to not adding tryptone. However adding tryptone to the process will add to the operational costs in full scale operation
- The propionic acid produced was used as the carbon and energy source for the biological sulphate removal.
- Part of the rumen microorganism consortia were sulphate reducers
- When 30, 60 and 90 g grass/ℓ were fermented by SRB, the fastest sulphate removal occurred in the reactor containing the highest grass concentration. This result showed a clear relationship between the cellulose concentration, the COD/VFA produced and the sulphate removal.
- Not all produced VFA was used for sulphate removal in the reactor with the highest grass concentration, thus grass and sulphate should be added proportionally to the reactor for the most efficient technology.
- Total sulphate removal was achieved in batch reactors, when using rumen fluid micro organisms as the fermentation bacteria and the SRB as the sulphate removers in one reactor,
- When 4x150 g grass cuttings were added to the fermentation/sulphate removing reactor (FR), an average of 86 % sulphate removal efficiency was

observed, during an experimental period of 77 days, using synthetic sulphate rich feed water.

- When pretreated AMD was used as feed water for the same reactor configuration the highest sulphate removal efficiency was 78%.
- The technological description, developed on the basis of the described process indicated that the amount of grass needed to remove 1.5 g SO_4/ℓ at a flow of 2000 m^3/day.
- Bacteria obtained from the rumen fluid from ruminants operated efficiently at 36-39 ℃
- Applying the described technology will be expensive when mine effluents functioning as feed water need to be heated prior to biological treatment to 36-39 ℃, to accommodate the rumen incocula.
- The described SO_4 removal technology can most likely compete with other South African developed SO_4 removal technologies, after showing that rumen microbes can adapt to lower operating temperatures degrading cellulose.

APPENDIX A

When the SRB use propionate and butyrate for the respiration to reduce sulphate to sulphide, reactions (1) and (2) can be applied:

$Propionate^- + ¾\ SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + ¾\ HS^- + ¼\ H^+$ (1)

$Butyrate^- + ½\ SO_4^{2-} \rightarrow 2\ Acetate^- + ½\ HS^- + ½\ H^+$ (2)

Table: The theoretical ratio between the $VFA/COD_{utilized}/SO_4/_{removed}$

Acetate

$CH_3COOH + 2O_2 \longrightarrow 2CO_2+2H_2O$

60 g acetate needs 64 g oxygen for total oxidation

60 g acetate provides 64 g COD

1 g acetate provides 64/60 = 1.07 g COD

1 mol acetate (60 g) to reduce 1 mol of sulphate (96 g)

per g reduced SO4 needed 60/96*1.07 = 0.67 g COD

Propionate CH_3CH_2COOH +1.75 SO_4

74 g prop needed to reduce 168 g SO_4

CH_3CH_2COOH +3 1/2 $O_2 \longrightarrow 3\ CO_2 + 3H_2O$

74 g prop provides (3.5*32)112 g COD

1 g prop provides 112/74 = 1.51 g COD

1 mol propionate(74g) to reduce 1.75(168 g) mol SO_4

per g reduced SO_4 needed 74/168*1.51= 0.67 g COD

Butyrate $CH_3CH_2CH_2COOH$ +2.5 SO_4

1 mol butyrate to reduce 2.5(240 g) mol SO_4

88 g butyric needed to reduce 240 g SO_4

CH_3CH_2COOH +5 $O_2 \longrightarrow 4\ CO_2 + 4H_2O$

88 g butyric provides (5*32)160 gr COD

1 g butyric provides 160/88 = 1.8 g COD

1 mol butyrate(88g) to reduce 2.5(240 g) mol SO_4

per g reduced SO4 needed 88/240*1.8= 0.66 g COD