CELLULOSE FERMENTATION PRODUCTS AS AN ENERGY SOURCE FOR BIOLOGICAL SULPHATE REDUCTION OF ACID MINE DRAINAGE TYPE WASTEWATERS

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

INTRODUCTION

Large volumes of acid mine drainage (AMD) are generated from the mining operations as well as from waste rock piles and from tailing dams. This water usually contains high concentrations of acid, salts and metals, resulting in a low pH and often corrosive wastewater, which, because of its polluting properties, needs to be treated before releasing it in rivers and dams. The study described in this report presents an active treatment method, which, with a few alterations, can possibly also be applied for passive treatment options.

OBJECTIVES

The first objective of this study was to investigate whether the microorganisms from rumen fluid (RI) could be adapted to ambient temperature (22-25°C), with the aim to degrade grass-cellulose (GC) to produce volatile fatty acids (VFA) and other intermediates, which could be used as the carbon and energy sources for the biological sulphate removal technology. The second objective was to investigate the effect of autoclaved and non-autoclaved grass on the degradation of GC on the biological sulphate removal as well as the effect on the diversity, structure and function of the microbial communities in sulphate removing bioreactors using t-RFLPs as a functional microbial analyses tool.

LITERATURE REVIEW

The literature review provides a reflection of the AMD generated from the different activities relating to mining as well as the composition thereof. It is clear that most mine water seepage, plumes and discard water need to be treated before it can be released in the environment due to its acidity, salinity and metal content. Different mine water technologies do exist and are applied, both nationally and internationally. The main chemical treatment method described is the neutralisation technology, both in ponds as in full scale reactors, while the emphasis using physical methods is presently concentrated on Reverse Osmosis, which technology is at the moment applied to mine water originating from different mines, located in the Witbank coal mining area of South Africa, operated by different mining houses. It is envisaged that once the plant is in operation, the treated water will be used as potable water.

Several active biological treatment systems are presently being investigated at pilot scale in South Africa, e.g. the Paques system at Navigation Mine and the Biosure

system at Erwat (Springs), while a passive treatment system is under investigation at one of the BHPBilliton mines. The main advantage of the biological sulphate removal technology is that the low pH of the mine water will increase in the treated water, due to the oxidation of the carbon source(s) and to the subsequent alkalinity production, that the metals from the mine water will be removed due to metal sulphide precipitation, since sulphide is the reduction product of sulphate. Furthermore, the sulphate concentration can be reduced to values < 500-200 mg/ℓ, as stipulated by the Department of Water Affairs (DWAF). Biological sulphate reduction only needs the addition of a carbon and energy source, which should be cost effective, such as grass-cellulose.

Cellulose degradation to polysaccharides and monosaccharides and to ultimately VFA by natural occurring microorganisms from the rumen of ruminants has been described. It was shown that biological sulphate removal can be achieved using the produced degradation products after hydrolyses and fermentation of cellulose and hemicellulose. Different reactors systems have been applied for the treatment of sulphate rich mine water, e.g. Upflow Anaerobic Sludge Blanket, Anaerobic Filter Reactors and Fluidized Bed Reactors.

OUTCOME OF INVESTIGATIONS

The overall results from the investigations described in the report showed that grass cuttings can be degraded by rumen microbial flora to organic material, such as VFA, which can be used as the carbon and energy sources for the biological sulphate reduction technology. The findings showed that the rumen microbes, outside the rumen of ruminants, could be adapted to ferment grass cellulose at 25°C which resulted in high sulphate reduction rates at this decreased operational temperature. This outcome will make the technology more cost effective for implementation at mining sites.

Different studies were conducted with the aim to obtain a better understanding of the biological processes when RI and sulphate reducing bacteria (SRB) were added to tap water and to synthetic sulphate rich water. The results obtained after supplementation reactors containing tap water with RI suggested that an increased COD and VFA concentration was achieved compared to the reactor which did not receive additional microorganisms. This result indicated that the RI seemed responsible for active degradation of GC. When tap water was replaced by sulphate rich feed water, and when at first the reactor was supplemented with RI and later with

SRB, increased sulphate removal rates were achieved. However, the results seemed to indicate that supplementing the reactors with RI and SRB had a more significant effect on the sulphate reduction then when the reactor was supplemented with only RI. The findings of these batch studies were applied to a larger scale continuous stirred reactor (10 ℓ) (first at batch scale and later continuously operated), however the results of those investigations showed a high COD concentration and low VFA concentrations, which resulted in a low sulphate removal efficiency. This outcome could possibly be ascribed to the stirring of the reactor contents, which may negatively affect the RI adhering to the grass cuttings. Optimal results, however, were achieved, when operating a hybrid reactor, which contained the SRB at the bottom of the reactor, immobilised on ceramic rings, where a thick SRB biofilm was formed. The top part of the reactor contained the grass-cellulose, mixed with RI. When subjecting grass pieces from the reactor to light microscopy, it seemed that the RI used the grass pieces as immobilisation material to form a biofilm. This hybrid reactor was at first fed with sulphate rich synthetic feed water and later with pre-treated mine water. The hydraulic retention time (HRT) in both instances was 2.4 days. During the experimental period of 100 days feeding synthetic water, an average percentage sulphate removal efficiency of 82% was achieved. This efficiency increased to an average of 92% when pre-treated mine water was fed to the reactor. The pre-treated mine water consisted of 1 part AMD from a coal mine in the Witbank area and 1 part effluent originating from the biological sulphate reducing reactor system. The advantage of using pre-treated AMD rather than raw AMD is that the metals in the AMD are precipitated with the sulphide in the treated reactor water before entering the reactor and that the low pH of the AMD will be increased due to the alkalinity concentration in the treated reactor water. The experimental period during this part of the study was 33 days. Subsequent studies have shown the repeatability of this work over longer periods (not shown in this report).

When investigating the effect of autoclaved and non-autoclaved grass on the degradation of grass cellulose it was determined that GC need not be autoclaved prior to adding GC to a sulphidogenic bioreactor for the purpose of cellulose degradation to provide the energy sources for biological sulphate removal. The population in the reactor using the autoclaved GC was not subjected to competition from additional microbes added with grass supplementation and reduced sulphate efficiently under ideal conditions, namely when sufficient GC substrate was available. The native grass microorganisms did not contribute vastly to GC degradation, but in addition did not hinder the VFA formation by the rumen associated bacteria.

The molecular studies provided insight into dynamics of sulphidogenic bioreactors. In general, when the microbial diversity decreased, sulphate removal efficiency decreased, which verifies that microbial diversity is related to optimal ecosystem functionality. Community composition in both the autoclaved and non-autoclaved reactors comprised of a relatively low diversity of SRB, while high sulphate reduction rates were achieved. Similar observations were made by Rose et al. in their sulphate removing studies (personal communication P. Rose, May 2008). The majority of possible genera appeared to be cellulose degraders and fermentative microorganisms. This result emphasised the critical roles that other microbial members, such as cellulose degraders play in the full cellulose to sulphate removal process.

CONCLUSION

The results of the batch operated studies showed that the degradation products of non-autoclaved GC and RI can function as the carbon sources for biological sulphate removal, feeding both sulphate rich synthetic and pre-treated mine water at 25°C. When operating continuously run reactor systems, it was observed that remarkably improved sulphate removal rates were achieved when operating a hybrid reactor system as compared to the completely mixed reactor operation. High sulphate removal efficiencies of 82 and 92%, respectively, were achieved treating synthetic feed water and pre-treated acid mine water, obtained from a closed mine in the coal mining district from the Witbank area. The microbial studies showed a diverse population of cellulose degrading microorganisms in both reactors. The bacterial community structure was dynamic and varied constantly in the non-autoclaved reactor, while remaining fairly stable in the autoclaved reactor system. Although an efficient sulphate reducing capacity in the reactors was observed, a relative low diversity of SRB was detected.

With the findings that the rumen microbial population can be adapted to 25°C fermenting grass cellulose to VFA and other intermediates as carbon sources for the biological sulphate removal this technology is ready to be tested at pilot scale. Operating a pilot scale reactor at 25°C rather than at 37-39°C is a far more attractive and cost effective option for the mining industry to contemplate.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AB	Acetogenic Bacteria
AF	Anaerobic Filter
AMD	Acid Mine Drainage
bp	Base-pair
C2 Acid	Acetic Acid
C3 Acid	Propionic Acid
C4 Acid	Butyric Acid
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
FB	Fluidized Bed
g	Gram
GC	Grass-cellulose
H_2	Hydrogen
HFS	Hybrid Fermentation Sulphate reactor system
HMB	Hydrogen Utilising Methanogenic Bacteria
HSRB	Hydrogen Utilising Sulphate Reducing Bacteria
IF	Innovation Fund
Kb	Kilobase
ℓ	Litre
MB	Methanogenic Bacteria
PAGE	Polyacrylamide Gel Electrophoresis
PAT	Phylogenetic Assignment Tool
PCR	Polymerase Chain Reaction
PS	Primary Sludge
rDNA	Ribosomal DNA
RI	Rumen (fluid) Inoculum
RO	Reversed Osmosis
SDS	Sodium Dodecyl Sulphate
SOB	Sulphide Oxidising Bacteria
Spp	Species
SRB	Sulphate Reducing Bacteria
SRBR	Sulphate reducing Bio Reactors
t-RF	Terminal Restriction fragment

t-RFLP	Terminal Restriction Fragment Length Polymorphism
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WRC	Water Research Commission

CELLULOSE FERMENTATION PRODUCTS AS ENERGY SOURCE FOR BIOLOGICAL SULPHATE REDUCTION

BACKGROUND TO WRC REPORT K5/1728

Cellulose degradation with the aim to produce volatile fatty acids (VFA) and other intermediates for the removal of sulphate from acid mine drainage water was studied at the CSIR from February 2004 to March 2007. The project was funded by 1) BioPAD, which received money from the Department of Science and Technology (2004-2006) and 2) CSIR Parliamentary Grant (April 2004-March 2008).

Motivation for K5/1728

The mining industry has a marked impact on water pollution in South Africa, producing vast volumes of mine effluent being produced every year. For instance, at least 200 ML/day of mining effluent is discharged annually into the water bodies of the Gauteng region, which accounts for sulphate loads of approximately 73 000 tonnes/annum, while this contribution is estimated to be 12 000 tonnes/annum in Mpumalanga. Mine water in the order of 50 ML/d is discharged in the Upper Olifants River Catchment in Mpumalanga (upstream of Loskop Dam), resulting in local acidification and regional salination of surface water resources (Maree et al., 2004). Globally, researchers are concentrating on finding a solution for the treatment of the different mine waters. Two biological treatment processes are in operation in South Africa, namely the Paques demonstration plant at Navigation, Witbank (Anglo Coal), which uses waste ethanol as the carbon and energy source and the Rhodes BioSure process, which is in operation at Grootvlei mine (Rose, 2000; Joubert, 2005). This plant uses primary sludge (PS) as the carbon and energy source. Prior to the Paques technology, the CSIRosure demonstration plant was in operation at Navigation Colliery, removing high sulphate concentrations, however the total sulphide produced resulted in higher concentrations than could be precipitated by the metals present in the mine water (Maree *et al*., 1997). The excess sulphide was biologically oxidised by sulphide oxidising bacteria (SOB), resulting in a sulphur film on the top of the clarifier.

An alternative biological sulphate removal process has been developed at the CSIR over the past four years, using the degradation products of plant biomass as the carbon source (Greben *et al*., 2007). The outcome of the study has shown that VFA, when using grass-cellulose (GC) as the plant biomass, can be produced. The produced VFA can subsequently be used as the carbon and energy source for the

biological sulphate removal process. Natural occurring fermentation microorganisms, sourced from rumen fluid were used to degrade GC to VFA and other intermediates as energy source for the sulphate reducing bacteria (SRB) to biologically reduce sulphate to sulphide.

Main outcomes of previous research studies

Previous results showed that when grass was added to a continuous operated reactor, fed with synthetic sulphate rich water and with pre-treated acid rich mine effluent, the sulphate was reduced and the acidity neutralised, due to the alkalinity produced. Continuous sulphate reduction was observed from concentrations of 1 500- 3 000 mg/ℓ SO_4 to values < 200 mg/ℓ SO_4, at a reactor temperature of 37°C and at a pH of 6.6-6.9. It was also shown that fresh grass needed to be added regularly to maintain a high reactor chemical oxygen demand (COD) concentration.

Plant biomass is a sustainable source of energy, depending on sunlight, which is abundantly available in South Africa. Moreover, grass can be cultivated by irrigating it with partially treated mine water (Jovanovich *et al*., 1998, 2002). The novelty of this way of mine water treating is that grass fermentation and sulphate reduction occur in one reactor, that grass can be grown at every mining site, thus omitting transport costs for the carbon and energy source, which will result in a cost effective manner of treating mine effluents.

The rumen microbes fermented cellulose/hemicellulose from GC to VFA and other intermediates and to hydrogen, which was utilised by the SRB to reduce sulphate from synthetic feed water and from pre-treated acid mine drainage (AMD). The disadvantage of the proven technology, however, was that the rumen fluid inoculum (RI) and SRB microbes operated at mesophilic temperatures. It can be imagined that heating vast volumes of mine water (e.g. 2-10 Mℓ/d) would make the technology very expensive and thus not feasible. It was therefore proposed for the studies conducted in 2006/07 to decrease the operating temperature step wise by 1°C each time. The results of that study indicated that the to 39°C adapted biomass was able to produce and utilise VFA till 35°C, but that the sulphate reduction efficiency at 34 and 33°C decreased to 70 and 57%, respectively, while this was 95% at 39°C (Table 1.1).

It was furthermore observed from the study in 2006/07 that when the reactors were operated at room temperature, VFA production and utilisation resulted in biological sulphate removal. To investigate whether these results were reproducible Water

research Commission (WRC) funding was granted for the 2007-08 financial year. Studies were conducted to establish whether particular microorganisms obtained from rumen fluid can adapt to converting plant biomass to VFA at ambient temperatures. Molecular studies will be conducted to investigate whether a shift in the rumen population could be observed during and after the experiments.

Table 1.1. SO_4 removal rates during decreasing temperatures

Temperatures in °C	SO_4 removed g/d	SO_4 removal rate g SO_4/(ℓ.d)	% SO_4 removal	Amount of grass needed to remove 1g SO4
39	17.5	0.88	95	2.00
38	14.35	0.72	68	3.98
37	15.85	0.79	94	5.36
36	15.50	0.76	66	4.71
35	18.96	0.95	83	3.30
34	12.45	0.62	70	4.82
33	10.44	0.52	57	6.46

REFERENCES

Greben, H.A., Baloyi, L.M. and Venter, S.N. (2007). Grass cellulose as cost effective energy source for biological sulphate removal. Water SA, Vol 33, No 5.

Joubert, J. (2005). Acid mine drainage: Sludge as an electron donor and carbon source in a biological sulphate reducing processsludge as an electron donor and carbon source in a biological sulphate reducing process. *Proc. Management of Residues Emanating from Water and Waste Water treatment.* Sandton Convention Centre, Johannesburg, South Africa, 9-12 August, 2005.

Jovanovic, N.Z., Barnard, R.O., Rethman, N.F.G and Annandale, J.G. (1998). Crops can be irrigated with lime-treated acid mine drainage. *Water SA.* **24** (2):113-123.

Jovanovic, N.Z., Annandale, J.G., Claassens, A.S., Lorentz, S.A., Tanner P.D., Aiken, M.E. and Hodgson, F.D.I. (2002) Commercial production of crops irrigated with gypsiferous mine water. *Water SA.* **28** (4): 413-423.

Maree, J.P., Dill, S., Van Tonder, D., Greben, H.A., Engelbrecht, C., Kehlenbeck, M., Bester, C., Adlem, C., Strydom, W., and de Beer, M. (1997). Removal of Nitrate, Ammonia and Sulphate from AECI Effluent*. Internal CSIR report*: ENV/P/C 97141/1.

Maree, J.P, Hlabela, P., Nengovhela, A.J., Geldenhuys, A.J., Mbhele, N., Nevhulaudzi, T. and Waanders, F.B. (2004) Treatment of mine water for sulphate and metal removal using barium sulphide. Mine Water and the Environment, 23(4), pp. 195-203.

Rose, P.D. (2000). The Rhodes Biosure Process: The piloting of an active process for the treatment of acid mine drainage wastewaters. *Proceeding Y2K Millennium Meeting*. Grahamstown 23-28 January, 2000: 605-606.

CHAPTER 1

INTRODUCTION

1.1 MINE EFFLUENTS, THE ENVIRONMENT, TREATMENT

AMD is generated from the mining operations as well as from waste rock piles and from tailing dams. This water usually contains high concentrations of acid, salts and metals, resulting in a low pH and often corrosive waste water, which when released in receiving water will negatively affect the aquatic life.

Water has been identified as South Africa's most limited natural resource. Due to the growing population, its upliftment and urbanisation, 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 indicate that the demands for water in each sector of the economy will increase (Ashton and Haasbroek, 2002).

Water management efforts should strongly focus on the re-use of industrial and mining effluents, since re-use of these effluents may 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. AMD from coal mines in the Witbank area (South Africa) is treated with reverse osmosis (RO) membrane technology, resulting in potable water for the Emalahleni (Witbank) Municipality, which has a shortage of drinking water (Günther, 2006). This way of treating mine water is very attractive from a water management point of view, since low quality polluted mine effluents, can be rendered potable for human consumption. In 2007, Rose *et al*., showed that the treated mine water generated at Grootvlei mine has the potential to be used for vegetable cultivation by means of hydroponic systems to be operated by the populations of informal settlements in the vicinity of Grootvlei mine. This endeavour could result in poverty alleviation for the mainly unemployed residents from these (ex) mining communities.

There is a wide range of conventional treatment methods for mine water effluents due to the variety of mine waters encountered in nature (Younger *et al*., 2002). Mine water treatment can be achieved through active, semi-passive or passive methods.

There are a variety of biotic and abiotic processes available. Passive and semi-passive treatment systems are often used, where mining has stopped but where mine water is still generated (Pulles, 2000). Active treatment processes are mainly used at mines, which are still in operation (Skousen, 1998, Brown *et al*., 2002). The preference for passive or semi passive treatment at closed mines is economically driven, since active mine water treatment is far more expensive.

Active treatment is the improvement of water quality by methods, which require ongoing inputs of artificial energy and/or (bio) chemical reagents (Younger *et al*., 2002), and refers to physical, chemical and biological systems, e.g. RO, neutralisation using an alkali and precipitation, using organic polymers. Usually the quality of the polluted mine water, the costs associated with the treatment and the demand for the potential re-use of the treated water all contribute to the chosen treatment option. Mine effluents can be neutralised by applying limestone (Maree *et al*., 2003) and/or lime, however the residual sulphate in the form of gypsum ($CaSO_4$) is dependent on the solubility of gypsum, which is typically about 1 500 mg/ℓ as sulphate (SO_4). For removal of SO_4 to below 1 500 mg/ℓ, the biological sulphate reduction technology can be applied (Greben *et al*., 2000). In order to achieve biological sulphate reduction, anaerobic conditions, favoured by SRB and the presence of suitable carbon and energy sources, have to be adhered to. Successful SO_4 reduction is typically associated with a pH increase due to alkalinity production. Therefore, the biological sulphate reduction technology is particularly beneficial to industries experiencing AMD problems, as it results in the removal of sulphate, an increase in the pH of the treated water and often in the removal of metals as a result of the formation of sulphides, followed by metal precipitation as metal-sulphides.

To avoid incurring high treatment costs, the idea of an integrated treatment system was conceived, in which initially the high acidity and sulphate load is treated chemically with limestone until the water pH is neutral and the sulphate concentration is reduced to approximately 1 500 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).

Biological sulphate reduction is carried out by SRB, which use organic carbon sources (for biomass) and sulphate as electron acceptors for their metabolism. In active treatment system, bulk solvents, such as ethanol (De Smul *et al*., 1997) and

methanol (the latter at increased operating temperature) (Weijma, 2000) were used. Hydrogen in the combination with carbon-dioxide was used successfully in laboratory studies (Du Preez *et al*., 1992, Van Houten, 1996, Eloff *et al*., 2004). In passive or semi-passive treatment systems, the carbon source can be any carbon material, e.g. manure, sawdust, decaying roots of wetland plants or compost, grass or biosolids (e.g. primary sludge). The organic layers in an anaerobic biofilter have the advantage that they retain metal sulphides (Higgins and Hard, 2003).

The advantage of using plant biomass as the carbon and energy source for the treatment of SO_4 and metal rich mine effluents is the relative low costs of either waste plant biomass or of cultivated biomass, e.g. grass (Greben *et al*., 2007). Most land adjacent to the mining operations is used to grow grass for either grazing or to harvest hay, with the aim to sell it to interested farmers. Grass contains cellulose, hemi-cellulose and lignin, of which the first two components can be degraded to polymers, monomers and to VFA and other intermediates, sources of carbon for biological sulphate removal. Naturally occurring cellulose degrading microorganisms inhabit the rumen of ruminants. The fermentation of cellulosic material is a natural process, since cellulose is the major constituent of plant biomass, forming an important link in the carbon cycle. The carbon cycle is closed as a result of cellulose utilising microorganisms present in soil and in the gut of animals (Lynd *et al*., 2002).

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 carbon dioxide and water, while only 10% is converted by anaerobic microorganisms producing methane and carbon dioxide.

The aim of this study is to investigate whether sulphate removal in synthetic feed water and in AMD (as feed water) can be achieved, using the degradation products of grass-cellulose as the carbon and energy sources for the biological sulphate reduction process, operating at room temperature, utilising microorganisms originating from rumen fluid.

1.2 RESEARCH QUESTIONS

- What is the effect of the grass loading rate (daily/weekly supplementation) to the reactors on the COD/VFA concentration and on the sulphate removal rate?
- What is the effect of microorganism supplementation in the form of RI and SRB to the reactors on the COD/VFA concentration and on the sulphate removal rate?
- What is the effect of the indigenous microorganisms attached to the GC entering the reactors on the COD/VFA concentration and thus on the sulphate removal rate and how will the addition of autoclaved and non-autoclaved grass influence the composition of the reactor microbial populations?
- Can sustainable sulphate reduction be achieved and maintained at ambient temperature of 25°C?
- Which reactor configuration will provide the highest sulphate removal?

In order to answer these research questions the following study objectives were set:

1.3 OBJECTIVES

The objectives of this study were to investigate:

1. The effect of daily addition of GC and RI to batch reactors on the COD and VSS concentrations
2. The effect of daily addition of RI and GC to batch operated reactors, containing grass, RI, SO_4 rich feed water and SRB as opposed to daily addition of grass and *no* additional dosing of RI, on the COD/VFA, SO_4^{2-}, S^{2-} and VSS concentration in the reactors.
3. The effect of autoclaved and non-autoclaved grass on the microbial population in the bioreactors, by applying specialised molecular tools on microbial samples from the reactor.
4. To operate the reactor systems at ambient temperatures (22-25°C).
5. The effect of operating continuous fed reactors, containing GC, rumen fluid, SRB and SO_4 rich water, feeding initially synthetic feed water and later pre-treated AMD on the COD/VFA, SO_4^{2-} and VSS concentration in the reactors.

1.4 REFERENCES

Ashton, P.J. and Haasbroek, B. (2000). Hydropolitics in the Developing World – *A Southern African Perspective,* Chapter 14. Anthony Turton and Roland Henwood (Ed.): 187-205. AWIRU, Pretoria, 2002, 269 pp.

Brown, M., Barley, B. and Wood, W. (2002). Mine water treatment, technology, application and policy. IWA Publishing, London, UK.

Coughlan, M.P and Mayer, F. (1992). The Cellulose-Decomposing Bacteria and Their Enzyme Systems. In: The Prokaryotes: Handbook on the biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Volume 1. 2nd edition, Editors: A Balows, H.G Truper, M Dworkin, W Harder and K-H Schleifer. Springer-Verlag, Berlin.

De Smul, A., Dries, J., Goethals, L., Grootaerd, H. and Verstraete, W. (1997). High rate of microbial sulphate reduction in a mesophile ethanol fed expanded-granular-sludge-blanket reactor. *Appl. Microbiol. Biotechnol.* **48:** 297-303

Du Preez, L.A., Odendaal, J.P., Maree, J.P. and Ponsonby. G. (1992). Biological removal of sulphate from industrial effluents using producer gas as energy source. *Environ.Technol*. **13:** 875-882.

Eloff, E., Greben, H.A., Maree, J.P., Radebe, B.V. (2004). Biological sulphate removal using a mixture of hydrogen and carbon dioxide gas as the energy/carbon source. Proc. IWA YRC 2004 May 2004 at the Agricultural University Wageningen, The Netherlands, pp 307-317.

Greben, H.A., Maree, J.P., Singmin, Y. and Mnqanqeni, S. (2000). Biological sulphate removal from acid mine effluent using ethanol as carbon and energy source. *Water Sci.Technol*. **42**(3-4)**:** 339-344.

Greben, H. A., Kahlo, D. M., Maree J. P. and Hagger M. (2003) The biological treatment of a nickel and copper mine effluent to render it suitable for irrigation of agricultural crops. Presented at the IWA conference Cape Town September 12-15: Water, the key to sustainability.

Greben, H.A., Baloyi, L.M. and Venter, S.N. (2007). Grass cellulose as cost effective energy source for biological sulphate removal. Water SA, Vol 33, No 5.

Günther, P. (2006). Emalahleni Mine Water Reclamation Project. In *proceedings* of Mine Water Drainage (South African Perspective). Randfontein 19-20 October, 2006

Higgins, J.P and Hard, B.C. (2003). Bioremediation of rock drainage using sulphate-reducing bacteria. Iscard Conference, Cairns, Australia, 2003.

Jovanovic, N.Z., Barnard, R.O., Rethman, N.F.G and Annandale, J.G. (1998). Crops can be irrigated with lime-treated acid mine drainage. *Water SA.* **24** (2):113-123.

Jovanovic, N.Z., Annandale, J.G., Claassens, A.S., Lorentz, S.A., Tanner P.D., Aiken, M.E. and Hodgson, F.D.I. (2002) Commercial production of crops irrigated with gypsiferous mine water. *Water SA.* **28** (4): 413-423.

Lynd, L.R., Weimer, P.J., Van Zyl, W. and Pretorius I.S. (2002). Microbial Cellulose Utilisation: Fundamentals and Biotechnology. *Microbiol. Mol.Biol. Rev.* **66** (3): 506-577.

Maree, J.P., Hagger, M.J., Strobos, G., Hlabela, P., Cronjé, H., Van Niekerk, A., Wurster, A and Nengovhela, R. (2003). Neutralization of acid leachate at a nickel mine with limestone. *Proc. Sudbury 2003 Mining and the Environment, 28th CLRA Meeting,* Laurentian University, Sudbury, 25 to 28 May, 2003.

Maree, J.P., Greben, H.A. and de Beer, M. (2004) Treatment of acid and sulphate-rich effluents in an integrated biological/chemical process. *Water SA*, **30:**(2): 183-191.

Pulles, W. (2000). Development of passive mine water treatment technology. *Proceeding Y2K Millennium Meeting*, Grahamstown 23-28 January, 2000: 600-601.

Rose, P.D., Moffett, M., Pulles, W, Nell, J.P., Louw, D., Melville, A., Leucona, A., Kumalo, S and De Wet. (2007). Integrated beneficiation of mine waters. Volume 2. WRC Project K5/1456.

Skousen, J. (1998).Overview of passive systems for treating acid mine drainage. In: Acid Mine Drainage control and treatment. American Society for Agronomy and the American Society for Surface mining and reclamation. http://www.wvu.edu/-agexten/landrec/passtrt/passtrt.htm

Van Houten, R.T. (1996). Biological sulphate reduction with synthesis gas. PhD Thesis, Agricultural University, Wageningen, The Netherlands.

Weijma, J. (2000). Methanol as electron donor for thermophilic biological sulfate and sulfite reduction. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

Younger, P.L., Banwart, S.A. and Hedin, R.S. (2002). Mine Water. *Hydrology, Pollution, Remediation.* Kluwer Academic Publishers. Dordrecht, Boston, London.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

Internationally, coal is the most widely used primary fuel and accounts for approximately 36% of the world's electricity production. It is predicted that the generation of coal to supply the energy demand will continue till the year 2020. In South Africa, 77% of the country's energy demand is supplied by coal, which makes it the fifth largest coal producer in the world and the third largest exporter, since 25% of the production is exported internationally (Eskom, 2006). These statistics imply that South Africa is faced with severe pollution of water and air due to the act of coal mining as well as due to the burning of coal to generate electricity. However, not only coal mining produces contaminated water, but other mineral mining activities e.g. the mining of platinum and gold contribute to the overall mine water pollution. For this literature survey, the emphasis will focus on polluted mine water generated from the mining of coal.

2.1.1 Generation of 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 pyrite, existing in the coal seam comes into contact with oxygen and water. 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). Studies carried out by the Council of Geosciences on behalf of the Department of Minerals and Energy, in South Africa have shown the ingress of ground water into defunct and closed mines. One result indicated that volumes as high as 70% of the water in the Wits (gold) mines originate from groundwater. This implies that unpolluted groundwater was mixed with the AMD present, thus resulting in polluted groundwater. Not only groundwater is getting contaminated this way, but also surface water, finding its way into old mine shafts.

2.1.2 Prevention of mine water pollution

In order to prevent the polluted AMD from getting to the surface, most defunct and closed mines, as well as mines in operation have to pump vast volumes of water from the underground vaults. This is a costly operation, since the pumped water has to be collected in a dam, from where it needs to be neutralised or treated for sulphate and metal removal. It is therefore important that both groundwater and surface water are prevented from entering the underground basin. In some mines, it was determined that surface/ground water most likely entered the mined-out shafts through stopes, sinkholes and shafts, created by the act of mining over the years of active mining (WRC, 2005). Thus to prevent mine water pollution, it is important to avoid the ingress of water into the mines, which will reduce the volume of contaminated water.

2.1.3 Active AMD treatment technologies

Before 1980, the only proven technologies for mine water treatment were the active treatment methods, i.e. conventional waste water engineering applied to mine waters (Younger *et al*., 2002). In most cases mine effluents can be treated following the design of infrastructure for similar unit processes in ordinary waste water treatment plants. 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 and other pollutants from AMD. Physical (R.O., electrodialysis and ion exchange) and chemical methods (precipitation with barium salts and limestone

neutralisation followed by lime precipitation, for Ca and Mg removal) have been tested and applied.

2.1.3.1 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 (Maree *et al*., 2003), resulting in a 50% saving in operating costs. 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.

2.1.3.2 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-sulphide precipitation, e.g. FeS, since AMD contains 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 initially use limestone as a pre-treatment step rather 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.

2.1.4 Passive AMD treatment

Passive treatment of poor quality waste water can be attractive for the mining industry, especially for closed mines (Pulles *et al*., 2001). 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, a relatively cheap passive treatment system can be operated with low maintenance and little supervision. Passive treatment technologies use ecological 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).

2.1.4.1 Anaerobic wetlands

Anaerobic wetlands, also referred to as Sulphate Reducing Bioreactors (SRBR) or compost wetlands, are mainly used for the treatment of AMD with a low pH and high metal concentration. Although these constructed wetlands have the potential to decrease the metal and acidity concentrations, they have not been in operation for a long time. There is no information available on long-term performance of full-scale anaerobic systems, operating for longer than 10 years. The longest anaerobic wetland designed for mine water treatment has been in operation for approximately 5 years. Thus long-term costs and requirements for substrate management, which are important for successful continuation of the operation there of are uncertain (Kuyucak *et al*., 2006).

Criteria for the successful operation of anaerobic passive treatment systems are the nutrients and the organic substrate required by the SRB for the reduction of sulphate. 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). The alkalinity produced after biological SO_4 removal results in a pH increase of the AMD while the sulphide produced is beneficial for metal precipitation (reactions 2.1 and 2.2).

$$2CH_2O + SO_4^{2-} \rightarrow HS^- + 2HCO_3^- + H^+ \qquad (2.1)$$

$$Me + H_2S \longrightarrow MeS \quad (2.2)$$

A low reduction-oxidation potential (redox value, expressed in mV) is beneficial for the sulphate reduction process. Other parameters for a successful operation of the anaerobic wetlands include the hydraulic design of the system, such as channelling the flow as well as operating with controlled hydraulic and metal loading rates (Gusek, 2004, Kuyucak, 2002).

2.1.4.2 Combined aerobic-anaerobic wetland systems

Kuyucak *et al*. (2006) suggested that the most effective passive treatment method for treating metal ions in AMD is the combination of an anaerobic system with an aerobic system to create complete, integrated systems. This may not be the required treatment method for the reduction of sulphate, since the sulphide produced can be oxidised back to sulphate, unless the wetlands are constructed such that the sulphide oxidation stops at sulphur, as is the case in the proposed systems by Pulles *et al*. (2001). Over the past 5-10 years, numerous research studies have been carried out with the aim of finding passive mine water treatment solutions for the South African mine effluents funded by the WRC and the Innovation Fund (IF). This has resulted in a pilot scale passive treatment wetland system, the degrading packed bed reactor system (DPBR) constructed at Middelburg Mine (BHP Billiton), treating 200 m^3/day of AMD.

2.1.4.3 Integrated Managed PassIve Treatment System (IMPI)

The principle of this passive treatment system (IMPI) is based on the use of four ponds, in which high levels of SO_4 reduction can potentially be achieved as well as the removal of metals (87-95% removal efficiency), resulting in an effluent with a neutral pH, based on the results obtained from the operation of smaller units. When these were operated, the oxidation of carbon sources occurred in the first pond, resulting in VFA production, followed by SO_4 reduction. In the second pond the sulphide oxidised to sulphur, which as the end product in the reduction and oxidation process can be removed. This oxidation pond was followed by a secondary sulphate reducing reactor and a secondary sulphide oxidising reactor. The process used naturally available energy sources, supplemented with readily available carbon, the land gradient and microbial energy through photosynthesis and other symbiotic microbial interactions (Heath, 2002, 2007).

2.1.4.4 Advantages and disadvantages of the passive treatment systems

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 social and ecological values (Hawke & José, 1996, Younger 1998). The plant-microbe associations in wetlands can serve as source of carbon for the sulphate reduction and water quality improvement (Batchelor *et al.*, 1998). Some plants in wetland systems have the advantage that they provide a level of phytoremediation through adsorption of dissolved metals on the surfaces of their rhizomes (sub-surface root nodules). These processes can be enhanced by selecting wetland plants with phytoremediating properties, which allow them to take up and metabolize organics and heavy metals from AMD passing through their root systems (Higgins and Hard). Ernst (1998) described phytoremediation (or "phytoextraction") of sulphate (as source of sulphur), which can be taken up by the roots of higher plants. The *Gypsophila* species are well adapted to extract sulphur from gypsiferous soils/waters, in case the soil contains enough nitrate and phosphate. In the past, plants such as peat moss (*Sphagnum*) have been used in wetland systems, since these plants can hold large volumes of water inside their cells. Furthermore, *Sphagnum* can take up Ca and Mg (metals associated with mine water pollution), releasing hydrogen, a potential energy source for the biological sulphate removal process. Cattails (*Typha latifolia*) showed similar properties, taking up polluted mine water and were responsible for metal removal (Pulles *et al*., 2001). The plant containing wetlands are usually aerobic and are typically applicable to AMD containing Fe, Mn and acidity at relative low concentrations (Gusek *et al.*, 2006). The advantages and disadvantages of passive treatment systems are listed in Table 2.1 (Younger 2002).

Table 2.1. The advantages and disadvantages operating a passive mine water treatment system

Advantages	Disadvantages
Low operating costs and usually low capital costs (depending on the mine water flow)	Passive treatment technology is relatively new, reliable expertise is scarce
Use of non-hazardous materials	Precise control of treatment effluent quality is not feasible, since there is no day-to-day supervision
Passive treatment systems can work for long period of time (with little supervision) when well designed and constructed	Depending on plume/seepage water flow, a high surface area of land is needed
Passive treatment systems can be integrated with surrounding ecosystems	
Passive treatment systems are more attractive in appearance than active systems	

From the information as presented in Table 2.1, it can be observed that Younger *et al*. (2002) provided more advantages than disadvantages for the use of passive mine water systems. They do, however, indicate that the large land footprint is probably the principle drawback for passive treatment systems. That observation, however, may be less of an obstacle in South Africa, especially in the more rural areas, where the mine water plumes and seepages typically occur. Since reactions in passive treatment systems take place at lower pH and at lower temperatures than active systems, the retention times are usually longer to achieve the same results.

2.1.4.5 Application of passive treatment systems

Passive treatment of polluted mine water has already a track record in the USA, treating AMD generated in the eastern coalfields. Other similar treatment systems occur in Europe (UK and Spain). Passive treatment can function as the technology of choice for long-term use, wherever the hydrogeochemical prognosis is favourable and where land space is available. However, it has to be taken into account that mine water treatment in the USA is mainly focussed on the removal of metals and acidity, while in South Africa the removal of salinity (SO_4^{2-}) is a requirement stipulated by Department of Water Affairs and Forestry (DWAF).

The status of wetlands for the mine water treatment can be summarised as follows:

- Passive treatment systems for the removal of iron from acidic and alkaline waters can be implemented with confidence.
- For the removal of other contaminants active research is still ongoing for full-scale implementation.
- North America retains a leading role in the development of passive treatment technologies, mainly focussed on aerobic wetlands to remove metals
- Technical advantages on passive treatment are achieved in Australia and Canada for some of the more challenging mine water types and for operating at lower temperature, respectively.

2.1.4.6 Pitfalls of passive mine water treatment

Johnson and Hallberg (2002) make the observation that the biogeochemistry and microbiology of the constructed wetlands are highly complex. There is limited detailed knowledge of many of the basic natural processes occurring in these passive treatment systems. They, however, conclude by remarking that those mentioned problems are not insurmountable and can be solved by further research into these complex ecosystems. Coetser (2003) concluded from her study that after 200 days of operation the wetland ceased to operate due to lack of substrate, thus resulting in a low COD concentration in the systems.

2.1.5 Active biological sulphate removal

So far not many full scale biological sulphate removing reactors are in operation in South Africa, nor in other parts of the world. The waste water treatment technologies company Paques BV, based in Balk, The Netherlands, has constructed and commissioned a 3 ML/day biological sulphate reduction demonstration plant. This plant is in operation at Landau Colliery in Witbank, South Africa, where it treats AMD with a SO_4 concentration of 2.4 g/ℓ and a pH of 2.9. Waste ethanol is used as the carbon and energy source in this system. Prior to the construction and operation of the Paques plant, the CSIR showed that sulphate could be removed at 12.4 g SO_4(ℓ.d) operating a one stage completely mixed demonstration reactor (designed for 100 ℓ/d) at Navigation, Anglo Coal, Witbank, South Africa (Maree *et al*., 2004). This reactor system used a combination of mainly technical ethanol and a small amount of sugar (Greben *et al*., 2002) as the electron donor and was able to operate at a HRT of 6 hours.

More recently a 10 Mℓ/day full-scale plant treating AMD from Grootvlei goldmine was commissioned at Erwat's Ancor Waste water treatment works in Springs, South Africa (Joubert, 2005). This plant uses primary sewage sludge as the carbon and energy source. The mine water enters the plant through a pipeline and is then mixed with primary sewage sludge in a mixing tank, where after the mixture enters the biological sulphate removing reactors. The collected effluents from these reactors are mixed with iron slurry and transported to clarifiers to remove the sulphide in the form of iron sulphide precipitates. The clarifier overflow, with reduced sulphate and sulphide concentrations, but with a residual COD concentration is pumped back to the sewage works with the aim to remove the remaining COD concentration.

2.2 CARBON SOURCES FOR THE BIOLOGICAL SULPHATE REMOVAL TECHNOLOGY

The emphasis of this WRC research study 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. 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, they can also use propionate, butyrate, higher and branched fatty acids, lactate, ethanol and higher alcohols, fumarate, succinate, malate and even 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. Although the biological sulphate removal is an attractive option, worldwide not many active full scale treatment systems are in operation, however, as indicated, several demo-scale plants have been constructed in South Africa. Additional research is still being conducted to find the optimal reactor configuration (UASB, EESB, Completely Mixed Systems), the maintenance of biomass and a cost effective carbon and energy source, of which the latter is the purpose of the project described in this report.

In full scale operations, the addition of a carbon and energy source will add to the overall running costs of the operation. Therefore, the research focus from using the traditionally used carbon sources (ethanol, sucrose, lactate etc) has shifted to the use of the degradation products of complex organic products, such as primary sludge

(Rose, 2000 and Joubert, 2005), hay and grass (Coetser *et al.*, 2000). Dill *et al.* (2001) achieved a 99% SO_4 removal efficiency when using hay as the carbon and energy source, while this was 97.8% when using Kikuyu grass.

In the previous century, 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). During the anaerobic degradation of cellulose, VFAs and other intermediates are formed, which can function as the carbon and energy source for the biological sulphate removal technology.

2.2.1 Anaerobic degradation of cellulose

Plant biomass is constructed of polysaccharides, of which cellulose and hemicellulose are the most prominent. It is easily available and is a rich, renewable energy source and is due to its origin of photosynthesis a product of solar energy (Sheehan and Himmel, 1999). Cellulose is the most prominent single organic compound on earth. It can easily be mineralised and hydrolysed by enzymes, which makes the hydrolysis of polysaccherides one of the most important enzymatic processes on earth (Schwarz, 2001). Numerous bacteria have been reported to ferment plant biomass, such as from the rumen of ruminants, from the gut of termites and other insects and from compost, soil or mud (Coughlan and Mayer, 1992; Lynd *et al*., 2002). The rumen, especially is a highly cellulytic ecosystem with a complex microbial population of Bacteria, Archaea, Protozoa and Fungi. This consortium produces VFA for the ruminants as source of energy, biomass as the source of protein, while the final degradation products are gases (CH_4 and CO_2).

Cellulose fibres 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 derivatives of cellulose. 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).

2.2.2 Production of Volatile Fatty Acids from complex organic material

VFA 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 describing the following processes:

- *Hydrolysis*

During the hydrolysis process, complex, non-soluble organic compounds are solubilized by exoenzymes. 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.

2.2.3 Anaerobic oxidation of Long-Chain Fatty Acids

The anaerobic degradation of long-chain fatty acids occurs by β-oxidation (Jeris and 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 hydrogen consuming SRB (HSRB) is higher than that of hydrogen consuming methanogenic bacteria (HMB), and therefore the HMB are out-competed by HSRB in environments where a sufficient amount of sulphate is present (Robinson & Tiedje, 1984). The SRB keep the hydrogen partial pressure low in presence of sulphate, since as soon as hydrogen is produced in the acetogenesis process, the hydrogen will be utilised by SRB for the reduction of sulphate.

2.2.4 The oxidation of organic compounds in a sulphidogenic reactor

The SRB are very diverse in terms of their metabolic capabilities. Acetate is the product, when oxidizing propionic (C3) acid and butyric (C4) acids, as is the case in the hydrolysis of the C3 and C4 acids. The hydrolysis products as well as the oxidation products in the presence of sulphate, propionate and butyrate are acetate and hydrogen. Both autotrophic and heterotrophic growth on hydrogen is possible. The *Desulfovibrio* sp. requires acetate as carbon source, whereas e.g. Desulfobacterium sp. can use CO_2 as the only carbon source (Van Houten, 1996).

2.2.5 Fatty Acids in the sulphidogenic reactor

Widdel and Pfenning (1977) showed 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.

An important factor in the competition of SRB, methanogenic bacteria (MB) and acetogenic bacteria (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.

2.2.6 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).

2.2.7 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. 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. Visser (1995) showed 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 limiting conditions.

2.2.8 Cellulose degrading microorganisms

Anaerobic degradation of plant material can be executed efficiently using the bacteria, fungi and protozoa occurring in the rumen of ruminants 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.

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).

Ljungdahl and Eriksson, (1985) described the fermentation of sugars to produce carbon dioxide and hydrogen according to Equation (2.3):

$$C_6H_{12}O_6 + 6\ H_2O \rightarrow 6\ CO_2 + 12\ H_2 \qquad (2.3)$$

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).

2.2.8.1 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.

2.2.8.2 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).*

2.2.8.3 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.*

2.2.8.4 *Clostridium* species

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

2.2.8.5 *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.

2.2.8.6 *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).

2.2.9 Competition for the same substrate in the bio-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*., 1998). 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). 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 HSRB gain more energy

from the consumption of molecular hydrogen, have a higher substrate affinity, growth rate and cell yield than the 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.

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) confirmed those by Harada *et al*. (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.

2.2.10 Reactor configuration

When the rumen microorganisms ferment GC to VFA and other intermediates, such as hydrogen, it is important that the SRB are present to utilise the products of the cellulose fermentation for the biological sulphate reduction. By using the hydrogen as soon as it is produced the SRB keep the hydrogen partial pressure low, thereby allowing the rumen microbes to continue degrading the grass-cellulose. It is therefore important that the different species of microorganisms function in one reactor. Different reactor configurations can be used for this purpose, such as completely mixed reactors. However, in order to retain the microorganisms, a settler is needed from where the biomass can be recycled to the bio-reactor. Another reactor system suitable for the syntrophy of the different microorganisms and to retain the biomass inside the reactor could be the use of a hybrid reactor. A hybrid reactor provides suspended growth in the sludge layer and biofilm formation on the packing material. Most anaerobic hybrid reactors are a combination of Upflow Anaerobic Sludge Bed (UASB) and Anaerobic Filter (AF) reactors, thereby promoting the advantages of both reactor systems (Buyukkamaci & Filibeli, 2002). Operating a hybrid reactor provides suspended growth in the sludge layer and biofilm formation on the packing material, which prevents washout of biomass.

2.2.11 The challenges associated with AMD treatment

Mining contributes positively to the economy, but negatively to the environment, due to the production of contaminated effluents in the form of AMD and due to the often polluted leachate of the coal discard heaps. These polluted streams should be treated so that the treated water can either be discharged to a river system or re-used in the coal processing plant. Several treatment methods have been developed, both in active treatment plants as well as under passive conditions in the different mining area around the world. In South Africa, 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. Several full-scale plants have been constructed for the neutralisation of AMD. However this technology does not remove the sulphate concentration. In order to remove both the sulphate and the metals, the biological sulphate removal technology can be applied, producing sulphide and alkalinity. Sulphide causes heavy metals in the mine water to precipitate as metal-sulphides (MeS), while alkalinity results in a pH increase of the treated water.

An important factor in applying the biological sulphate removal technology is the need for a cost effective carbon and energy source (electron donor), while sulphate is the electron acceptor. To date, the use of 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 that it can be used as energy source through the fermentation of cellulose to oligomers, monomers and ultimately VFA, which then can be used as energy sources for biological sulphate removal. This way of utilising a garden waste product is more environmental friendly than placing the biowaste in landfill, producing leachate and methane gas, as is the practise in most regions of South Africa

2.3 CONCLUSIONS

The information collected from the literature study showed 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 gained knowledge is thus important for the successful outcome of the proposed WRC K5/1728 study. 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. From previous studies it has been shown that the rumen fluid microorganisms can degrade grass-cellulose to the required carbon/energy sources for the biological sulphate removal. Therefore, these microorganisms will solely be used to achieve the set objectives and will be adapted to room temperature for the purpose of the studies, described in the following chapters.

2.4 REFERENCES

Batchelor, A., Maree, J.P., Dill, S. and Dingemans, D. (1998). Approaches to Sulphate Removal: Chemical to Ecotechnological. *CSIR internal report compiled for Anglo American Coal Division*.

Bryant, M.P. (1986. Genus Ruminococcus In Bergey's Manual of Systematic bacteriology, Vol2 Ed: PHA Sneath, NS Mair, ME Sharp and JG Holt. p1093-1097.

Buyukkamaci, N. and Filibeli, A. (2004). Volatile fatty acid formation in an anaerobic hybrid reactor. Process Biochemistry **39:**1491-1494.

Carlile, M.J. and Watkinson, S.C. (1997). The fungi. *Academic Press, New York, N.Y.* pp: 269-275.

Coetser, S.E., Cloete, T.E. and Zdyb, L. (2000). Biological sulphate reduction in artificial acid mine drainage using different carbon sources. *Proceeding Y2K Millennium Meeting, Grahamstown 23-28 January, 2000*: 606.

Coetser, S.E. (2003). Microbial sulphate reduction in passive acid mine drainage treatment systems. Ph.D Thesis in Waster Resource Management. University of Pretoria, South Africa.

Colleran, E., Finnegan, S. and Lens, P. (1995). Anaerobic treatment of sulphate-containing waste streams. *Antonie van Leeuwenhoek* **67:** 29-46.

Coughlan, M.P and Mayer, F. (1992). The Cellulose-Decomposing Bacteria and Their Enzyme Systems. In: The Prokaryotes: Handbook on the biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Volume 1. 2nd edition, Editors: A Balows, H.G Truper, M Dworkin, W Harder and K-H Schleifer. Springer-Verlag, Berlin.

Dill, S., Cloete, T.E., Coetser, L. and Zdyb, L. (2001). Determination of the suitability of alternative carbon sources for sulphate reduction in the passive treatment of mine water. *WRC Report*: 802/1/01.

Domka, F., Gasiowek, J. and Klemm, A. (1977). Processing of sulphate wastes using municipal sewage. *Gaz. Wodka Techn. Sanit*; **51:** 179-180.

Ernst, W.H.O. (1998). Sulfur metabolism in higher plants: potential for phytoremediation. *Biodegradation* **9**: 311-318.

Eskom publication (2006). African Wildlife: Special edition: Energy. Autumn 2006.

Greben, H.A., Bologo, H. and Maree, J.P. (2002). The effect of different parameters on the biological volumetric and specific sulphate removal rates. *Water SA*. *Special Edition,* WISA Biennial Conference 2002, pp: 33-37

Gusek, J. (2004). Scaling Up Design Challenges for Large Scale Sufate Reducing Bioreactors. National Meeting of the American Society of Mining and Reclamation the 25th West Virginia Surface Mine Drainage Tsak Force, April 18-24 2004. pp.752-765.

Gusek , J.J., Wildeman, T.R. and Conroy, K.W (2006). Conceptual methods for recovering metal resources from passive treatment systems. In: Proceedings 7th

International conference on Acid Rock Drainage (ICARD), March 26-30, 2006, St. Louis MO.

Hanaki, K., Matsuo, T. and Nagase, M. (1981). Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. *Biotechnol. Bioeng.* **23** p:1591.

Harada, H., Uemura, S. and Monomoi, K. (1994) Interaction between Sulphate-Reducing Bacteria and Methane-Producing Bacteria in UASB Reactors fed with Low-Strength Wastes containing different levels of Sulphate. *Wat. Res.* **28:** 335-367.

Harmsen, H.J.M. (1996). Detection, phylogeny and population dynamic of synthrophic propionate-oxidizing bacteria in anaerobic sludge. *PhD thesis*, Wageningen Agricultural University, Wageningen.

Hawke, C.J. and José, P.V. (1996). Reedbed Management for Commercial and Wildlife Interest Royal Society for the Protection of Birds, Sandy Bedfordshire. pp 212

Heath, R. (2002). IMPI for mine water treatment. *Water Wheel*. **28** (4):27-29.

Heath, R. (2007). Passive treatment for active solutions. *Water Sewage & Effluent* **27** (5):22-25.

Higgins, J.P and Hard, B.C. (2003). Bioremediation of rock drainage using sulphate-reducing bacteria. Iscard Conference, Cairns, Australia, 2003.

Hungate, R.E. (1966). The rumen and its microbes. Academic Press Inc. New York, USA

Jeris, J.S. and McCarthy, P.L. (1965). The biochemistry of methane fermentation using ^{14}C tracers, *J. Water Pollut. Control Fed.* **37:**178.

Johnson, D.B. (2000). Biological removal of sulphurous compounds from inorganic wastewater. Environmental Technologies to Treat Sulphur Pollution, Principles and Engineering (P. Lens and L Hulshoff Pol eds). IWA Publishing London, UK: 175-205.

Johnson, D.B. & Hallberg, K.B. (2002). Pitfalls of passive mine water treatment. *Re/vieuws in Environ. Sc & Bio/Technol.* **1:**335-343.

Joubert, J. (2005). Acid mine drainage: Sludge as an electron donor and carbon source in a biological sulphate reducing processsludge as an electron donor and carbon source in a biological sulphate reducing process. *Proc. Management of Residues Emanating from Water and Waste Water treatment.* Sandton Convention Centre, Johannesburg, South Africa, 9-12 August, 2005.

Koster, I.W. (1988). Microbial, Chemical and Technological aspects of the anaerobic degradation of organic pollutants. In Wise D.L. (ed): *Biotreatment systems, Vol. lll,* CRC Press Inc., Boca Raton, USA : pp 285-316.

Kuyucak, N. (2002). Microorganisms in Mining:Generation of Acid Rock Drainage, its Mitigation and Treatment. *EJMP&EP* **2**: (3)

Kuyucak, N., Chabot, F and Martschuk, J. (2006). Successful implementation and operation of a passive treatment system in an extreme cold climate, Northern Quebec, Canada. In: Proceedings 7th International conference on Acid Rock Drainage (ICARD), March 26-30, 2006, St. Louis MO.

Lee, S.S., Ha, J.K. and Cheng, K.J. (2000). Relative contribution of bacteria, protozoa and fungi to in vitro degradation of orchard grass cell walls and their interaction. *App. Environ Microbiol.* **66** (9): 3807-3813.

Lens. P.N.L., Visser, A., Janssen, A.J.H., Hulshoff Pol, L.W. and Lettinga, G. (1998). Biotechnological Treatment of Sulphate-Rich Wastewaters. *Crit. Rev. Environ. Sci. Technol.* **28**(1): 41-88**.**

Ljundahl L.G. and Eriksson, K-E. (1985) Ecology of microbial cellulose degradation. In: Advances in microbial ecology. Editor: K.C. Marshall. Plenum Press, New York.

Lovley, D.R. (1985). Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl. Environ. Microbiol*. **49:**1530-1531.

Lynd, L.R., Wyman, C.E and Gerngross, T.U. (1999). Biocommidity engineering. *Biotechnol. Prog.* **15**:777-793.

Lynd, L.R., Weimer, P.J., Van Zyl, W. and Pretorius I.S. (2002). Microbial Cellulose Utilisation: Fundamentals and Biotechnology. *Microbiol. Mol.Biol. Rev.* **66** (3): 506-577.

Madigan, M.T., Martinko, J.M. and Parker, J. (1997) Brock: *Biology of Microorganisms.* Eighth ed. Prentice-Hall, Inc.

Marchessault, R.H., and Sundararajan, P.R. (1993). Cellulose. 11-95. In G.O. Aspinall (ed). *The polysaccarides*, Vol 2. Academic Press, Inc, New York, N.Y.

Maree, J.P., Hagger, M.J., Strobos, G., Hlabela, P., Cronjé, H., Van Niekerk, A., Wurster, A and Nengovhela, R. (2003). Neutralization of acid leachate at a nickel mine with limestone. *Proc. Sudbury 2003 Mining and the Environment, 28th CLRA Meeting,* Laurentian University, Sudbury, 25 to 28 May, 2003.

Maree, J.P., Greben, H.A. and de Beer, M. (2004). Treatment of acid and sulphate-rich effluents in an integrated biological/chemical process. *Water SA*. 30**(2)**: 183-189

McAnally, R.A. (1942). Digestion of straw by the ruminant. *Biochem.J.* **36**: 392-399

McKinney, R.E. and Conway, R.A. (1957). Chemical oxygen in biological waste treatment. *Sewage & Ind. Wastes*. **29:** 1097-1106.

O'Flaherty, V., Lens, P., Leahy, B and Colleran, E. (1998). Long-term competition between Sulphate-Reducing and Methane-Producing Bacteria during full-scale Anaerobic Treatment of Citric Acid Production Wastewater. *Wat. Res*.**32**(3): 815-825.

Omil, F., Lens, P., Visser, A., Hulshoff Pol, L.W. and Lettinga, G. (1997). Long term competition between Sulfate Reducing and Methanogenic Bacteria in UASB reactors treating Voltile Fatty Acids. *Biotechnol. Bioeng.* **57:**667-685.

Oude Elferink, S.J.W.H., Visser, A., Hulshoff Pol, L.W. and Stams, A.J.M. (1994). Sulphate reduction in methanogenic bioreactors. *Fems Microb. Rev.* **15:**119-136.

Oude Elferink, S.J.W.H. (1998). Sulphate-reducing Bacteria in Anaerobic Bioreactors. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

Pipes, W.O. Jr. (1960). Sludge digestion by SRB, *Proc. Ind. Waste Conf*., Purdue Univ, West Lafayette: 871-877

Postgate, J.R. (1984). The sulphate-reducing bacteria. *Second edition*. Cambridge University Press, Cambridge pp 208.

Pulles, W., Van Niekerk, A., Wood, A., Batchelor, A., Dill., S., du Plessis, P., Howie, D. and Casey, T. (2001). Pilot scale development of integrated passive water treatment systems for mine effluent streams. WRC Report No 700/1/01

Raskin, L., Rittmann, B.E. and Stahl, D.A. (1996). Competition and coexistence of sulphate-reducing and methanogenic populations in anaerobic biofilms. *Appl. Environ. Microbiol.* **60**: 1241-1248.

Reynolds P .H.S., Sisson, C.H., Daniel, R.M. and Morgan, H.W. (1986) Comparison of cellulolytic activities in *Clostridium thermocellum* and three cellulolytic anaerobes. *Appl. Environ. Microbiol.* **51**: 12 – 17.

Rinzema, A. and Lettinga, G. (1988). Anaerobic treatment of sulfate containing wastewater. In: Biotreatment systems, **3:** (Wise, DL, Ed). CRC press, Inc., Boca Raton, Florida. Pp 65-109

Robinson, J.A. and Tiedje, J.M. (1984). Competition between sulphate reducing and methanogenic bacteria for H_2 under resting and growing conditions. *Arch Microbiol*. **137**: 26

Rose, P.D. (2000). The Rhodes Biosure Process: The piloting of an active process for the treatment of acid mine drainage wastewaters. *Proceeding Y2K Millennium Meeting*. Grahamstown 23-28 January, 2000: 605-606.

Schwarz, W.H. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. *Appl Microbiol Biotechnol* **56**:634-649.

Sheehan, J. and Himmel, M. (1999). Enzymes, energy and the environment: a strategic perspective on the U.S. department of energy's research and development activities for bioethanol. *Biotechnol Prog* **15**:817-827.

Stams, A.J.M., Kremer, D.R., Nicolay, K., Weenk, G.H. and Hansen, T.A. (1984). Pathway of propionate formation in *Desulfobulbus propionicus*. *Arch.Microbiol*. **139**:167-173.

Van Houten, R.T. (1996). Biological sulphate reduction with synthesis gas. PhD Thesis, Agricultural University, Wageningen, The Netherlands.

Visser, A. (1995). The anaerobic treatment of sulphate containing wastewater. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

Widdel, F and Pfennig, N. (1977). A new anaerobic, sporing, acetate-oxidizing, sulphate-reducing bacterium, Desulfotomaculum (emend) acetooxidans, *Arch, Microbiol.* **112,** 119-122.

WRC. (2005). Mine Water Pollution. The Water Wheel. March/April 2005.

Younger, P.L. (1998). Adit hydrology in the long-term: observations From Pb-Zn mines of northern England. In *Proceedings of the International Mine Water Association Symposium on "Mine water and Environmental Impacts,* Johannesburg, South Africa, 7-13th September, 1998. Volume ll, pp 347-356.

Younger, P.L., Banwart, S.A. and Hedin, R.S. (2002). Mine Water. Hydrology, Pollution, Remediation. Kluwer Academic Publishers. Dordrecht, Boston, London pp 442

CHAPTER 3

THE EFFECT OF DAILY ADDITION OF RUMEN INOCULA ON THE FERMENTATION OF GRASS-CELLULOSE

3.1 INTRODUCTION

Cellulose is the major constituent of plant biomass, forming an important component in the carbon cycle and is formed through 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, producing 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 grass to VFA was enhanced when sulphate-adapted biomass was added to the fermentation process, even without sulphate 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 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). Cellulose in the ruminant feed is converted into microbial cells and compounds such as CO_2, CH_4, and acetic, propionic and butyric acids during the fermentation process. The rumen is inhabited by between 10^{10}-10^{11} bacteria and 10^6 protozoa per mℓ rumen fluid (Hungate, 1966). The rumen houses a complex ecosystem where microorganisms live in symbiotic relationships that facilitate fibre digestion. It was, therefore, hypothesized that anaerobic degradation of plant material may be executed 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 objective of the studying this chapter was to investigate the effect of daily addition of RI on the degradation of grass-cellulose on COD, VFA and Volatile Suspended Solids (VSS) concentrations.

3.2. MATERIALS AND METHODS

3.2.1 Feed water

Tap water to which micro nutrients were added (Table 3.1) was used as feed water.

Table 3.1. Composition of micro nutrients

Compound	Nutrient	Concentration
KCl	K	0.52 mg/ℓ
$FeCl_3$ $4H_2O$	Fe	0.1 mg/ℓ
$CoNO_3.6H_2O$	Co	0.21 mg/ℓ
$MnCl_2.4H_2O$	Mn	0.28
NH_4VO_3	V	0.44
$NiCl_2.6H_2O$	Ni	0.25
$ZnCl_2$	Zn	0.48
$Na_2MoO.2H_2O$	Mo	0.40
H_3BO_3	B	0.18
$CuCl_2.2H_2O$	Cu	0.37

3.2.2 Reactor systems and biomass

Two stirred batch reactors, called RD (investigative reactor) and RO (control reactor) with a volume of 2 ℓ were operated. Both RO and RD received 100 ml RI (VSS 17.2 g/ℓ) at the start of the experiment. Thus the rumen biomass occupied 5% of the total reactor volume. The rumen fluid was obtained from fistulated ruminants (University of Pretoria, South Africa) and stored in an incubator at 37°C at the CSIR laboratories, prior to use.

3.2.2 Carbon and energy source

Cut grass was used as the source of cellulose, of which the formed fermentation products served as the carbon and energy source in the reactor. For this experiment autoclaved Kikuyu grass was used with the rationale that the natural occurring grass degrading microbes should be eliminated, since the purpose of the study was to investigate the effect of the daily addition of RI. Kikuyu grass was obtained from the CSIR, Pretoria Garden Service. After cutting, the cut grass was collected and kept at 4°C. Grass used for the experiments in the following chapters came from the same stockpile from the cold room. The size of the cut grass was between 1-2 cm. The weight of the grass in the text refers to air dried grass, due to storage at 4°C (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.

3.2.3 Experimental

Both RO and RD received 70 g dried autoclaved GC. Thereafter, autoclaved GC (5 g/d) was added daily to RO and RD. In addition RD received 15 mℓ RI (VSS 2.4 g/ℓ) on a daily basis. The experimental period for both reactors was 53 days. Daily samples (50 mℓ) were taken. The sample volume was replaced with feed water (3.2.1). The reactors temperature was ambient at 24-25°C and the pH in both reactors was controlled at and 6.7-6.9, using ATI pH controller. To adjust the pH, the pH controller was connected to a solution of 0.1 N NaOH and 0.1N HCl.

3.2.4 Analytical

Daily samples were analysed for COD, pH, VFA and VSS. All analyses were carried out according to standards analytical procedures as described in Standards Methods (APHA, 1985). The nitrate (NO_3^--N) and the phosphate (PO_4^{3-}-P) concentrations in the batch reactors were analysed using the Hach Spectrophotometer DR/2010. The presented graphs in the following figures represent the results of the daily analyses, while the data in the tables represent the average values of the results of the daily analyses. The sulphate, sulphide, alkalinity, COD and pH were determined manually according to analytical procedures as described in Standards 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 (FID), while the data analyses

were done using the Chem Station software package, supplied by Hewlett Packard. The column used was a HP-FFAP, 15 m x 0.53 nm, 1 micron. The GC/FID programme can be summarized as follows: initial oven temperature 30°C, for 2 min., temperature programmed to increase thereafter from 30°C to 200°C at 25°C/min, with temperature hold for 1 min at 200°C, FID temperature 240°C. The carrier gas (N_2) flow rate was set at 1 mℓ/min.

3.3 RESULTS

3.3.1 COD Concentration

The COD concentrations in RD and RO during the total experimental period are presented in Figure 3.1, which showed that the COD concentration in RD and RO increased from day 1 (ca. 3 000 mg/ℓ) till day 37. On that day the COD concentration was 9290 and 13290 mg/ℓ in RO and RD, respectively, which reflects a difference in COD concentration between RD and RO of 4 000 mg/ℓ. This initial COD concentration increase can be ascribed to the initial GC addition (70 g). During the period from day 37 to day 53, the COD concentration in RO stabilized till the end of the experiment (d 53). This result indicated that the daily addition of 5 g GC was sufficient to maintain a steady COD concentration in RO. The results in RD till day 42 showed that due to the daily addition of RI, the COD concentration in RD was higher than in RO, thus the addition of RI in RD resulted in an increased COD concentration. This result indicated that a *higher* biomass concentration resulted in a *higher* COD concentration.

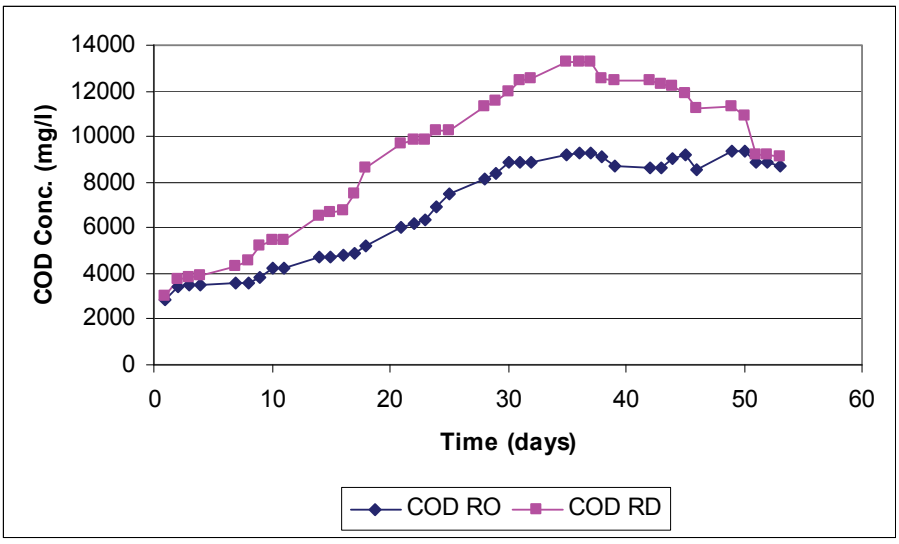

Figure. 3.1. COD concentration in RD and RO

On day 42 biomass loss was recorded (described below) resulting in a decrease in COD concentration in RD. The COD decreased from 12 420 mg/ℓ to 9 140 mg/ℓ from day 42 till day 53. Although 15 mℓ RI was added on a daily basis, no further increase in the COD concentration was observed in RD, which again was ascribed to the biomass loss.

3.3.2 VSS Concentration

The VSS concentrations in RD and RO during the total experimental period are given in Figure 3.2. It can be observed that although the VSS concentration in RD and RO was the same at the beginning of the experimental period, the VSS concentration in RD rapidly increased till day 39, when the VSS concentration was 6 224 mg/ℓ. However on day 42, biomass was lost, due to gas build up in the reactor and consequently the VSS concentration decreases from 6 224 mg/ℓ to 5 634 mg/ℓ. The VSS concentration in RO increased gradually until day 30, thereafter it stabilised at ± 3 000 mg/ℓ till day 50. This result showed that initially the RI population increased due to the fermentation of the cut grass from which the RI obtained energy in the form of COD/VFA. Since autoclaved grass was used, the increase of the indigenous grass microorganisms can be neglected. The VSS concentration in RD was approximately 3 000 mg/ℓ higher than that compared to RO, which was due to the daily addition of RI. Not only was the existing RI population supplemented with additional microbes, the microorganisms present in the reactor increased due to the addition of substrate in the form of GC. This finding indicated a clear relationship between the supplementation of additional substrate and RI and the VSS concentration in the reactor (RD) as can be observed from Figures 3.1 and 3.2.

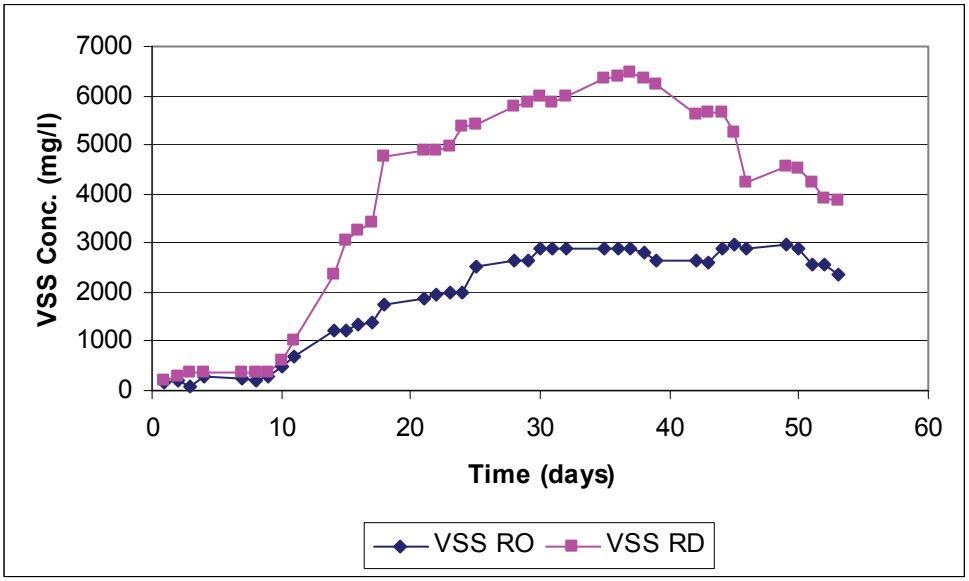

Figure 3.2. VSS Concentration in RD and RO

3.3.3 VFA Concentration

3.3.3.1 Propionic acid concentration in RD and RO

From Figure 3.3, it can be observed that the overall propionic concentration was higher in RD than in RO. The propionic acid concentrations in both reactors were the same (258 mg/ℓ) on day 1. However throughout the duration of the experiment, the propionic acid concentration was higher in RD than in RO. The highest measured propinic acid concentration in RD was 1 191 mg/ℓ, while this was 845 mg/ℓ in RO. The difference in propionic acid concentration between RD and RO was 31% (based on average data). These results indicated that adding RI on a daily basis as opposed to a once off addition was favourable for an increased propionic acid concentration in the reactor. The propionic acid concentration increase can be attributed to the higher microbial population in RD as can be observed from the VSS concentration (Figure 3.2). These results showed a clear relationship between the higher microbial population and the higher propionic acid concentration between RD and RO.

3.3.3.2 Butyric acid concentration in RD and RO

The overall butyric acid concentration in RD was higher than in RO (Figure 3.4). The highest measured butyric acid concentration in RD was 1403 mg/ℓ, while this was 896 mg/ℓ in RO. The difference in butyric acid concentration between RO and RD was on average 33%. The higher butyric acid concentration in RD can be ascribed to the daily addition of RI similar to the propionic acid concentration result.

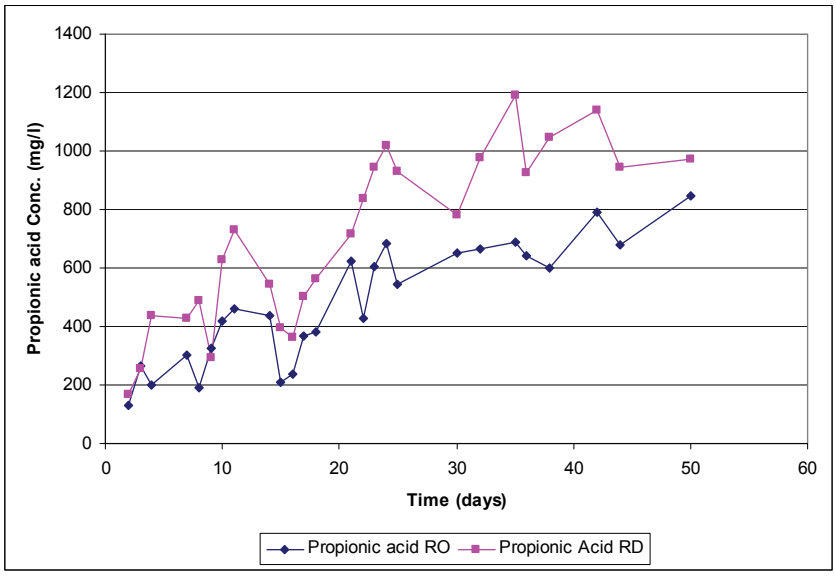

Figure 3.3. Propionic acid concentration in RD and RO

Microorganisms hydrolyse cellulosic material to glucose and cellobiose. In the subsequent fermentation process, the sugars are transformed to VFA (predominantly acetate, propionate and butyrate), carbon dioxide and hydrogen. MB utilise hydrogen and carbon dioxide to form methane, which maintains a low hydrogen level in the rumen of a ruminant, while in the sulphidogenic reactors the SRB utilise the hydrogen as energy source for the sulphate removal and thus keep the partial pressure of hydrogen low (Visser, 1995). Acetate, propionate and butyrate typically represent around 65%, 21% and 12% of VFA formed in the rumen environment, respectively (Barnes and Keller, 2003).

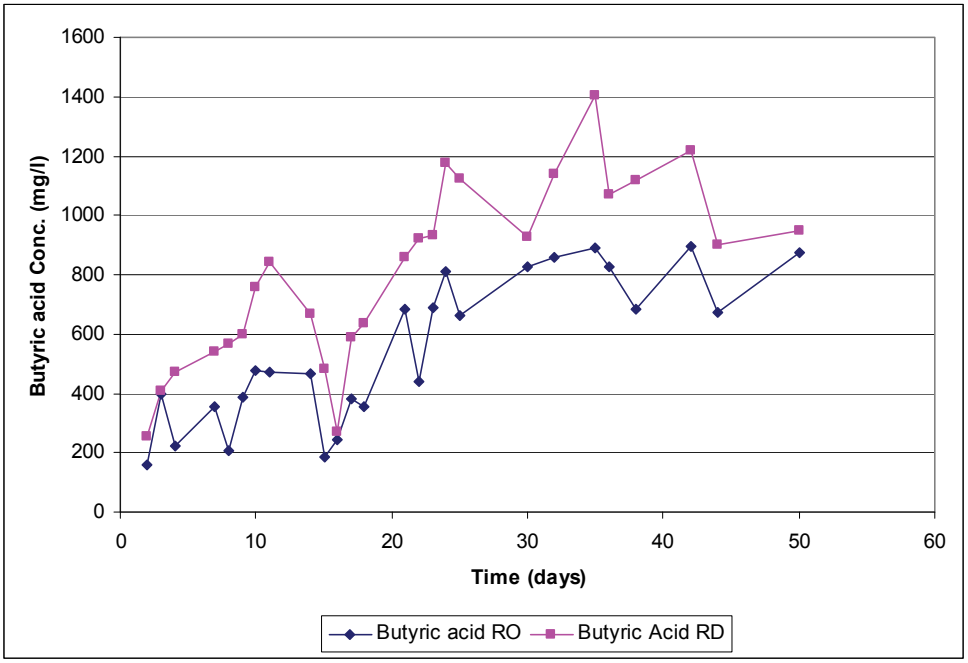

Figure 3.4. Butyric acid concentration in RD and RO

3.3.3.2 Acetic acid concentration in RD and RO

The acetate acid concentration measured in was also higher in reactor RD compared to reactor RO. The acetic, propionic and butyric acid concentrations in RD were distributed as 68%, 15% and 17%, respectively. The higher acetate concentration in RD was, in addition to hydrolysis and fermentation from cellulose, ascribed to the hydrolysis of propionic and butyric acids (Equations 3.1 and 3.2)

$$C_2H_5COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2 \quad (3.1)$$

$$C_3H_7COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2 \quad (3.2)$$

3.3.4 Average concentrations of the main experimental parameters

From the daily analyses results, the average values of the parameters were calculated (Table 3.2). The average COD concentration in RD (9 044 mg/ℓ) was higher than in RO (6 766 mg/ℓ) as were the VSS concentrations (3 866 and 1 881 mg/ℓ in RD and RO, respectively) and the VFA concentrations. The acetic and butyric acid concentrations were higher than the propionic acid concentration in both reactors, while theoretically more propionic than butyric acid is formed (Barnes and Keller, 2003). The increased concentrations in RD were ascribed to the daily addition of RI.

Table 3.2. Average results of COD, VSS and VFA in RO and RD

Parameters	**RO**	**RD**
COD (mg/l)	6 766	9 044
VSS (mg/l)	1 881	3 866
Acetate	1 277	1 578
Propionic	476	701
Butyrate	543	801

3.4 CONCLUSIONS

The following conclusions were drawn from this study:

1. The daily supplementation of GC in both reactors maintained a stable substrate level for the RI.
2. The daily supplementation of RI to RD was beneficial for improved cellulose degradation since it resulted in higher COD, VFA and VSS concentrations. Since the grass was autoclaved prior to use the increase of VSS due to the indigenous grass microbes could be ignored.
3. The daily addition of rumen bacteria enhanced the cellulose degradation and resulted in increased VFA production. This observation is beneficial for the biological sulphate reduction, since the SRB utilize the VFA produced as the carbon and energy sources.

3.5 REFERENCES

APHA, (1985) Standard Methods for the Inc. Examination of Water and Wastewater 16th Edition, Washington DC.

Barnes, S.P. and Keller, J (2003). Cellulosic waste degradation by rumen-enhanced anaerobic digestion. Water Science and Technology. IWA Publishing, Vol. **48:** No. 4, 155-162.

Greben, H.A. and Baloyi, J. (2004). The beneficial use of a bio-waste product in the biological sulphate removal technology. Wisa Biennial Conference and Exhibition, Cape Town, South Africa, May 2-6, 2004.

Harmsen, H.J.M. (1996). Detection, phylogeny and population dynamic of synthrophic propionate-oxidizing bacteria in anaerobic sludge. *PhD thesis*, Wageningen Agricultural University, Wageningen.

Hungate, R.E. (1966). The rumen and its microbes. Academic Press Inc. New York, USA

Lee, S.S., Ha, J.K. and Cheng, K.J. (2000). Relative contribution of bacteria, protozoa and fungi to in vitro degradation of orchard grass cell walls and their interaction. *App. Environ Micrbiol.* **66** (9): 3807-3813.

Lynd, L.R., Weimer, P.J., Van Zyl, W. and Pretorius I.S. (2002). Microbial Cellulose Utilisation: Fundamentals and Biotechnology. *Microbiol. Mol.Biol. Rev.* **66** (3): 506-577.

Oude Elferink, S.J.W.H. (1998). Sulphate-reducing Bacteria in Anaerobic Bioreactors. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

Partridge, I. (2003) Better pastures for the tropics and subtropics. http://www.tropicalgrasslands.asn.au/pastures/kikuyu.htm

Sonakya, V., Raizada, N., Dalhoff, R and Wilderer, P.A. (2003). *Wat., Sci. and Technol*. Vol **48 (**8): 255-259

Visser, A. (1995). The anaerobic treatment of sulphate containing wastewater. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

CHAPTER 4.

THE EFFECT OF DAILY SUPPLEMENTATION OF MICROORGANISMS ON BIOLOGICAL SULPHATE REMOVAL

4.1 INTRODUCTION

The metallurgical and mining industries produce wastewater containing high concentration of sulphate. The biological sulphate reduction technology is a known anaerobic, microbial decomposition process, which is dependent on the presence of sulphate and a suitable carbon and energy source (Hedin *et al*., 1989; Dvorak *et al*., 1991). A disadvantage of this particular mine water 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). The hydrolysis of organic waste products produce soluble intermediates due to the presence of exoenzymes e.g. cellulases, amylases, proteases and lipases (Sonakya *et al.,* 2001).

VFA are products of the anaerobic digestion of complex organic material, such as grass-cellulose. The effective conversion of complex organic material into the final product e.g. methane depends on the combined activity of a diverse microbial population consisting of various genera of obligate and facultative anaerobic bacteria. When sulphate is present, the anaerobic degradation of organic wastes 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).

The aim of the study was to investigate the effect of:

1. Daily addition of RI
2. Daily addition of additional SRB and RI
3. No addition of RI nor SRB to the reactor
4. Weekly addition of GC rather than daily

on the sulphate removal rate in a bio reactor, containing grass cuttings (GC), rumen fluid, sulphate feed water, SRB and nutrients.

In order to reach the objectives, four studies were carried out, viz.:

Study 1: The effect of the daily addition of GC and RI on biological sulphate reduction.

Study 2: The effect of the additional supplementation of GC, RI and SRB on biological sulphate reduction.

Study 3: The effect of omitting the daily addition of RI and SRB on the biological sulphate reduction.

Study 4: The effect of reducing GC feed from daily to weekly on biological sulphate reduction.

4.2. MATERIALS AND METHODS

4.2.1 Feed water

Artificial feed water (SO_4 concentration of 2.5 g/ℓ, using $MgSO_4$, Crest Chemicals, Johannesburg) to which micro nutrients (Chapter 3, Table 3.1) were used. A stock solution with the same SO_4 concentration (2.5 g/ℓ) was used to replace the 100 mℓ sample volume.

4.2.2 Reactor systems and biomass

Study 1 – Period 1

Two stirred batch reactors, ED (investigative reactor) and OO (control reactor) (Vol. of both ED and OO: 2.5 ℓ) were operated. Both reactors received 150 g autoclaved grass cuttings and 15 ml concentrated RI. More grass cuttings were added in this study as compared to the study in Chapter 3, since the higher substrate level would result in a higher initial COD concentration and the reactors were slightly larger (by volume) (20%). Both ED and OO received 5 g/d autoclaved GC to maintain the high COD concentration and ED received 15 mℓ/d RI. The RI was obtained from fistulated ruminants and stored in an incubator at 37°C at the CSIR laboratories, prior to use. The composition of the RI changed as soon as it was removed from the ruminant and underwent several changes before it entered the bio-sulphate removing reactors at the CSIR. Since the aim of the study is to adapt RI to the conditions in these bioreactors, this change is to be expected. The reactors were operated at ambient temperature (22-25°C) throughout the experimental period.

Study 2 – Period 2

After 55 days of operation, both ED and OO received an additional 5 mℓ SRB to investigate whether the additional daily supplementation of SRB would enhance the biological sulphate reduction efficiency in the reactors.

Study 3 – Period 3

On day 68 the daily addition of RI and SRB were stopped to investigate whether the developed populations of cellulose-degrading and sulphate reducing microbes populations could be maintained under the prevailing conditions in the bioreactor. GC was added as before.

Study 4 – Period 4

The effect on the daily addition of GC (on day 102) was changed to weekly addition with the aim to investigate whether sufficient COD concentration could be maintained in the bioreactor. Due to a fault in the pH controller (day 76), reactor OO received an oversupply of acid and could not be used for further studies.

4.2.3 Carbon and energy source

Grass cuttings were used as the source of cellulose, of which the formed fermentation products served as the carbon and energy source in the reactor.

4.2.4 Experimental

The four studies as executed in ED and OO during the four periods are presented in Tables 4.1 and 4.2 respectively.

Table 4.1. Experimental periods Studies 1- 4 for ED.

Period (days)			
Day (1-55)	**Day (55-68)**	**Day (68-75)**	**Day (102-132)**
Study 1	Study 2	Study 3	Study 4 (ED only)
1. Daily addition of GC 2. Daily addition of RI	1. Daily addition of GC 2. Daily addition of RI 3. Daily addition of SRB	1. Daily addition of GC	1. Weekly addition of GC

Table 4.2. Experimental periods Studies 1- 3 for OO.

Period (days)		
Day (1-55)	**Day (55-68)**	**Day (68-75)**
Study 1	Study 2	Study 3
1. Daily addition of GC	1. Daily addition of GC 2. Daily addition of SRB	1. Daily addition of GC

4.2.5 Sampling

Daily samples (100 mℓ/d), except during the weekends, were taken from the two reactors throughout the study period. In order to replace the sample volume 100 mℓ of the SO_4 rich stock solution (see 4.2.1) was added to the reactors of which the volume was 2.5 ℓ. The additional daily sulphate load added to the reactor was thus 100 mℓ/2 500 mℓ * 2 500 mg/ℓ = 100 mg SO_4/d (except weekends).

4.2.6 Analytical

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

4.3 RESULTS

4.3.1 Sulphate removal

Study 1: Daily addition of RI in ED and GC to ED and OO

The results of Study 1 are presented in Figure 4.1 and Table 4.3. It can be observed from Figure 4.1 that the sulphate reduction in both reactors improved with time. Initially it took 14 and 18 days respectively to obtain total SO_4 removal. However during the subsequent periods, the sulphate was reduced in ED over periods of 8, 5, 4 and 5 days, respectively. The three shorter periods coincided with the increase in the VSS concentrations (Figure 4.2). This result indicated that an increase in the biomass resulted in a faster cellulose degradation and in an increased sulphate reduction.

The average improved SO_4 removal rate (mg $SO_4/ℓ/d^{-1}$) in ED (Table 4.3) was due to the daily addition of RI, which degraded grass-cellulose to VFA and other intermediates for the biological sulphate removal. It can, however, been seen from Table 4.3 that the difference in removal rate between ED and OO is initially similar at 164 versus 150 mg $SO_4/ℓ/d^{-1}$ and 117 versus 94 mg $SO_4/ℓ/d^{-1}$ from day 1-14 and from

day 15-33, respectively. This may be ascribed to the effect of adding GC and RI to the reactors at the start of the experiment. The decrease in removal rate with time between ED and OO (Table 4.3) can be ascribed to the decrease in cellulose degradation due to no further addition of RI to OO. When observing the data in Table 4.3 from day 33 to day 55, it can be noted that in both reactors the sulphate removal rate increased, albeit at lower rates in the control reactor (OO) than in the investigative reactor (ED) which implied that when the reactors were supplemented with RI a faster cellulose degradation, followed by higher sulphate removal rates could be observed. The highest sulphate removal rate observed in ED was 570 mg $SO_4/\ell/d^{-1}$, while this was 420 mg $SO_4/\ell/d^{-1}$ in OO.

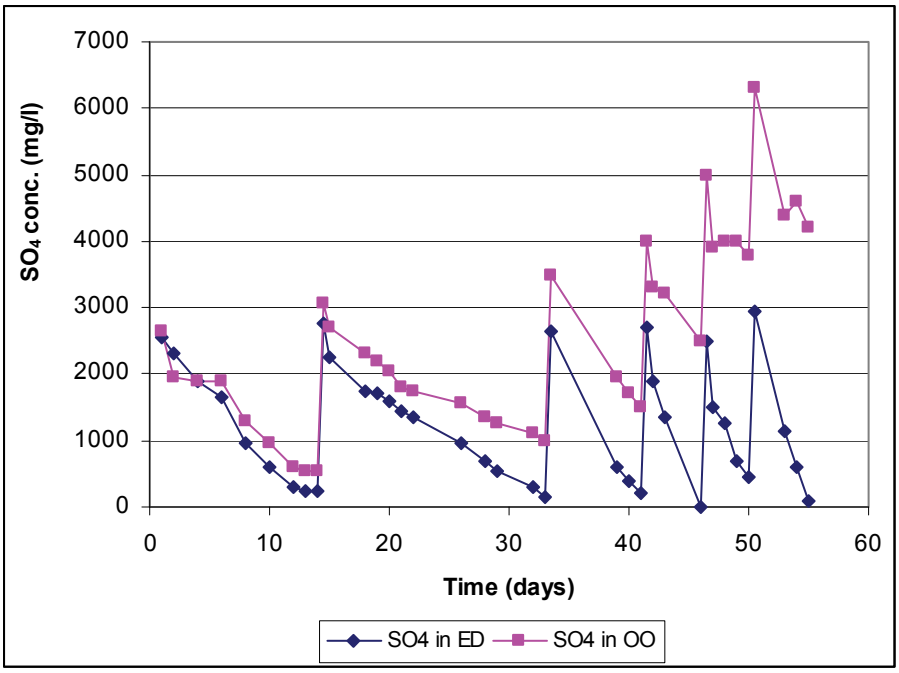

Figure 4.1. Sulphate reduction during Study 1 in ED and OO

Table 4.3. Sulphate removal in ED and OO during Study 1

Period (days)	SO_4 removal (mg $SO_4/\ell/d^{-1}$)		Total SO_4 removal(days) in ED
	ED	OO	
1-14	164	150	14
15-33	117	94	18
33-41	306	250	8
41-46	540	160	5
46-50	512	300	4
50-55	570	420	5

Study 2: **Daily addition of RI to ED and of SRB and GC to ED and OO**

The results of Study 2 are presented in Figure 4.2 and Table 4.4. The graphs in Figure 4.2 showed that although the overall SO_4 concentration in ED was lower than in OO, the SO_4 removal trend seemed similar in both reactors. It can be observed from Table 4.4 that the initial SO_4 removal rates in both reactors were considerably higher (46 and 47.5%, respectively) during Study 2 as compared to Study 2 at 1 050 and 800 mg $SO_4/\ell/d^{-1}$, respectively. Total sulphate removal in ED was achieved in short periods of 2, 7 and 4 days. It can be observed that when the duration for total sulphate removal was 7 days, the sulphate removal rates in ED and OO were very similar at 457 and 471 mg $SO_4/\ell/d^{-1}$. During the period day 65-68, the SO_4 removal rate in ED was higher compared to that in OO and it took only 4 days to achieve total SO_4 removal.

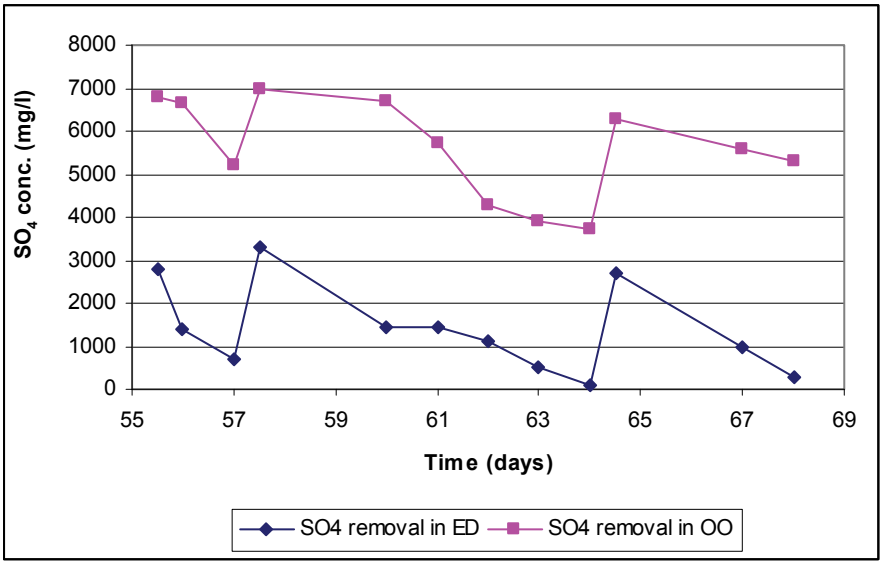

Figure 4.2. Sulphate reduction during Study 2 in ED and OO

Table 4.4. Sulphate removal in ED and OO during Study 2

Period (Days)	SO_4 removal (mg $SO_4/\ell/d^{-1}$)		Total SO_4 removal (days) in ED
	ED	OO	
55-57	1050	800	2
57-64	457	471	7
65-68	600	250	4

The obtained results indicated that SRB supplementation resulted in higher SO_4 removal rates, at least initially. The difference in SO_4 removal rate in ED and OO during period day 65-68 can possibly be ascribed to the fact that OO was not

supplemented with RI, which implied that the additional cellulose degrading microorganisms added to ED resulted in a higher VFA production in ED. However, when comparing the results in OO of Study 2 with that of Study 1, it was noted that the sulphate removal rate in OO generally improved, especially from day 55-57, when the sulphate removal rate was 800 mg $SO_4/\ell/d^{-1}$. This result indicated that the addition of SRB most likely contributed to the degradation of polymers to VFA or due to the presence of additional SRB, the sulphate reduction progressed faster. SRB are known to assist in the degradation of organic matter to produce propionic acid in the presence of sulphate as the energy source for the biological sulphate removal (Harmsen, 1996; Oude Elferink, 1998).

Study 3. **Daily addition of GC in ED and OO**

The results of Study 3 are given in Figure 4.3 and Table 4.5, respectively. It can be observed from Figure 4.3 that the sulphate reduction progressed in ED at a longer time of 7, 10 and 13 days, respectively. The sulphate removal rates from day 68-75 in ED were similar to that in OO (393 and 364 mg $SO_4/\ell/d^{-1}$, respectively), most likely due to the fact that ED did not receive additional RI anymore during this period. The SO_4 removal rates in OO could thereafter not be monitored anymore, due to a faulty pH controller. During the following periods, the SO_4 removal rate in ED decreased to 185 and 205 mg $SO_4/\ell/d^{-1}$over periods of 10 days to achieve total SO_4 removal. The lower SO_4 removal rates from day 75-99 can be ascribed to the cessation of adding microbial populations. This result is disappointing as it was expected that the addition of RI during the first two studies would have resulted in a strong and healthy RI population in the reactor.

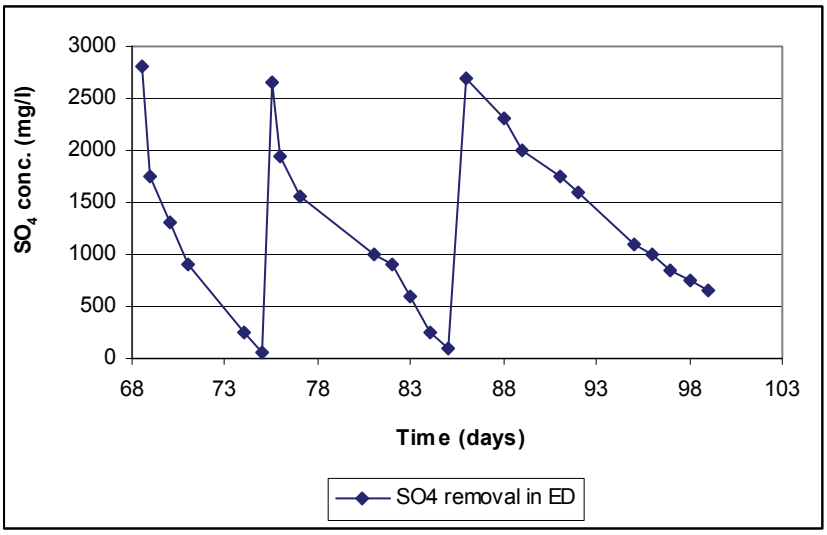

Figure 4.3. Sulphate reduction during Study 3 in ED

The VSS concentrations, presented in Figure 4.6, showed that the biomass concentration decreased on day 70, which coincided with the first period of Study 3. After day 90, the VSS concentration decreased till values <5 g/ℓ, which resulted in the slower sulphate removal during Study 3.

Table 4.5. Sulphate removal from ED and OO during Study 3

Period (days)	SO_4 removal (mg SO_4/ℓ/d^{-1})		Total SO_4 removal (days) in ED
	ED	OO	
68-75	393	364	7
75-85	185		10
89-99	158		>13

Study 4: **Weekly addition of grass cuttings to ED reactor**

The data obtained from Study 4 are given in Table 4.6, from where it can be observed that the sulphate removal was 144 mg SO_4/ℓ/d^{-1}. During this period a total of 2 600 mg/ℓ sulphate removal was achieved over a period of 18 days (day 102-132). The longer duration of sulphate removal was ascribed to lower concentration of microorganism (Figure 4.6) when after day 100, the VSS concentration in ED decreased to less than 5 g/ℓ, while after biomass supplementation the VSS concentration in ED increased to concentrations of 25 g/ℓ. The lower sulphate removal over a longer period of 18 days might indicate that the microorganisms had adapted to the daily addition of GC as had occurred in Studies 1-3.

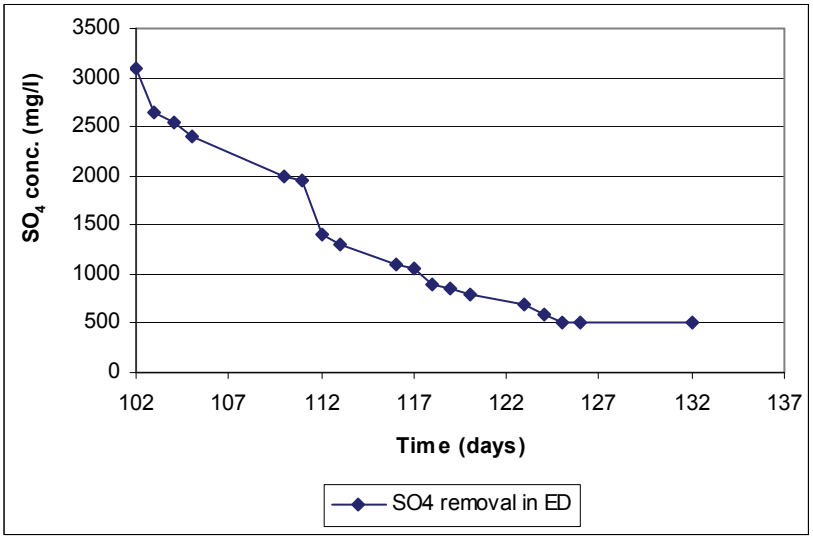

Figure 4.4. Sulphate reduction during Study 4 in ED

Table 4.6. Sulphate removal from ED during Study 4

Period (days)	SO_4 removal (mg $SO_4/\ell.d^{-1}$)	Duration
102-132	144	18 days

4.3.1.1 The comparison of the four studies

The highest sulphate removal rates achieved during the four studies are presented in Table 4.7. It can be observed from the data obtained from the four studies that the highest and fastest sulphate reduction in ED was achieved during Study 2, when ED received daily supplementation of GC, RI and SRB. In ED the highest removal rate obtained was 1050 mg $SO_4/\ell.d^{-1}$ during Study 2, while this was 420 mg $SO_4/\ell.d^{-1}$ in the control reactor, which also did receive SRB. The addition of the SRB favoured the sulphate reduction, more than the supplementation of the fermentation bacteria. The sulphate removal rates during Study 1 were higher than during Studies 3 and 4, which most likely can also be ascribed to the initial addition of the GC and microorganisms. The obtained results furthermore showed that during Study 1, the SO_4 removal rate in ED increased from 164 to 570 mg $SO_4/L/d^{-1}$, while it was as high as 1050 mg $SO_4/\ell.d^{-1}$ during Study 2, to decrease to 158 and 144 mg $SO_4/\ell.d^{-1}$, respectively, in Studies 3 and 4, when it also took longer to achieve total SO_4 removal.

Table 4.7. The highest sulphate removal rate from ED and OO during the four studies

Supplementation	SO_4 removal (mg $SO_4/\ell/d^{-1}$)		Total SO_4 removal (days) in ED
	ED	OO	
GC/d, RI	570	420	7
GC/d, RI, SRB	1050	800	2
GC/d	393	364	7
GC/w	144		18

4.3.2 Sulphate removed/GC used

From the sulphate removal data obtained and the known amounts GC added daily it could be calculated how much SO_4 could be reduced from 1g GC over the experimental period of the four studies each. The results are presented in Table 4.8. It was observed that the highest amount of SO_4 reduced using 1 g GC was during Study 2 in both ED and OO, the period that RI as well as SRB supplementation was

added to the reactors. During Study 4, 1 g of GC removed 0.1 SO_4, which was due to the fact that only 5 g GC was added on a weekly basis to the reactors during Study 4. It can also be observed from Table 4.8 that in general a higher removal was observed in ED than in OO, except during Study 2, when an almost equal amount of SO_4 was removed from both reactors.

Table 4.8. The amount of SO_4 removed using 1 g GC

Studies	ED	OO
1 (GC+RB)	0.07	0.05
2 (GC+ RB + SRB)	0.13	0.14
3 (GC daily)	0.04	0.02
4 (GC weekly)	0.10	

4.3.3 COD utilization during Studies 1-4

The COD concentrations in both reactors are presented in Figure 4.5. The results showed that at the start of the total experimental period the COD concentration in ED was similar to that in OO. However, on day 5, the COD concentration in ED increased faster than in OO, till day 26, when in both reactors the COD concentration decreased remarkably. This drop in COD concentration could not be ascribed to an increased sulphate removal, since the data in Table 4.3 indicated that the lowest SO_4 removal rate was achieved at 117 and 94 mg $SO_4/\ell.d^{-1}$ over a period of 18 days. The average COD concentration in ED during Study 1 was 7 907 mg/ℓ, while this was 6 933 mg/ℓ in OO. From day 26, it decreased in both ED and OO from 9 000 and 7 000 mg/ℓ, respectively, till ± 2 000 mg/ℓ over a period of 17 days. This reduction in COD concentration was explained by its utilization for the sulphate removal and possibly to a lower production of COD. These lower COD concentration results may indicate that the available COD was faster utilised for the biological sulphate removal than it was produced by the cellulose degrading microorganisms. The average residual COD concentration in ED was 5 884 and in OO was 5 069 mg/ℓ during Study 1. The difference in COD concentration in the two reactors was only 14%, although ED received cellulose degrading microorganisms (RI) on a daily basis (till day 68). When both reactors received SRB during Study 2 (and ED still received the RI), the average COD concentrations in ED and OO were 2 865 and 2 218 mg/ℓ, which resulted in a 23% higher residual COD concentration in ED. Thus due to the increased SO_4 removal rate in OO during Study 2, the residual COD concentration in OO was < than that in ED.

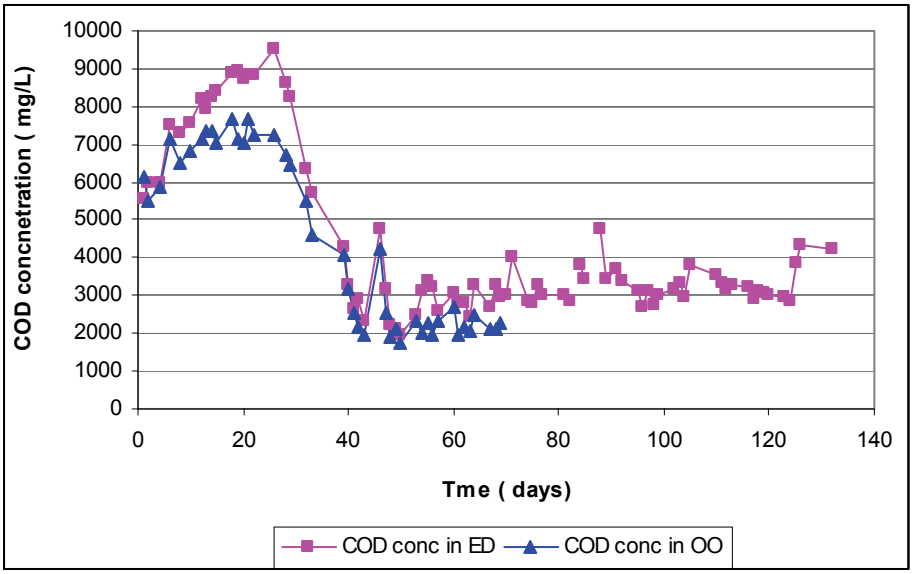

Figure 4.5. The COD concentrations in ED and OO during Studies 1-4.

4.3.4 VSS concentration during studies 1-4

The VSS concentrations during the four studies are given in Figure 4.6. It can be observed that the VSS concentration in ED and OO was similar at the beginning of the experiment till day 40. From day 40 to 50 the VSS concentration in ED increased and stabilized till day 60, where after it steeply increased. This sharp increase can be ascribed to the supplementation of SRB on day 55. The VSS concentration in OO decreased slowly till the end of Study 3, even though SRB were added to OO from day 55 to day 68. The decrease in VSS concentration in OO can not be explained, especially since both reactors received SRB during Study 2, which in ED resulted in an increased VSS concentration during Study 2 and partly Study 3. After day 69 (Study 3), a steep decrease of the VSS concentration in ED was observed. This decrease in VSS concentration occurred just after the start of Study 3 (day 68), when the supplementation of RI and SRB was stopped and decreased further into Study 4, when the daily addition of substrate (GC) was changed to weekly additions. These results were not expected since it was expected that sue to the daily addition of RI and SRB as well as the daily addition of GC (Substrate) a healthy microbial reactor population would have been established. Grass cellulose is the substrate for cellulose degrading microorganisms. The results seem to indicate that when substrate addition comes to a halt, the microbial population decreased.

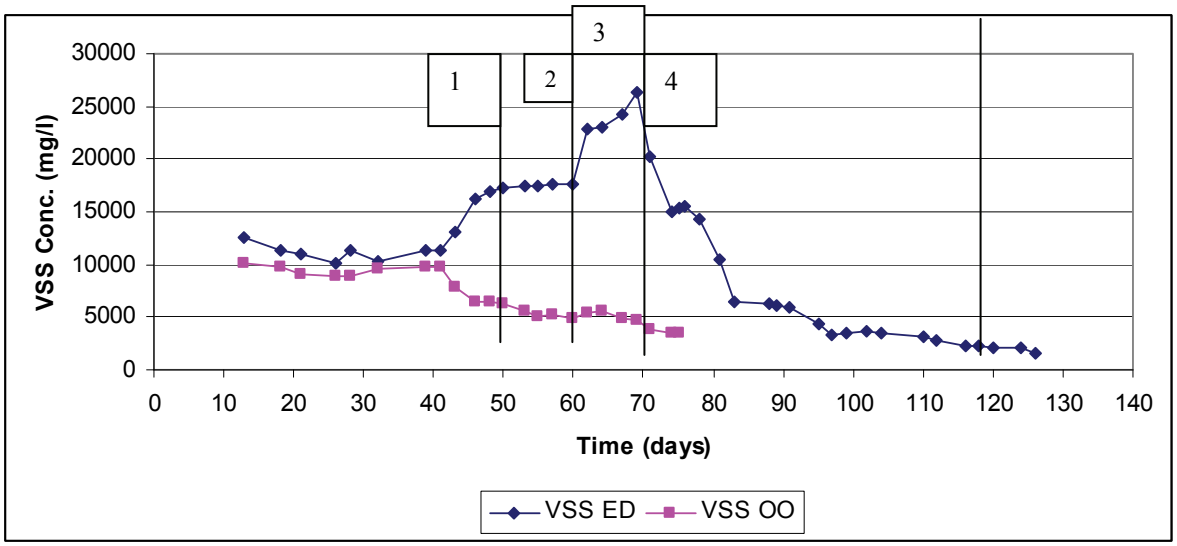

Figure 4.6. The VSS concentration in ED and OO during Studies 1-4.

4.3.5 VFA concentration in ED and OO

Cellulose degradation results in VFA production, which serves as the carbon and energy source for the biological sulphate reduction. It was observed during Study 1, that the SO_4 removal rate in ED increased with time, which implied that sufficient VFA was present for an increased SO_4 removal rate. An average residual propionic and butyric acid concentration of 40 and 53 mg/ℓ, respectively, were detected in ED during Study 1, while the acetate concentration was 158 mg/ℓ Table 4.9).

The higher acetic acid concentration in ED can be ascribed to two factors:

1) The acetate production from cellulose is higher than the propionic and butyric acid production (see Chapter 3).
2) Acetate is the oxidation product of butyric and propionic acid, according to Equations 4.1 and 4.2:

$$Propionate^- + ¾\ SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + ¾\ HS^- + ¼\ H^+ \quad (4.1)$$

$$Butyrate^- + ½\ SO_4^{2-} \rightarrow 2\ Acetate^- + ½\ HS^- + ½\ H^+ \quad (4.2)$$

During Studies 2-4 the average propionic and butyric acid concentrations decreased in ED and OO due to total utilisation of these acids during the biological sulphate reduction process. When SRB participate in the degradation of polymers and monomers, the final product of this fermentation process is acetate (Visser, 1996, Oude Elferink, 1998). The data in Table 4.9 indicate that the average acetic acid concentration in ED fluctuated, from 158 to 60 to 111 and 23 mg/ℓ, possibly indicating

that more acetate was produced than utilised for SO_4 removal. The C2 acid concentration was unexpectedly high in OO. This can possibly be ascribed to equations 4.1 and 4.2, but furthermore showed that in OO the C2 acid was not utilised for increased SO_4 removal, while this could be the case in ED. This obtained result might indicate that due to the daily addition of RI to ED, a different SRB population developed in ED as opposed to OO. Rinzema and Lettinga (1988) have described a specific acetate utilising SRB (*Desulfotomaculum acetoxidans*), which has been isolated from manure, rumen content and fresh water sediments contaminated with manure. Thus the frequent addition of RI might have resulted in the growth of a strong *D.acetoxidans* population in ED. This bacterium has a temperature optimum at 36°C and does not grow at a temperature < 10°C, but can possibly adapt at the operating reactor temperature of 25°C.

Different authors (Colleran, 1995, Omil *et al*., 1997, Lens *et al*., 1998 and Greben *et al. (*2000a, 2000b, 2004) have referred to the residual acetate concentrations in sulphidogenic bio-reactors, which was not utilised for increased SO_4 removal. The non-preference for acetate is a nutritional characteristic of SRB and even ASRB, such as *Desulforhabdus amnigenus*, still prefer propionate and butyrate to acetate (Oude Elferink, 1998).

Table 4.9. VFA concentrations in ED and OO during Studies 1-4.

Parameter (mg/ℓ)	**Period (d 1-55)**		**Period (d 55-68)**		**Period (d 68-75)**		**Period (d 75-131)**
	Study 1		**Study 2**		**Study 3**		**Study 4**
Reactors	**ED**	**OO**	**ED**	**OO**	**ED**	**OO**	**ED**
Acetate	158	**639**	60	**350**	111	**471**	23
Propionate	40	17	5	8	0	3	2
Butyrate	53	30	1	8	0	4	1

4.3.6 Sulphide, Alkalinity, Redox Potential and pH values in ED and OO

4.3.6.1 Sulphide

Sulphide is the product of the biological sulphate removal process according to equation 4.3, showing that for every mole sulphate removed, one mole sulphide is produced.

$$2CH_2O + SO_4^{2-} \rightarrow S^{2-} + 2H_2O + 2CO_2 \quad (4.3)$$

It is observed from Table 4.10, that the average sulphide concentration measured in ED was during Study 2, followed by Study 1, 3 and 4, respectively. Higher sulphide concentrations were observed in ED than in OO, which results are in agreement with the sulphate removal rates. The sulphide concentration in OO was the highest during Studies 1 and 2.

4.3.6.2 Alkalinity

As is the case with sulphide, alkalinity is the product of biological sulphate reduction. The higher alkalinity concentrations in ED compared to OO can be explained due to the higher sulphate removal rates in ED.

4.3.6.3 Redox Potential

The redox potential results decreased at higher sulphide concentrations. The pH in both reactors was controlled in the range of 6.9–7.0 as can be observed from the results in Table 4.10.

Table 4.10. Sulphide, Alkalinity, Redox and pH values in ED and OO

	Sulphide (mg/L)		**Alkalinity (mg/L)**		**Redox (mV)**		**pH (value)**	
Reactors → / **Studies ↓**	**ED**	**OO**	**ED**	**OO**	**ED**	**OO**	**ED**	**OO**
1	194	106	2460	1408	-140	-99	6.96	6.92
2	301	97	1402	313	-166	-78	7.00	7.00
3	155	70	667	154	-117	-2	6.91	6.91
4	95		278		-24		7.00	

4.4 CONCLUSIONS

The following conclusions were made of studies 1-4:

1. The daily addition of GC and RI showed an increase in the VSS (biomass) concentration, which resulted in increased sulphate removal rates, most likely due to an increased cellulose degradation and VFA production.

2. The effect of the additional supplementation of SRB in ED and OO enhanced the sulphate reduction efficiency, achieving a sulphate removal rate of 1 050 mg $SO_4/\ell.d^{-1}$ in ED, while this was 8 00 mg $SO_4/\ell.d^{-1}$ in OO. Total SO_4 removal was achieved over 2 days in ED. These results seemed to indicate that the SRB assisted in the grass-cellulose degradation to produce additional VFA for increased biological sulphate reduction.

3. When the microbial supplementation to ED and OO was ceased, the sulphate removal rate decreased in both reactors, while the COD concentration remained stable. Furthermore the sulphate reduction was observed over longer periods (in days), which indicated that the sulphate removal seemed dependant on the supplementation of maybe the RI and surely the SRB.

4. The VSS concentrations in both reactors provided unexpected results, although during Studies 1 and 2, the VSS concentration increased due to the daily supplementation of the biomass. As soon as this supplementation was stopped in ED, the VSS decreased sharply, indicating that no stable population had formed in the reactor during the periods that additional biomass was added. When the substrate supplementation changed from daily to weekly, the biomass (VSS) concentration decreased even further.

5. When the daily addition of GC changed to weekly addition, the sulphate removal rate of 144 mg $SO_4/\ell.d^{-1}$ was obtained over a period of 18 days. During that period, the VSS concentration in ED decreased further.

6. Higher residual acetate concentrations were noted in OO than in ED. This could be ascribed to a specific acetate utilising SRB bacterium *Desulfotomaculum acetoxidans (*possibly added to ED with the daily addition of RI), which originates from rumen and which can use acetate for biological sulphate removal.

7. The highest sulphate reduction rate of 1 050 and 800 mg $SO_4/\ell.d^{-1}$ in ED and OO, respectively, was achieved with daily supplementation of GC, RI and SRB to the reactor.

4.5 REFERENCES

Coetser, S.E., Cloete, T.E. and Zdyb, L. (2000). Biological sulphate reduction in artificial acid mine drainage using different carbon sources. *Proceeding Y2K Millennium Meeting, Grahamstown 23-28 January, 2000*: 606.

Colleran, E., Finnegan, S. and Lens, P. (1995). Anaerobic treatment of sulphate-containing waste streams. *Antonie van Leeuwenhoek* **67:** 29-46.

Dill, S., Cloete, T.E., Coetser, L. and Zdyb, L. (2001). Determination of the suitability of alternative carbon sources for sulphate reduction in the passive treatment of mine water. *WRC Report*: 802/1/01.

Dvorak, D.H., Hedin, Edenborn, H.M., and P.E (1991). Treatment of metal-contaminated water using bacterial sulphate reduction: results from a pilot scale reactor, *Biotech. Bioeng*. **40 :** 609.

Greben, H.A., Maree, J.P. and Mnqanqeni, S. (2000a). The comparison between sucrose, ethanol and methanol as carbon and energy source for biological sulphate reduction. *Water Sci.Technol*. **41**(12)**:** 247-253.

Greben, H.A., Maree, J.P., Singmin, Y. and Mnqanqeni, S. (2000b). Biological sulphate removal from acid mine effluent using ethanol as carbon and energy source. *Water Sci.Technol*. **42**(3-4)**:** 339-344.

Greben, H.A., Tjatji M.P. and Maree, J.P. (2004). Biological sulphate removal at different feed COD/SO_4 ratios using acetate and propionate as the carbon and energy source. (2004). Proceedings Mine water 2004, Process, Policy and Progress, Newcastle-upon-Tyne, UK. 19-23 September 2004.

Harmsen, H.J.M. (1996). Detection, phylogeny and population dynamic of synthrophic propionate-oxidizing bacteria in anaerobic sludge. *PhD thesis*, Wageningen Agricultural University, Wageningen.

Hedin, R.S., Hammack, R.W., Hyman, D.M., 1989. Potential importance of sulfate reduction processes in wetlands constructed to treat mine drainage. In: Hammer, D.A. (Ed.) Constructed Wetlands for Wastewater Treatment. Lewis Publishers, Chelsea, MI. 508-514.

Lens, P.N.L., Van den Bosch, M.C., Hulshoff Pol, L.W. and Lettinga, G. (1998). Effects of staging on Volatile Fatty Acid Degradation in a Sulfidogenic Granular Sludge Reactor. *Wat.Res*. **32** (4):1178-1192.

Omil, F., Lens, P., Visser, A., Hulshoff Pol, L.W. and Lettinga, G. (1997). Long term competition between Sulfate Reducing and Methanogenic Bacteria in UASB reactors treating Voltile Fatty Acids. *Biotechnol. Bioeng.* **57:**667-685.

Oude Elferink, S.J.W.H. (1998). Sulphate-reducing Bacteria in Anaerobic Bioreactors. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

Rose, P.D. (2000). The Rhodes Biosure Process: The piloting of an active process for the treatment of acid mine drainage wastewaters. *Proceeding Y2K Millennium Meeting*. Grahamstown 23-28 January, 2000: 605-606.
Rinzema, A. and Lettinga, G. (1988). Anaerobic treatment of sulfate containing wastewater. In: Biotreatment systems, **3:** (Wise, DL, Ed). CRC press, Inc., Boca Raton, Florida. Pp 65-109

Sonakya, V., Raizada, N. and Kalia V.C. (2001). Microbial and Enzymatic Improvements on Anaerobic Digestion of Waste Biomass. *Biotechnol. Letters*. **23** (18):1463-1466.

CHAPTER 5

THE EFFECT OF AN INDIGENOUS MICROBIAL POPULATION IN THE CELLULOSE DEGRADING/SULPHATE REMOVING BIOREACTORS

5.1 BACKGROUND

A biological sulphate removal technology has been developed, using the degradation products of plant biomass (GC) as carbon sources. The rumen is a highly cellulosic ecosystem with a complex microbial population able to utilise the cellulose in grass cuttings for anaerobic fermentation, producing VFA (Barnes and Keller, 2003). The produced VFA can be used as the carbon and energy sources for SRB, which facilitate biological sulphate removal.

From a previous study, it was observed that a consortium of 6 isolated compost bacteria were out-competed when grass cuttings were added to GC degrading and sulphate removing bioreactors. Therefore, in order to investigate whether a similar competition occurred using rumen fluid microorganisms, the emphasis in this study was on the use of ***autoclaved*** and ***non-autoclaved*** GC within the reactors. Alternatively, the native grass microbes added to the reactor with the cut grass, may assist in grass fermentation, which would be very beneficial for the degradation process. In order to obtain an understanding of the microbial ecology of these systems, the utilisation of ***autoclaved*** and ***non-autoclaved*** grass cuttings should be investigated.

Terminal restriction fragment length polymorphism (t-RFLP) is a sensitive technique used for strain identification and comparative bacterial community analysis (Marsh, 1999). T-RFLP results in fingerprint profiles of microbial communities consisting of labelled terminal restriction fragments (t-RF). These community profiles can easily be compared to other community profiles examined over specified time periods to observe changes within the community. A clear understanding of the variation in the microbial community due to changing external factors may provide answers to questions relating to the function of the different microbial populations and consortia in the described bio-reactors.

Two studies were executed with the following objectives:

Study 1

1. To investigate the effect of autoclaved and non-autoclaved grass on the degradation of grass cellulose (by means of COD, VFA, SO_4 and VSS concentration observations).

2. To investigate the effect of the addition of different amounts of GC on biological sulphate removal

Study 2

1. To investigate the diversity, structure and function of the microbial communities in sulphate removing bioreactors using t-RFLPs.

5.2 MATERIALS AND METHODS

STUDY 1

5.2.1 Autoclaved Grass

Grass was autoclaved for 30 minutes to remove microorganisms. Autoclaved and non-autoclaved GC were placed on nutrient agar (Biolab) and allowed to incubate for 48 hrs at 37°C. Autoclaved grass plates were compared to the control non-autoclaved grass plates to determine whether 30 minutes autoclave time was sufficient to sterilize the grass.

5.2.2 Reactors and Biomass

Two batch reactors A and NA, with a volume of 2.5 ℓ each, were operated. The reactors were treated identically with the exception of the addition of GC, which was non-autoclaved to NA and autoclaved to A for the duration of the experiment. At the start of the experimental period, both A and NA received:

150 g GC
100 mℓ RI
350 mℓ SRB
2,5 g/ℓ $MgSO_4$ diluted in tap water

5.2.3 Experimental

The reactor temperatures were maintained at 25°C by heating jackets surrounding the reactors, connected to a recycling water bath. 1N HCl (Merck) and 1N NaOH (Merck) were used to maintain the pH of the reactors between 6.7 and 7.0. The reactors were filled with a 2.5 g/ℓ $MgSO_4$ (Saarchem) solution in order to compensate for loss of volume due to sampling. When the sulphate concentration was reduced to below 500 mg/ℓ, 16 g of $MgSO_4$ dissolved in the liquid from the reactor contents was added to the reactors. On day 25, 15 mℓ SRB was added and on day 28, 15 mℓ RI was added to both reactors. Four experimental periods were observed as indicated in Table 5.1. During Period 1, (day 1-29) there was no GC addition. During Period 2 (day 30-58), 35 g of GC were added every 7 days to maintain a high COD concentration for sustained sulphate (SO_4) reduction. During Period 3, (day 59-105), 50 g of grass was added every 7 days and during Period 4 (day 105-151), 20 g of grass was added every 7 days. The GC loading for periods 2-4 were approximately 5 g/day, 7 g/day and 3 g/day respectively.

Table 5.1. Grass addition during experimental periods

Period	1	2	3	4
Day	1-29	30-58	59-104	105-151
GC/week	0	35 g	50 g	20 g

5.2.4 Analytical

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

STUDY 2

5.2.5 Molecular Analysis

Samples were selected for molecular analysis based on the occurrences of chemical or biological changes within the reactors, described in Study 1.

5.2.5.1 Total Genomic DNA Extraction

The total genomic DNA was extracted from the samples by means of an adapted CTAB method (personal communication, Mrs E. Eloff, CSIR 2005). Before collecting cells by centrifugation, the samples were placed on ice and sonicated for 30 seconds using a sonication probe. Biomass (1.5 mℓ) collected from the bio-reactors was centrifuged at 13 000 rpm. The supernatant was discarded and the biomass pellet was re-suspended in 565 µℓ of TE buffer (10 mM Tris-HCl; 1mM EDTA; pH 8.0) and

5 µℓ of 50 mg/mℓ lysozyme was added. The suspension was incubated at 37°C for 30 minutes. Thirty µℓ of 10% sodium dodecyl sulphate (SDS) and 5 µℓ of 20 mg/mℓ Proteinase K were added and the suspension was mixed and incubated at 45°C for 1 hour. After incubation, 100 uℓ of 5M NaCl was added. The suspension was mixed thoroughly and 80 uℓ of CTAB/NaCl solution was added. The suspension was mixed and incubated at 65°C for 25 minutes. After incubation, an extraction was performed using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The suspension was centrifuged at 13 000 rpm for 5 minutes to separate the phases and the top aqueous phase was transferred to a new Eppendorf tube. This step was repeated until the interface dividing the phases appeared clean. Precipitation of the genomic DNA was performed by adding 0.6 volume of 96% ethanol to the aqueous phase followed by incubation on ice for 10 minutes. After centrifugation for 20 minutes the supernatant was removed and the DNA pellet was washed by adding 70% ethanol and centrifuged again at 13000 rpm for 5 minutes. The supernatant was removed, the DNA pellet air-dried and re-suspended in 50 µℓ of nuclease free water (Roche).

5.2.5.2 16S Ribosomal Gene Amplification

Polymerase chain reaction (PCR) amplification of the 16S rDNA gene was performed using Eubacterial universal primers (Table 5.2).

Table 5.2. 16S PCR primer sequences

Primer	Sequence
341F	5'CCTACGGGAGGCAGC3'
16R1522pH	5'ACGCCGACCTAGTGGAGGA3'

PCR reactions contained 25 mM $MgCl_2$, 5 µℓ of reaction buffer (10 X), 1 unit of taq DNA polymerase (Southern Cross), 2.5 mM dNTPs, and 10 µM of each primer indicated in Table 1. Genomic template DNA was diluted 1:15 to 1:20 times. Of these dilutions, 5 µℓ was used in the PCR reaction and the total reaction volume was made up to 50 µℓ with nuclease free water (Roche). A hot start PCR was performed at 94°C for 3 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. A final extension step at 72°C for 4 minutes was included.

5.2.5.3 16S Ribosomal Gene Purification

Successful amplification of 16S ribosomal DNA (rDNA) fragments was followed by purification with the Zymo Research Kit (Inqaba Biotechnologies), to remove excess primers and PCR reagents. A final volume of 30 µℓ purified 16S rDNA was retained. The concentration of the 16S ribosomal DNA products was determined by comparison of the purified PCR products to a 1 Kb DNA ladder (New England Biolabs).

5.2.5.4 Restriction Enzyme Digestions

Five different restriction enzymes (RsaI, MspI, HhaI, HaeIII and Sau96I) were selected to digest the purified 16S ribosomal DNA. The restriction enzymes are four base-pair (bp) cutters. The total volume of each restriction enzyme reaction was 25 µℓ. Each reaction contained 1 ng of purified 16S rDNA, 2.5 µℓ of 10 X reaction buffer and 1 unit of restriction enzyme and was made up to a final volume with nuclease free water. The digestions were performed at 37ºC for 1 hour. Digestion reactions were placed on ice until loading on the LI COR DNA sequencing system.

5.2.5.5 Polyacrylamide Gel Electrophoresis (PAGE)

After digestion of the 16S rDNA with the respective restriction enzymes, the terminal restriction fragments generated were separated using PAGE and the IR^2 Global Edition DNA Analyser (LI COR, Lincoln, USA). An 8% polyacrylamide gel was prepared using 20 mℓ of gel solution (Long Range Gel), 150 µℓ of 0.1 g/mℓ APS and 15 µℓ of TEMED. The gel solution was poured between glass plates, which were washed with a 1% SDS solution and the gel was allowed to set for 1 hour. The solidified gel was mounted in the LI COR sequencer and the buffer tanks were filled with 0.8 X TBE buffer. The gel was subjected to a 30 minute pre-run with the following settings: Set Voltage: 1500 V; Set Current: 35 mA; Set Power: 35 W; and Set Temperature: 45ºC. After the pre-run, 0.8 to 1.5 µℓ of the digested 16S rDNA was loaded onto the gel with a 700 bp standard marker. Prior to loading, the digested 16S rDNA was denatured at 90ºC for 3 minutes and then kept on ice until loaded. Electrophoresis was performed at the same settings as the pre-run for a period of 4 hours. A control was included for each gel run with a known terminal restriction fragment band pattern.

5.2.5.6 PAGE Gel Image Analysis

The generated PAGE image was analysed using Bionumerics® computer software. The identification of the terminal restriction fragments was based on the visualisation

of the specific terminal restriction fragment. Bionumerics® calculates the sizes of the unknown terminal restriction fragments by using the percentage migration of each band and the size of each band from the internal size standard.

5.2.5.7 Data Analysis

The Phylogenetic Assignment Tool (PAT), provided by Wisconsin University, was used to assign bacterial species to specific terminal restriction bands. The terminal restriction band length values were entered into the program in the form of tab delimited Excel files. The software used restriction data from all five enzymes to compute all possible combinations and represented bacterial species for each set of values.

5.3 RESULTS AND DISCUSSION

STUDY 1

5.3.1 Autoclaved and Non-autoclaved Grass

After 48 hours of incubation, no growth was observed on the plates with the grass autoclaved for 30 minutes in comparison to the non-autoclaved grass. This result indicated that 30 minutes was sufficient sterilization time to remove the microorganisms from the grass cuttings.

5.3.2 Sulphate Reduction and COD

Period 1

From day 11, the sulphate reduction occurred much faster in reactor NA than A (Figure 5.1, showing the actual sulphate removal (mg/ℓ) in the reactors), while the COD concentration was noticeably lower in NA than A (Figure 5.2, showing the actual COD concentration (mg/ℓ) in the reactor), indicating that the COD was being used for sulphate reduction. SRB were added on day 25 and RI on day 28, to stimulate microbial activity, however, these additions had little effect on the COD concentration increase (Figure 5.2). During this period, the sulphate removal efficiency was on average 20% higher in reactor NA than A.

Period 2

On day 30, 35 g GC was added to both A and NA, which was repeated every 7 days over a period of 28 days (Table 5.1) with the aim to increase the COD concentration. An increase in sulphate removal and in COD was observed in both reactors (Figures

5.1 and 5.2). The sulphate removal efficiency continued to be 7% higher in reactor NA when compared to A.

Period 3

The addition of 50 g GC to both A and NA on day 59 and every 7 days thereafter over a period of 45 days (Table 5.1) resulted, from day 63 in sulphate reduction, occurring much faster in A than NA during this period (Figure 5.1). A higher COD concentration was observed in reactor NA than reactor A, due to the higher COD utilisation in A than NA (Figure 5.2). Reactor A removed on average 29% more sulphate than NA.

Period 4

From day 105, the addition of 20 g of GC resulted in a faster sulphate reduction in NA than A during this period (Figure 5.1), with NA removing 30% more sulphate than reactor A. In both reactors a decrease in COD concentration was observed, however the COD concentration gradually increased in reactor A (Figure 5.2). The large difference between A and NA could be attributed to the stirrers tripping on day 134 resulting in the pH controllers pumping excess acid to pH 3.3 in A. The low pH could have affected the microbial population in reactor A negatively and could have accounted for the decrease in sulphate removal. However, from day 106, the activity of the microbial population in A seemed to show a gradual trend of decreasing sulphate removal (Figure 5.1).

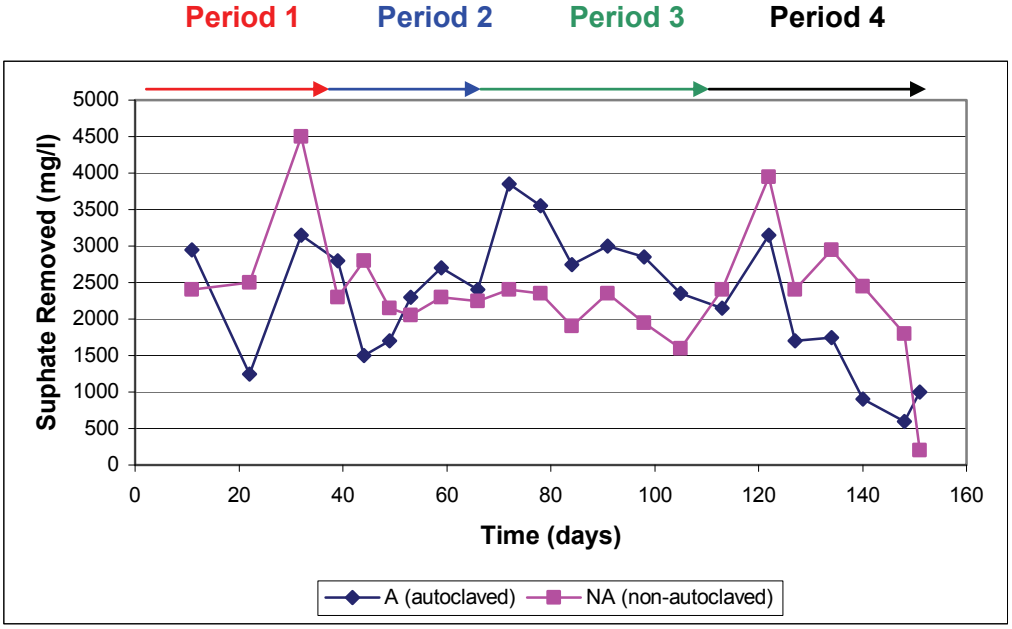

Figure 5.1. Sulphate removal in reactors A and NA

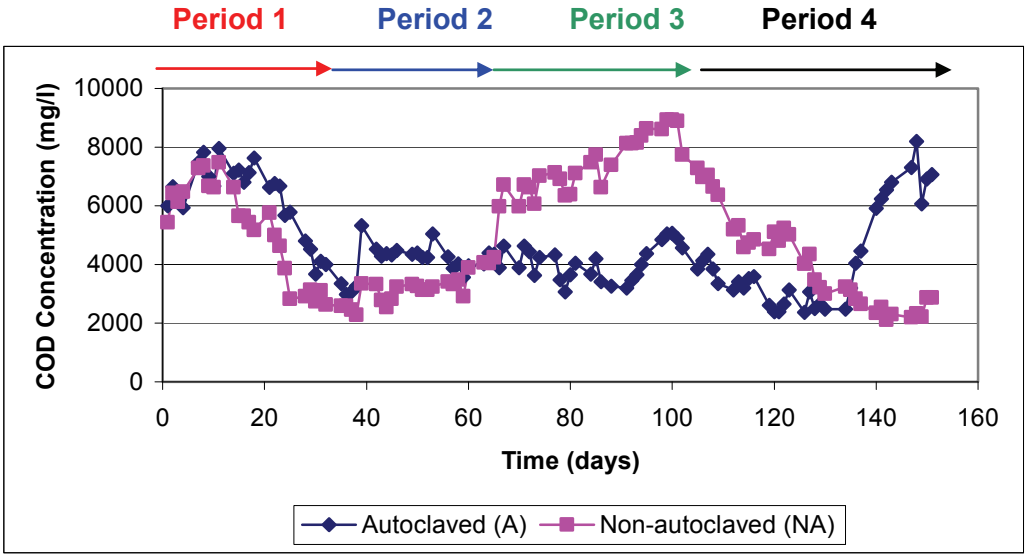

Figure 5.2. COD concentration in reactors A and NA

The presented results showed that improved sulphate reduction was obtained when higher concentrations of COD were available. The addition of SRB and RI did not increase the COD concentration when insufficient grass substrate was available. Although NA displayed increased sulphate removal efficiency in Periods 1 and 2, A showed increased sulphate removal during Period 3, when a higher GC concentration was added. During Period 4, reactor A showed a gradual decrease in sulphate reduction during this period to below that of reactor NA. The COD in both reactors decreased due to lowering the concentration of GC. It was observed during the total experimental period, that when high sulphate removal efficiency was obtained in the reactors, the reactors' contents appeared more liquefied, whereas when the sulphate removal decreased, the reactors contents appeared thicker. This could be due to the grass degrading microbes performing more efficiently in a liquefied environment, and therefore providing more readily available substrate for the SRBs to remove sulphate.

In total, over the 151 days of operation, reactor NA removed 2% more sulphate than reactor A. Initially, the native microorganisms on the non-autoclaved grass could support a larger variety of microorganisms, which could play a role in the enhancement of cellulose degradation, producing higher VFA concentrations, which were then more readily available to the SRB. Over time, the naturally occurring grass microbes may have competed for GC or for cellulose degraded intermediates, resulting in a change in members of the community. The addition of autoclaved grass may have resulted in lower COD concentration and thus a slower sulphate removal initially, while the population of microorganisms was allowed to establish. Once

established, a system could be obtained where the necessary nutrient and organics levels within the reactor were not subjected to competition from additional microbes added with the grass. The higher substrate (GC) addition during Period 3 seemed to have enhanced the substrate for the microbial population in A more efficiently than in NA. Decreasing the grass concentration to 20 g per week resulted in NA out-performing A in terms of sulphate removal. This result indicated that although the established population of microbes in reactor A may have performed better under ideal conditions, when sufficient COD was available (i.e. 50 g GC/week), the microbial population in reactor NA may possibly have contained a more robust and dynamic population due to the constant addition of native grass microorganisms, which enabled the community to adapt and survive when subjected to stress under low nutrient conditions.

The overall 2% increase in sulphate removal over the experimental period of 151 days appeared insignificant and, therefore shows that adequate sulphate removal was obtained using either non-autoclaved GC or autoclaved GC. The rumen microbes occur in significant numbers and have growth rates sufficient to counteract the constant dilution due to turnover of rumen contents. Therefore rumen fluid microbes have a competitive advantage in that they are rapidly able to fill niche environments (Mould *et al*., 2005).

5.3.3 Volatile Suspended Solids

The VSS concentration in the reactor is a good indication of the amount of microorganisms present. It was shown in a previous study (Greben *et al*, 2006) that there is a linear relationship between VSS concentration and the number of cells present in the reactor. When the cell count increased, so did the VSS concentration. The average VSS concentration in A and NA during the total experimental period is shown in Table 5.3. The VSS concentration increased significantly in both reactors during Periods 2 and 3, when the GC concentration was increased and decreased in Period 4 in both reactors when lower concentrations of GC were added. This result indicated the relationship between the amount of carbon present and the cell growth. Rumen bacteria obtain energy for growth by anaerobically fermenting carbohydrates. The highest GC load was supplied to both reactors during Period 3 (50g/week), which resulted in the highest VSS concentration. The highest sulphate removal was obtained in the same period, especially in reactor A only containing RI microbes and no additional native grass microbes. This finding indicated that the RI consortium thrive on a high carbon load. The chief energy supply for anaerobic microorganisms

is carbohydrate and as diet composition and nutrient availability are the largest factors affecting microbial growth in the rumen, this will have a major impact on the microbial activity of this inoculum (Mould *et al*., 2005).

Table 5.3. Average VSS concentration in A and NA

Period	Autoclaved (A)	Non-autoclaved (NA)
1	15042	10587
2	17588	18292
3	25234	24343
4	23533	21401

5.3.4 Volatile Fatty Acids

5.3.4.1 Acetate

The acetate concentration reached a maximum of about 3 700 mg/ℓ in NA and 2 800 mg/ℓ in A in Period 1 (Figure 5.3), which decreased in both reactors as a result of utilisation during Period 2, with the exception of an increase in acetate on day 53. The highest concentrations of acetate were observed during Period 3, when higher amounts GC (50 g/week) were added to the reactors. During Period 3, the concentrations of acetate were considerably higher in NA (reaching concentrations of 9 000 mg/ℓ) than in reactor A, indicating that the acetate was being utilised by the microbes in A to provide a faster rate of sulphate removal. During Period 4, the concentration of acetate gradually decreased in NA compared to A, possibly as more acetate was utilised for increased sulphate removal during this period by NA. The decreased acetate concentrations in NA during Period 4 can be attributed to the lower amounts of GC (20 g/week) added to the reactors. The higher acetate concentrations in comparison to that of propionate and butyrate levels (Figures 5.4 and 5.5) can be attributed to the fact that the acetate production from cellulose is higher than the propionic and butyric acid production or when sulphate is reduced using butyrate and propionate as carbon sources, acetate is the oxidation product. This occurs according to equations 5.1 and 5.2:

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

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

Coetser *et al. (*2000) showed that propionic, butyric and lactic acids were the preferred carbon sources to give effective sulphate reduction. Visser (1995) indicated

that hydrogen is the energy source most favoured by the SRB, which is the reason that little methane is produced from a sulphidogenic reactor system.

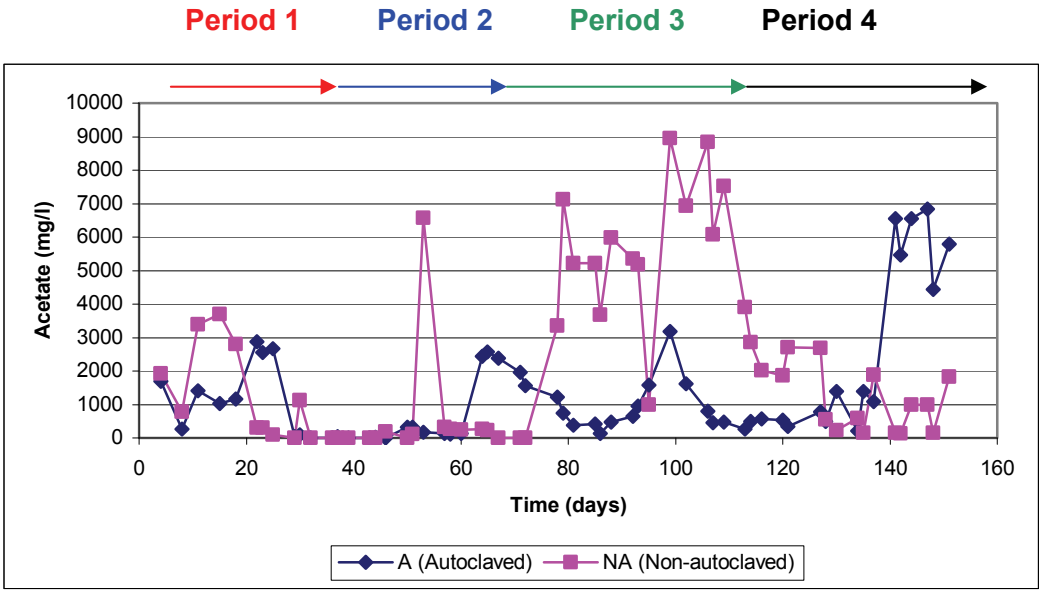

Figure 5.3. Acetate concentration in reactors A and NA

5.3.4.2 Propionate

Very low levels of propionate were observed during Periods 1 and 2 in both reactors, indicating that the propionic acid produced was utilised for sulphate removal in both reactors (Figure 5.4). Concentrations of propionate increased during Period 3 due to the addition of a higher amounts of GC (50 g), but remained higher in reactor NA, indicating that the propionate was being more efficiently utilised by the microbes in reactor A to provide a faster rate of sulphate removal during this period. The results during Period 3 clearly indicated the relationship between a high GC load and the VFA production and utilisation. When an "over-supply" of GC was added to the reactors, a residual VFA concentration was observed. In Period 4, the concentrations of propionate decreased slowly in NA due to the lower addition of grass cuttings (20 g) supplemented to the reactors during this period, but remained high in A as a poorer sulphate removal was obtained during this period by A. The accumulation of propionate in Period 4 for reactor A indicated poor utilisation of the VFA for sulphate removal.

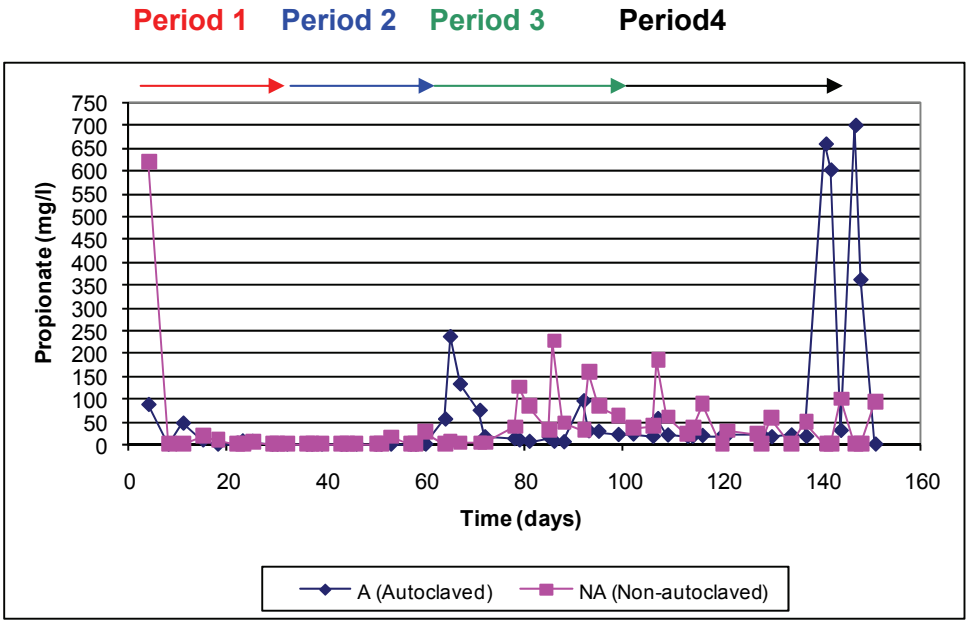

Figure 5.4. Propionate concentration in reactors A and NA

5.3.4.3 Butyrate

Very low levels of butyrate were observed during Periods 1 and 2, indicating that butyrate produced was utilised for sulphate removal in both reactors (Figure 5.5). Concentrations of butyrate increased during Period 3 due to the addition of a higher amount of GC (50 g), but remained slightly higher in NA, indicating that the butyrate was efficiently utilised by the microbes in A to provide a faster rate of sulphate removal during this period. During Period 4, the concentration of butyrate was higher in A indicating insufficient utilisation of butyrate during this period as sulphate removal efficiency was lower in this reactor.

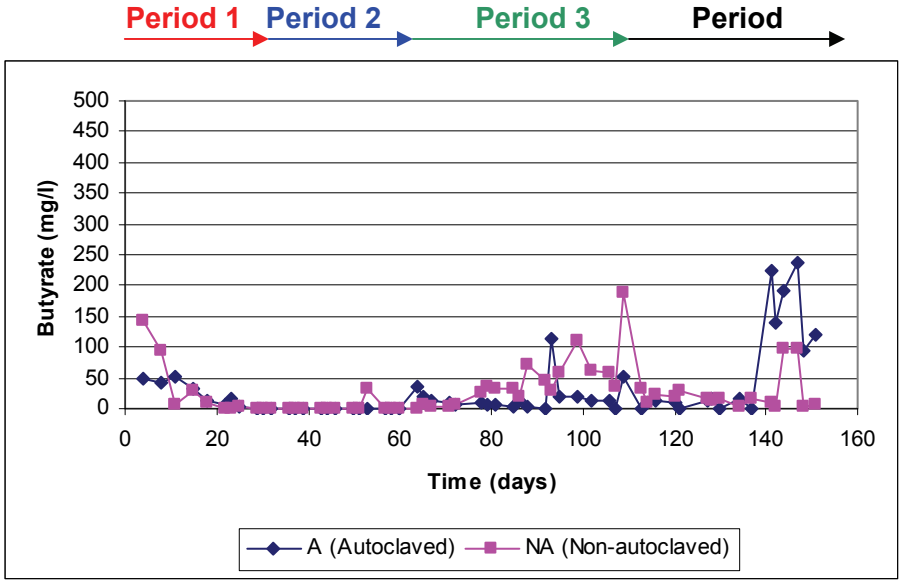

Figure 5.5. Butyrate concentration in reactors A and NA

STUDY 2

5.3.5 Molecular Analysis

5.3.4.1 Preparation of DNA for Molecular Analysis

The genomic DNA extraction method was optimised and genomic DNA was successfully extracted from reactor samples (Figure 5.6).

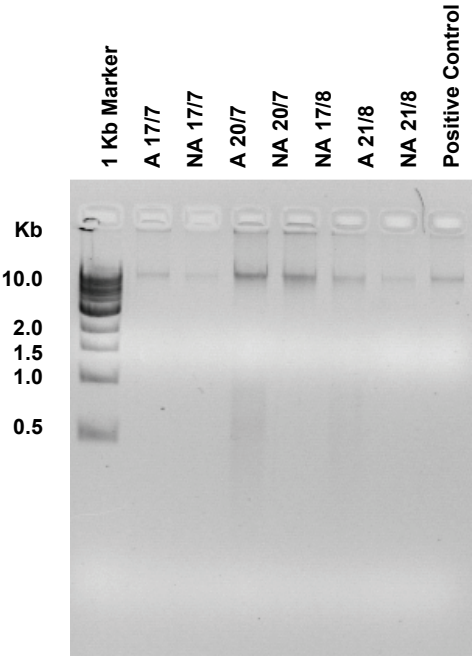

Figure 5.6. Genomic DNA extraction of samples from A and NA

After obtaining genomic DNA, 16S amplification was carried out. Positive PCR reactions showed a DNA band size of approximately 1181 bp which was followed by purification as indicated in Figure 5.7.

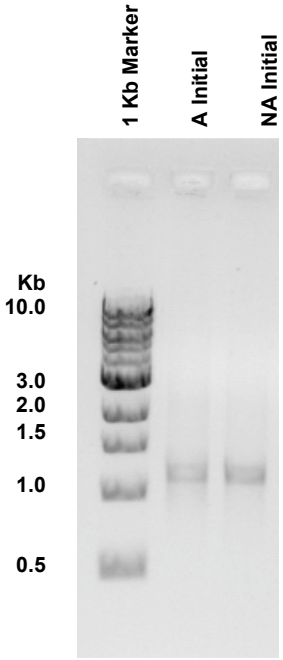

Figure 5.7. Purified 16S PCR products

5.3.5.1 Microbial Community Composition and Dynamics

The observed changes in the chemical composition of the reactor contents can possibly be explained by the microbiological population shift observed in the reactors. T-RFLP analysis can be seen as a fingerprint of the microbial communities present in the reactors. Each t-RF represents a possible bacterial member of the community. The presence of many bands or t-RFs on the gel indicates high bacterial diversity. The following t-RFLP results indicated significant changes in both the autoclaved and non-autoclaved reactors during the 151 days of operation.

Day 1

On day 1, the bacterial diversity was initially high in both A and NA reactors (Figure 5.8). The majority of possible genera present in the reactor determined by the PAT online analysis tool appeared to be cellulose degraders and fermentative microorganisms, although the presence of the SRB Desulfonatronovibrio and Desulfosporosinus were observed in reactor A and Desulfonatronovibrio and an uncultured Desulfovibrio was observed in NA (Figure 5.14 and 5.15). On visually observing the most dominant terminal restriction fragment band patterns (Figure 5.8), the following genera were determined to be the most dominant possible genera on day 1:

Reactor A: *Marine Bacterium*, *Marinobacter*, *Sulfitobacter*

Reactor NA: *Alteromonas*, *Arthrobacter*, *Azotobacter*, *Burkholderia*, *fenthion degrading bacteria*, *gamma Proteobacteria*, *Proteobacteria*, *Pseudomonas*, *Saccharospirillum, swine manure bacterium*, *uncultured soil bacterium*.

Day 32

A large proportion of the bacterial population remained on day 32 from day 1 in both reactors A and NA (Figure 5.14 and 5.15). Although the t-RF band patterns of A and NA on day 32 appeared similar (Figure 5.9), the online PAT revealed a higher bacterial diversity in reactor NA in comparison with A (Figure 5.14, 5.15 and 5.16, 15.17). At this point the sulphate removal efficiency was higher in NA than A. On visually observing the most dominant terminal restriction fragment band patterns (Figure 5.10), the following bacterial members were determined to be the most dominant possible genera on day 32:

Reactor A: *Desulfotomaculum*, *Heliorestis*

Reactor NA: *Arthrobacter*, *Azospirillum*, *Burkholderia, fenthion degrading bacterium, gamma Proteobacterium, Lactococcus*, *Marinobacter*, *Proteobacteria*, *Pseudomonas, swine effluent bacterium, swine manure bacterium.*

Day 64:

The population changed substantially from day 32 to day 64 in both reactors. Only 1 of the initial possible bacterial members from day 1, the uncultured rumen bacterium, remained on day 64 in Reactor A (Figure 5.14 and 5.16). None of the initial bacterial members from day 1 remained in NA (Figure 5.15 and 5.17). On day 64, the sulphate removal efficiency was higher in reactor A and the t-RFLP results indicated a higher level of population diversity in reactor A (Figure 5.10) in comparison to NA. In reactor NA, the population diversity decreased substantially from day 32 to day 64 (Figure 5.17). It is possible that this shift in population resulted in a decrease in sulphate removal efficiency. On visually observing the most dominant terminal restriction fragment band patterns (Figure 5.10), the following bacterial members were determined to be the most dominant possible genera on day 64:

Reactor A: *Acetobacter*, *Acidiphillum*, *Acidomonas*, *Gluconobacter, Roseomonas, uncultured alpha Proteobacterium, uncultured rumen bacterium*

Reactor NA: *Uncultured rumen bacterium*

Day 99:

From day 64 to day 99, the population in reactor A remained fairly stable (Figure 5.16). The bacterial community in reactor NA displayed variability as new possible genera were observed (Figure 5.17). Reactor A presented higher sulphate removal efficiency during this period than NA, however, reactor NA displayed higher bacterial diversity than A in all enzyme t-RFs with the exception of enzyme HhaI (Figure 5.11). The higher bacterial diversity could have been facilitated by the addition of a higher amount of grass (50 g) with native grass microbes during this period. This could indicate that a shift in the bacterial population had already begun to occur, allowing for reactor NA to remove more sulphate than A in period 4. On visually observing the most dominant terminal restriction fragment band patterns (Figure 5.11), the following bacterial members were determined to be the most dominant possible genera on day 99:

Reactor A: *Clostridium*, *uncultured Dehalococcoides*, *uncultured sludge bacterium*

Reactor NA: *uncultured Dehalococcoides, uncultured sludge bacterium*

Day 133:

From day 99 to day 133 the bacterial population in reactor A again remained fairly stable (Figure 5.16), whereas the population in NA was accompanied by the addition of new possible genera (Figure 5.17). On day 133, a much higher diversity of bacteria was observed in reactor NA, when compared to reactor A (Figure 5.12) and reactor NA removed more sulphate during this period. A large number of bacterial members initially present in reactor NA reappeared on day 133, while a large number of new possible bacterial members were also observed (Figure 5.15 and 5.17). The bacterial community structure in reactor A, again remained fairly stable (Figure 5.16). On visually observing the most dominant terminal restriction fragment band patterns (Figure 5.12), the following bacterial members were determined to be the most dominant possible genera on day 133:

Reactor A: *Cyanothece*, *Dactylococcopsis*, *Desulfotomaculum*, *Euhalthece, uncultured Eubacterium*

Reactor NA: *Clostridium*, uncultured rumen bacterium

Day 151:

From day 133 to day 151 the bacterial diversity remained higher in reactor NA than A (Figure 5.13). The sulphate removal efficiency decreased dramatically for A during this period and slightly for NA. On day 105, the GC concentration was reduced to 20

g per week, which may have resulted in a depletion of certain nutrients leading to shift in certain members of the community. This bacterial population shift was observed more readily in reactor NA (Figure 5.17) as more variability occurred in reactor NA due to the addition of un-autoclaved grass. The lower concentration of GC may have not been efficient for sustained sulphate removal in reactor A, when additional native grass microbes were not included on the autoclaved grass. On visually observing the most dominant terminal restriction fragment band patterns (Figure 5.13), the following bacterial members were determined to be the most dominant possible genera on day 133:

Reactor A: *Cattellicoccus*, *Dezemzia, Enterococcus,Tetragenococcus*, *uncultured soil bacterium*

Reactor NA: *Alteromonas*

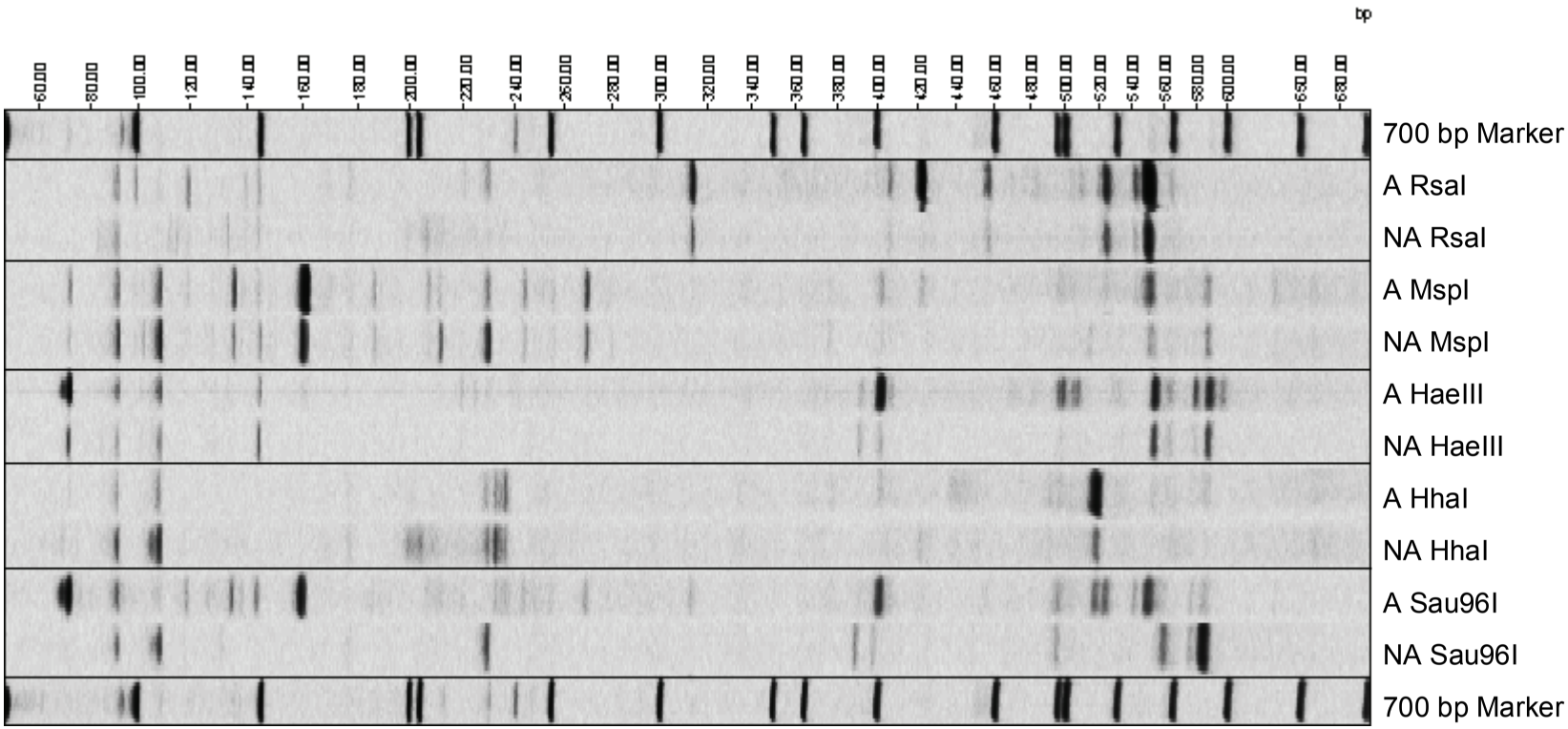

Figure 5.8. Day 1 t-RF pattern obtained for reactors A and NA

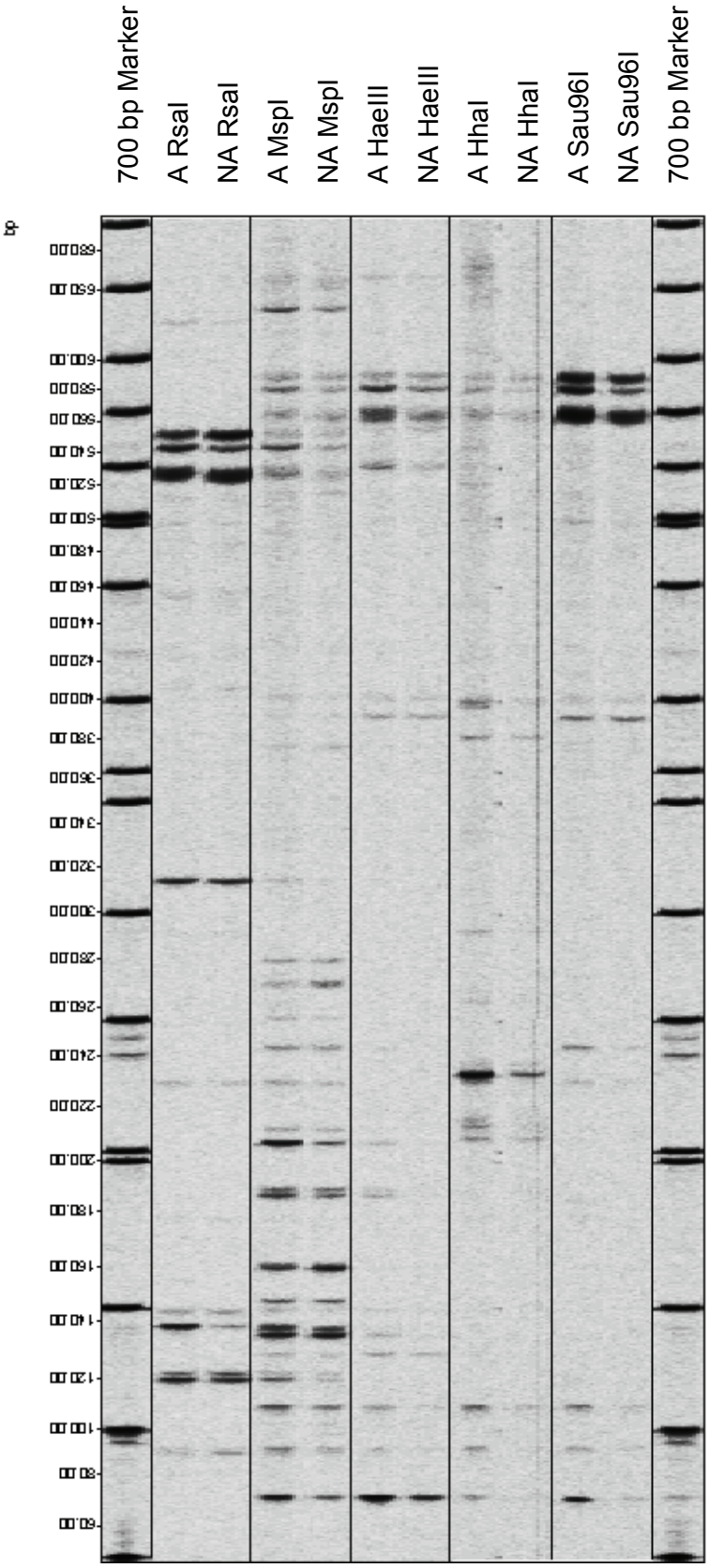

Figure 5.9. Day 32 t-RF pattern obtained for reactors A and NA

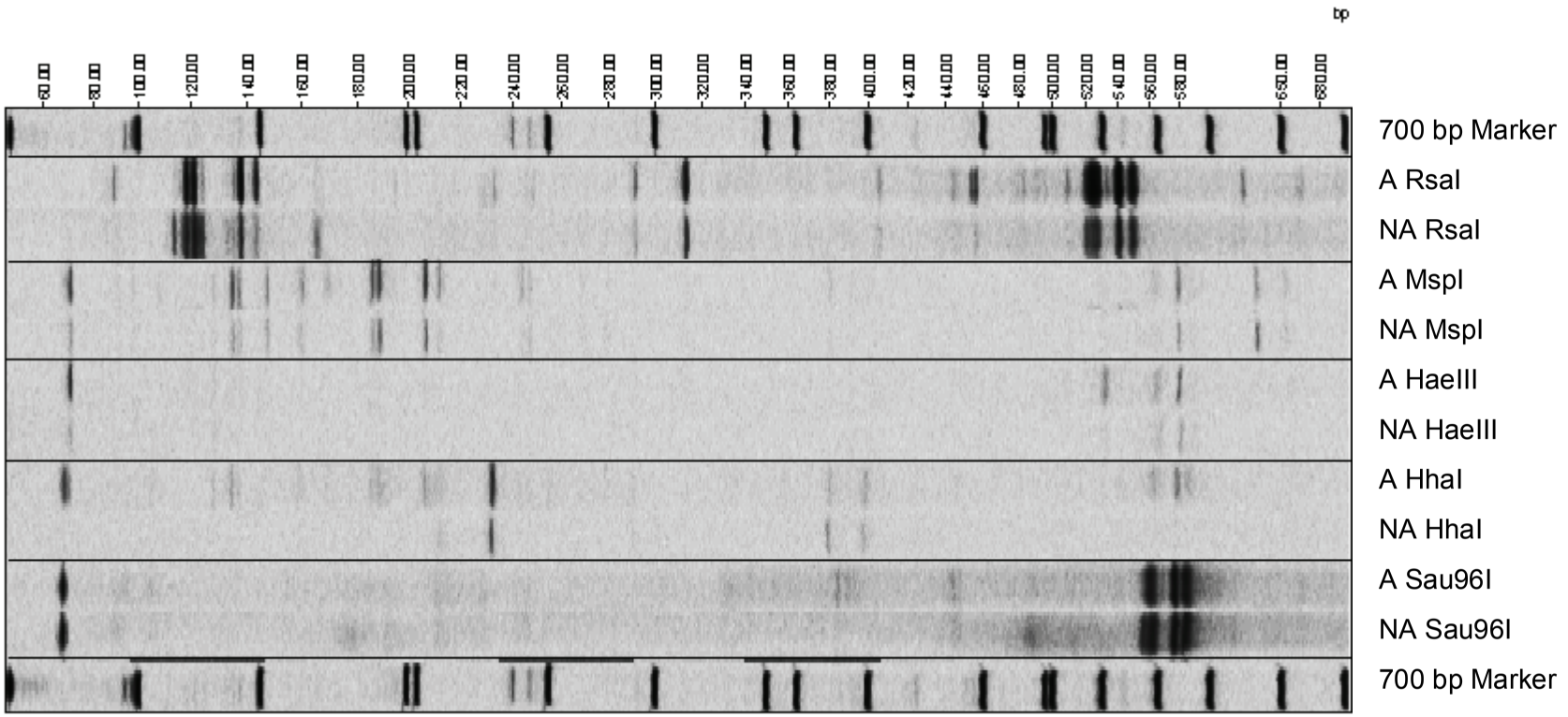

Figure 5.10. Day 64 t-RF pattern obtained for reactors A and NA

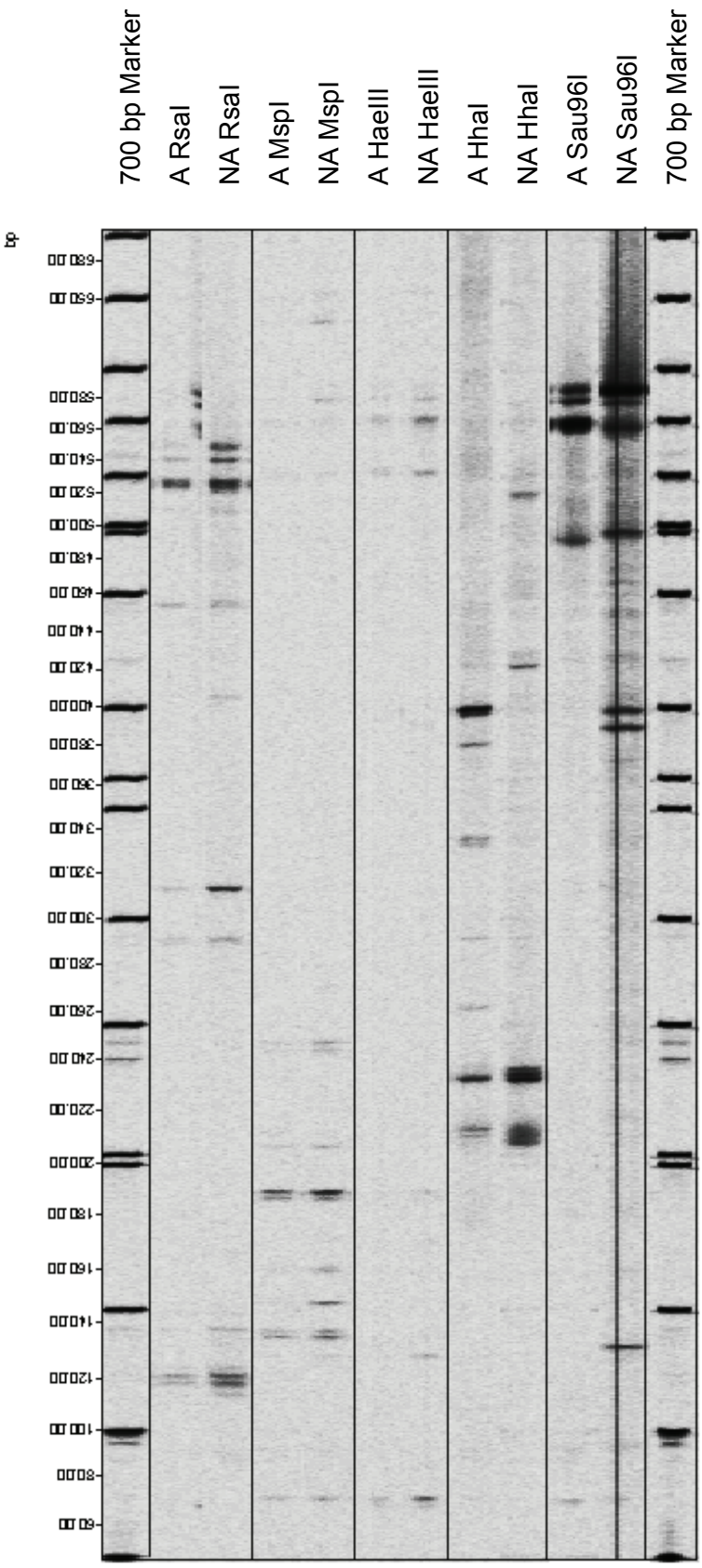

Figure 5.11. Day 99 t-RF pattern obtained for reactors A and NA

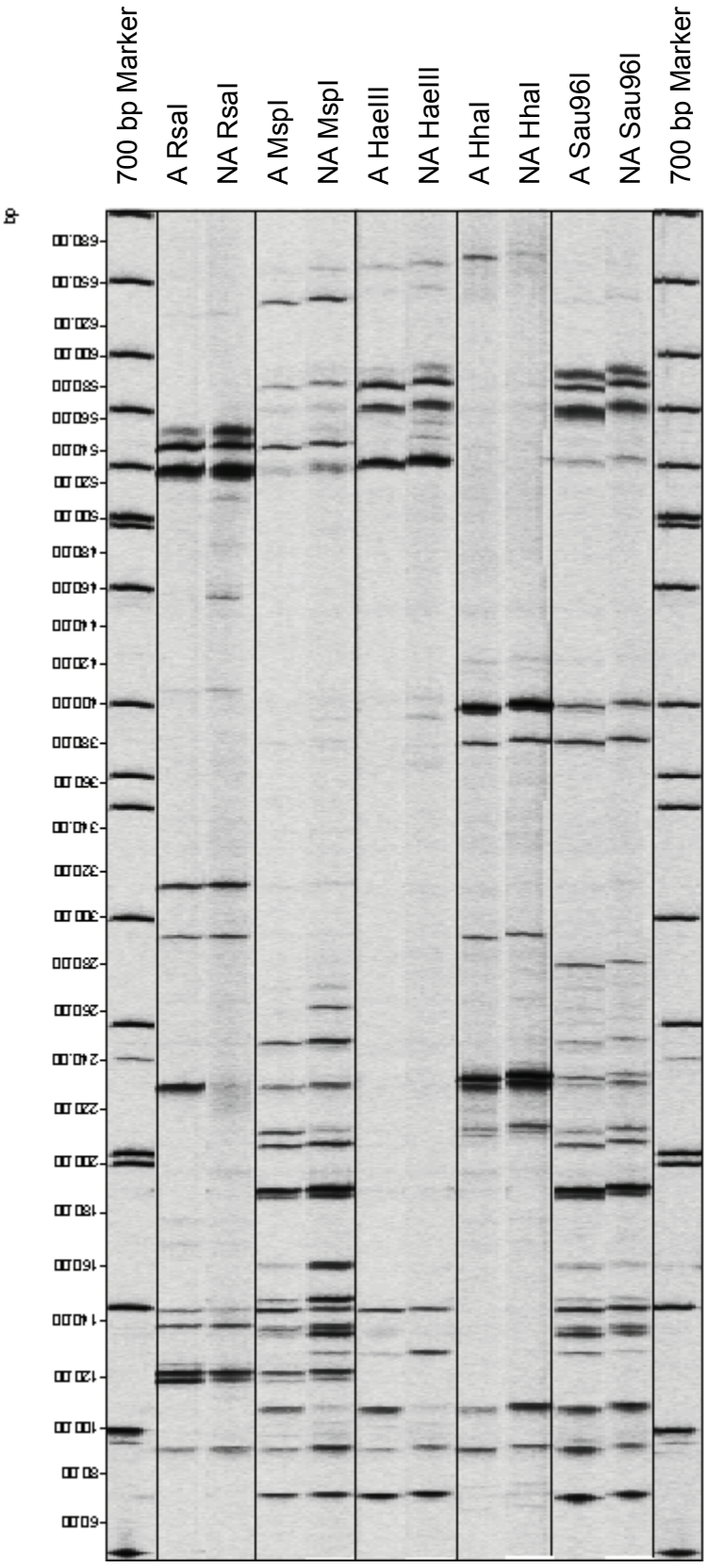

Figure 5.12. Day 133 t-RF pattern obtained for reactors A and NA

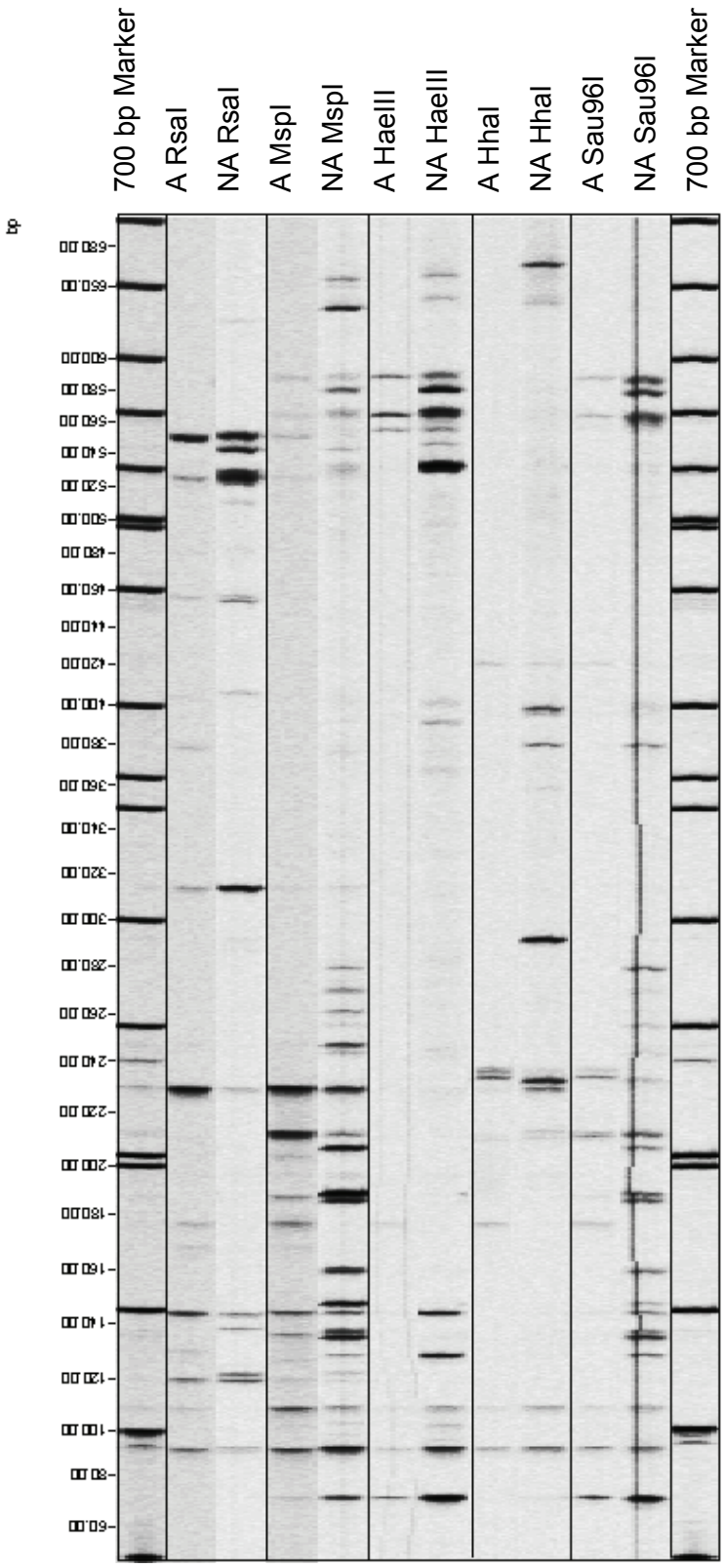

Figure 5.13. Day 151 t-RF pattern obtained for reactors A and NA

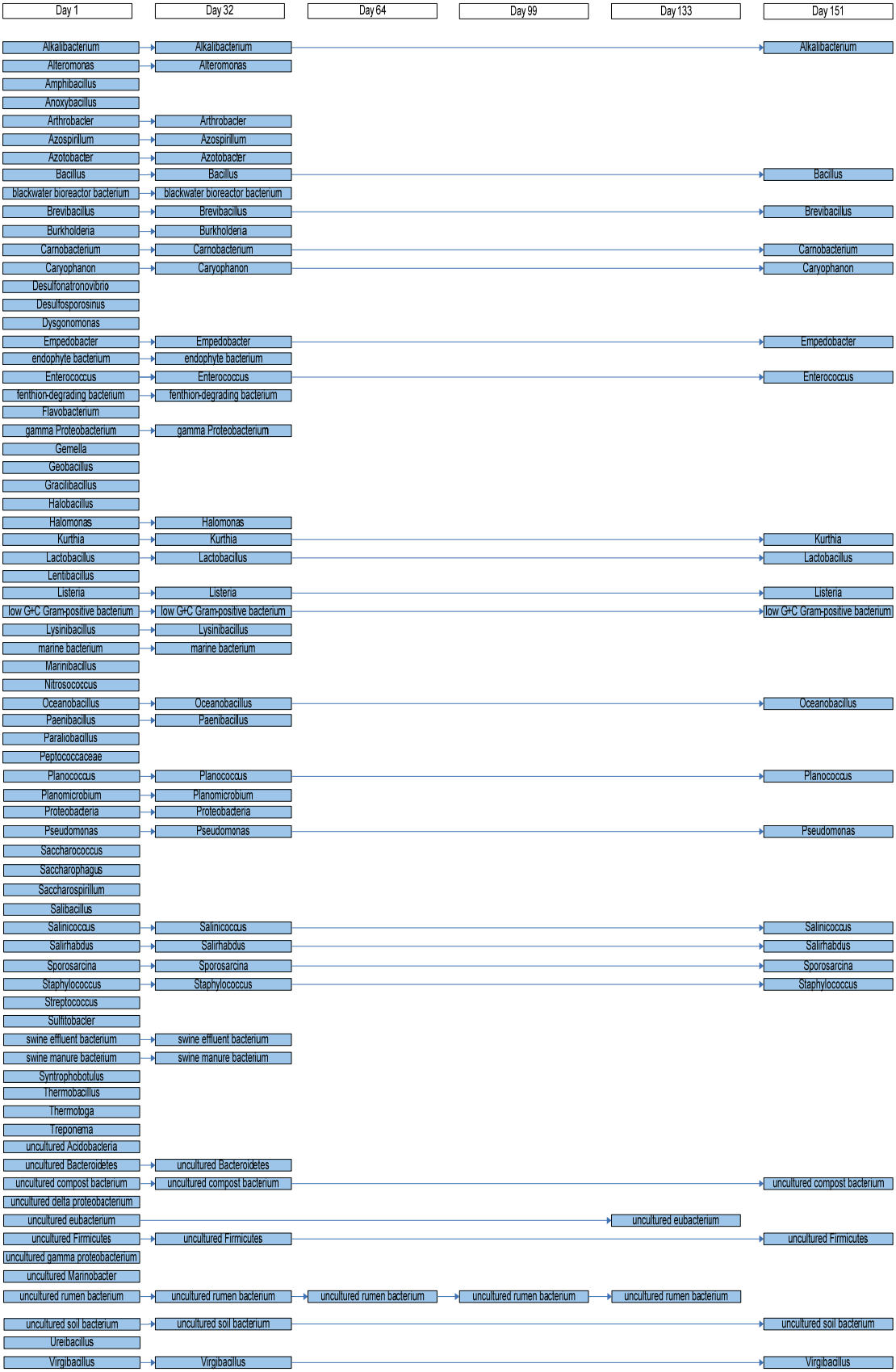

Figure 5.14. Flow diagram of initial possible genera in reactor A from Day 1 persisting throughout the entire reactor operation period

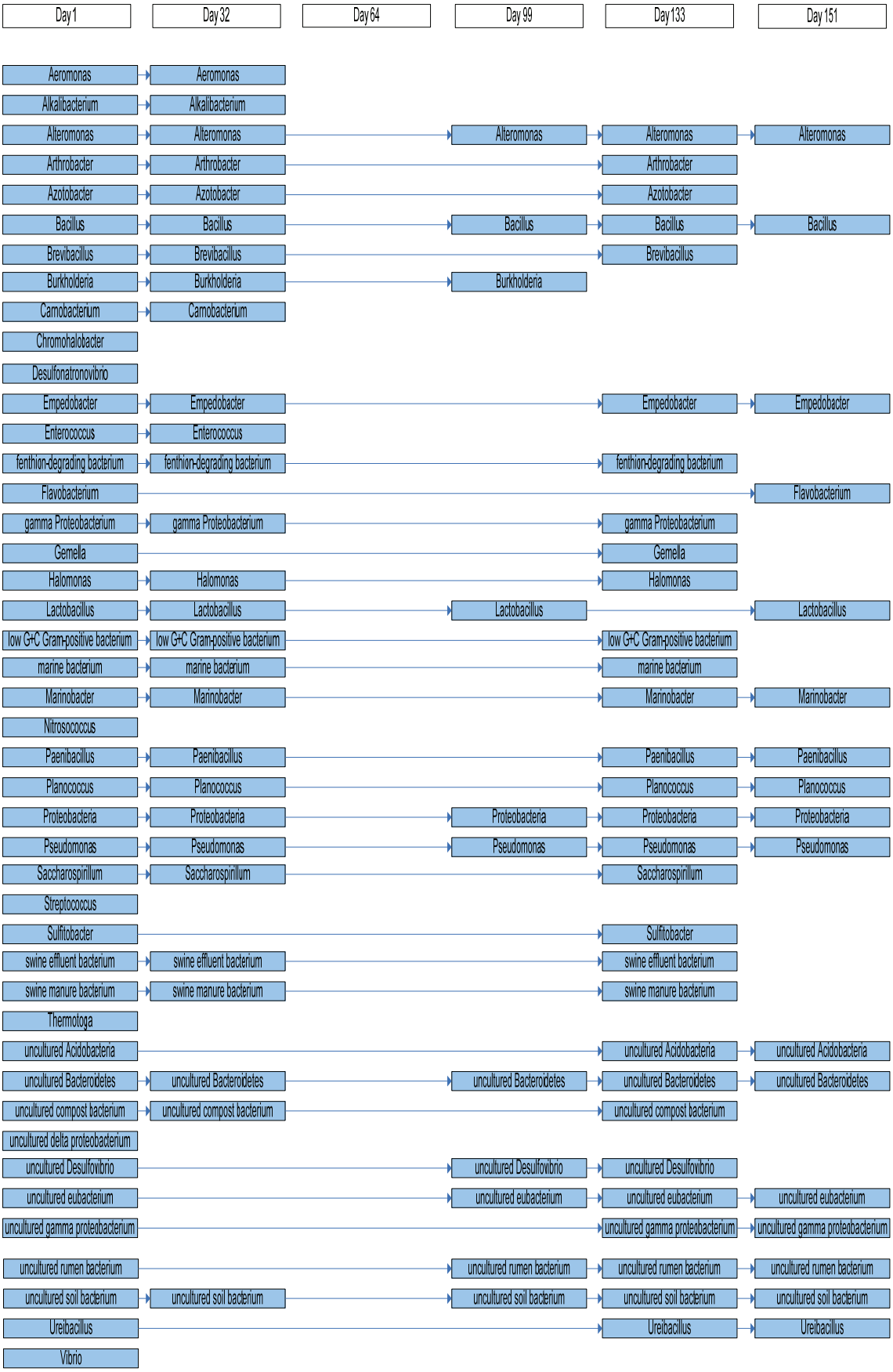

Figure 5.15. Flow diagram of initial possible genera in NA from Day 1 persisting throughout the entire reactor operation period

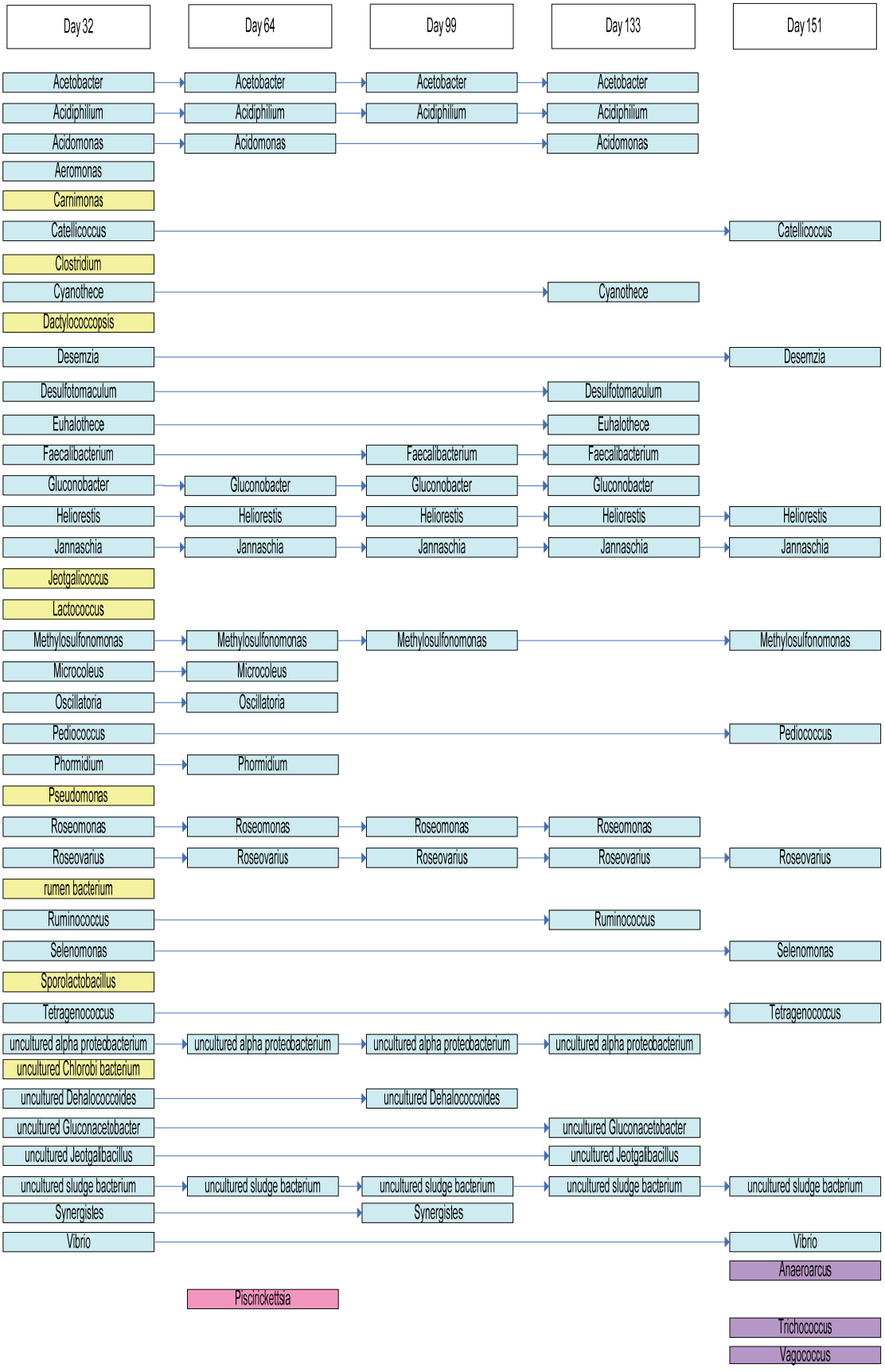

Figure 5.16. Flow diagram of new possible genera introduced on Day 32 and their persistence throughout the reactor A operation period

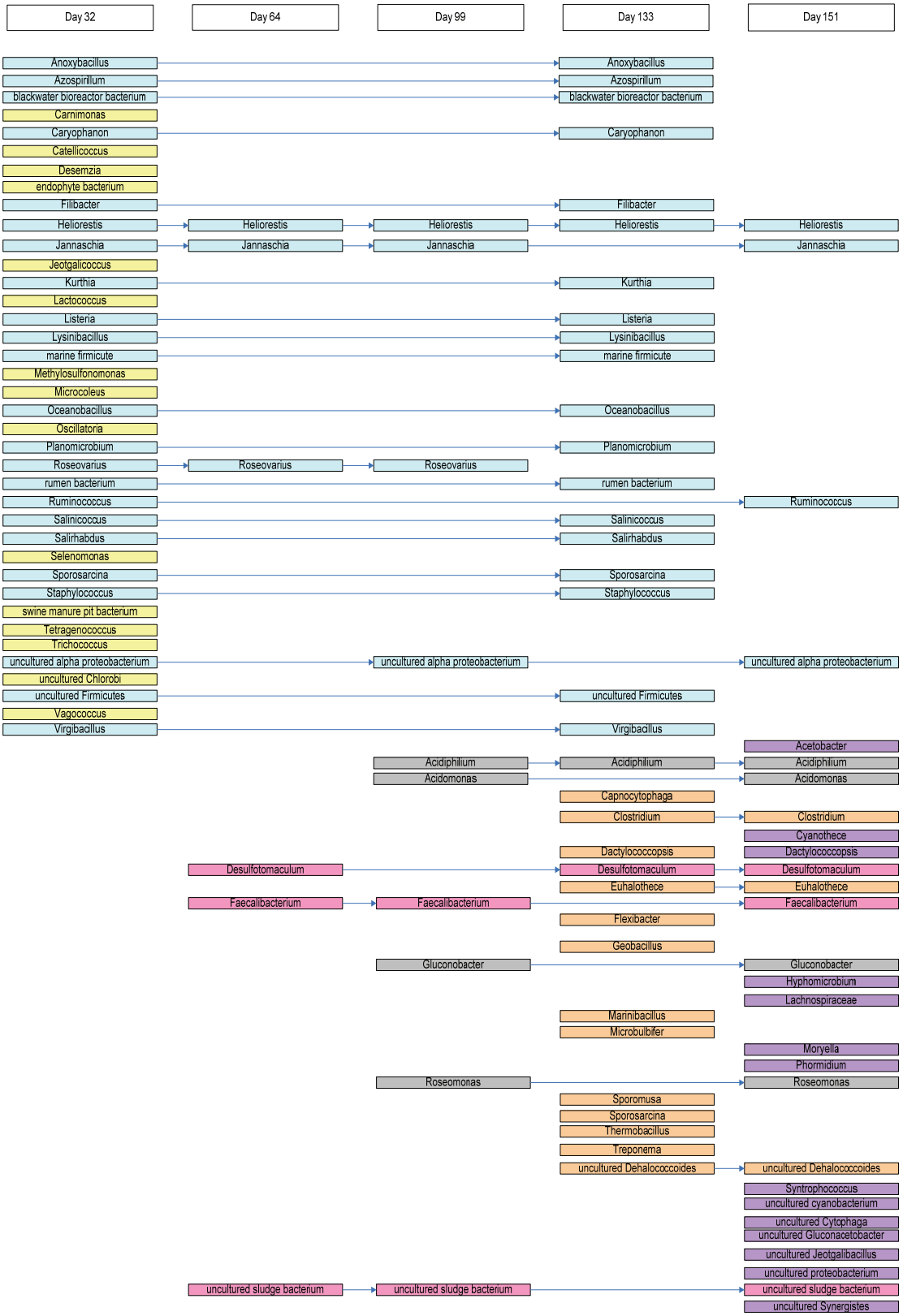

Figure 5.17. Flow diagram of new possible genera introduced on Day 32 and their persistence throughout the reactor NA operation period

The results clearly illustrated the rich diversity of microorganisms present in both the reactors (Figure 5.14, 5.15, 5.16 and 5.17). Aerobic genera were not eliminated from the PAT analysis as the batch reactors were not completely anaerobic as an overhead stirrer was required to mix the contents, which possibly allowed for oxygenated zones. The presence of a large number of uncultured bacterial genera highlights the functional importance of these members in sulphate removing bioreactor communities. A brief description of the metabolism of the visually determined dominant bacteria is given below. It appears that most of the dominant bacteria play a role in cellulose degradation and fermentation.

Acetobacter:

Members of this *genus are* obligately aerobic and never fermentative. They are chemoorganotrophs and oxidise ethanol to acetic acid. Acetate and lactate are oxidised to carbon dioxide and water. They do not hydrolyse starch or lactose and are prevalent in fruits (Krieg, 1984)

***Acidiphillum*:**

This genus is aerobic, acidophilic, mesophylic and chemoorganotrophic. They are common in acidic mineral environments such as pyritic mine drainage (Staley, 1984)

***Acidomonas*:**

This genus is commonly distributed in activated sludge. It produces acid from sugars and is aerobic. Acetate is oxidised, but lactate is not or only weakly oxidised (Yamashita *et al*., 2004).

***Alteromonas*:**

This genus is aerobic and chemoorganotrophic. They are not capable of fermentative metabolism. They are commonly found in marine environments (Krieg, 1984)

***Arthrobacter*:**

Although they are usually regarded as obligately aerobic, some species of this genus utilise nitrate ammonification and lactate, acetate and ethanol producing fermentation processes for anaerobic growth. They occur in soil (Eschbach *et al*., 2003)

***Azospirillum*:**

This genus has a mainly respiratory type metabolism but weak fermentative ability may occur and is associated with soil and plant roots. They are chemoorganotrophic and some strains are facultative hydrogen autotrophs. Fructose and certain sugars can be used as carbon sources, and salts of organic acids such as malate succinate, lactate or pyruvate are utilised (Krieg, 1984).

***Azotobacter*:**

This genus is aerobic but can grow under decreased oxygen tensions. They are chemoorganotrophic using sugars, alcohols and salts or organic acids for growth. They are nitrogen fixers occurring in soil and water (Krieg, 1984).

***Burkholderia*:**

This genus is usually associated with the rhizospere of grasses and assimilates sugars as a sole carbon source (Viallard *et al*., 1998).

***Cattellicoccus*:**

This genus is facultatively anaerobic, ferments sugars and is closely related to *Entereoccus* spp. (Lawson *et al*., 2006)

***Clostridium*:**

This genus produces mixtures of organic acids and alcohols from carbohydrates or peptones. They are obligately anaerobic and may be saccharolytic (Sneath, 1984).

***Cyanothece*:**

This genus is a cyanobacterium and can synthesize nitrogenase under anaerobic conditions. Most strains tolerate high salinities. They are found in fresh water or marine environments (Staley, 1984)

***Dactylococcopsis*:**

This genus is a cyanobacterium. Photosynthesis occurs under anaerobic conditions in the presence of sulphide (Walsby, 1983)

***Desulfotomaculum*:**

Members of this genus are spore forming, strict anaerobes and chemoorganotrophs. Sulphates and sulphites act as electron acceptors and are reduced to hydrogen sulphide and oxidation is incomplete and leads to the formation of acetate and carbon dioxide (Sneath, 1984).

Dezemzia:

Members of this genus are microaerophylic, display fermentative metabolism, produce acids from sugars, and were previously known as the genus *Brevibacterium* (Stackebrandt *et al.,* 1999).

Enterococcus:

This genus consists of facultative anaerobes. They have a fermentative metabolism and convert carbohydrates to lactic acid. They are found in soil, surface water and the gastrointestinal tract of humans and of animals (Salminen *et al*., 2004).

***Uncultured Eubacterium*:**

This genus is obligately anaerobic. They are chemoorganotrophs and can be saccharoclastic. They produce organic acids from carbohydrates often including large amounts of butyric, acetic or formic acids (Sneath, 1984).

***Euhalothece*:**

A halotolerant Cyanobacteria identified in hypersaline microbial mats (Garcia-Pichel *et al*., 1998)

***fenthion degrading bacteria*:**

A bacterium possessing degradative enzymes possibly belonging to the genera *Alcaligenes*, *Flavobacterium*, *Pseudomonas* or *Rhodococcus* (Aislabie and Lloyd-Jones, 1995)

***gamma Proteobacterium*:**

A class of Proteobacteria

Gluconobacter:

This genus is chemoorganotrophic and obligately aerobic. They oxidise ethanol to acetic acid, do not oxidise acetate or lactate to carbon dioxide. They occur in garden soil and fruits and form acid from D-glucose and D-xylose (Krieg, 1984).

***Heliorestis*:**

This genus is alkaliphylic, obligately anaerobic, and grows photoheterotrophically on organic compounds such as acetate and pyruvate. Sulphide is oxidised to elemental sulphur and polysulphides under photoheterotrophic conditions. Its habitat is alkaline soils or soda lakes (Bryantseva *et al*., 1999).

***Lactococcus*:**

This genus produces lactic acid from fermentation of carbohydrates. They are facultatively anaerobic (Facklam and Elliot, 1995)

***Marine Bacterium*:**

The marine environment is rich in sulphides and sulphates, therefore this member could play a role in the sulphur cycle.

***Marinobacter*:**

This genus uses organic acids, alcohols, amino acids and hydrocarbons as carbon and energy sources. They do not utilise carbohydrates or degrade polysaccharides. They grow anaerobically by denitrification and are mesophilic and halotolerant (Brenner *et al*., 2005).

***Proteobacteria*:**

This Phylum encompasses Gram-negative bacteria in the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, deltaproteobacteria and Epsilonproteobacteria (Brenner *et al*., 2005).

***Pseudomonas*:**

This genus is aerobic, but nitrate can be used as alternate electron acceptor allowing growth to occur anaerobically. They perform starch hydrolysis due to amylolytic activity (Krieg, 1984).

Roseomonas:

An oxidative genus that utilizes acetate and produces acid from methanol. The closest genotypic and phenotypic relative is *Methylobacterium* spp. (Rihs *et al*., 1993)

***Saccharospirillum*:**

This genus exhibits aerobic to microaerophilic growth and does not grow anaerobically on glucose in the absence of nitrate. They grow on various sugars and carboxyllic acids. Their closest relatives include *Oceanospirillum*, *Pseudospirillum*, *Marinospirrilum*, *Halomonas*, and *Chromohalobacter* (Labrenz *et al*., 2003).

***Sulfitobacter*:**

This genus occurs in marine environments and represent strictly aerobic, heterotrophic bacteria. Oxidation of sulphite occurs and glucose is not fermented. (Pukall *et al*., 1999)

swine effluent bacterium:

Bacteria occurring in swine effluent possibly having a cellulolytic function

***swine manure bacterium*:**

Bacteria occurring in swine manure possibly having a cellulolytic function

***Tetragenococcus*:**

Members of this genus are facultatively anaerobic. They have a fermentative Metabolism, produce acid from carbohydrates, and were previously known as *Pediococcus* spp. (Facklam and Elliot, 1995).

uncultured alpha Proteobacterium:

An uncultured class of Proteobacteria

uncultured Dehalococcoides:

Members of this genus survive in a wide range of geographical locations and are associated with environmental dechlorination (Hendrickson *et al*., 2002)

uncultured rumen bacterium:

Bacteria originating from the rumen possibly involved in cellulose degradation. *Ruminococcus* is the most active cellulolytic bacterial species in rumen microflora (Latham *et al*., 1971). They ferment cellulose and various sugars, to produce acetate, formate, succinate, ethanol, hydrogen and carbon dioxide as major end products (Sneath, 1986).

uncultured sludge bacterium:

Bacteria that are possibly anaerobic and depolymerise organic materials, and could also include denitrifying bacteria or methanogenic bacteria.

***uncultured soil bacterium*:**

Bacteria occurring in soil, which may play a role in organic matter decomposition. The most abundant bacterium in soil is *Pseudomonas* spp. (Janssen, 2006)

The presence of *Desulfotomaculum* was observed in reactor A on days 32, 99, 133 and in reactor NA on days 64, 99, 133, 151 (Figure 5.14, 5.15 and 5.16, 5.17). While some SRB are capable of complete oxidation of organic compounds to carbon dioxide, others carry out incomplete oxidations and usually produce acetate as an end-product. The genus *Desulfotomaculum* is an incomplete oxidiser (Sneath, 1984). The higher acetate concentrations in comparison to that of propionate and butyrate levels (Figures 5.5 and 5.6) can possibly be attributed to the presence of this organism.

In general, at each sampling point in the non-autoclaved reactor the bacterial community structure was dynamic and varied constantly (Figure 5.15 and 5.17), while that of the autoclaved grass reactor remained more stable (Figure 5.14 and 5.16). This was possibly due to the addition of non-autoclaved grass to NA, which therefore resulted in the addition of native grass microbes. When the microbial diversity decreased, the sulphate removal efficiency decreased, indicating that microbial diversity is possibly related to optimal ecosystem functionality. A microbial community of high diversity may provide a more robust population that is capable of adjusting to changing environmental conditions. Community composition in both A and NA reactors comprised of a relatively low diversity of SRB. It has been observed that SRB represent a relatively low percentage of the microbial community in other sulphate reducing mine drainage treatment systems (Johnson and Hallberg, 2003; Morales *et al*., 2005; Pruden *et al*., 2007). This result emphasised the critical roles that other microbial members, such as cellulose degraders play in the full cellulose-sulphate removal process. It has been demonstrated that cellulose degradation rather than sulphate reduction is a rate limiting step in sulphate reducing systems (Logan *et al*., 2005).

In this study, the t-RFLP method, based on 16S gene sequence and restriction site data was used for the identification of bacterial members within the reactor communities. Similar 16S ribosomal sequences and restriction sites are shared between many different genera, therefore species present or not present within the reactors may have been overlooked. The t-RFLP analysis does, however, provide a good overview of the community dynamics and demonstrates the loss and gain of bacterial members in changing reactor conditions. The main objective of this study was to investigate whether the microorganisms present on the GC out-competed the rumen fluid associated microorganisms within the reactor, or assisted in grass fermentation. The bacterial community structure in reactor A remained more stable.

Although the established population of microbes in reactor A may have performed better under ideal conditions, when sufficient COD was available (i.e. 50 g GC/week), the molecular results indicated that the microbial population in reactor NA contained a more dynamic population due to the constant addition of native grass microorganisms. This could have enabled the community to adapt and survive when subjected to stress under low nutrient conditions (i.e. 20g GC/week), possibly due to a more robust population. In total, over the 151 days operation, the non-autoclaved grass reactor removed 2% more sulphate than the autoclaved grass reactor. The overall 2% increase in sulphate removal over the experimental period of 151 days is insignificant and, therefore showed that adequate sulphate removal was obtained using either non-autoclaved GC or autoclaved GC and that the native grass microorganisms added to the non-autoclaved grass reactor did not contribute significantly to GC degradation. In previous studies, an isolated compost bacterial consortia of six different members was used as the main degradation microbes for the production of VFAs. It was reported that this isolated compost consortium was not robust enough and was out-competed by the grass microbes (Greben, 2007). Sonyka *et al* (2003) have also shown that anaerobic digestion of grass showed better degradation with rumen content as inoculum than with cattle dung slurry. Rumen fluid microbes occur in considerable numbers and have high growth rates in order to counteract the constant dilution due to turnover of rumen contents, providing the rumen fluid microbes with a competitive advantage (Mould *et al*., 2005). The results show that when this technology is applied at a larger scale, the GC need not be autoclaved as the GC consortia did not hinder the VFA formation by the rumen associated bacteria.

5.4 CONCLUSIONS

Throughout the 151 days of reactor operation, higher COD concentration in the reactors resulted in improved sulphate reduction. The addition of SRB and RI did not increase the COD concentration when insufficient GC substrate was available. A higher addition of GC resulted in a higher VFA production. Mainly propionate and butyrate were utilised for SO_4 reduction producing acetate. It was observed that the acetate concentrations were lower when sulphate reduction was more efficient, which indicated that acetate was possibly utilised as an energy source. A rich diversity of microorganisms was present in both reactors. When the microbial diversity decreased, sulphate removal efficiency decreased, which verifies that microbial diversity seemed closely related to optimal ecosystem functionality. The bacterial community structure in the non-autoclaved reactor was dynamic and varied constantly, while that of the autoclaved grass reactor remained more stable. NA contained a more dynamic population due to the constant addition of native grass microorganisms, which may have enabled the community to survive and adapt when subjected to stress. The population in A, once established, was not subjected to competition from additional microbes added with the GC and reduced sulphate efficiently under ideal conditions, when sufficient GC substrate was available. Community composition in both reactor A and NA comprised of a relatively low diversity of SRB. Although NA represented a more dynamic microbial population, the overall sulphate removal efficiency was only 2% higher than in A, during the full 151 days of operation. The native grass microorganisms did not contribute vastly to GC degradation, but in addition did not hinder the VFA formation by the rumen associated bacteria. From the above findings, it can be concluded that GC need not be autoclaved prior to adding GC to a sulphidogenic bioreactor for the purpose of cellulose degradation to provide the energy sources for biological sulphate removal.

5.5 REFERENCES

Aislabie, J., Lloyd-Jones G. 1995. A review of bacterial degradation of pesticides. *Australian Journal of Soil Research* **33**(6): 925-942

Barnes, S.P., Keller, J. 2003. Cellulosis waste degradation by rumen-enhanced anaerobic digestion. *Water Science and Technology* **48**(4): 155-162

Brenner, D.J., Krieg, N.R., Staley, J.T. (eds.) Bergey's Manual of Systematic Bacteriology, 2nd edition, vol. 2, Springer, New York

Bryantseva, I.R., Gorlenko, V.M., Kompantseva, E.I., Achenbach, L.A., Madigan, M.T. 1999. *Heliorestis daurensis*, gen. nov. sp. nov., an alkaliphilic rod-to-coiled-shaped phototrophic heliobacterium from a Siberian soda lake. *Archives of Microbiology* **172**: 167-174

Coetser, S.E., Cloete, T.E., Zdyb, L. 2000. Biological sulphate reduction in artificial mine drainage using different carbon sources. Proceeding Y2K Millenium Meeting, Grahamstown 23-28 Jan, 2000: 606.

Eschbach, M., Möbitz, H., Rompf, A., Jahn, D. 2003. Members of the genus *Arthrobacter* grow anaerobically using nitrate ammonification and fermentative processes: anaerobic adaptation of aerobic bacteria abundant in soil. *FEMS Microbiology Letters* **223**: 227-230

Facklam, R., Elliot, J.A. 1995. Identification, classification, and clinical relevance of catalase-negative gram-positive cocci, excluding the Streptococci and Enterococci. *Clinical Microbiology Reviews* **8**(4): 479-495

Garcia-Pichel, F., Nübel, U., Muyzer, G. 1998. The phylogeny of unicellular extremely halotolerant cyanobacteria. *Archives of microbiology* **169**: 469-482

Greben, H., Botha, A., Joubert, L., Baloyi, J., Tjatji, M., Matshusa, P., Brown, N. 2006. The production and utilisation of bio-energy sources for the reduction of sulphate in industrial and mining wastewaters. Final report: BioPAD BRIC BP008

Greben, H.A. 2007. Internal CSIR report. Fermentation of biomass and VFA production. January 2007.

Hendrikson, E. R., Payne, J.O., Young, R.M., Starr, M.G., Perry, M.P., Fahnstock, S., Ellis, D.E., Ebersole, R.C. 2002. Molecular analysis of *Dehalococcoides* 16S Ribosomal DNA from chloroethane-contaminated sites throughout North America and Europe. *Applied and Environmental Microbiology* **68**(2): 485-495

Internal CSIR report (2006).

Janssen, P.H. 2006. Minireviews: Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology* **72**(3): 1719-1728

Johnson, D.B., Hallberg, K.B. 2003. The microbiology of acidic mine waters. *Research in Microbiology* **154** (7): 466-473.

Krieg, N.R. (ed.) Bergey's Manual of Systematic Bacteriology, vol. 1, Williams & Wilkins, Baltimore, 1984

Labrenz, M., Lawson, P.A., Tindall, B.J., Collins, M.D., Hirsch, P. 2003. Saccharospirillum impatiens gen. nov., sp. nov., a novel γ-Proteobacterium isolated from hypersaline Ekho Lake (East Antartica). *International Journal of Systematic and Evolutionary Microbiology* **53**: 653-660

Latham, M.J., Sharpe, M.E., Sutton, J.D. 1971. The microflora of the rumen of cows fed hay and high cereal rations and its relationship to the rumen fermentation. *Journal of Applied Bacteriology* **34**: 425-434

Lawson, P.A., Collins, M.D., Falson, E., Foster, G. 2006. *Catellicoccus marimamalian* gen. nov., sp. nov., A novel Gram-positive, catalase-negative, coccus-shaped bacterium from porpoise and grey seal. *International Journal of Systematic and Evolutionary Microbiology* **56**: 429-432

Logan, M.V., Reardon, K.F., Figuero, L., Mclain, J.E.T., Ahmann, D. 2005. Microbial community activities during establishment, performance, and decline of bench-scale passive treatment systems for mine drainage. *Water Research* **39**: 4537-4551.

Marsh, T.L. 1999. Terminal Restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Microbiology* **2**: 323-327.

Morales, T. A., Dopson, M., Athar, R., Herbert Jr, R.B. 2005. Analysis of bacterial diversity in acidic pond water and compost after treatment of artificial mine drainage for metal removal*. Biotechnology and Bioengineering* **90** (5): 543-551

Mould, F.L., Kliem, K.E., Morgan, R., Mauricio., R.M. 2005. In vitro microbial inoculum: A review of its function and properties. *Animal Feed Science and Technology* **123-124**: 31-50

Pruden, A., Messner, N., Peryra, L., Hanson, R.E., Hiibel, S.R., Reardon, K.F. 2007. The effect of inoculum on the performance of sulphate-reducing columns treating heavy metal contaminated water. *Water Research* **41**: 904-914.

Pukall, R., Buntefuß, D., Frühling, A., Rohde, M., Kroppenstedt, R.M., Burghardt, J., Lebaron, P., Bernard, L., Stackebrandt, E. 1999. *Sulfitobacter mediterraneous* sp. nov., a new sulphite oxidising member of the *α-Proteobacteria*. *International Journal of Systematic Bacteriology* **49**: 513-519

Rihs, J.D., Brenner, D.J., Weaver, R.E., Steigerwalt, A.G., Hollis, D.G., Yu, V.L. 1993. *Journal of Clinical Microbiology* **31**(12): 3275-3282

Saliminen, S., von Wright, A., Ouwehand, A. 2004. Lactic acid bacteria: microbiological and functional aspects. CRC Press, New York.

Sneath, P.H.A. (ed.) Bergey's Manual of Systematic Bacteriology, vol. 2, Williams & Wilkins, Baltimore, 1984

Sonakya, V., Raizada, N., Dalhoff, R., Wilderer, P.A. 2003. Elucidation mechanism of organic acids production from organic matter (grass) using digested and partially digested cattle feed. *Water Science and Technology* **48**(8): 255-259

Stakebrandt, E., Schumann, P., Swiderski, J., Weiss, N. 1999. Reclassification of *Brevibacterium incertum* (Breed 1953) as *Dezemzia incerta* gen. nov., comb. nov. *International Journal of Systematic Bacteriology* **49**:185-188

Staley, J.T. (ed.) Bergey's Manual of Systematic Bacteriology, vol. 3, Williams & Wilkins, Baltimore, 1984

Viallard, V., Poirier, I., Cournoyer, B., Haurat, J., Wiebkin, S., Ophel-Keller, K., Balandreau, J. 1998. Burkholderia graminis sp. nov., a rhizosperic *Burkholderia* species, and reassessment of [*Pseudomonas*] *pyrrocinia* and [*Pseudomonas*] *glathei* as *Burkholderia*. *International Journal of Systematic Bacteriology* **48**: 549-563

Visser, A. (1995). The anaerobic treatment of sulphate containing wastewater. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

Walsby, A. E., Van Rijn, J., Cohen, Y. 1983. The biology of a new gas-vacuolate cyanobacterium, Dactylococcopsis salina sp.nov., in soda lake. *Proceedings of the Royal Society of London* SeriesB, Biological Sciences **217**(1209): 417-447

Yamashita, S., Uchimura, T., Komagata, K. 2004. Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki, and Komagata 1989. *International Journal of Systematic and Evolutionary Microbiology* **54**: 865-870

CHAPTER 6

REACTOR CONFIGURATION

6.1 BACKGROUND

Since the biological sulphate reduction technology came to maturity, several reactor designs have been developed. Among these are the UASB Reactor (Lettinga, *et al*., 1980), the Fluidized Bed (FB) Reactor (Iza, 1991) and the AF (Young & McCarty, 1969). These reactor designs 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. 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, at the clarifier liquid/gas interchange, which resulted in the biological oxidation of the sulphide produced during the biological sulphate removing process.

The reactors used in this chapter are based on the completely mixed reactor and a hybrid reactor-system. Most anaerobic hybrid reactors are a combination of UASB and AF reactors, thereby promoting the advantages of both reactor systems (Buyukkamaci & Filibeli, 2002). Operating a hybrid reactor provides suspended growth in the sludge layer and biofilm formation on the packing material, which prevents washout of biomass.

The results of Chapter 4 showed that the daily supplementation of GC, RI and SRB to the test reactor, with a volume of 2.5 ℓ enhanced the biological sulphate reduction. In order to test whether these results were reproducible, the same experiment was repeated, using a completely mixed reactor (CMR) with a volume of 10 ℓ. Increasing the reactor volume was to investigate the effect of scale up of the reactor size. Subsequently, all other applicable parameters were increased 4 times for this investigation compared to Study 2, described in Chapter 4. This study forms Part 1 of Chapter 6. In Part 2 of Chapter 6 a hybrid reactor is used for the formation and utilisation of VFA as substrate for the biological sulphate removal process treating initially synthetic sulphate rich feed water and later pre-treated sulphate rich AMD.

The aims of the studies in Chapter 6 are to investigate:

1. The effect of daily addition of GC, RI and SRB on the biological sulphate removal, operating a completely mixed reactor (Vol. 10 ℓ) in batch and continuous modes at room temperature (20°C) and at 25°C.
2. Whether sustained biological sulphate removal could be obtained operating a continuously fed hybrid reactor (Vol. 20 ℓ) at ambient temperature of 25°C, treating sulphate rich synthetic and pre-treated AMD feed water.

6.2 MATERIALS AND METHODS

Study 1: The operation of a completely mixed reactor (CMR)

Study 1a: Batch mode operation CMR

6.2.1 Feed water

Synthetic sulphate rich water with a concentration of approximately 2 500 mg/ℓ SO_4, ($MgSO_4$, Merck Chemicals, Ltd, Johannesburg) was used as feed water. When the sulphate concentration in the reactor was <100 mg/ℓ, fresh sulphate was added to the reactor, which occurred on days 13, 17, 28, 42 and 63. CMR was operated in batch mode over a period of 78 days.

6.2.2 Reactor Configuration

A completely mixed reactor system (CMR), comprising a reactor and a clarifier with a volume of 10 and 15 ℓ, respectively, (Figure 6.1) was used.

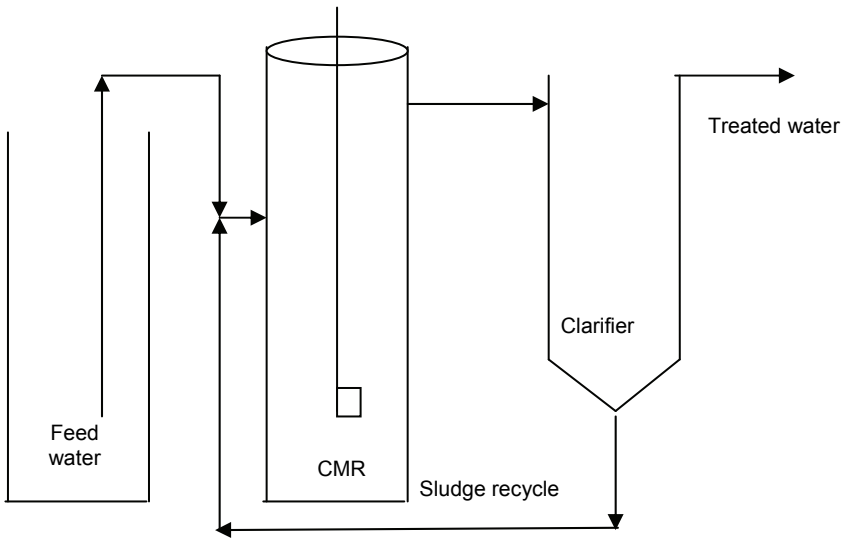

Figure 6.1. CMR reactor system

6.2.3 Biomass

The reactor was initially seeded with 60 mℓ RI and 20 mℓ l SRB. From day 1-42, CMR received 60 mℓ/d RI and 20 mℓ/d SRB, while from day 42-78 the biomass supplementation was given weekly, since the biomass increase resulted in a high VSS increase. The biomass supplementation was added at the top of the reactor.

6.2.4 Carbon and energy source

The degradation products of GC were used as the carbon and energy sources for the biological sulphate removal. At the start of the experiment 600 g GC were added, while in addition 20 g GC/d were added to maintain a high COD concentration in the reactor.

Study 1b: Continuous operation CMR

6.2.5 Feed water, reactor configuration, biomass, carbon and energy source

The same reactor system (CMR), as presented in Figure 6.1 was operated in continuous mode. During continuous operation the 10 ℓ reactor was fed 5 ℓ/d synthetic feed water (with the same chemical composition as during Study 1a), resulting in a HRT of 2 days. The pH of the feed water was adjusted with 0.1 N HCl to a pH of 6.6-6.9 to accommodate the rumen inoculum. All parameters regarding biomass and carbon and energy source were the same as in the batch mode operation of CMR. From day 1-24, the temperature of the reactor was not controlled and decreased from 22 to 18°C, due to the start of winter, while from day 25-33, the reactor temperature was controlled at 25°C, by way of electrical wires around the reactor. The total experimental period lasted 33 days.

Study 2: Continuous operation of Hybrid Fermentation Sulphate reactor (HFS)

6.2.6 Feed water

At the start of the experimental period, synthetic feed water, of which the composition is based on the composition of AMD (see 6.2.5) was used for the continuous operation of HFS. Later it was changed to pre-treated mine water, consisting of one part mine water and one part effluent of HFS after the biological sulphate reduction. This pre-treated mine water is made by mixing one part sulphide- and alkali-rich effluent after biological sulphate reduction with one part mine water, obtained from a coal mine in the Witbank area. Thus the pre-treated mine water consisted of a higher pH and no metals in the feed water, due to the alkalinity and sulphide concentrations,

respectively, in the after biologically treated water. The mine water was obtained from a closed mine in the Witbank area. The feed rate was 5 ℓ/d, which resulted in a HRT of 2.4 d. Micro nutrients were added to the feed water of which the chemical composition is presented in Chapter 3 (Table 3.1).

6.2.7 Reactor configuration and biomass

A one stage anaerobic reactor system, HFS (Vol.: 20 ℓ) was operated (Figure 6.2). The bottom part of the reactor contained SRB, obtained from a biological sulphate removing demonstration plant (Witbank, South Africa). Ceramic rings were added as immobilisation material for the SRB to form a biofilm, thus to prevent SRB wash out. The top part of the reactor contained GC to which RI, obtained from fistulated ruminants (University of Pretoria) was added. The active volume of HFS was 12 ℓ.

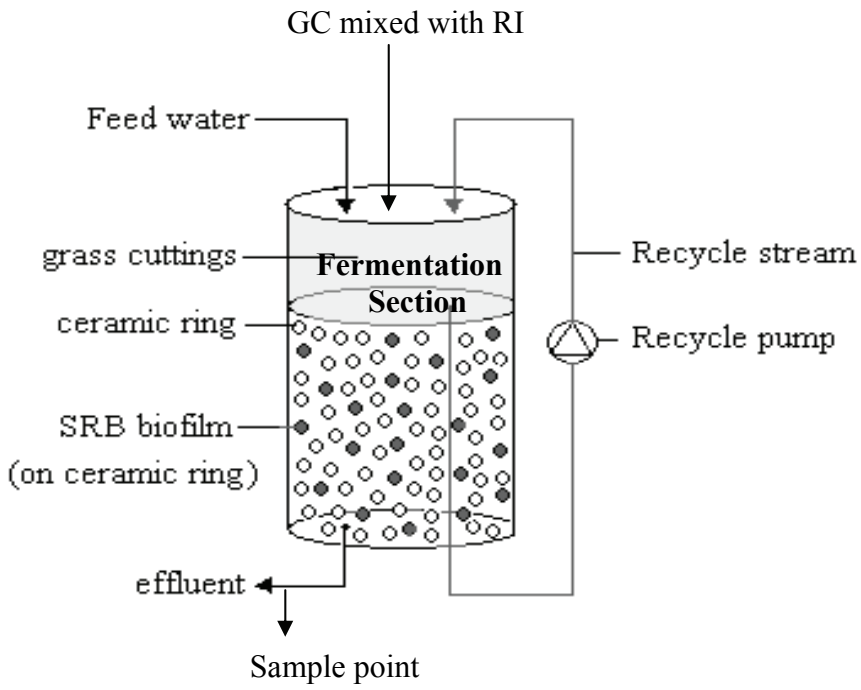

Figure 6.2. Schematic overview of HFS reactor system.

6.2.8 Experimental

The feed water entered HFS at the top (Figure 6.2). The recycle (360 ℓ/d) was installed from the fermentation part of the reactor to the top, for mixing purposes. The effluent left the HFS reactor at the bottom. The HFS reactor was operated at 25°C with water recycling from a water bath set at 25°C through a water jacket surrounding the reactor. Three different experimental periods were determined by adding GC and

microorganisms. Initially there were two short periods, period 1 and 2 consisting of 10 to 20 days (Table 6.1) followed by one long period (period 3) of 100 days. During the third experimental period (day 35-135), 150 GC were added weekly to the reactor, to maintain the COD concentration in the reactor, except on day 128, when the COD concentration in HFS was low and 200 g/wk GC were added. During Period 3, no biomass supplementation was added to the reactor.

Table 6.1. Experimental periods 1, 2 and 3 operating HFS

Period	**1**	**2**	**3**
Days	1-19	20-33	35-135
GC	300 g	150 g	150
SRB	200 ml, VSS 5.3 g/L	100 ml, VSS:3.6 g/L	None
RB	240 ml, VSS 38.7 g/L	120 ml, VSS: 25.5 g/L	None

6.2.9 Sampling

Daily samples (weekends excluded) were taken from HFS from the effluent (sample point: Fig. 6.2) during the different experimental periods. The results presented in the graphs are related to the daily sampling, while the data in the tables reflect the average of the results of the daily samples.

6.2.10 Analytical methods

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

6.3 RESULTS

STUDY 1A: CMR IN BATCH MODE.

6.3.1 Sulphate removal

The sulphate removal in CMR is presented in Figure 6.3. The graphs showed the removal of sulphate during the different periods after a fresh sulphate solution has been added to CMR (Figure 6.3). Fresh sulphate solutions were added on days 13, 17, 28, 42 and 63 (arrows). It can be observed from Figure 6.3 that during the first period from day 1-13, the sulphate was removed in 13 days and that total sulphate removal was achieved. During the following period the sulphate was removed faster, from day 14-17, on which day the SO_4 concentration was 650 mg/ℓ. After adding fresh sulphate on day 17, the SO_4 removal was almost removed on day 28, when the SO_4 concentration was 500 mg/ℓ. On day 41, the SO_4 concentration in CMR was 50

mg/ℓ and on day 42 it was 0 mg/ℓ, thus it took 14 days for total SO_4 removal. After a fresh SO_4 solution was added to the reactor on day 42, it took 20 days for total removal on day 62. From day 68-78, the SO_4 removal was very slow, which resulted in stopping the batch operated reactor.

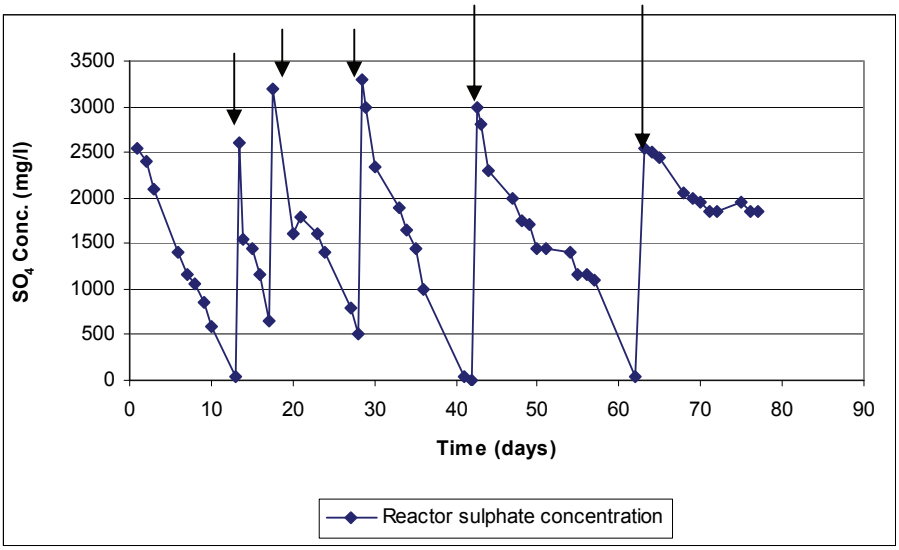

Figure 6.3. The sulphate concentration in CMR, batch operated

Thus although GC, RI and SRB were added daily to CMR, the sulphate reduction was relatively poor, especially towards the end of the experimental period when the sulphate concentration in the reactor remained at values of 2 000 mg/ℓ, over a period of 10 days. The COD concentration in CMR (Figure 6.4) was >3 000 mg/ℓ, which as was shown in previous experiments (see previous chapters) was high enough for continuous sulphate removal. The obtained sulphate removal results do not correspond with the results of period 2 in Chapter 4, where it was shown that daily addition of GC, RI and SRB resulted in fast and total SO_4 removal. The results presented seem to indicate that SO_4 removal inhibition occurred. The average sulphide concentration in CMR was 292 mg/ℓ, which is usually not inhibitory for the SRB to reduce SO_4 (Greben *et al*., 2005). However, the average reactor pH during the total experimental period was 7.0, which may result in the partly occurrence of H_2S in gas form, which may be inhibitory for the RI. The preferred reactor pH in a sulphate removing reactor is between 7.5 and 8.5 (Visser 1995), which is, however, too high for the RI, for which the reactor pH should not be >7.0 (Hungate, 1966).Thus to accommodate the RI population, the reactor pH is maintained at 6.6-6.9, but at this lower reactor pH, a higher percentage of H_2S is likely in gas form, which is more inhibitory to the different microbial populations. Weast (1981) described that the pK_a value of the dissociation equilibrium of H_2S is 7.04 at 18°C. 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).

6.3.2 COD concentration

The reactor COD concentration is presented in Figure 6.4, which shows that the COD concentration in CMR was higher than 3 000 mg/ℓ throughout most of the experimental period. Usually a sufficient high COD concentration in the reactor results in continuous sulphate removal. However, the results in the previous paragraph showed that although initially total sulphate removal was achieved in a reasonable short period, the results towards the end of the experimental period showed a delay in sulphate reduction as well as a high residual sulphate concentration in the reactor.

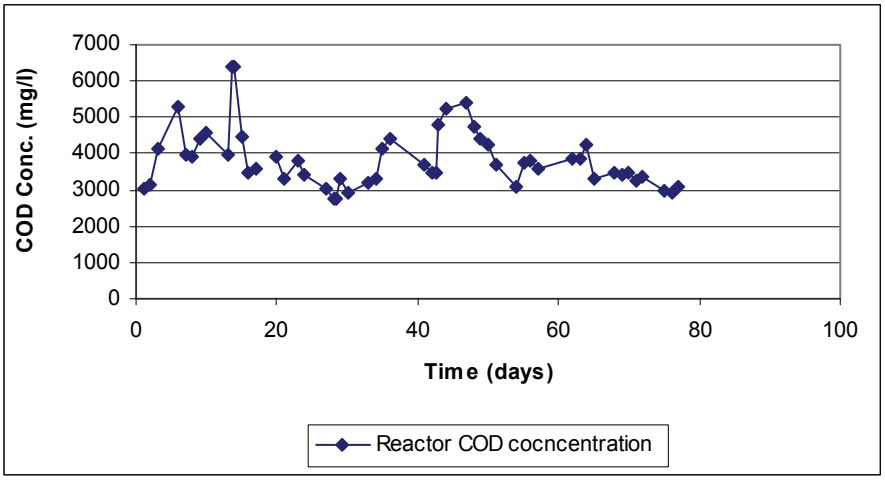

Figure 6.4. The COD concentration in CMR

6.3.3 VFA concentration

The high average COD concentration of 3 835 mg/ℓ thus seemed to indicate that the RI was able to ferment the grass-cellulose to high levels of COD. When observing the graphs in Figures 6.5 and 6.6, showing the VFA concentrations, it can be seen that these concentrations were very low. The average propionate and butyrate concentrations were both 10 mg/ℓ, while the acetate concentration was as low as 25 mg/ℓ. These low VFA results may indicate that all VFA produced was used for sulphate removal, or more likely that the VFA production was too low to sustain continuous sulphate removal. Although GC, RI and SRB were added daily, The VFA production as well as the sulphate removal was low.

From the low VFA concentrations and from the poor sulphate removal relative to the high COD concentration, especially towards the end of the experimental period, it can be deduced that the RI seemed inhibited and thus were not producing the right substrate for the SRB to adequately remove the available sulphate. When grass-cellulose is degraded, macro nutrients in the form of NO_3-N and PO_4 are released, which is important for cell growth and cell activity. The amount of ATP (Adenosine tri phosphate) generated in SRB is 1 mol of ATP per mol sulphate removed (Schlegel, 1993). For every mol of ATP produced, 1 mol of biomass was delivered. Thus in order for cell growth to proceed, the presence of sufficient phosphate is needed. In addition, microorganims are in need of trace elements (micro nutrients) for enzymatic activities within the cell. The average residual PO_4 concentration in the treated water throughout the total experimental period was 157 mg/ℓ, while the average residual NO_3-N concentration was 3.9 mg/ℓ, which should be sufficient for cell growth. When observing the VSS results (Figure 6.7), it can be seen that cell growth was indeed not inhibited.

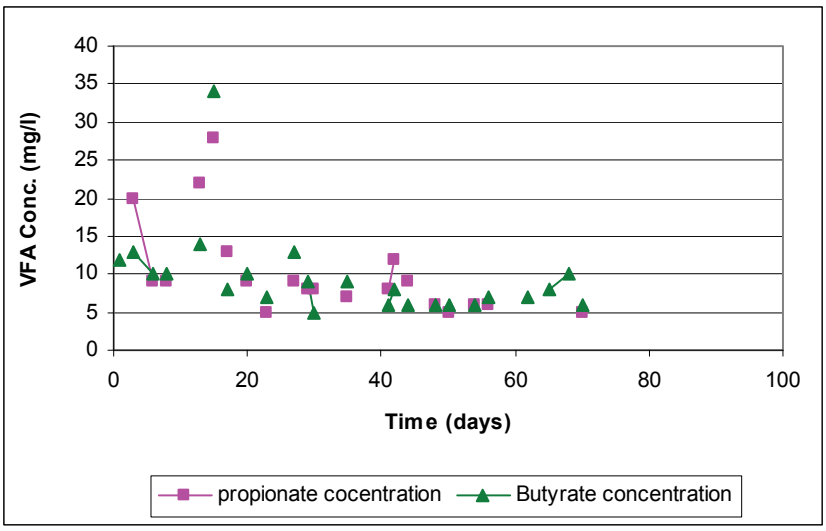

Figure 6.5. The C3 and C4 acids concentration in CMR

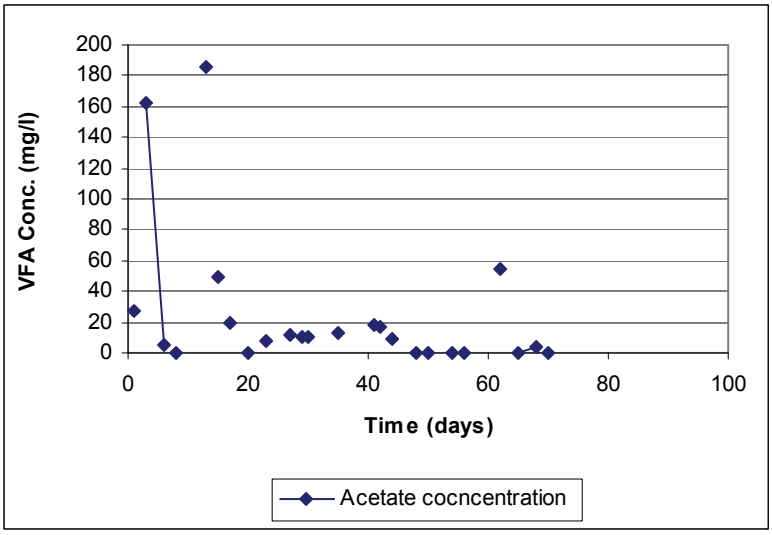

Figure 6.6. The acetic acid concentration in CMR

6.3.4 VSS concentration

The VSS concentration presented in Figure 6.7 shows that the VSS concentration initially increased slowly (between days 20 and 40), and then it increased sharply between days 40 and 60 and even more rapidly after day 60. The increase in biomass can be ascribed to the daily addition of RI and SRB and to the regular addition of GC. However, the remarkable increase in VSS concentration does not explain the poor sulphate removal. This result showed that the VFA production is not only reliant on substrate (GC) and high concentrations of biomass (VSS), but also on the ability of the cells to efficiently produce the required substrate for sulphate removal. Since the reactor was operated in batch mode, the waste products in the reactor were not removed and thus it can be hypothesized that these products accumulated, which may have been toxic to the VFA producing microorganisms, since that the VFA concentrations in the reactor were low, while the COD concentration was higher than 3 000 mg/ℓ. Thus the COD concentration produced was not ascribed to VFA production, but to other intermediates, which seemingly were not the preferred carbon sources for sulphate removal.

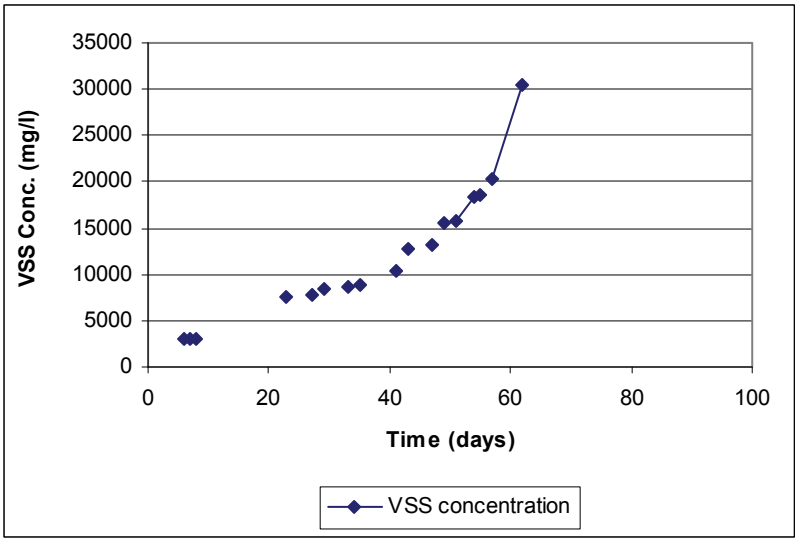

Figure 6.7. The VSS concentration in CMR.

STUDY 1B: CMR IN CONTINUOUS MODE.

6.3.5 Sulphate, COD and VFA concentrations

The sulphate concentrations in the feed and in the treated water are presented in Figure 6.8, as well as the COD concentration in the treated water. The graphs show that the feed water sulphate concentration was stable at about 2 600 mg/ℓ, except towards the end of the experimental period, when it decreased to 2 300 mg/ℓ. The COD concentration was higher than 1 500 mg/ℓ, during the total experimental period and was mainly higher than 2 000 mg/ℓ. The average COD concentration during this experimental period was 2 185 mg/ℓ. The relative high COD concentration did not result in sulphate removal as can be seen by the sulphate concentration in the treated water, which except for day 8, was 2 000 mg/ℓ (Figure 6.7). The high COD concentration of 3 000 mg/ℓ on day 8 seemed to result in total SO_4 removal. As was observed from the batch operation in CMR, the VFA production/sulphate reduction system seemed inhibited. The VFA concentrations (acetic-, propionic and butyric acids) were not detectable. This result may indicate that VFA were not produced at high enough concentrations. The relative high COD concentration resulted in a 21% sulphate removal during the 33 day experimental period. This finding may indicate that fermentation products other than VFA e.g. ethanol or/and H_2, were utilised for the biological sulphate reduction, which are produced in the degradation of polymers and monomers.

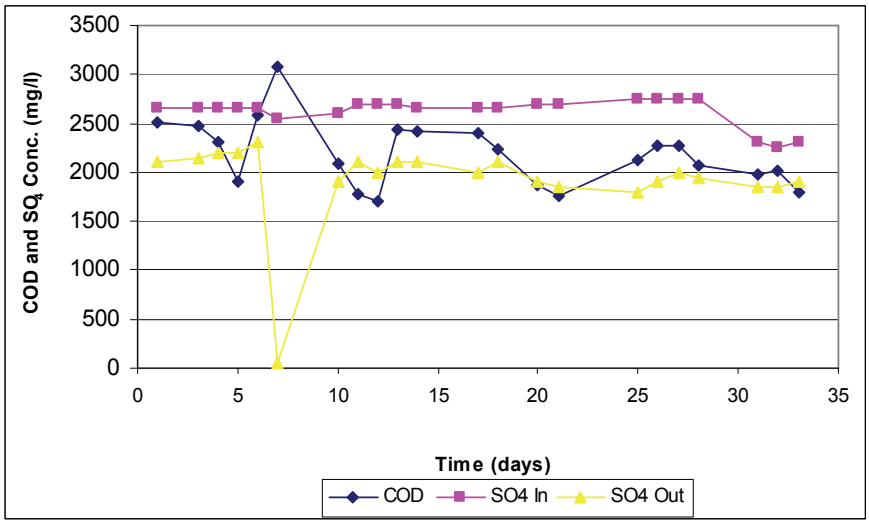

Figure 6.8. The SO_4 and the SO_4 and COD concentration in the feed and treated water, respectively.

6.3.6 VSS concentration

The VSS concentration in CMR during this experimental period was measured from day 1 till day 21, during which period it increased from 4.5 to almost 7 g/ℓ, due to the weekly addition of RI and SRB as well to due expected growth of the cells. Towards the end of the 21 day period, the VSS concentration decreased from about 7 g/ℓ to just over 4 g/ℓ. This period coincided with a cold period, which may have affected the reactor contents. From day 25 onwards, the reactor was heated till 25°C, however no further VSS samples were analysed. Another reason for the biomass loss can be ascribed to the settling of sludge in the clarifier. Normally the settled sludge is recycled from the bottom of the clarifier to the top of the reactor, thus the biomass remains active in the reactor. In this reactor, however, the GC settled partly in the clarifier as well, which resulted in blockages between the settler and the reactor and thus the recycle was stopped before the experiment was stopped. Therefore, the biomass concentration in the settler likely increased, while it decreased in CMR.

STUDY 2: HFS IN CONTINUOUS MODE AT 25°C FEEDING SYNTHETIC FEED WATER

6.3.7 Sulphate and COD concentrations.

The sulphate concentrations in the feed water and in the treated water during Periods 1 and 2 (divided by the vertical bar) are presented in Figure 6.9. It can be observed from the graphs that the COD concentration in the treated water of the HFS reactor was generally >1000 mg/ℓ, which resulted in a high sulphate removal during both periods. After the addition of fresh GC and microorganisms, the COD concentration

(Period 2) increased again to concentrations > 2 000 mg/ℓ, resulting in total SO_4 removal. The percentage SO_4 removal efficiency during Period 1 was 90%, while this was 99% during Period 2. The relationship between the COD and SO_4 concentration in the treated water can be observed from Figure 6.9: when the COD concentration was high, the sulphate concentration was low. Thus it is important to maintain a high COD concentration in the reactor, which can be achieved by adding GC and by maintaining a healthy cellulose degrading community.

The sulphate and COD concentrations during Period 3 are shown in Figure 6.10, from where it can be observed that the feed SO_4 concentration was stable after day 75 at approximately 2 700 mg/ℓ. At the same time, the residual COD concentration in the reactor decreased from >1 000 mg/ℓ to <1 000 mg/ℓ, which resulted in a residual SO_4 concentration of >500 mg/ℓ. The higher feed SO_4 concentration required more COD for the SO_4 reduction, which resulted in a lower COD concentration in the treated water. Although 150 g GC was added weekly, it was a challenge to keep the COD concentration in the reactor at high enough concentrations for a high SO_4 removal efficiency. In order to keep the COD concentration at higher concentrations, more GC should be added to the reactor. However, the size of the reactor is the limiting factor. Thus to remove a higher sulphate load, a different reactor design may be required.

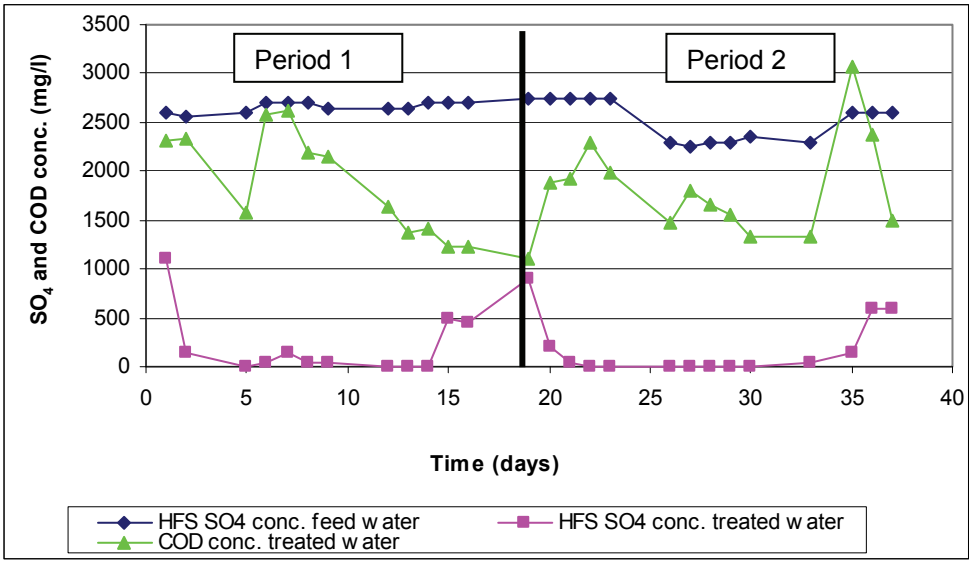

Figure 6.9. The SO_4 and COD concentrations in the feed and in the treated water operating HFS during Periods 1 and 2.

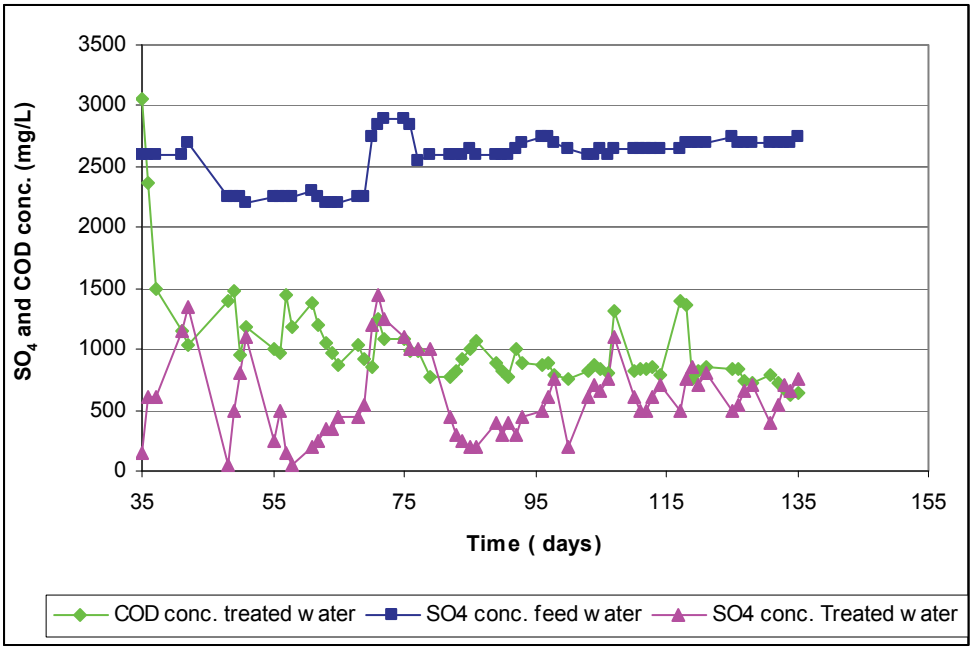

Figure 6.10. The SO_4 and COD concentrations in the feed and in the treated water operating HFS during Period 3.

The average percentage SO_4 removal efficiency during Period 3 was 77%, while for the total period of 100 days (thus including Periods 1 and 2) this was 82%. The percentage SO_4 removal efficiency is depicted in Figure 6.11. It can be observed that from an initial high percentage sulphate removal, during Periods 1 and 2, it decreased and was irregular till ca. day 80-100, when the percentage SO_4 removal was stable at an average of >80%, after which it decreased from day 100-135 at a percentage between 70-80%.

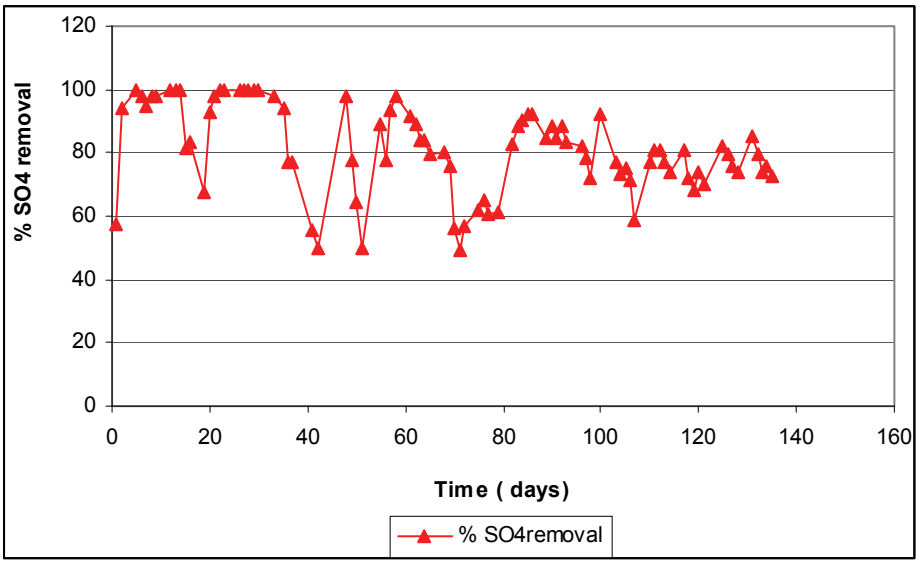

Figure 6.11. Percentage sulphate removal

The average SO_4 removal (g/d) data and the average COD concentrations as obtained from the studies during the three periods and during the total period are presented in Table 6.2.

Table 6.2. The experimental data during Periods 1, 2, 3 and total period

Period	**1**	**2**	**3**	**Total**
Parameter				
Residual COD (mg/ℓ)	1 825	1 725	1 015	1 228
SO_4 removed (g/d)	12.0	12.3	9.9	12.1
SO_4 removed (%)	90	99	77	82
S^{2-} (mg/ℓ)	425	494	436	441
$S^{2-}_{produced}$/ $SO_{4\ removed}$ ratio	0.18	0.20	0.22	0.21
Alkalinity produced (mg/ℓ)	2669	2842	2199	2351
$Alk_{produced}$/ $SO_{4\ removed}$ ratio	1.11	1.16	1.12	1.12
1 g GC/g SO_4 removed	0.52	0.82	0.29	0.41
NO_3-N in treated water (mg/ℓ)	62	100	97	83
PO_4 in treated water (mg/ℓ)	93	159	133	136

From the data in Table 6.2, the relationship between the higher COD concentration in the treated water and the SO_4 removal data is evident. At a higher residual COD concentration in the reactor (Period 2), the highest sulphate removal was observed. Due to the high sulphate removal efficiency, high concentrations of sulphide were noticed in the treated water. Since no metals were added to the synthetic water, no sulphide was removed from the reactor.

The experimental $S^{2-}_{produced}$/ $SO_{4\ removed}$ ratio of 0.18, 0.20 and 0.22 are very similar throughout the three experimental periods. Although the theoretical $S^{2-}_{produced}$/ $SO_{4\ removed}$ ratio is 0.33, the experimentally obtained ratios are mostly ± 0.20, due to partly sulphide to sulphur oxidation, to other intermittent sulphur species and to the formation of H_2S gas. Similar results were obtained for the experimental $Alk_{produced}$/ $SO_{4\ removed}$ ratio, which was rather stable at 1.11, 1.16 and 1.12 and deviated 7% from the theoretical ratio of 1.04.

The $GC_{added}/SO_{4\ removed}$ ratio is presented in Table 6.2, showing that 1 g GC removed the highest sulphate concentration during Period 2, when 1 g GC removed 0.82 g SO_4. The $GC_{added}/SO_{4\ removed}$ ratio over the full experimental period was 0.41. Thus at

the described reactor conditions, the sulphate removal from 1 g GC is ± 0.5 g SO_4 at a HRT of 2.4 days and a reactor temperature of 25°C.

6.3.8 Nutrients

Due to the anaerobic degradation of GC, macro nutrients, such as nitrate (NO_3^--N) and phosphate (PO_4^{3-}-P) are released, which are used by the microorganisms for sustainability. Most rumen bacteria obtain their energy for growth through anaerobic fermentation of carbohydrates, while nitrogen is utilised for cell structure. Generally, bacteria use up carbon 35-30x faster than they use nitrogen. This implies that the C:N ratio should be in the order of (25-30):1 (Polprasert, 2007). The NO_3-N and the PO_4 concentrations in the treated water are listed in Table 6.2. It can be observed that the residual carbon and nitrogen ratio during Period 1 adhere to the prescribed ratio. However, the actual ratio in the reactor can not be measured. The nutrient concentrations in the treated water are too high for discharge. In case the treated water after sulphate reduction is used for land irrigation, the increased levels of macro nutrients as well as the residual COD concentration in the treated water can be used as fertiliser and soil conditioner, respectively.

6.3.9 VFA

The residual VFA concentrations in the treated water are presented in Table 6.3. It can be noted that both the propionic and butyric acid concentrations were low and that the residual acetate concentration was higher. This higher C2 acid concentration can be explained by the fact that acetate is formed when propionic and butyric acids are utilised for biological sulphate reduction and the SRB population is only utilise acetate for sulphate reduction, when no other energy sources are available. This observation was discussed in the previous chapters. The lower residual C3 and C4 acid concentration in the treated water can be ascribed to the utilisation of these energy sources for the sulphate reduction.

Table 6.3. The VFA concentrations during Periods 1, 2, 3 and total period

Period	**1**	**2**	**3**	**Total**
Parameter				
Acetate	74	363	80	113
Propionate	6	2	18	14
Butyrate	11	3	12	11

6.3.10 pH

The reactor pH was manually controlled, by trying to keep the reactor pH below 7. Since the reactor pH increased due to SO_4 removal process, this was attained by controlling the feed water pH. The pH values in the feed- and treated water are depicted in Figure 6.12. It can be observed that with time, the feed water pH needed to be maintained at pH 5, in order to keep the reactor pH at approximately 7, since the required pH for the RI is 6.6-6.9.

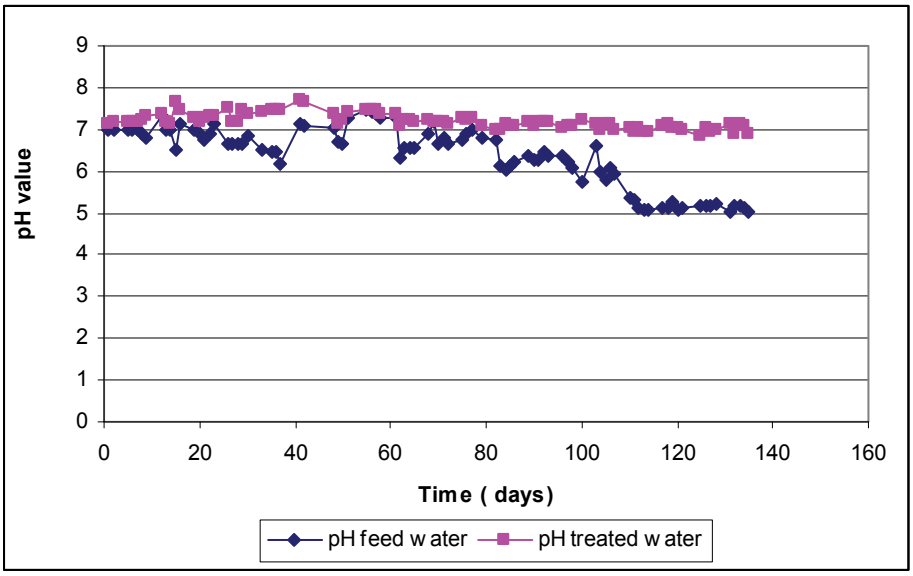

Figure 6.12. The pH of feed and treated water

STUDY 2B: HFS IN CONTINUOUS MODE AT 25°C FEEDING PRE-TREATED MINE WATER

6.3.11 Sulphate removal as function of COD concentration

The sulphate concentrations in the feed and treated water as well as the COD concentration in the treated water during the total experimental period of 33 days are depicted in Figure 6.13. It can be observed that the sulphate concentration in the feed water was stable at an average of 2 613 mg/ℓ, while the sulphate concentration of the treated water was mainly <500 mg/ ℓ, at an average of 206 mg/ℓ. This obtained result was ascribed to the relative high COD concentration in the treated water (at an average of 951 mg/ℓ). These results showed that hybrid reactor HFS performed well treating pre-treated mine water, which indicated that this type of reactor system can be advised for continuous sulphate reduction at a HRT of 2.4 days and at a temperature of 25°C.

The sulphate removal results operating the hybrid reactor can be observed from Figure 6.14, which showed the percentage sulphate removal efficiency. The average percentage removal efficiency was 92%.

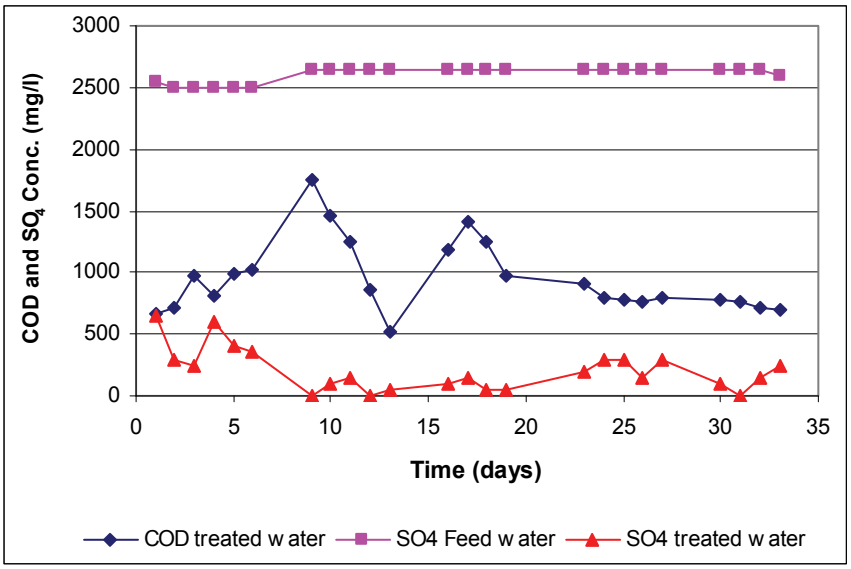

Figure 6.13. The sulphate concentration in feed and treated water and COD concentration in treated water.

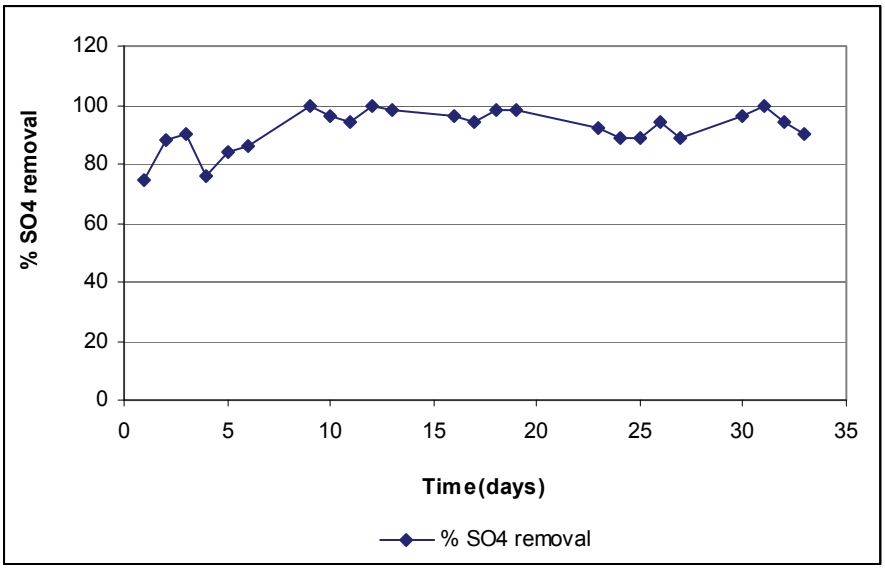

Figure 6.14. The % sulphate removal efficiency

6.3.12 The average experimental data as obtained from the treatment of pre-treated mine water is presented in Table 6.4.

The VFA results showed that the average residual acetate concentration was 148 mg/ℓ, while the propionic and butyric acids were low at 26 and 22 mg/ℓ, respectively. The additional data presented in Table 6.4 indicated an average sulphate removal of 12 g/d and in a total sulphate removal of 396 g sulphate over the 33 day

experimental period. During that period 750 g GC were added, thus 1 g GC removed 0.53 g sulphate. This result was comparable to the obtained $GC_{added}/SO_{4\ removed}$ ratio during the first part of this experiment, feeding synthetic feed water, when 1 g GC removed an average of 0.41 g SO_4. Thus, the improved percentage sulphate removal efficiency obtained when feeding pre-treated mine water, was reflected in the improved $grass_{used}/sulphate_{removed}$ ratio, which improved from an average of 0.41 to 0.52. The high sulphide concentration of 486 mg/ℓ and the relative low redox potential of -191 mV both indicated that the reactor performed well removing the sulphate from the waste water. This was also observed from the $S^{2-}_{produced}/ SO_{4\ removed}$ ratio data, which was 0.20 during this experimental period, which was similar to the finding when HFS functioned on synthetic feed water. The experimental $Alkalinity_{produced}/ SO_{4\ removed}$ ratio of 1.05 feeding pre-treated AMD was close to the theoretical value of 1.04. The average nutrients (NO_3-N, NH_3-N and PO_4-P) concentrations in the treated water were 36, 85 and 155 mg/ℓ, respectively, while the VSS concentration was 1.16 g/d. These values are too high for discharge, but the nutrients and organic content in the treated water can make it suitable for irrigation of grass and/or crops.

Table 6.4. The experimental data feeding pre-treated mine water

Parameter	Value
Residual COD (mg/ℓ)	951
SO_4 removed (g/d)	12.0
SO_4 removed (%)	92
S^{2-} produced (mg/ℓ)	486
$S^{2-}_{produced}$/ $SO_{4\ removed}$ ratio	0.20
Alkalinity produced (mg/ℓ)	1 864
$Alk_{produced}$/ $SO_{4\ removed}$ ratio	1.05
pH feed water	4.99
pH treated water	7.01
Acetate	148
Propionate	26
Butyrate	22
1 g GC/SO_4 removed	0.52
Redox Potential (mV)	-191
NO_3-N in treated water (mg/ℓ)	36
NH_3-N in treated water	85
PO_4 in treated water (mg/ℓ)	155
VSS in treated water (g/d)	1.16

6.3.13 Metal removal

Samples were taken from the AMD as obtained from the mine, from the pre-treated AMD and from the effluent after sulphate removal in HFS. The results are presented in Table 6.5, from where it can be observed that except for the Chromium (Cr) concentration all metals were removed to values <1, except for Manganese (Mn), which decreased from 9 to 1 mg/ℓ. Most of the iron concentration of 11 mg/L in the AMD decreased to 0.33 mg/ℓ in the pre-treated AMD, due to precipitation with the sulphide concentration in the effluent after sulphate reduction. The Zinc (Zn) concentration decreased from 1.4 mg/ℓ in the AMD to 0.28 mg/ℓ after pre-treatment and decreased further to values < 0.04 after the sulphate removal in HFS. The average lead (Pb) concentration remained similar during the sulphide precipitation process.

Table 6.5. Metal removal from AMD during pre-treatment and SO_4 removal

Metal (Mg/ℓ)	AMD	Pre-treated AMD	HFS Treated water
Cr	0.1	0.09	0.11
Fe	11	0.33	0.3
Pb	0.25	0.34	0.22
Mn	9	8	1
Ni	0.53	0.4	0.09
Zn	1.4	0.28	< 0.04

6.3.14 Economics of the technology using degradation products of GC as the carbon and energy sources

Initial calculation towards the economics of the use of grass cuttings versus the use of ethanol as the carbon and energy source for the biological sulphate removal have been undertaken. In these calculations it was assumed that 1 000 m^3/d AMD with a concentration of 2.5 kg/m^3 SO_4 needed to be treated. Thus 2.5 tons of SO_4 per day needed to be treated for this assumption (which was based on AMD, used in the previous study). The grass requirements for this treatment process were 660 058 kg dry GC per year, for which 43.5 ha land was needed. In order to mow this grass 3 361 MJ diesel per ton SO_4 removed is needed. In the case that ethanol was used as the carbon and energy source a similar calculation was executed and it was found that 28 750 MJ (ethanol) per ton SO_4 removed was needed. Thus when taking the energy-economics of the technology into account, it is more favourable to use the degradation products of GC rather than ethanol as the carbon and energy source for the described biological sulphate reduction.

When a biological sulphate removal reactor is operated using technical ethanol as the carbon and energy source, the running costs of operating such a reactor will increase with the increase of the petrol price, both from the perspective of the product as well due to the transportation costs. The COD of pure ethanol theoretical is ± 2 000 mg/ℓ, the empirical value of technical ethanol is ≈ 1 250 mg/ℓ. Thus when the SO_4 concentration in AMD is 2 500 mg/ℓ, for every litre of AMD treated 2 ml ethanol are needed. When treating 1 000 m^3/d AMD, the ethanol requirement amounts to 2 m^3/d. The price of technical ethanol in April 2007 was increased to R2 858/m^3. Thus the ethanol costs treating 1 000 m^3/d AMD with a concentration of 2.5 kg/m^3 SO_4 will amount to R5 716/d. The costs associated with the cultivation and fertilisation of grass lies mainly in preparing the land, sowing the grass seeds,

irrigation, fertilisation and cutting. In order to irrigate the land, treated AMD can be used, which still contains nitrate and phosphate in the effluent as the degradation products of grass, which can supply the nutrients to the land. Once the preparation of the land and the sowing is done, then the land can produce grass cuttings for a full season and the surplus of grass generated through the summer can be stored for the winter months or alternatively the grass can be irrigated throughout the winter months resulting in additional cut grass albeit at lower yields as during the summer period.

6.4 CONCLUSIONS

It can be concluded from the studies relating to CMR that:

- The daily addition of biomass (RI and SRB) and of GC to CMR resulted in a high concentration of COD
- Although the COD concentration in CMR was high, the VFA concentration was low, which was not conducive for efficient sulphate reduction
- The VSS concentration in CMR increased with supplementation of RI and SRB, but did not result in increased sulphate reduction
- CMR was not the chosen reactor system for biological sulphate removal, using the degradation products of GC as the carbon and energy sources, since the high COD concentration did not result in efficient sulphate reduction.

It can be concluded from the studies relating to HFS that:

- The percentage SO_4 removal efficiency varied from 90 to 99 to 77% during periods 1, 2 and 3, respectively, when feeding synthetic feed water. This removal percentage increased to 92%, when feeding pre-treated mine water.
- The higher sulphate SO_4 efficiency was directly related to the COD concentration in the treated water.
- That 1 g GC could remove 0.52, 0.82 and 0.29 g SO_4 during periods 1, 2 and 3, respectively feeding synthetic feed water. This value was 0.52 g SO_4 from 1 g GC when feeding pre-treated mine water.
- The high SO_4 removal efficiency resulted in high sulphide concentrations, which may with time have affected the COD/VFA production, since the SO_4 removal efficiency decreased with time.
- The overall SO_4 removal efficiency was 82% when operating the HFS reactor at a HRT of 2.4 days and a temperature of 25°C, feeding synthetic feed water and it was 92% when feeding the HFS reactor with pre-treated mine water.

- ❖ Using the degradation products of GC and RI is a promising option for treating high sulphate concentrations in mine and other sulphate rich industrial waste waters.
- ❖ The energy-economics of the study indicated that the use of the degradation products of grass cuttings is more favourable than using technical ethanol as the carbon and energy sources for the technology.
- ❖ Grass can be grown at mining sites, using the treated water for irrigation, since this water contains nutrients (NO_3-N and PO_4) as degradation products of GC. The residual COD concentration is beneficial as soil improver.

6.5 REFERENCES

Buyukkamaci, N. and Filibeli, A. (2004). Volatile fatty acid formation in an anaerobic hybrid reactor. Process Biochemistry **39:**1491-1494.

Greben, H.A, Maree,J.P, Eloff, E and Murray, K. (2005). Improved sulphate removal rates at increased sulphide concentration in the sulphidogenic bioreactor. Water SA, Vol. 31, No 3.

Hungate, R.E. (1966). The rumen and its microbes. Academic Press Inc. New York, USA

Iza, J. (1991). Fluidized bed reactors for anaerobic waste water treatment. *Water Sci.Technol*. **24:**109-132.

Lettinga, G., Van Velssen, A.F.M., Hobma, S.W., de Zeeuw, W. and Klapwijk, A. (1980). Use of the Upflow Sludge Blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnol. Bioeng*. **22:** 669-734.

Maree, J.P., Dill, S., Van Tonder, D., Greben, H.A., Engelbrecht, C., Kehlenbeck, M., Bester, C., Adlem, C., Strydom, W., and de Beer, M. (1997). Removal of Nitrate, Ammonia and Sulphate from AECI Effluent. *Internal CSIR report*: ENV/P/C 97141/1.

O'Flaherty, V. and Colleran, E. (2000). Environmental Technologies to treat sulphur pollution. Principles and Engineering. Ed. Lens, P.N.L and Hulshoff Pol L). IWA London.

Polprasert, C. (2007). Organic Waste Recycling. Third edition. IWA publishing, Alliance House, London, UK. ISBN:184339121X

Schlegel, H.G. (1993). General microbiology, Seventh Edition, Cambridge University Press.

Visser, A. (1995). The anaerobic treatment of sulphate containing wastewater. *PhD Thesis*, Wageningen Agricultural University, Wageningen, The Netherlands.

Weast, R.C. (1981). Handbook of Chemistry and Physics, 62th ed., CRC Press Inc., Boca Raton, USA.

Young, J.C. and McCarty, G.Y. (1969). The anaerobic filter for waste water treatment. *J.Wat. Poll. Control Fed.* **41**:160-173.