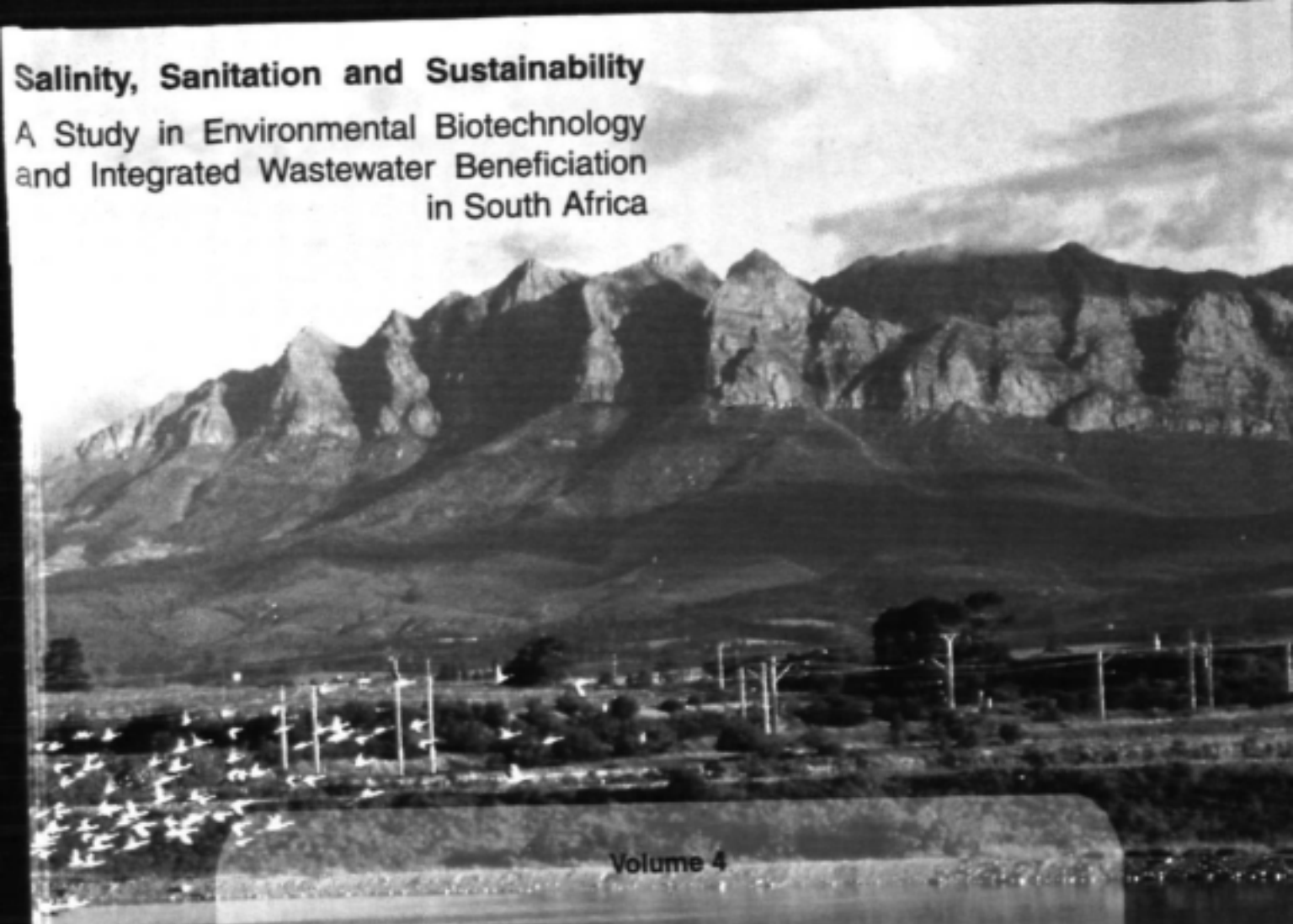
Salinity, Sanitation and Sustainability

A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa



Volume 4

THE RHODES BioSURE PROCESS®

Part 2: Enhanced Hydrolysis of Organic Carbon Substrates - Development of the Recycling Sludge Bed Reactor

K Whittington-Jones, C J Corbett, P D Rose

WRC Report No: TT 196/02

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Water Research Commission

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Cover Photograph:

Flamingoes on tannery wastewater ponds at Mossop Western Leathers Co., Wellington, South Africa. The presence of Phoenicopteridae, including both the Greater and Lesser Flamingo, is an important indicator of healthy and naturally functioning saline aquatic ecosystems. This flock occupied the ponding system shortly after commissioning the novel Spirulina-based Integrated Algal Ponding System which had been developed for the treatment of tannery wastewaters. This apparent seal of environmental approval became an icon for the studies which followed in this series.

Photograph by Roger Rowswell, whose observation of this system, over a number of years, was instrumental in the initiation of these studies.

Salinity, Sanitation and Sustainability:
A Study in Environmental Biotechnology and
Integrated Wastewater Beneficiation in South Africa

Volume 4

THE RHODES BioSURE PROCESS®

Part 2: Enhanced Hydrolysis of Organic Carbon Substrates – Development of the Recycling Sludge Bed Reactor

Report to the Water Research Commission
By

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Environmental Biotechnology Group
Rhodes University
Grahamstown

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Report to the Water Research Commission on Projects K5/ 869 'Biological sulphate desalination and heavy metal precipitation in industrial and mining effluents using the IAPS'; and K5/972 'Process development and system optimisation of the integrated algal trench reactor process for sulphate biodesalination and heavy metal precipitation in mining and industrial effluents'.

Project Leader: Prof P.D. Rose

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FOREWORD

The work presented in this series covers a decade of concerted research into critical sustainability issues in the water-scarce Southern African situation. The provision of safe and adequate drinking water and sanitation services to all our people remains a challenge. Pervasive salination from a range of mining, industrial and agricultural activities threatens the quality of our water resources. Simultaneously, the complex ecological needs of the aquatic environment are being understood with ever-increasing clarity.

Significant progress has been made in meeting some of these challenges. In the years since the democratic elections of 1994, millions of previously unserviced South Africans have been supplied with safe drinking water and sanitation services. The problem of increasing salinity of our water resources, with its direct economic impacts and future threat to sustainability, is being addressed at policy and implementation levels, for example by reduction-at-source measures. The ecological needs of the aquatic environment have been recognised by the provision in our water law of a prioritised ecological reserve, to be managed by the catchment management agencies being formed.

Such promising developments notwithstanding, ultimately sustainable resolution of these issues depends crucially also on acquiring appropriate and affordable technologies that provide physical solutions to our water-related challenges. It is in this context that the research described in this series deserves special commendation for the highly innovative biotechnological linkage developed between the treatment of saline wastewaters on one hand and domestic sewage and sludges on the other.

In the novel approach followed, salinity and sanitation issues are each viewed essentially as a resource base (rather than simply as "waste problems") in a suite of integrated process schemes which can be variously manipulated to deliver products of treated water, recovered nutrients and metals, and algal biomass. The paradigm is consequently changed from one of "managing problems" to one of "engineering opportunities", with the potential of offering a major contribution towards the management of water and sanitation in the RSA - some applications have already been taken to full scale implementation, for example in the accelerated digestion of sewage sludge. Significantly, the achievements of this research add weight to biotechnology as "the" technology of the 21st century.

So, as we approach the World Summit on Sustainable Development, we can reflect on the provisions of Agenda 21 adopted after the Earth Summit some 10 years ago, and note that in this time we have ourselves in various ways "done something" about our own situation. And we can therefore point with a justifiable sense of pride and achievement to the body of work presented here as being "Made in South Africa", at a time when social, environmental, political and economic calls are being made to all of Africa to stand up in the continental and global communities of nations.

My deep thanks and appreciation go to the Water Research Commission for the foresight in funding this work, and, in particular, to Prof Peter Rose and his research team at Rhodes University, for the vision, purposefulness, innovation and application with which this work has been conceived and executed.

Ronnie Kasrils

Minister of Water Affairs and Forestry
Pretoria
31 July 2002

EDITOR'S NOTE

In 1990 the Water Research Commission, under the (then) Executive Director Dr Piet Odendaal, appointed the Environmental Biotechnology Group at Rhodes University, led by Prof Peter Rose, to carry out a one-year feasibility study to evaluate the potential of a biotechnological approach to the linked treatment and management of saline and sanitation wastewaters with recovery of useful components such as nutrient bio-products.

In the intervening years, this seminal project has resulted in a rich research programme, managed initially by Dr Oliver Hart, subsequently by Zola Ngcakani, and latterly (since 1997) by myself. The progression of the research programme is reflected in this series of reports. Report 1 critically reviews the main arguments considered in the sustainability discourse and their relation to salinity and sanitation, and presents an overview of the work covered in the individual Reports 2 – 12, each of which deals with specific aspects of the research programme. The reports are also to be issued on CD.

The research period concerned spans approximately the decade between the Rio Earth Summit in 1992 and the imminent World Summit for Sustainable Development in Johannesburg. During this time, international concern has been expressed about the limited extent to which the sustainability objectives formulated at Rio, as captured for example in Agenda 21, have been followed through to implementation.

By contrast, it is a noteworthy achievement of this research programme that the "sustainable biotechnology" originally conceptualised by the researchers has in fact, by dint of rigorous research development, experimentation and testing, been translated into a suite of practicable processes for delivering treated water as well as value-adding organic and inorganic co-products. In some applications, full-scale plants are already being installed, fulfilling the cycle of research → development → implementation.

It is probably fair to say that the full potential of the original work initiated twelve years ago, with its various applications as they have been developed since then, could at inception only have been dimly foreseen – which, with hindsight, underscores the clarity, breadth and depth of the originators' vision.

It has been a pleasure and a privilege to be involved with this work, as Research Manager and now as Editor of this series. I am confident that you, the reader, will find the contents both informative and as stimulating as I have.

Greg Steenveld
Water Research Commission
Pretoria
31 July 2002

PREFACE

This report is one of a series of twelve Water Research Commission studies undertaken by the Environmental Biotechnology Group at Rhodes University, on biotechnology and integration in the management of saline and sanitation wastewater systems. Environmental problems in these areas are reckoned to be responsible for six of the seven priority pollution issues undermining the sustainable development project in Southern Africa. While both salinity and sanitation have separately been the subject of quite extensive investigation, relatively little has been reported on the potential linkage of these systems in meeting sustainable development objectives.

At the time these studies commenced, in 1990, focus on the operationalisation of the sustainability idea had identified 'integrated waste resource management' as a key requirement for progress towards 'closed systems' production. Here human activities, and the associated technological environment, would be detached as far as possible from the bio-physical environment related to natural systems. Waste recovery, recycle and reuse had emerged as major strategies for achieving the radical shift to new technologies which would enable societies to live off nature's income, rather than consuming its capital. Waste beneficiation (a term still more common in the traditional resources sector, and referring to operations that add value by transforming raw material into finished products), was seen as a means of placing treatment operations on an economic footing, with value added in the form of products and services accrued in the waste management operation.

To meet the time-scale of the sustainability agenda, the breakthroughs in technology required would have to be initiated now to guarantee their availability in the next 2 to 4 decades. This led to widespread use of technology-push approaches in sustainable technologies research.

The principal aim of this programme was thus to investigate potential in environmental biotechnology for the development of technological enablement in the linkage of saline and sanitation wastewater management. This involved initial studies in the biology of organic saline wastewater impoundments and an evaluation of the recovery of nutrient values in these wastes in the form of high-value bio-products produced by halophilic micro-organisms. Integrated Algal Ponding Systems were investigated as a 'core technology' in delivering these objectives.

A critical path research methodology was used to identify technological constraints in the organic saline wastewater treatment operation and served to prioritise the research inputs required to underpin bioprocess development. Studies in the microbial ecology and environmental biotechnology of these systems provided the basis for bio-process innovation, and the subsequent development of treatment processes to full-scale engineered applications.

This series includes an introductory volume which provides an overview of the twelve-year programme to date. The reports are listed inside the front cover, and each study in the series is identified by a 'racing flamingo' number, which also appears on the outside cover. This relates to the appearance of a large flock of flamingoes, which

took up residence on tannery wastewater ponds following the installation of the *Spirulina*-based Integrated Algal Ponding System developed in the initial studies in this series. The development of the 'Salinity, Sanitation and Sustainability' programme is outlined below in Figure P1, and shows studies in the integrated algal ponding of saline, and domestic and industrial wastewaters, leading to the Rhodes BioSURE Process®, which provides linkage in the treatment of sulphate saline wastewaters and sewage sludge disposal.

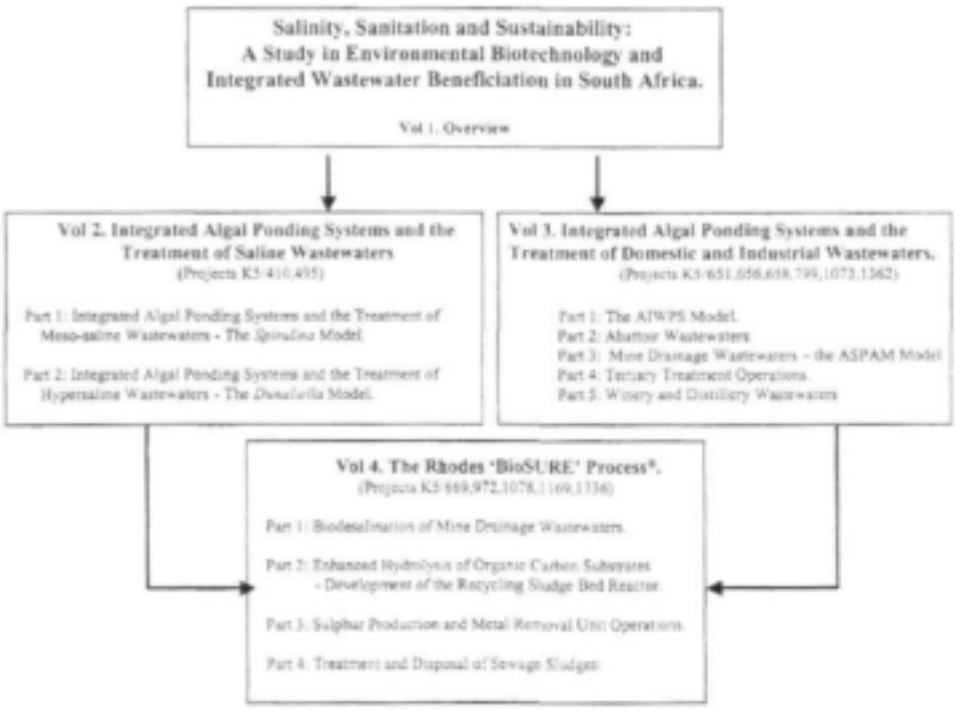

Figure P1. Research projects undertaken as components of the Water Research Commission study 'Salinity, Sanitation and Sustainability'.

A large number of people have assisted generously in many ways in the development of these studies, and are thanked under Acknowledgments. The support of former Water Research Commission Executive Director, Dr Piet Odendaal, is noted in particular. His vision of research needs in water resource sustainability, in the period leading to the Rio Earth Summit in 1992, not only contributed to this study, but also initiated early contributions to sustainable development research in water and sanitation service provision to developing communities. His inputs, together with Research Managers Dr Oliver Hart, Mr Zola Ngcakani, and Mr Greg Steenveld, have made substantial contributions to the development of the ideas investigated in these studies. The contribution and enthusiasm of my post-graduate research students is beyond measure.

Peter Rose
Environmental Biotechnology Group
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Grahamstown

CONTENTS

EXECUTIVE SUMMARY

1 BACKGROUND

Highly saline wastewaters are one of the biggest threats to freshwater resources in South Africa, and originate from a range of industrial activities including mining operations and tanneries. In 1990, the Rhodes Environmental Biotechnology Group (EBG) commenced a WRC-sponsored investigation into the possibility of using Integrated Algal Ponding Systems (IAPS) to treat tannery effluents. Based on the success of a pilot plant, a full-scale IAPS system was constructed on-site in Wellington, and included the establishment of a Primary Facultative Pond (PFP).

During this phase of the study, it was observed that the solubilisation of particulate organic solids leaving the PFP was near complete (Dunn, 1998). Biosulphidogenic activity in the PFP was high and it was proposed that reciprocal upwelling events in the PFP and shallower evaporation ponds served to counteract mass transfer limitations between the water column and the sediment. This movement of particulate organic matter through sulphide gradients, established in these systems, seemed to play an important role in their biodegradation. Particulates not subjected to this passage became compacted into sediments which were sulphate mass transfer limited, and remained undegraded (Rose *et al.*, 2002b).

The study on an IAPS-based application in acid mine drainage (AMD) treatment commenced in 1995, and following an investigation of the unit operations involved, at both laboratory- and technical-scale the project resulted in the development of the Algal Sulphate Reducing Ponding Process for Treating Acid and Metal-containing Wastewaters – ASPAM (Rose *et al.*, 2000a). This then led directly to the conceptualization of the Rhodes BioSURE Process®, the design of which is described in detail in Part 1 of this report (Rose *et al.*, 2002b). A dual-stage process allows for separate optimisation of firstly hydrolysis in the novel Recycling Sludge Bed Reactor (RSBR) and then sulphate reduction in the second-stage Anaerobic Baffle Reactor (ABR).

In order to prove the feasibility of the Process for the remediation of large volume flows of AMD, it was necessary to demonstrate the effective use of a number of complex carbon substrates. Previous studies had shown that tannery effluent and primary sewage sludge (PSS) could be used as sources of electron donors to drive biological sulphate reduction in both laboratory-scale and in $1m^3$ reactors (Dunn, 1998; Rose *et al.*, 2002a). Again, results indicated that the hydrolysis of these complex carbon sources was enhanced under biosulphidogenic conditions, but no studies examined the role of the recycling sludge bed.

Although biological options for AMD remediation have a number of advantages over chemical alternatives, the availability of a readily available carbon source limits its feasibility for large volume flows. Published yields of

soluble carbon from complex sources such as PSS rarely exceed 30% under conventional methanogenic reactor systems. The observations made in the above studies carried out by the EBG suggest that this could be exceeded under sulphidogenic conditions, and would greatly enhance the feasibility of utilizing biological sulphate reducing systems. However, the concept of enhanced hydrolysis of PSS under sulphate reducing conditions still remained to be proved and quantified. Furthermore, if the underlying mechanism could be elucidated, it may be possible to further optimise the process through reactor design and operational control.

2 RESULTS FROM LABORATORY-SCALE REACTORS

Four laboratory-scale reactors were built from 2L vessels in order to prove the concept of enhanced hydrolysis in a RSBR. The reactor configuration allowed for influent sludge to enter near the top of the vessel, and while the soluble fraction passed forward, the heavier particulate matter settled into the base. The sludge from the base was recycled continuously to combine with fresh influent sludge and sulphate (Figure 1a). Each reactor consisted of one or more interconnected units (Figure 1b) that enabled evaluation of both a single and a dual-stage process. The performance of methanogenic and sulphidogenic single and dual-stage processes were also compared. The COD:sulphate ratio of the feed was maintained at 1:1 for the duration of the experimental period. All four reactor systems were operated at a hydraulic retention time (HRT) of 2 days, at room temperature (22-25°C).

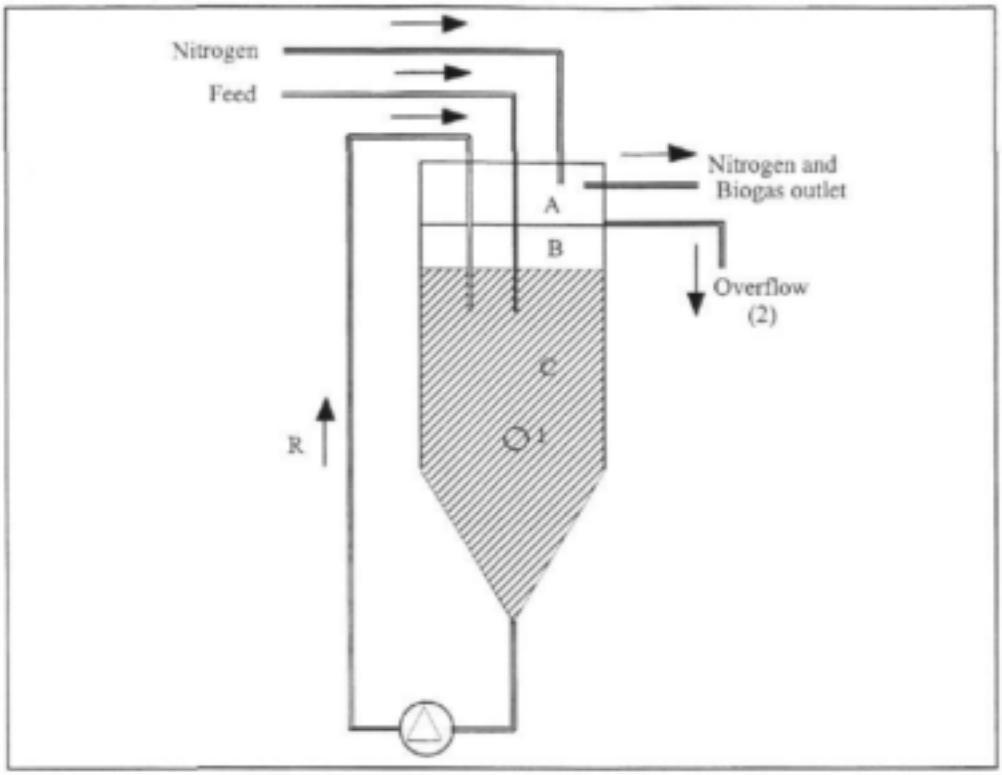
Results from a laboratory-scale RSBR confirmed that complex carbon source such as PSS could be used to drive biological sulphate reduction and that the hydrolysis of PSS was enhanced in a recycling biosulphidogenic environment. The sludge flocs in the sulphidogenic reactor were significantly smaller than those in the methanogenic control. Although it is thought that this plays a role in enhanced hydrolysis by reducing mass transfer limitations, it also increased the susceptibility of washout of associated bacteria and hydrolytic enzymes. A comparison of single- and multiple-stage reactors indicated that the total percentage of sulphate reduced was improved in the latter, and this was taken into account when finalising the design of the dual-stage BioSURE Process®.

3 DEVELOPMENT OF A DESCRIPTIVE MODEL

The solubilisation of the particulate fraction of PSS was followed in flask studies, and was enhanced in the presence of sulphide at both pH 7 and pH 10. A more detailed examination of the mechanism of sulphide-enhanced hydrolysis confirmed that both the protein and carbohydrate fractions were affected. The extent of protein hydrolysis was followed using a new SDS-PAGE technique and showed that a sulphide concentration of 100mg.L^{-1} was required to achieve enhanced hydrolysis of this organic fraction. The exact mechanism by which sulphide enhanced the hydrolysis of the carbohydrate fraction is not understood at this stage, although it may involve removal of lignin either due to the action of sulphide and alkalinity or through direct degradation by SRB. The use of inhibitors, such as toluene to block the uptake

of soluble sugars and molybdate to inhibit sulphate reduction, provided further evidence that the hydrolysis of the lignocellulosic component of PSS was enhanced in the presence of biological sulphate reduction.

a)



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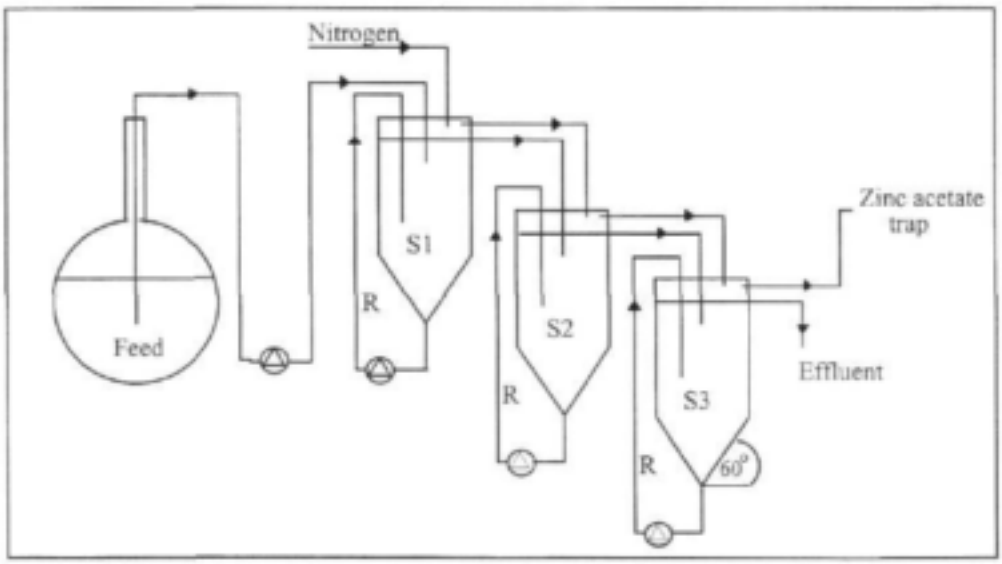
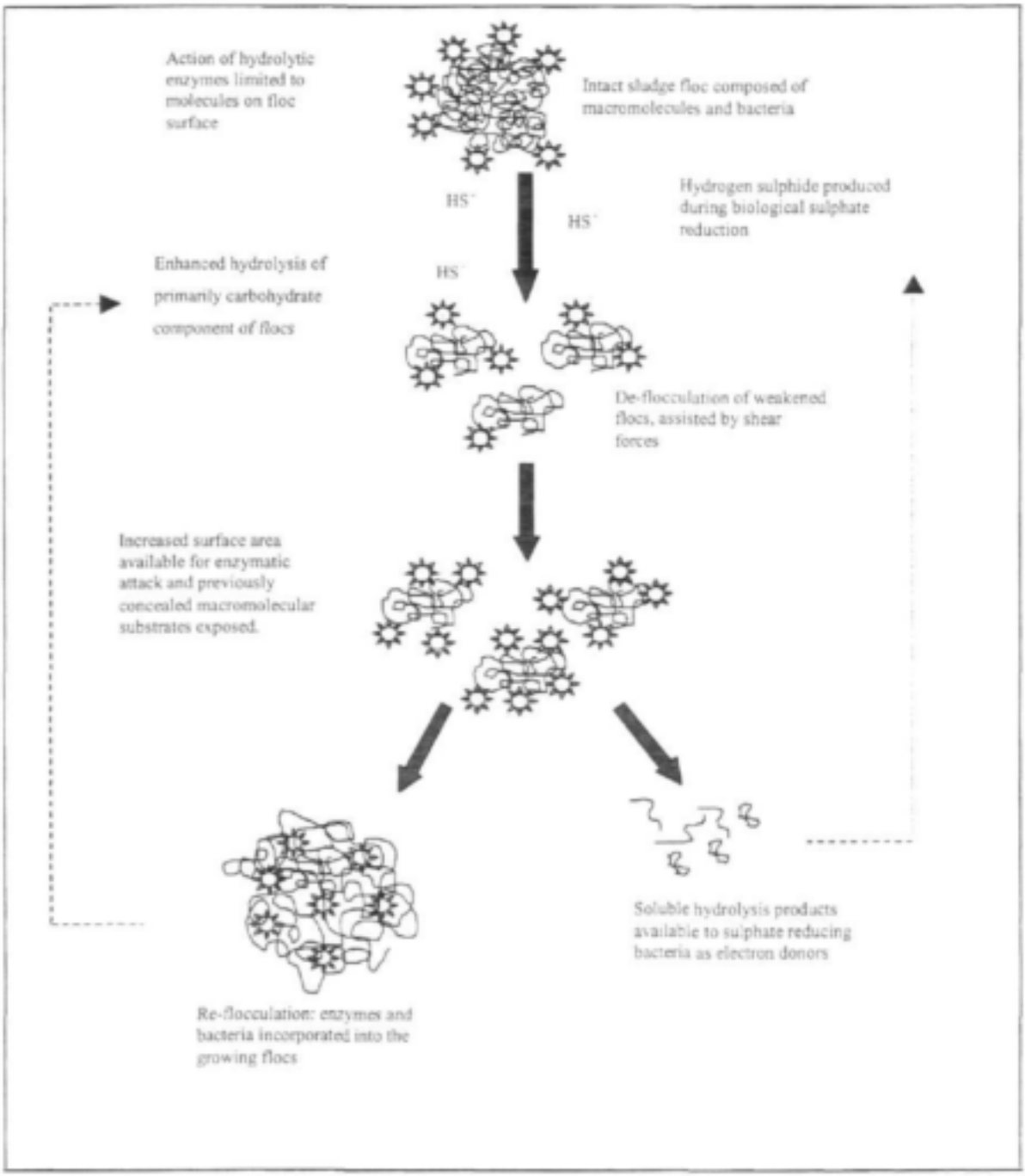


Figure 1. The laboratory-scale reactors used in the study. a) Detail of the single stage reactor (A = headspace, B = Top zone, C = Bed) showing the location of sample ports (1 = Bed, 2 = Effluent). R = recycle. The position of the gas inlets and outlets is also shown. b) Multiple-stage reactor system (S = stage).



Based on the above findings, a descriptive model was developed to explain the phenomenon of enhanced hydrolysis in the RSBR (Figure 2). It is proposed that sulphide has a negative affect on the physical integrity of the sludge flocs, and during the recycling events, large flocs are fractured. This promotes the release of soluble hydrolysis products and sulphide from the floc. The smaller flocs are recycled to the inlet of the reactor where they come into contact with fresh substrate, and reflocculate in the upper settling zone of the RSBR. During reflocculation, fresh substrate, sulphate, bacteria and associated hydrolytic enzymes are incorporated into the center of the flocs. These then

settle into the base of the reactor and as hydrolysis proceeds, the products of the process stimulate further hydrolysis.

4 RESULTS FROM A TECHNICAL-SCALE PILOT-PLANT

Scale-up of the RSBR proceeded through 2L, 10L, $1m^3$ to $23m^3$ units. Oxidation of sulphide in the Perspex 10L RSBR reactor was found to be extremely rapid and resulted in the formation of a floating sulphur biofilm covering the entire surface of the reactor within 24 hours. The mechanism, rates and microbial ecology involved in the formation of these films have been studied and will be the subject of a future report.

The dual-stage pilot plant Rhodes BioSURE Process® (Figure 3 shows the process flow diagram) was constructed on-site at Grootvlei Mine and was run for a period of 18 months. This report is, however, restricted to the performance of the RSBR and details of the piloting exercise are described in Part 1 of this series (Rose *et al.*, 2002b). A longitudinal cross-section of the RSBR is shown in Figure 4. The diagram clearly shows how particulate matter settles into the base of the reactor while soluble product flows forward in the clear upper zone.

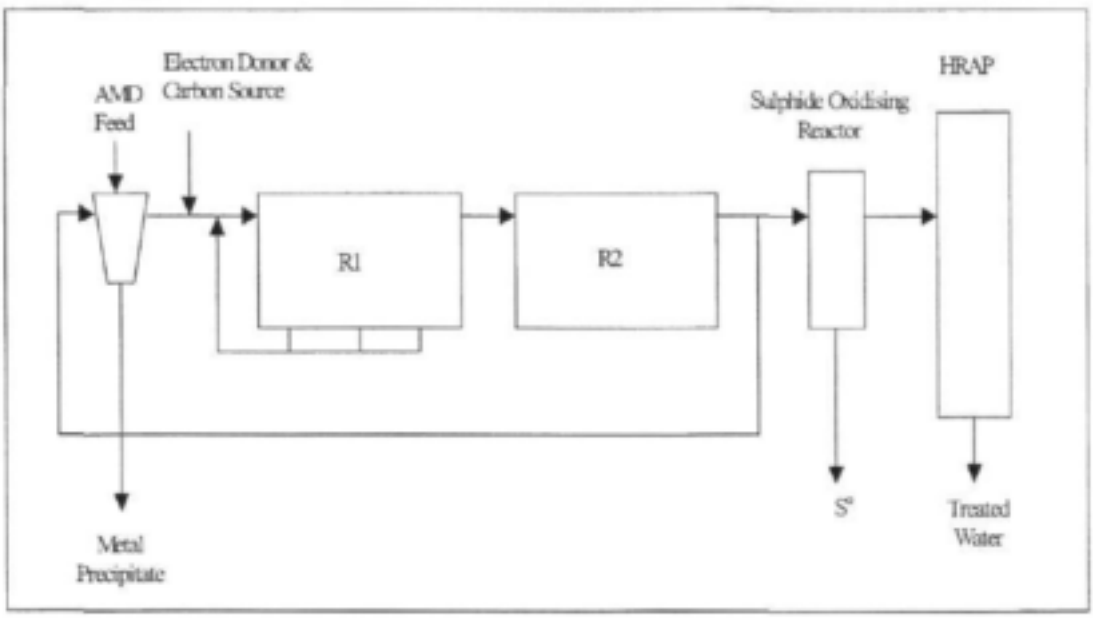


Figure 3. Process flow diagram of the Rhodes BioSURE Process® applied to the treatment of acid mine drainage wastewater. R1 = Recycling Sludge Bed Reactor; R2 = anerobic baffle reactor; HRAP = High Rate Algal Pond; PSS = primary sewage sludge.

Based on the quantity of sulphate reduced, it was concluded that a minimum of 52% of the particulate carbon entering the RSBR had to have been solubilised to its readily available form. This value exceeded all previous published values by approximately 20% and interms of proof-of-concept demonstrated the effectivity of the recycling sludge bed unit operation. Sludge depth profiles were conducted on three separate occasions over the study

period and it was shown that accumulation of sludge within the RSBR was minimal.

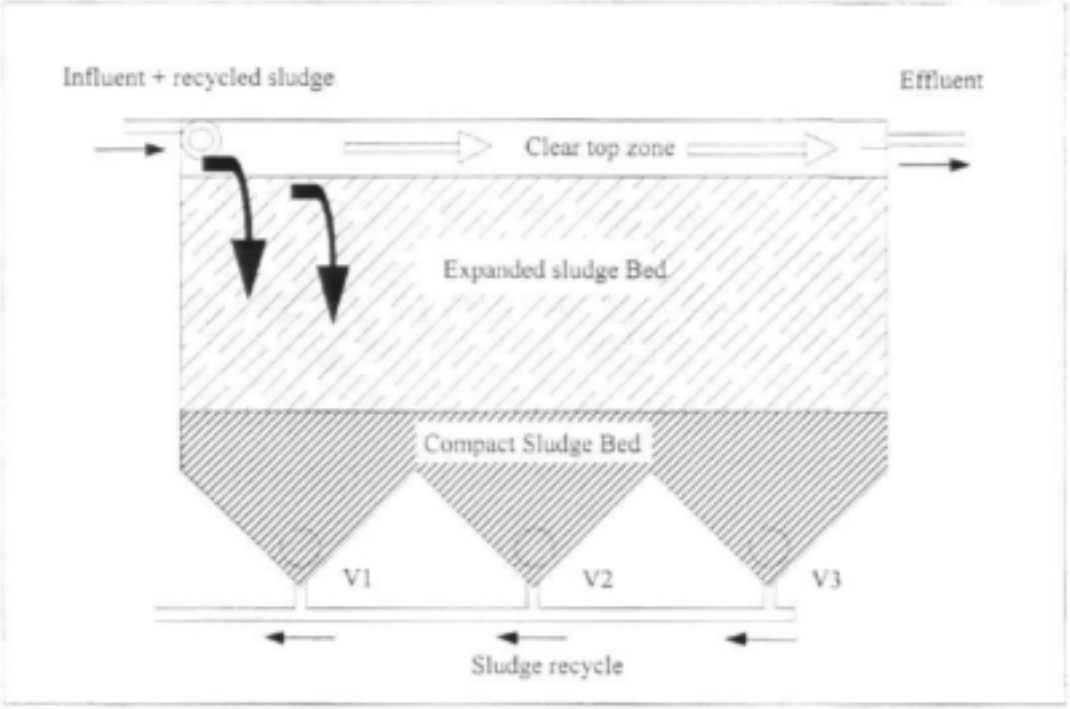


Figure 4. The Recycling Sludge Bed Reactor showing segregation of settled sludge, the recycle of the sludge to blend with incoming flow, and the supernatant liquid carrying the solubilised product to the second stage in the BioSURE Process®.

5 CONCLUSIONS

The aim of the current study was to provide proof of the concept of the enhanced hydrolysis of complex carbon sources within a biosulphidogenic RSBR, to develop of descriptive model of the underlying mechanism and to evaluate the performance of a technical-scale pilot plant.

- Results from a laboratory-scale RSBR confirmed that a complex carbon source such as PSS could be used to drive biological sulphate reduction and that the hydrolysis of PSS was enhanced in a recycling biosulphidogenic environment.

- A comparison of single- and multiple-stage reactors indicated that the total percentage of sulphate reduced was improved in the latter, and this was taken into account when finalizing the design of the dual-stage BioSURE Process®.

- A descriptive model was developed to explain the phenomenon of enhanced hydrolysis in the RSBR. It is proposed that sulphide has a negative affect on the physical integrity of the sludge flocs, and during the reciprocation events, large flocs are fractured. This promotes the release of soluble hydrolysis products and sulphide from the floc. The

smaller flocs are recycled to the inlet of the reactor where they come into contact with fresh substrate, and reflocculate in the upper settling zone of the RSBR. During reflocculation, fresh substrate, sulphate, bacteria and associated hydrolytic enzymes are incorporated into the center of the flocs. These then settle into the base of the reactor and as hydrolysis proceeds, the products of the process stimulate further hydrolysis.

- Scale-up of the RSBR proceeded through 2L, 10L, $1m^3$ to $23m^3$, and based on the quantity of sulphate reduced, it was concluded that a minimum of 52% of the particulate carbon entering the RSBR had to have been solubilised to its readily available form. This value exceeded all previous published values by approximately 20% and demonstrated the efficacy of the recycling sludge bed concept.

6 RECOMMENDATIONS

A number of recommendations relating to the sulphidogenic enhanced hydrolysis concept emerged from the combined research programme undertaken in WRC Projects K5/869 and K5/972.

1. Where the descriptive model had provided evidence for the concept of sulphidogenic enhanced hydrolysis, process development to industrial-scale application would require a mathematical modeling approach to predictive quantification of the relationship between physico-chemical and biological parameters within the reactor. The initiation of these studies was recommended.

2. While a provisional investigation of the enzymology in the RSBR was undertaken in this study, it was apparent that more detailed quanitative studies were required to adequately describe the underlying events. Follow-up studies in this area were recommended.

3. Deflocculation events and the release of soluble products were found to be central to the enhanced hydrolysis reactions in the RSBR. Further studies on the solubilisation process were proposed.

7 RESEARCH PRODUCTS

The combined investigation undertaken in WRC Projects K5/869 and K5/972 led to a number of follow-up studies based on the above recommendations. These are the subject of separate investigations and project reports, are noted below under follow-up actions and are listed in Appendix 1.

Student training associated with these projects has included 1 Post-Doctoral Fellow, 6 PhD and 8 MSc students. Publication of the results of these studies is ongoing but currently includes 7 patents, 9 journal articles and 2 reports. Publication in conference proceedings includes 5 plenary and key note

lectures, 13 international and 20 local conference presentations. Student training and publication outputs are reported in Appendix 2.

Industrial technology transfer has been undertaken, involving the products of these studies, and is noted in Appendix 3. In addition, research spin-off developments which have resulted in associated follow-up research projects have been noted below and in Appendix 4 of this report.

8 FOLLOW-UP ACTIONS

Although it has been proved over a range of scales, from laboratory experiments to a technical-scale RSBR pilot plant, that the hydrolysis of a complex carbon source such as PSS is enhanced significantly under biosulphidogenic conditions, a number of aspects relating to the Rhodes BioSURE Process® are still under investigation. The principles involved also provided the basis for a number of follow-up studies.

8.1 Enhanced Hydrolysis of Other Complex Carbon Sources

Although PSS is readily available in large quantities, in some circumstances it may be preferable to utilize other "cleaner" carbon sources to drive sulphate reduction. This would be particularly relevant when the effluent from the Process is required for the cultivation of products destined for human consumption eg. crop plants. Alternative carbon sources being studied at present include maize waste, wood chips and grass. The studies are being undertaken in collaboration with DACST Innovation Fund project lead by Pulles Howard and De Lange and also with Eskom in active and passive approaches to coal mine wastewater treatment.

8.2 The Role of Sulphate Reducers and Sulphide

The results of the study supported the general mechanism for enhanced hydrolysis in the RSBR. However, the exact role of sulphide, sulphate and SRB in the destabilization of the floc integrity is not clearly understood. Current research is focusing on the effect of sulphide on hydrolytic enzymes in sewage sludge as well as the ability of sulphate reducers to directly enhance the hydrolysis of lignocellulosic material. This work is being undertaken as a component of a Department of Arts Culture Science and Technology (DACST) Innovation Fund Project and in WRC follow-up project K5/1170 by Prof. C. Whiteley's Environmental Enzymology Group in the Dept. Biochemistry, Microbiology and Biotechnology, Rhodes University.

8.3 Oxidation of Sulphide

Biodesalination implies the final removal of sulphate salinity from the waste stream. The concentrations of sulphide in the effluent of the BioSURE Process® can exceed 200mg.L^{-1} and the EBG is investigating the oxidation of this compound to elemental sulphur which may be precipitated and removed from the system.

8.4 Metal Precipitation

The mine wastewater used in the pilot study passed through a High Density Sludge (HDS) process to remove the majority of the iron prior to it entering the RSBR. However, the Rhodes BioSURE Process® allows for metal removal by sulphide precipitation and the control of this step is still under investigation. It is envisaged that a small stream from the ABR will be mixed with the influent metal-rich and the metal-sulphide precipitate will be settled out of solution prior to it entering the RSBR. The chemistry of the process is under investigation in collaboration with Prof. R. Loewenthal of the Dept. Civil Engineering at the University of Cape Town.

8.5 Modeling of the Recycling Sludge Bed Reactor

Although a descriptive model has been proposed to describe enhanced hydrolysis within the RSBR, a mathematical model will provide a greater understanding of the relationships between physico-chemical and biological parameters within the reactor. This information is critical for the optimisation of the process and is being conducted in collaboration with Prof. G. Hansford and Dr A. Lewis in the Dept. Chemical Engineering at the University of Cape Town.

8.6 Disposal of PSS and Biological Nutrient Removal

The disposal of large quantities on undigested PSS is a global problem. Only a limited quantity may be disposed of on agricultural land and the disposal of the remainder, usually by incineration or dumping, is costly. The concept of enhanced hydrolysis in a RSBR offers a novel alternative and the soluble product may be used to drive BNR processes. This project involves collaboration with ERWAT and a full-scale reactor for the enhanced disposal of PSS is currently being investigated at ANCOR sewage disposal works, Springs.

8.7 Interrogation of the Descriptive Model

Although enhanced hydrolysis of complex carbon sources under biosulphidogenic conditions has been demonstrated at both laboratory and technical-scale, and has resulted in the development of a descriptive model, certain aspects of the model require further examination. According to the model, deflocculation events are central to enhanced hydrolysis of complex carbon sources. Destabilization of the sludge flocs allow release of soluble products of the anaerobic digestion process, primarily volatile fatty acids and reducing sugars, which could otherwise inhibit hydrolysis through negative feedback on the hydrolytic enzymes.

Subsequent experimental work conducted by J. B. Molwantwa (2002) confirmed that the maximum percentage solubilisation of PSS was significantly greater under sulphidogenic (63%) than methanogenic conditions (31%). This suggests that a certain organic fraction that would be considered

recalcitrant under methanogenic conditions is susceptible to microbial degradation under sulphidogenic conditions. The rates of hydrolysis were investigated using highly selective metabolic inhibitors, and it was shown that the rate of hydrolysis, measured as the rate of production of reducing sugars, was three times greater under sulphidogenic than methanogenic conditions. The rate of utilization of soluble products was also more rapid under the former, and was thought to be as a direct result of the ability of SRB use a wider range of compounds as electron donors than methanogenic populations.

The results of this study confirmed that both the yield and rate of hydrolysis of PSS were enhanced under sulphidogenic conditions and that this was most likely as a result of reduced accumulation of soluble products. Within the Recycling Sludge Bed Reactor, enhanced hydrolysis through the alleviation of product accumulation is achieved through floc fracture and rapid utilization of released products by resident SRB.

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ABBREVIATIONS

AMD	Acid Mine Drainage
ABR	Anaerobic Baffle Reactor
ASPAM	Algal Sulphate Reducing Ponding Process for Acidic Metal Wastewater Treatment
BNR	Biological Nutrient Removal
BT	Blend Tank
COD	Chemical Oxygen Demand ($mg.L^{-1}$)
COD_f	Filtered COD ($mg.L^{-1}$)
COD_p	Particulate COD ($mg.L^{-1}$)
COD_t	Total COD ($mg.L^{-1}$)
DACST	Department of Arts Culture Science and Technology
EBG	Environmental Biotechnology Group
EPS	Extracellular Polymeric Substances
GDW	Grahamstown Disposal Works
HDS	High Density Sludge
IAPS	Integrated Algal Ponding Systems
LIRI	Leather Industries Research Institute
HDPE	High Density Polyethylene
HRT	Hydraulic Retention Time
MS	Multiple-Stage
MWR	Mine Water Reservoir
PE	Polyethylene
PFP	Primary Facultative Pond
PSS	Primary Sewage Sludge
RSBR	Recycling Sludge Bed Reactor
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SHT	Sludge Holding Tank
SRB	Sulphate Reducing Bacteria
SRT	Sludge Retention Time
SS	Single-Stage
STR	Stirred Tank Reactor

UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids

1 ENHANCED HYDROLYSIS AND THE RECYCLING SLUDGE BED CONCEPT

1.1 BACKGROUND

Highly saline wastewaters are one of the biggest threats to freshwater resources in South Africa, and originate from a range of industrial activities including mining operations and tanneries. In 1990, the Rhodes Environmental Biotechnology Group (EBG) commenced a WRC-sponsored investigation into the possibility of using bioprocess applications to address this issue. Initial studies focused on the use of Integrated Algal Ponding Systems (IAPS) to treat tannery effluents at Mossop-Western Leathers tannery in Wellington (Western Cape Province) and at the Leather Industries Research Institute (LIRI) in Grahamstown (WRC Project K5/495 'A Biotechnological Approach to the Removal of Organics from Saline Effluents'). Based on the success of a pilot plant, a full-scale IAPS system was constructed on-site in Wellington, and included the establishment of a Primary Facultative Pond (PFP).

During this phase of the study, it was observed that the solubilisation of particulate organic solids entering the PFP was near complete (Dunn, 1998). This was unexpected as the tendency in the past was for these ponding systems to fill over time with undigested solid sediments. Biosulphidogenic activity in the PFP was high and it was proposed that reciprocal upwelling events in the PFP and shallower evaporation ponds served to counteract mass transfer limitations between the water column and the sediment. This movement of particulate organic matter through sulphide gradients, established in these systems, seemed to play an important role in their biodegradation. Particulates not subjected to this passage became compacted into sediments which were sulphate mass transfer limited, and remained undegraded (Rose *et al.*, 2002b).

Against the background of a growing awareness of the extent of the acid mine drainage (AMD) problem to be managed in both coal and gold mines in South Africa, a 3-year WRC-sponsored study (Project K5/656), was undertaken by the EBG in 1995, to evaluate the feasible development of IAPS in AMD treatment. The Wellington studies had indicated the potential value in linking co-disposal of complex organic waste streams with AMD treatment, and that an enhanced hydrolysis of complex organic carbon could provide cost-effective substrates for SRB-based remediation systems. Utilization rates comparable to those reported for refined carbon substrate such as ethanol and lactate could be considered (Rose *et al.*, 1998).

The study on an IAPS-based application in AMD treatment commenced in 1995, and following an investigation of the unit operations involved, at both laboratory- and technical-scale the project resulted in the development of the Algal Sulphate Reducing Ponding Process for Treating Acid and Metal-containing Wastewaters – ASPAM (Rose *et al.*, 2002a). This then led directly to the conceptualization of the Rhodes BioSURE Process®, the design of

which is described in detail in Part 1 of this report (Rose *et al.*, 2002b). In order to prove the feasibility of the Process for the remediation of large volume flows of AMD, it was necessary to demonstrate the effective use of a number of complex carbon substrates. Previous studies had shown that tannery effluent and primary sewage sludge (PSS) could both be used as sources of electron donors to drive biological sulphate reduction in both laboratory- and in $1m^3$ reactors (Rose *et al.*, 2002a, 2002b). Again, results indicated that the hydrolysis of these complex carbon sources was enhanced under biosulphidogenic conditions, but no studies examined the role of the recycling sludge bed.

1.2 BIOREMEDIATION OF AMD USING SULPHATE REDUCING BACTERIA

Biological treatment systems have a number of advantages over traditional chemical treatment methods, predominantly financial ones. Costs associated with chemical reagents, labour and sludge removal are negligible. Instead, costs are usually measured in terms of land (Gazea *et al.*, 1996), making biological treatment particularly attractive in developing countries such as South Africa. Biological processes include treatment by wetlands and by bacterial consortia, both *in situ* and in specially designed reactors.

Sulphate reducing bacteria (SRB) are found in a wide range of anaerobic environments, particularly in the anoxic sediments of freshwater (Elsgaard *et al.*, 1994) and marine (Marty, 1981; Hines and Buck, 1982) systems. Recently, they have even been found in anoxic micro-environments in aerobic wastewater treatment systems (Lens *et al.*, 1995) and in highly acidic environments (Johnson, 1995; Elliot *et al.*, 1998). However, these anaerobic organisms generally function best at pH values of 6-7 and at temperatures of around 30°C (Widdel, 1988). While the upper limit of their tolerance to sulphate is reported to be approximately $2600mg.L^{-1}$ (Maree *et al.*, 1986), their tolerance to sulphide is approximately an order of magnitude lower. The overall reaction carried out by SRB, such as *Desulfovibrio* sp. is as follows:

$$2CH_2O + SO_4^{2-} \leftrightarrow 2HCO_3^- + H_2S \qquad (1)$$

This generalised equation shows how the reduction of sulphate leads to the production of hydrogen sulphide and bicarbonate. The sulphide will react with dissolved heavy metals to produce insoluble metal sulphides, depending on the pH and the solubility of the particular metal sulphide. Bicarbonate is also important for the remediation of AMD as it will act to increase the pH of the solution.

The potential involvement of these microbes for the bioremediation of AMD and other sulphate- and metal-rich industrial effluents has also been realised (Tuttle *et al.*, 1969; Maree and Strydom, 1985; Maree *et al.*, 1986, 1987; Maree and Hill, 1989; Widdel and Hansen, 1992). However, one of the most important obstacles to the implementation of these biological treatment systems on a large-scale is the availability and cost of a suitable carbon source

and electron donor. SRB are able to oxidise hydrogen, a range of organic acids (such as lactate, acetate and propionate) (Widdel, 1988) and may even utilize compounds that were previously thought to be either toxic or highly recalcitrant, such as phenolic derivatives (Holliger *et al.*, 1988; Widdel, 1988; Drzyzga *et al.*, 1993; Kuever *et al.*, 1993; Pavlosthathis, 1994). They are, however, not able to hydrolyse large, complex organic molecules such as proteins and carbohydrates, and are thus reliant on a combination of hydrolytic and acidogenic bacteria to provide suitable electron donors.

These substrates are, however, too expensive to be used on full-scale remediation efforts, where large volumes of water are to be treated. Inexpensive carbon sources that have been evaluated for their ability to sustain biological sulphate reduction include cattle waste (Ueki *et al.*, 1988), molasses (Maree and Hill, 1989), lactate and cheese whey (Oleszkiewicz and Hilton, 1986; Herrera *et al.*, 1991) and producer gas (Du Preez *et al.*, 1992; Du Preez and Maree, 1994). More recently, research conducted by the EBG at Rhodes University demonstrated that sulphate reduction could be driven by other complex carbon sources such as tannery effluent, microalgal biomass and PSS (Rose *et al.*, 2002a). The possibility of using sewage sludge as a carbon source for biological sulphate reduction has also been reported by other authors (Butlin *et al.*, 1956; Burgess and Wood, 1961; Conradie and Grutz, 1973).

Davison *et al.*, (1989) attempted *in situ* treatment of AMD in a shallow lake using sewage as a carbon source. After increasing the pH of the water by adding hydrated lime, it was expected that the sewage would drive sulphate reduction that, in turn, would neutralise any further acid that entered the lake. The pH of the system increased to 10 after the addition of the lime, but after two years had decreased to its former value of less than 3. The failure of this attempt to treat AMD using SRB was ascribed to the shallow nature of the lake. A combination of wind and wave action was able to scour the sewage from the lake bottom, and it was removed from the system.

This example highlights a number of important points regarding the remediation of AMD by SRB. A wide range of substrates may be used successfully in bench-scale experiments, but may fail to produce satisfactory results on scale-up. Although one of the advantages of the natural treatment system vs. active treatment is its self-regulating capacity and low maintenance requirements, it is clear that some control is required in order to achieve satisfactory results. Thus, a higher quality, yet relatively inexpensive, biological treatment may best be achieved through the use of carefully designed reactors. A prerequisite for the bioremediation of large volumes of AMD using SRB is the availability of a large quantity of inexpensive electron donor material. PSS meets the above requirements but must be converted to a soluble form before it can be used by populations of SRB.

1.3 PRIMARY SEWAGE SLUDGE AS AN ELECTRON DONOR FOR SULPHATE REDUCTION

Pipyn and Verstraete (1979) set four questions that should be answered when evaluating the potential of a substrate for methane production. If slightly modified, these will also apply to substrates being considered as a potential energy source for sulphate reduction, and are:

1. To what extent is the material convertible to a substrate that can be used by SRB?
2. What is the maximum rate at which the process can proceed?
3. What is the best process in terms of pre-treatment, digestion and handling of the residue?
4. What are the overall economics in terms of waste treatment on the one hand, and capital expenditure on the other?

Current options for the disposal of PSS are costly and are unlikely to be sustainable over the long term. As worldwide production of PSS increases, novel and cost-effective methods for the disposal of PSS must be investigated. Thus, from an economic standpoint, the bioremediation of sulphate-rich mine waters using PSS as an electron donor is an attractive option. It is of particular importance to developing countries, where the use of relatively expensive electron donors, such as ethanol, for bioremediation of any sulphate-rich waters is impractical. Not only is PSS readily available, but by using it, two hazardous effluents will be treated simultaneously. The only major cost that may be incurred is the piping of PSS from a collection point to the site where it is to be used. Recent studies have shown that the performance of biological nutrient removal (BNR) processes may be enhanced by the addition of the soluble products of hydrolysis of PSS (Brinch *et al.*, 1994; Skalsky and Daigger, 1995; Canziani *et al.*, 1996; Hatziconstantinou *et al.*, 1996; Andreasen *et al.*, 1997; Banister and Pretorius, 1998). The success has, however, been limited despite the apparently large carbon source available. As yet, the optimisation of PSS hydrolysis for the production of large quantities of soluble products has not been used for biological sulphate reduction.

The majority of known SRB grow optimally on relatively simple molecules such as short-chain volatile fatty acids (VFA) (Widdel, 1988). Thus, a large proportion of the energy of PSS will remain trapped in complex molecules until freed by hydrolytic and acidogenic bacteria. The degradation of sewage sludge in anaerobic systems is slow. The value calculated for $t^{1/2}_{vs}$ (the half time of volatile solids) was 56 days (Pipyn and Verstraete, 1979). This was significantly higher than the values calculated for other high COD wastes such as piggery slurry (24 days), yeast production plant effluent (10 days) and industrial secondary sludge (16 days). The slow digestion was thought to be the result of the relatively high concentration (0.2-0.5g.L^{-1}) of mineral oil in the PSS. The concentration of cellulose, which is fairly resistant to degradation (Woodward, 1987), may have comprised a significant proportion of the dry matter. Ghosh (1991) suggested that a significant proportion of the

readily degradable COD is trapped by recalcitrant cell components such as the cell walls. Thus, cell lysis and/or hydrolysis would be required before the cell components could be utilised by the acidogenic bacteria.

The process of hydrolysis involves the cleavage of complex organic molecules (proteins, lipids and carbohydrates), to smaller compounds such as sugars, amino acids and peptides. Products of this reaction are then cleaved further during acidogenesis to long-chain fatty acids and VFA (Figure 1.1). Hydrolysis, the initial cleavage step, is considered to be rate-limiting (Nyns *et al.*, 1979; Eastman and Ferguson, 1981; Ghosh, 1991; San Pedro *et al.*, 1994; Eliosov and Argaman, 1995; El-Fadel *et al.*, 1996; Vavilin *et al.*, 1996; Penaud *et al.*, 1997). The rate at which hydrolysis proceeds is best described by first order kinetics (Eastman and Ferguson, 1981; Shimizu *et al.*, 1993) and may be strongly influenced by environmental and operational parameters. These include pH, temperature, microbial biomass, type and concentration of particulate substrate, particle size and product concentration (Eastman and Ferguson, 1981).

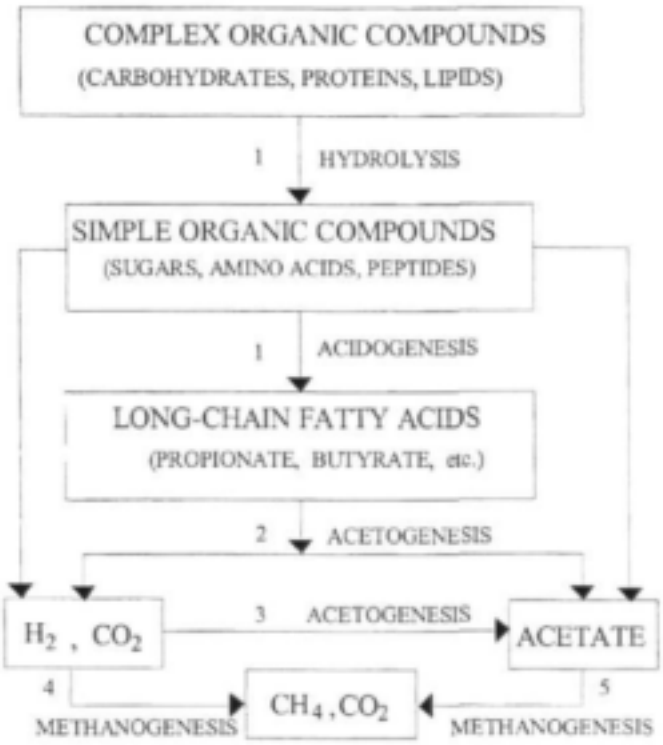


Figure 1.1. Metabolic steps and microbial groups involved in anaerobic digestion: fermentative bacteria; 2) H_2-producing acetogenic bacteria; 3) H_2-consuming acetogenic or homoacetogenic bacteria; 4) CO_2-reducing methanogenic bacteria; 5) acetoclastic methanogenic bacteria (Novaes, 1986).

Table 1.1 summarises rates and yields of soluble products for the anaerobic hydrolysis of PSS, as well as other organic molecules of varying complexity. Reported values for the rate of hydrolysis and the yields from primary sludge are limited, and comparison of published figures is complicated by the fact that the criteria by which the rates were calculated are often different.

Table 1.1. Summary of hydrolysis data obtained from reported studies.

Feed	k (day^{-1})	Yield (%)	Temp (°C)	Sludge recycle	Scale	pH	*HRT (hr	**SRT (day)	Reference
Primary sludge	-	15 - 35	150	none	full	<4	-	-	Karlsson & Göransson, 1993
Primary sludge	-	5	16-20	none	lab	6.7	15d	-	Canziani *et al.*, 1996
Primary sludge	-	Poor	16	yes	pilot	-	1.5	10	Canziani *et al.*, 1996
Primary sludge	-	10 - 15	25	none	Lab/ pilot	-	3d	10	Brinch *et al.*, 1994
Primary sludge	0.16	17	20	none	Lab (batch)	-	-	-	Lilley *et al.*, 1990
Primary sludge	-	9	18 - 28	none	Lab (batch)	-	6	-	Banister and Pretorius, 1998
Primary sludge	-	4.8 – 22.4 mean = 10	<20	none	Lab	-	6	-	Hatziconstantinou *et al.*, 1996
Primary sludge	-	5	18	None	Lab	-	2	2	Hatziconstantinou *et al.*, 1996
Primary sludge	-	35	24	10%	Pilot	-	2	4 –5	Hatziconstantinou *et al.*, 1996
Primary sludge	$0.12hr^{-1}$	27	35	none	lab	5.1	1.5	-	Eastman and Ferguson, 1981
Primary sludge	-	9 - 16	20	none	full	-	-	-	Andreasen *et al.*, 1997
Activated sludge	-	2.5	8 - 17	none	full	-	-	-	Andreasen *et al.*, 1997
Sludge mix	0.25	-	35	none	lab	-	10d	-	Siegrist *et al.*, 1993
Raw sewage	0.22	-	20	none	lab	-	5d	-	Balmat, 1957
Waste Activated Sludge	0.16	65	37	yes	lab	7	-	-	Shimuzu *et al.*, 1993
Released organics	1.2	90	37	yes	lab	7	10d	-	Shimuzu *et al.*, 1993
Starch	3.28	-	20	none	Lab (batch)	7	-	-	San Pedro *et al.*, 1994

HRT = Hydraulic Retention Time; **SRT = Sludge Retention Time

Calculations have been based on production of ammonia (Henze and Mladenovski, 1991), soluble products such as COD and VFA (Eastman and Ferguson, 1981; Lilley *et al.*, 1990; Brinch *et al.*, 1994, Canziani *et al.*, 1996; Hatziconstantinou *et al.*, 1996; Andreasen *et al.*, 1997; Banister and Pretorius, 1998), biogas production (Siegrist *et al.*, 1993) as well as the removal of particulate volatile suspended solids (VSS) (Shimizu *et al.*, 1993).

Calculation of the rate of hydrolysis from the production of soluble products may be misleading. As hydrolysis is the rate-limiting step, the process of acidogenesis and internalisation are relatively rapid (San Pedro *et al.*, 1994). Thus, rates calculated by this method probably describe the hydrolysis/acidogenesis step rather than hydrolysis alone. Futhermore, due to the rapid uptake of soluble products, especially VFAs, the rates and yields of the combined process are probably underestimates. Rates calculated from the removal of particulate VSS (Shimizu *et al.*, 1993) or specific complex molecules (San Pedro *et al.*, 1994) are probably more accurate.

The value of $0.12.hr^{-1}$ obtained by Eastman and Ferguson (1981) is considerably higher than that of $0.16.day^{-1}$ (Lilley *et al.*, 1990), although both rates were calculated from the production of soluble products. The difference may partially be a result of the different temperatures at which the two values were obtained (Table 1.1). Alternatively, such differences may have been due to differences in sludge composition. Shimizu *et al.* (1993) showed that different molecular components of PSS are degraded at significantly different rates. Lipids and cellulose were hydrolysed relatively slowly, at 0.76 and $0.52.day^{-1}$ respectively, while proteins and carbohydrates were both degraded at approximately $1.2.day^{-1}$. Less complex organics, such as starch (San Pedro *et al.*, 1994) and the organics released from sonicated activated sludge (Shimizu *et al.*, 1993) exhibited even higher rates of hydrolysis, with values of 3.28 and $1.2.day^{-1}$ respectively. Thus, as the rate of hydrolysis describes the overall degradation, the relative concentrations of slowly and more rapidly degradable compounds will influence the rate. Although the rate of hydrolysis will affect residence time and therefore, reactor size, percentage yield (expressed either as soluble products produced per unit of complex substrate, or percentage of the complex substrate solubilised) will determine the feasibility of downstream processes. Reported yields from hydrolysis of PSS range from 5% under psychrophilic conditions (Canziani *et al.*, 1996) to around 35% at 24°C (Hatziconstantinou *et al.*, 1996). Although yields are probably affected by environmental and operational parameters, expected yields are low, with the average being less than 20%. Some of the parameters affecting both yield and the rate of hydrolysis/acidogenesis are reviewed below.

The rates and yields reported in Table 1.1. are low and it is possible that inherent difficulties may be overcome by studying hydrolysis of complex substrates in natural aquatic and marine environments. Rose *et al.* (2002c) reported that the solubilisation of the complex organic fraction contained in tannery effluent appeared to be more rapid than would normally have been

expected. These observations were made when tannery effluent was digested in a semi-passive settler-type reactor.

1.4 NATURAL SYSTEMS AND THE CONCEPTUALIZATION OF THE RECYCLING SLUDGE BED REACTOR

The survival of entire aquatic and marine ecosystems relies on the ability of these systems to efficiently release simple compounds (that can be assimilated by the component organisms) from the complex forms in which they enter the system. For example, many deep water marine ecosystems rely on bacteria and other microbes to degrade dead algal biomass that sinks to the ocean bed. These systems are highly effective and the key to achieving enhanced hydrolysis of a complex organic substrate to provide soluble products for use by SRB may be to mimic them.

Jain *et al.* (1992) presented a mathematical model that accurately described the anaerobic digestion of cow dung, a complex substrate. The two factors that had the greatest impact on the rate-limiting hydrolytic step were the concentration of the hydrolytic enzymes and the contact between these enzymes and their substrates. Particle size has also been shown to have a profound impact on the rate of anaerobic digestion of complex substrates (Balmat, 1957; Levine *et al.*, 1985; Szikriszt *et al.*, 1988; Choi *et al.*, 1997; Madhukara *et al.*, 1997; Müller *et al.*, 1998). Vavilin *et al.* (1996) modeled the degradation of particulate substrates and proposed that the hydrolysis rate constant was a function of the ratio between the characteristic sizes of the hydrolytic bacteria and the substrate particles. Based on the above findings, any increase in the enzyme concentration in a digester, or a reduction in mass transfer limitations or particle size will result in an increase in the rate of hydrolysis of complex particulate substrates. The terms hydrolysis and solubilisation are used inter-changeably throughout the literature. To avoid confusion in this study, hydrolysis refers to the chemical cleavage of molecules, while solubilisation refers to the change of solids from the settleable to the suspened or soluble (i.e. <0.45μm) state.

To date, no information is available regarding the composition and formation of flocs in non-pellet forming anaerobic treatment systems. For the purpose of this study, it was assumed that the composition of the flocs in PSS is representative of the composition of the sludge, and contains roughly equal proportions of carbohydrates, lipids and proteins (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965; Levine *et al.*, 1985). It was also assumed that those factors known to be responsible for flocculation in non-bulking activated sludge systems, i.e. non-covalent bonds between bacteria, extracellular polymeric substances (EPS), and metal ions (Eriksson and Alm, 1991; Bruus *et al.*, 1992; Urbain *et al.*, 1993), are also essential for maintaining the integrity of the anaerobic flocs. For the purposes of this study, EPS is defined as being any extracellular organic molecules, including proteins and carbohydrates. Large undigested macromolecules present in the PSS would also be incorporated into the flocs.

Hydrolytic enzymes in biological treatment systems are thought to be bound to bacterial cell walls and the floc matrix, with minimal enzyme activity in the bulk solution (Frølund *et al.*, 1995; Confer and Logan; 1998; Goel *et al.*, 1998). Thus, flocculation is essential for the retention of both biomass and enzymes within the reactor system. However, it is proposed that flocculation results in severe mass transfer limitations, and will have a negative impact upon the rate of hydrolysis of complex macromolecules. Li and Ganczarczyk (1990) proposed that substrates and products diffusing to and from cells must overcome the diffusional resistance created by EPS. Indeed, any macromolecules within the floc could offer significant resistance to the passive movement of soluble molecules to and from the floc. If mass transfer to and from the floc is reduced, accumulation of product within the floc will result in product inhibition of enzyme production, and a severe reduction or cessation of hydrolysis. For this reason, it would be advantageous for cyclical floc fracture followed by reflocculation. Upon floc fracture, any products trapped within the flocs would be released, and upon reflocculation, hydrolytic enzymes would be concentrated around freshly incorporated substrate. As the mean size of flocs is dependent on the relative rates of flocculation and floc fracture, an increase in the rate of floc fracture would result in a smaller mean floc size. Thus, the area-to-volume ratio of the floc would be increased and would benefit hydrolysis of the floc components, assuming that the majority of the hydrolysis occurs on the floc surface.

Traditionally, anaerobic stabilisation of PSS is performed under methanogenic conditions, and rarely results in more than 30% solubilisation (Table 1.1). Pipes (1961) suggested that stabilization under sulphate reducing conditions might offer significant advantages for disposal of PSS. Indeed, more recent studies have shown that the rate and extent of the solubilisation of ligno-cellulose, an abundant compound in primary sludge (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965; Elefsiniotis and Oldham, 1994), was enhanced in the presence of sulphur compounds (Khan and Trottier, 1978; Kim *et al.*, 1997; Pareek *et al.*, 1998). Hydrolysis of large protein molecules may also be enhanced as sulphide is a strong reducing agent and is capable of reducing disulphide linkages that are essential for maintaining the three-dimensional conformation of large proteins. Although the concentration of lipids in PSS can be as high as that of the carbohydrate and protein components (Levine *et al.*, 1985), they are not degraded during the rate-limiting hydrolytic phase of anaerobic digestion (Eastman and Ferguson, 1981). Instead, they are degraded by β-oxidation in the acidogenic phase, and as such have not be considered during this study.

The main factors limiting the rate of hydrolysis of PSS appear to be the following:

- Mass transfer limitation due to floc size and structure;
- Reduced contact between hydrolytic enzymes and their substrates;
- Poor retention of biomass and enzymes due to low immobilisation efficiency;
- Inefficient separation of soluble products and undigested material.

Based on the above, it is proposed that the solubilisation of PSS will be enhanced under sulphate reducing conditions because of a decrease in both particle and floc size, as a result of enhanced hydrolysis of macromolecular proteins and carbohydrates. Furthermore, incorporation of a novel recycling and settling sludge bed will offer significant advantages in terms of cyclical flocculation and floc fracture, separation of the soluble products from undigested material and in the retention of small flocs, bacterial biomass and associated hydrolytic enzymes.

1.3 OBJECTIVES

The WRC study on the biodesalination of AMD wastewaters using PSS as an electron donor source for sulphate reduction was undertaken in the combined ProjectsK5/869 and K5/972. The following combined project objectives were identified:

1. To expand studies undertaken in elucidating the reaction mechanism involved in the phenomenon of biosulphidogenic enhanced hydrolysis of complex substrates;

2. To evaluate reactor design options in which the enhanced hydrolysis reaction may be applied in the treatment of AMD;

3. To undertake scale-up development of the AMD biodesalination process using complex carbon substrates as electron donor source for SRB-driven sulphate reduction;

4. An evaluation of the process at technical-scale, on-site at a mine, and using an AMD stream.

1.4 RESEARCH QUESTIONS

In order to use PSS as a carbon source and electron donor for biological sulphate reduction on the scale required for the remediation of large volumes of AMD in South Africa, the hydrolysis step of this complex substrate must be understood and optimised. Although work on natural systems suggests that hydrolysis of complex carbon sources is enhanced under sulphidogenic conditions, there is no direct evidence to support this theory. Thus, the questions to be answered during this component of the combined project were:

2. Is it possible to provide evidence for the phenomenon of enhanced hydrolysis of complex carbon substrates under biosulphidogenic conditions?

2. Does a recycling sludge bed offer any advantage to the above process?

3. Will the product of the reaction i.e. sulphide, enhance the hydrolysis of the substrate?

4. If so, what is the underlying mechanism involved in the sulphide-enhanced hydrolysis of PSS?

5. Does a settling sludge bed offer any advantage over conventional reactor systems?

6. By incorporating a recycling sludge bed under sulphidogenic conditions, can a complex carbon substrate, such as PSS, be used efficiently for the remediation of AMD, and can this be demonstrated on a technical-scale pilot plant?

2 THE EFFECT OF REACTOR DESIGN AND SULPHATE REDUCTION ON SOLUBILISATION OF PRIMARY SEWAGE SLUDGE: RESULTS FROM A LABORATORY-SCALE REACTOR

2.1 BACKGROUND

Recent work has demonstrated that inexpensive complex carbon derived from a range of sources, including tannery effluent, micro-algal biomass and PSS, may be used as electron sources to drive biological sulphate reduction (Rose *et al.*, 2002a). However, as sulphate reducers can only utilise simple organic compounds such as volatile fatty acids (VFA) (Widdel, 1988), they must rely on other microorganisms to undertake the initial hydrolytic and acidogenic steps. The influence of biological sulphate reduction on this step has not yet been investigated, although Dunn (1998) proposed that the hydrolysis of complex compounds under sulphate reducing conditions was enhanced.

Operational parameters such as mixing (Perot *et al.*, 1988; Banister and Pretorius, 1998), temperature (Gujer and Zehnder, 1983; Perot *et al.*, 1988; El-Fadel *et al.*, 1996; Van Lier *et al.*, 1997; Banerjee *et al.*, 1998) and sludge retention time (SRT) (Skalsky and Daigger, 1995) have all been shown to influence the rates and degree of hydrolysis of complex substrates. As described in section 1 of this report, Dunn (1998) proposed that a certain amount of mixing was required to overcome the mass transfer limitation of sulphate from the water column to the sediment, and that this was achieved in natural systems during reciprocal upwelling events. If the products of biological sulphate reduction are indeed required for enhanced hydrolysis to occur, then it unlikely that this could be achieved using current high rate anaerobic digester designs such as the Upflow Anaerobic Sludge Blanket (UASB). These systems are designed to achieve rapid and effective separation of undigested substrate from soluble products.

The incorporation of a zone of settling into the reactor design is also thought to be critical for the success of the process of enhanced hydrolysis for a number of reasons. Perhaps the most important advantage would be the facilitation of contact between fresh undigested complex substrate, resuspended partially digested matter, sulphate, sulphide and bacteria with their associated hydrolytic enzymes. The close proximity of all of these components is thought to be critical. The extent of washout of both biomass and soluble products from a settling sludge bed system could not be predicted. As washout of either of these components would have resulted in a reduction in the efficiency of the process, it is thought that this may be minimised by the introduction of a zone of settling. In order to maximise the efficiency of the process in terms of the percentage sulphate reduced and the amount of soluble carbon utilised, it was decided to investigate the use of a multiple-stage reactor.

The majority of the complex organic macromolecules found in PSS, including proteins and carbohydrates, are too large to pass through the bacterial cell membranes. Thus, before they can be internalised as a carbon source, they must be hydrolysed into smaller molecules by a range of extracellular bacterial enzymes (Eastman and Ferguson, 1981; Novaes, 1986; Jain *et al.*, 1992; Confer and Logan, 1997a, 1997b, 1998). Enzymatic "fingerprints" have been used to provide insight into the microbial activity in activated sludge systems (Nybroe et al., 1992). These "fingerprints" are affected by the availability of substrate and although they have never been used to study anaerobic systems, it is thought that they will provide information about the performance of hydrolytic populations under sulphate reducing conditions. Furthermore, bacterial activity and consequently enzyme "fingerprints" may differ among stages in a multiple-stage reactor system.

2.2 OBJECTIVES

1. To follow the hydrolysis of PSS in the presence of biological sulphate reduction in a novel Recycling Sludge Bed Reactor configuration.
2. To investigate whether sulphate reduction resulted in any physical changes in sludge morphology that could assist in explaining altered rates of sludge hydrolysis.
3. To determine whether a dual-stage process offered any advantages, in terms of separation and consumption of hydrolysis products or sulphate reduction, over a single-stage system.
4. To determine the effect of sulphate reduction and reactor configuration on the distribution and "activity" of hydrolytic enzymes using enzymatic "fingerprints".

2.3 MATERIALS AND METHODS

2.3.1 Reactor Design and Operation

The reactor was composed of one or more interconnected units (Figure 2.1b) with the feed of PSS and sulphate-rich water entering the first unit near the surface (Figure 2.1a). The particulate organic matter in the PSS settles into the base of the unit, and is then recirculated and re-enters the reactor adjacent to the influent stream, allowing a period of reaction. A portion of the influent water is drawn into the bed, while the remainder containing solubilised material flows horizontally. Solubilisation of the PSS and sulphate reduction occurs in the bed of the reactor. Although the retention of biomass is reliant on flocculation, the predominantly downward flow of liquid is thought to offer significant advantages, in terms of biomass retention, over the upward flow in the UASB-type reactors. The recirculation of sludge may be handled in single- or multiple-stage operations.

Two single-stage and two three-stage reactors were used, so as to allow for comparison of both reactor configurations under sulphidogenic (+ sulphate) and non-sulphidogenic (- sulphate) conditions. All reactor vessels were constructed from 2L vessels, with a neck angle of 60°. The overflow of the

reactors was positioned to allow for a working volume of 1.7L. Multiple-stage (MS) reactors (Figure 2.1b) were constructed from three single-stage (SS) reactors inter-connected by silicone rubber tubing (5mm i/d). Each of the three successive components was positioned on a marginally lower level to allow passive overflow from one stage of the reactor to the next. The headspaces of the 3 stages were interconnected by silicone rubber tubing so as to allow periodic purging of the headspace with nitrogen gas. The sludge that settled in the base of each reactor unit was continuously recycled to the top of the same reactor unit at a rate of 20% reactor volume/hour, using a variable speed peristaltic pump (Watson-Marlow 504S). Turbulence created by the inflow of recycled sludge resulted in the formation of a suspended sludge bed within the reactor. The height of the bed could be regulated by altering the recycle rate, and was initially maintained at approximately 50 mm below the overflow in the single-stage reactors and stage 1 of the MS systems. The positions of the sample ports in the sides of the reactor (Figure 2.1a) allowed samples to be drawn from the center of the sludge bed. The overflow of each reactor (effluent) was sampled via taps in the interconnecting tubes.

All four reactor systems were operated at a HRT of 2 days, at room temperature (22-25°C). Fresh PSS was collected every two days from Grahamstown Disposal Works (GDW). After collection, the PSS was passed through a sieve (2mm mesh size) and stored at 4°C until required. Fresh feed was made up every 48-hours to minimise build-up of large bacterial populations in the feed reservoirs. Sieved PSS was diluted with tap water to obtain a feed with a COD of 2000 $mg.L^{-1}$. Na_2SO_4 was added to the feed of the sulphidogenic system to obtain a final sulphate concentration in the feed of $2000mg.L^{-1}$. The ratio of COD: sulphate in the feed was thus 1:1. The glass feed reservoir was placed on a magnetic stirrer, and the contents stirred continuously, at low speed, to prevent settling of particulate organic matter. The neck of the feed reservoir was closed to limit oxygen transfer into the feed. The reactors were allowed to stabilize for 14 days, after which time it was assumed that steady state had been reached. Samples were drawn and analyzed at regular intervals for a period of 38 days after which time the experiment was terminated.

2.3.2 Analytical Procedures

2.3.2.1. Chemical Analysis

Merck Spectroquant® test kits were used to determine concentrations of sulphate (Merck # 14791) and COD (Merck # 14541). Filtered COD (COD_f) was determined by passing samples through a Glass Microfibre Filters (type GF/A; Whatman Ltd. # 1820 025), and calculating the COD of the filtrate. This method was modified from Lilley *et al.* (1990) and represented the soluble COD fraction. Prior to determination of COD, all samples were acidified with 32% HCl to pH<2 and shaken for 10 minutes, to remove any sulphide. Although the accuracy of COD quantification may have been improved if sludge samples were macerated prior to dilution and analysis, it

was felt that this might have resulted in an artificial underestimation of the particulate COD fraction (COD_p).

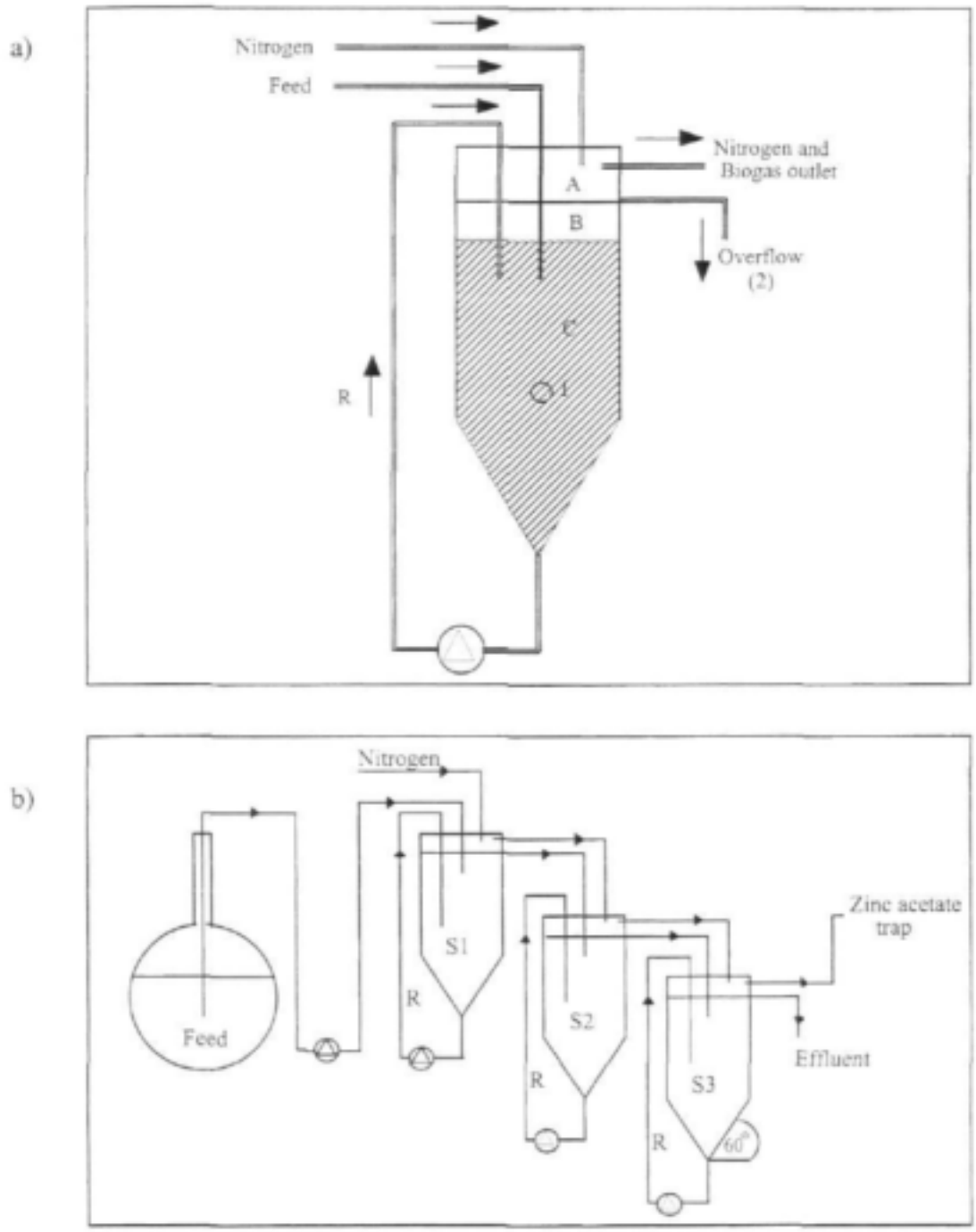


Figure 2.1. The laboratory-scale reactors used in the study. a) Detail of the single stage (A = headspace, B = Top zone, C = Bed) showing the location of sample ports (1 = Bed, 2 = Effluent). R = recycle. The position of the gas inlets and outlets is also shown. b) Multiple-stage reactor system (S = stage).

Instead, determination of total COD (COD_t) was carried out in triplicate and the mean value calculated, in an attempt to obtain accurate quantification of the COD_t of the highly particulate sludge samples. COD_p was calculated as the

difference between the COD_i and COD_f of a sample. The percentage sulphate removed was calculated as the difference between the concentration of sulphate in the feed and that in the effluent of particular reactor or reactor system. pH was measured with an electronic pH meter (Cyberscan 2000, Wirsam Scientific).

2.3.2.2. Floc Size

Sludge samples were drawn from various points within the reactor systems, and diluted 10X with a 10% (v/v) formalin solution. Formalin acts as a fixing agent and has been used successfully to preserve the three-dimensional structure of activated sludge flocs (Li and Ganczarczyk, 1990). Each sample was gently mixed and a drop placed onto a glass slide using an automatic pipette with an enlarged tip aperture. A glass coverslip was then placed over the sample, ensuring that minimal pressure was applied. Although the presence of a coverslip may have resulted in a flattening and expansion of the flocs, it was envisaged that this effect was minimized by the fixation step. The flocs were then viewed under a light microscope, photographed and the area of the flocs (mm^2) calculated using image analysis software (Sigma Scan Image™). Between 50 and 100 flocs per sample were measured. Statistical software (Statgraphics 7.0) was used to compare the mean floc size of the various samples.

2.3.2.3. Enzymology

API ZYM test kits were obtained from bioMérieux (# 2 520 0), and used according to the manufacturer's instructions. As these testes are qualitative and semi-quantitative, and all qualitative data should be interpreted with caution. All of the enzymes included in the kit are listed in Table 2.1. Milli-Q® water was used for dilutions and maintenance of humidity. 65µL of sample was placed into each cupule, and the strips incubated at 35°C for 4 hours. After incubation and addition of the two reagents (ZYM A and ZYM B), strips were held under a bright light for 5 minutes to remove any residual yellow colouration. This yellow colour is due to the presence of excess Fast Blue that did not react.

All activities are indicated as nmol of substrate hydrolysed. Due to the dark colouration and high viscosity of some of the sludge samples, accurate comparison of colour was impossible. These samples were diluted appropriately with sterile distilled water prior to analysis of enzyme activity. The dilution factor was taken into account when calculating the quantity of substrate converted.

Table 2.1. Enzymes tested for by the API ZYM test kit. The number of each enzyme is used in place of enzyme names in all subsequent figures.

Number	Enzyme	Number	Enzyme
1	Control	11	Acid Phosphatase
2	Alkaline Phosphatase	12	Naphthol-AS-BI-phosphohydrolase
3	Esterase (C 4)	13	α-galactosidase
4	Esterase lipase (C 8)	14	β-galactosidase
5	Lipase (C 14)	15	β-glucuronidase
6	Leucine arylamidase	16	α-glucosidase
7	Valine arylamidase	17	β-glucosidase
8	Cysteine arylamidase	18	N-acetyl-β-glucosaminidase
9	Trypsin	19	α-mannosidase
10	Chymotrypsin	20	α-fucosidase

2.4 RESULTS AND DISCUSSION

2.4.1 Solubilisation of Particulate Organic Matter

Due to periodic dilution of municipal PSS after rains, and inherent difficulties associated with obtaining an accurate measure of COD in a particulate sludge sample, influent COD to the bench-scale systems showed some fluctuation over the experimental period (Table 2.2, Figures 2.2a, 2.3). The concentration of COD_f in the feed to all four systems was, however, consistently low (Table 2.2, Figure 2.2, Figure 2.3) and rarely increased to above 500mg.L^{-1} at any stage of the reactor systems, except in the beds (stage 1) of the MS systems. The mean concentrations of COD_f in the beds (stage 1) were 711mg.L^{-1} and 950mg.L^{-1}, for the +sulphate and – sulphate MS systems, respectively (Table 2.2). In comparison, the COD_f in the beds of the SS systems were only 274mg.L^{-1} (+ sulphate) and 231mg.L^{-1} (- sulphate) (Table 2.2).

Sulphate reduction appeared to enhance the solubilisation of particulate organics in the multiple-stage reactor, but not to the same degree in the single-stage system. COD_p increased steadily in bed 1 of the – sulphate MS system, from 6862mg.L^{-1} on day 0, to 24027mg.L^{-1} on day 39 (Figure 2.3b). In the equivalent stage of the + sulphate system, periods of particulate accumulation were followed by periods of rapid removal of particulate COD (Figure 2.3b). The concentration of COD_p reached a maximum on day 4 (18171mg.L^{-1}) but then decreased to 6025mg.L^{-1} (day 13). COD_p began to accumulate again in the bed around day 30. The data suggests that there was, on average, comparatively little accumulation of COD_p within the beds of the SS systems (Figure 2.2b). This is misleading as the bed of the – sulphate SS system was very compact, with the top of the bed being situated below the lowest sample port. Although the actual concentration of COD_p in the bed of the – sulphate SS system was not determined, it was possibly far higher than indicated.

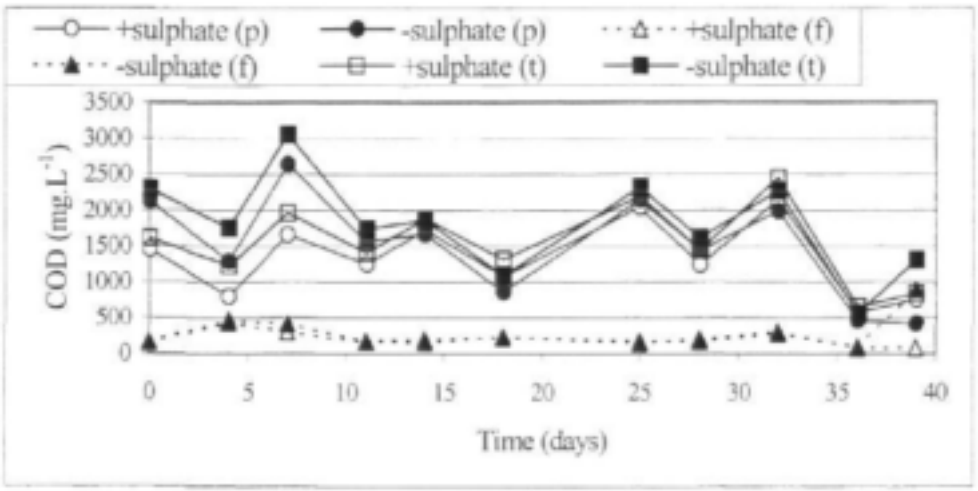
Table 2.2. Mean COD concentrations ($mg.L^{-1}$) for bench-scale reactors over the 38 day experimental period. SS = single-stage reactors; MS = multiple-stage reactors. Standard deviations are indicated in brackets.

	COD_t		COD_p		COD_f	
Sample	+ sulphate	- sulphate	+ sulphate	- sulphate	+ sulphate	- sulphate
Feed (SS)	1545 (548)	1808 (681)	1343 (525)	1511 (712)	205 (106)	296 (227)
Bed (SS)	5886 (3988)	4779 (2956)	4752 (3824)	4557 (2992)	274 (120)	231 (112)
Effluent (SS)	1271 (975)	1157 (1339)	1035 (1016)	964 (1375)	236 (120)	202 (152)
Feed (MS)	1674 (336)	1754 (452)	1479 (372)	1532 (423)	195 (98)	222 (116)
S1 Bed (MS)	10576 (5777)	20595 (7826)	9864 (5425)	19644 (7087)	711 (497)	950 (901)
S1 Effluent (MS)	2250 (1686)	834 (386)	1929 (1613)	535 (324)	321 (162)	299 (153)
S2 Effluent (MS)	1434 (1265)	415 (211)	1205 (1240)	250 (211)	229 (111)	161 (65)
S3 Effluent (MS)	1235 (956)	257 (141)	1044 (923)	128 (148)	190 (118)	129 (31)

Percentage COD removal data were transformed (arcsin$\sqrt{}$proportion) prior to statistical analysis, in an attempt to reduce the variability. Although the mean removal of COD_t was higher in the – sulphate systems (Table 2.3), the mean removals for the four reactor systems were not significantly different (ANOVA, df = 3, 44; $P>0.05$). The periodic increase in COD in the effluent of the systems (Figure 2.4) resulted in high standard deviations. Although this would have affected the statistical analysis, but should not detract from the significance of the results.

In the MS systems, 84% of the COD was removed in the – sulphate system compared with 21% removal in the + sulphate system. Removal in the SS systems was 67% and 42% for the – sulphate and + sulphate systems, respectively. The bed of the + sulphate reactors appeared to exhibit a cyclical accumulation of COD followed by a period of rapid hydrolysis (COD_p decrease). Interestingly, those periods of proposed rapid hydrolysis within the bed of stage 1 of the + sulphate system coincided with an increase in the COD_t in the effluent of stage 1 (Figure 2.4a). During these periods, there was thus a net loss of COD_t from stage 1 of the MS + sulphate system, as indicated by the mean change of 39% for that stage (Table 2.3). It is proposed that the formation of sludge flocs with good settling properties is essential if sludge is to be retained within the reactor. Furthermore, it is thought that the mean floc size is reduced during periods of rapid hydrolysis under sulphate reducing conditions, and that this results in the increased washout of COD that was observed.

a)



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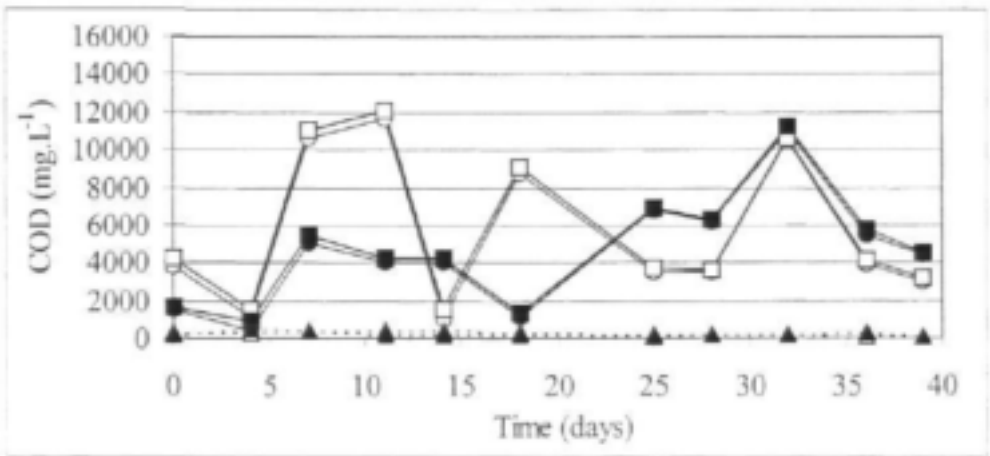

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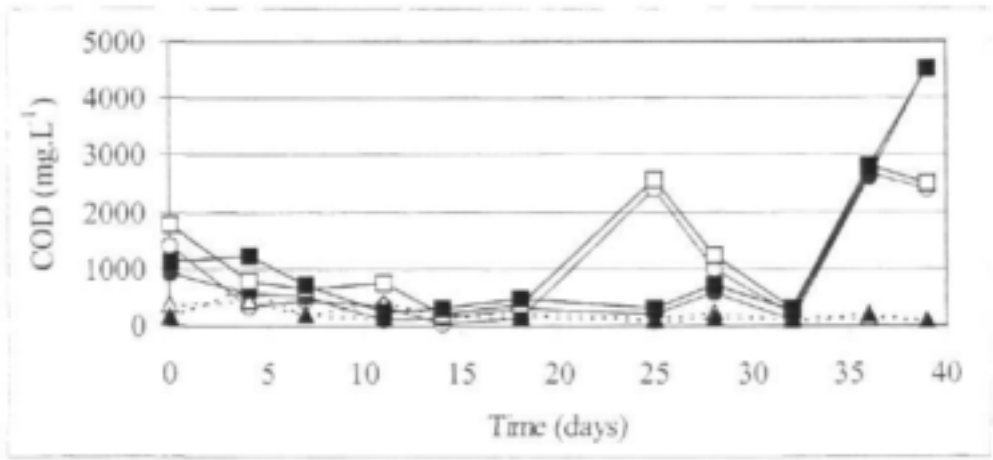
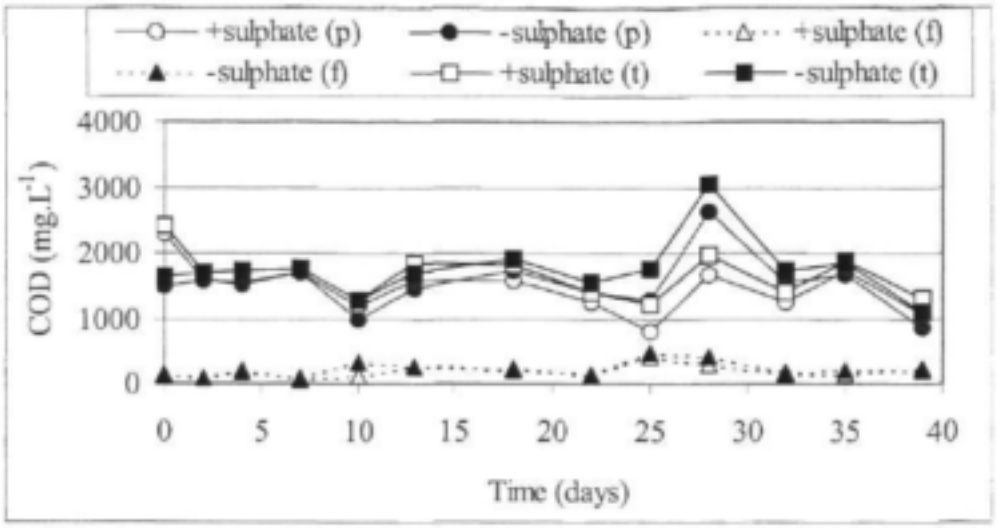


Figure 2.2. COD concentration in the single-stage reactors. a) Feed, b) Bed, c) Effluent. t = COD_t; p = COD_p; f = COD_f

a)



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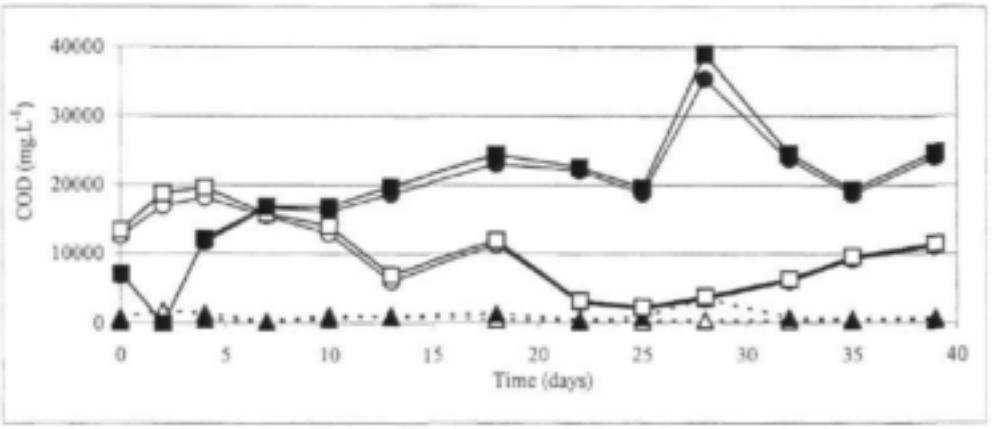


Figure 2.3. COD concentrations in stage 1 of the multiple-stage reactors. a) Feed, b) Bed. t = COD_t; p = COD_p; f = COD_f

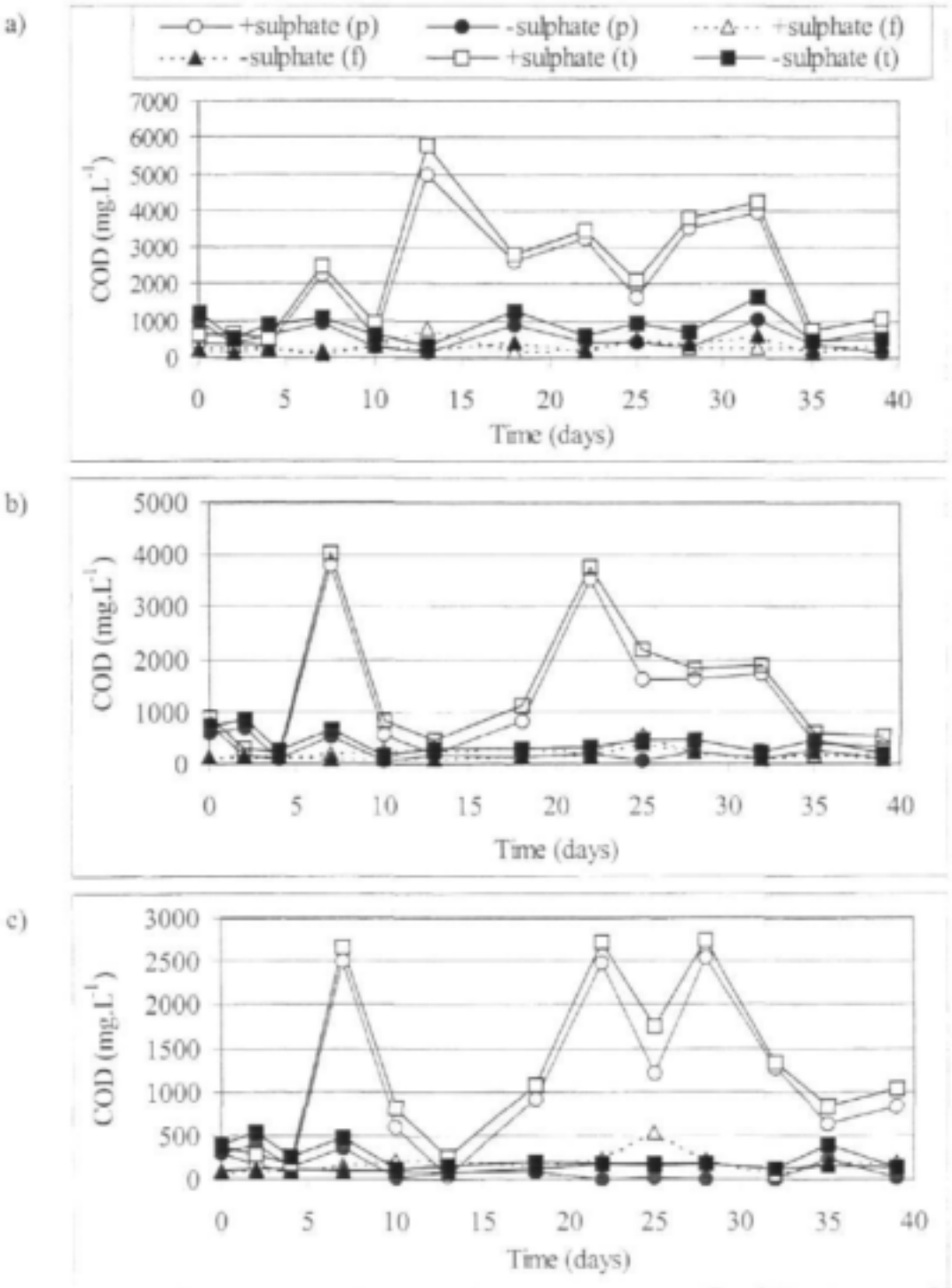


Figure 2.4. COD concentrations in the effluents of the multiple-stage reactor. a) Stage 1, b) Stage 2, c) Stage 3. t = COD_t; p = COD_p; f = COD_f

As similar washout was not observed in the – sulphate systems, it is thought that sulphate reduction plays a key role in enhanced hydrolysis of the PSS and in the destabilization of flocs.

The physical structure of the sludge beds within the –sulphate and + sulphate reactors were different. For the first 5 days both increased in depth and remained compact. After this initial period, the bed of the + sulphate system changed rapidly to a "fluffy" consistency and expanded to almost twice its original height. The bed in the – sulphate system remained relatively compact throughout the experimental period, although it was being recycled at the same rate as the + sulphate system. This event was evidence of the effect of sulphate reduction and/or sulphide on the physical property of the sludge.

Table 2.3. Comparison of mean percentage removal of sulphate and COD from bench-scale reactors. SS = single-stage reactors, MS = multiple-stage reactors. Standard deviations are indicated in brackets.

Sulphate	Sample	% COD change	% sulphate removal
+	Stage 1 MS	+39 (101)	34 (8)
+	Stage 2 MS	-8 (84)	50 (19)
+	Stage 3 MS	-21 (61)	59 (9)
-	Stage 1 MS	-50 (21)	-
-	Stage 2 MS	-75 (12)	-
-	Stage 3 MS	-84 (8)	-
+	SS	-42 (39)	21 (9)
-	SS	-67 (21)	-

2.4.2 Sulphate Reduction and pH

Overall, the mean percentage sulphate removal in the multiple-stage system (59%) exceeded that of the single-stage system (21%) (Table 2.3). The mean concentration of sulphate in the bed of the SS reactor (1076mg.L^{-1}) was lower than in the effluent (1537mg.L^{-1}) (Table 2.3), and indicated that sulphate reduction was higher in the bed than in the aqueous phase. Sulphate removal was highest in the first stage of the multiple-stage systems, although further removal took place in stages 2 and 3 (Tables 2.3, 2.4), and would have resulted in the consumption of a fraction of the COD_f that was produced during hydrolysis in the last 2 stages. Although the concentration of sulphate in the feed of the MS reactor was relatively constant, there was a large amount of variation in the sulphate concentration of the bed (stage 1). The percentage sulphate removal in the bed of stage 1 varied between 20 and 80%, and the concentration of sulphate between 400mg.L^{-1} and 1550mg.L^{-1} (data not shown). It is proposed that this variation in the percentage sulphate reduction was a result of variations in readily available soluble COD, produced during the recycling hydrolysis events, which is then available to sulphate reducers. Sulphate reduction in the last two stages of the MS reactor was low even though soluble COD was present. It is well known that SRB prefer certain VFAs over others (Omil et al., 1996; Lens et al., 1998) and it is proposed that the majority of the COD_f remaining in stages 2 and 3 were not suitable for SRB growth.

Table 2.4. Mean sulphate concentrations and pH in bench-scale reactors. SS = single-stage reactors; MS = multiple-stage reactors. Standard deviations are indicated in brackets.

	Sulphate ($mg.L^{-1}$)		pH	
Sample	**+ SO_4**	**- SO_4**	**+SO_4**	**-SO_4**
Feed (SS)	1858 (306)	<10	7.0 (0.2)	7.0 (0.2)
Bed (SS)	1076 (282)	<10	7.4 (0.3)	6.8 (0.3)
Effluent (SS)	1455 (274)	<10	7.4 (0.3)	6.8 (0.3)
Feed (MS)	2123 (185)	<10	6.9 (0.3)	6.9 (0.2)
Stage 1 Bed (MS)	844 (374)	<10	7.2 (0.4)	6.6 (0.3)
Stage 1 effluent (MS)	1378 (208)	<10	7.4 (0.3)	6.9 (0.2)
Stage 2 effluent (MS)	1039 (397)	<10	7.6 (0.3)	7.1 (0.2)
Stage 3 effluent (MS)	851 (199)	<10	7.5 (0.3)	7.0 (0.2)

The percentage sulphate removal calculated during these studies was lower than those recorded for the same substrate by Molipane (Rose *et al.*, 2002a). However, the presence of sulphur on the surface of the four reactors used in this study indicated that at least some of the sulphide produced was re-oxidised. Thus, it is likely that a significant proportion the sulphide was also oxidised to sulphate, and would have resulted in an artificially low percentage sulphate removal. Under optimal conditions, the amount of re-oxidation would be limited and the percentage sulphate reduction could be expected to be at least as high as that reported previously.

The mean pH values for the +sulphate systems exceeded those of the -sulphate systems, at all stages (Table 2.4), although the differences were not substantial. In both the single-stage (SS) and multiple-stage (MS) + sulphate systems, the pH within the reactors exceeded that of the feed, and was an indication of active sulphate reduction. In the – sulphate systems, the pH within the reactors was slightly lower than that of the feed, probably as a result of the production and accumulation of degradation products such as volatile fatty acids. The pH of the effluent of stage 3 of the MS + sulphate reactor was higher than that from the SS + sulphate reactor, as a result of more sulphate reduction in the MS system.

2.4.3 Floc Size

Microscopic examination of the flocs revealed the presence of dense particulate matter embedded within fine extracellular polymeric substances (EPS) (Figure 2.5). The floc-area data sets from most samples contained outliers, and resulted in large standard deviations (Figure 2.6). To reduce some of the variation, all data were log-transformed prior to statistical analysis. As the data were not normally distributed, means were analysed using a Kruskal-Wallis analysis for non-parametric data. The mean areas of the flocs from 18 samples were found to be significantly different ($P<0.001$; $df = 17$).

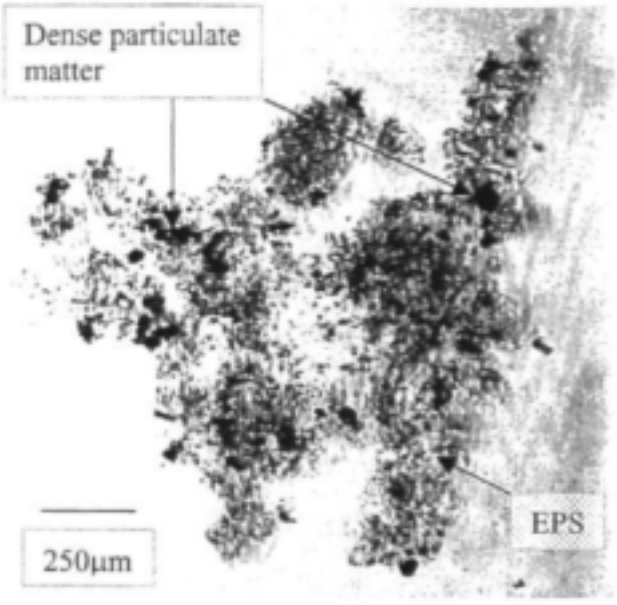
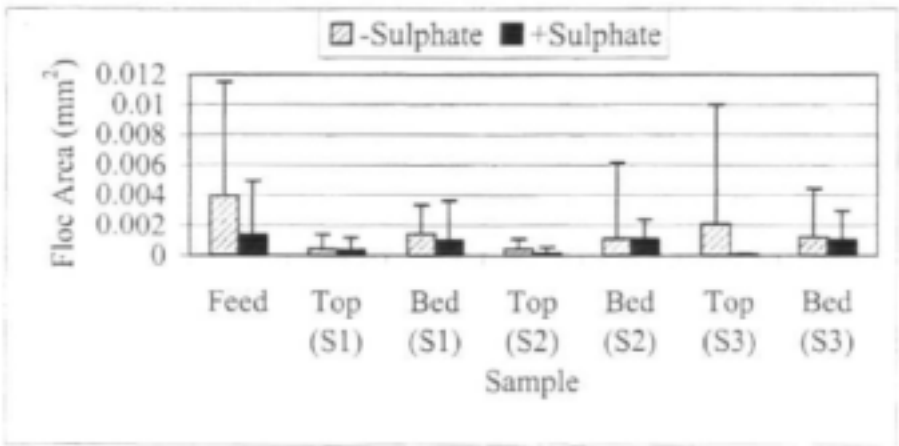


Figure 2.5. Floc morphology as seen under the light microscope (40x magnification). The dense particulate matter embedded within EPS is clearly visible.

As expected, the flocs in the upper zone of the reactors were smaller than those in the corresponding beds (Figure 2.6). This, alone, would have accounted for the significant finding. Thus, in order to determine whether sulphate reduction had any effect on floc size, it was necessary to analyse the data obtained from the reactor feed and beds separately from that of the upper zones. Similarly to previous observations, the differences observed in Figure 2.6 were significant ($P<0.001$; $df = 9$). Unfortunately, the Kruskal-Wallis test is unable to provide details regarding the exact position of the differences. From Figure 2.6, it appeared as if the flocs in the feed of the + sulphate system were significantly smaller than those in the feed of the – sulphate system. This was confirmed using a T-test ($P < 0.01$; $df = 132$). Thus, although the flocs in the beds of both the SS and MS systems were smaller in the + sulphate systems, this may have been a direct result of a significant difference in floc size within the feed of the two systems. Furthermore, the size of the flocs in the three stages of the MS systems appeared to remain constant. The relatively high mean value and standard deviation obtained for the top of stage 3 (- sulphate) was the result of 9 relatively large flocs in the sample. The presence of these could not be explained. As feed remained in the reservoir for a maximum of 24 hours, this may have been long enough for the initiation of sulphate reduction.

As floc size is dependent on the relative rates of flocculation and floc fracture, it is proposed that the process of biological sulphate reduction has two possible effects. It either reduces floc binding and reflocculation or is actively involved in the fracturing of the floc structure. The recycling nature of the solubilisation in the sulphidogenic systems implied that the effect of sulphate reduction on flocculation was not continuous and immediate, but that it required a period of incubation.

a)



b)

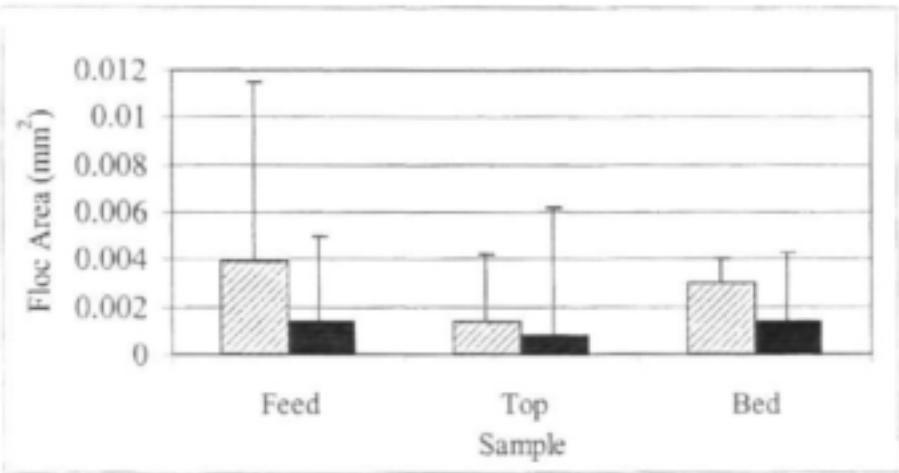


Figure 2.6. Mean area of flocs within the (a) Multiple- and (b) Single-Stage reactors, with and without sulphate.

Under the conditions used in this study, the MS reactor system would offer a significant advantage to the overall performance of the digestion process in terms of percentage removal of undigested but biodegradable matter. Small flocs of undigested organic matter that were washed from the first and second stages would have passed into the next stage of the reactor. There they may then have undergone flocculation and would have been retained within the reactor, where hydrolysis of organic particulate matter would have occurred. This process of floc fracture and reflocculation within the successive stages of the multiple-stage reactor would have extended the time that undegraded material was exposed to the solubilisation process, and thus increased the degree of degradation. More importantly, while deflocculation allows for the release of soluble products, reflocculation serves to facilitate the contact of undigested substrate, hydrolytic enzymes and bacteria.

2.4.4 Enzyme Analysis

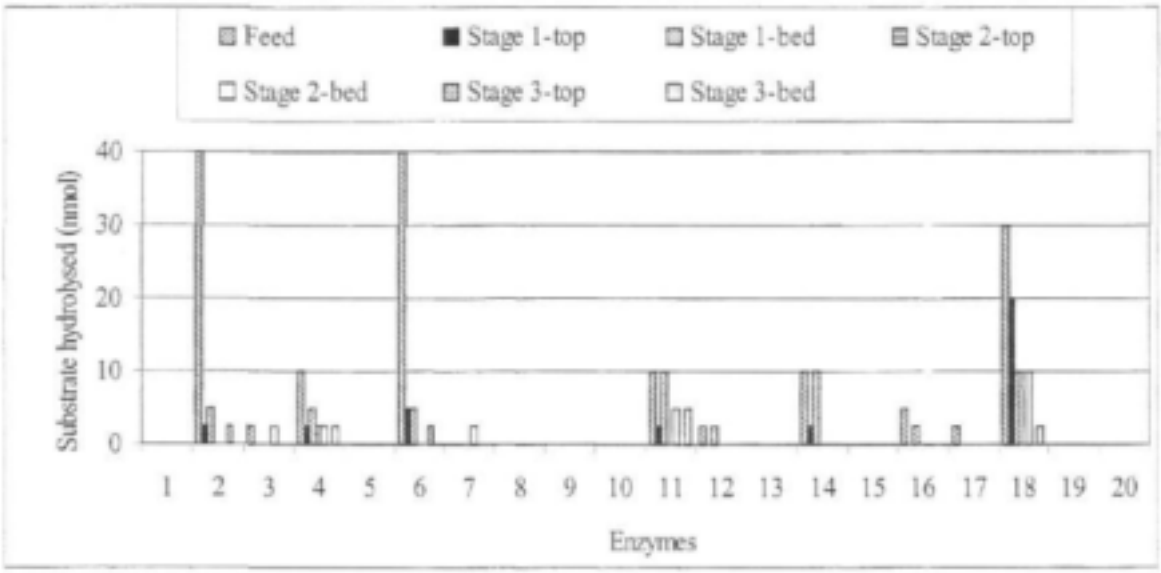
The API ZYM enzymes kits used in this study provide rapid, semi-quantitative colorimetric tests for a wide range of hydrolytic enzymes, including lipases, esterases, proteases, aminopeptidases, phosphatases and glucosyl hydrolases.

It has been used successfully to study the enzymology of activated sludge systems (Boczar *et al.*, 1992), as well as to identify anaerobic bacteria (Tharagonnet *et al.*, 1977). The results of this analytical system are however, semi-quantitative, and therefore caution must be exercised when comparing the relative activity of a particular enzyme either in different reactor systems or at different points within a single reactor.

It has already been shown that sludge flocs in sulphidogenic lab-scale reactors were smaller than those in the non-sulphidogenic systems. As a result, the sludge bed within the sulphidogenic system was less compacted, and the small flocs more susceptible to washout from the reactor. No information concerning the location of hydrolytic enzymes in anaerobic systems is available, but results from studies on activated sludge plants have shown that the bulk of hydrolytic enzymes are associated with the bacterial cells or sludge flocs (Boczar *et al.*, 1992; Frølund *et al.*, 1995; Confer and Logan, 1998; Goel *et al.*, 1998). Assuming that hydrolytic enzymes in anaerobic treatment systems are also closely associated with sludge flocs, a loss of small flocs (particularly in the + sulphate systems) would result in a loss of enzymes. This is supported by the analysis of enzymes at various stages of the lab-scale reactors (Figure 2.7, Figure 2.8).

Enzyme activity was detected in the effluent of the + sulphate single-stage system for 6 of the enzymes (alkaline phosphatase, esterase C4, esterase lipase C8, leucine arylamidase, acid phosphatase, n-acetyl-β-glucosamidase). In contrast, only slight esterase C4 (3) activity was detected in the effluent ("top") of the – sulphate single-stage reactor (Figure 2.8). The enzyme activity in the effluents ("top") of the multiple-stage systems was low, although a range of enzymes exhibited activity in the effluents of both the + sulphate and the – sulphate systems (Figure 2.7).

a)



b)

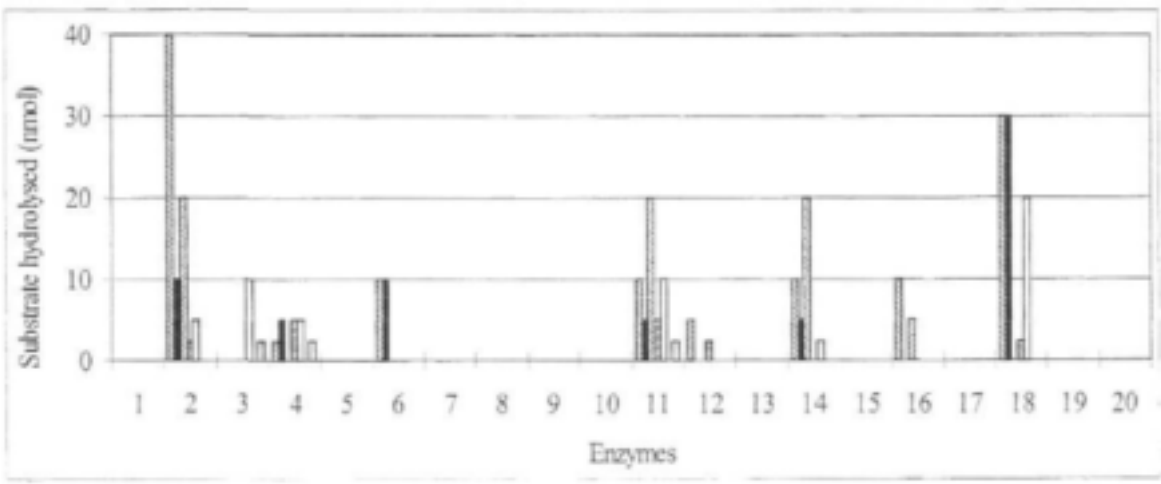
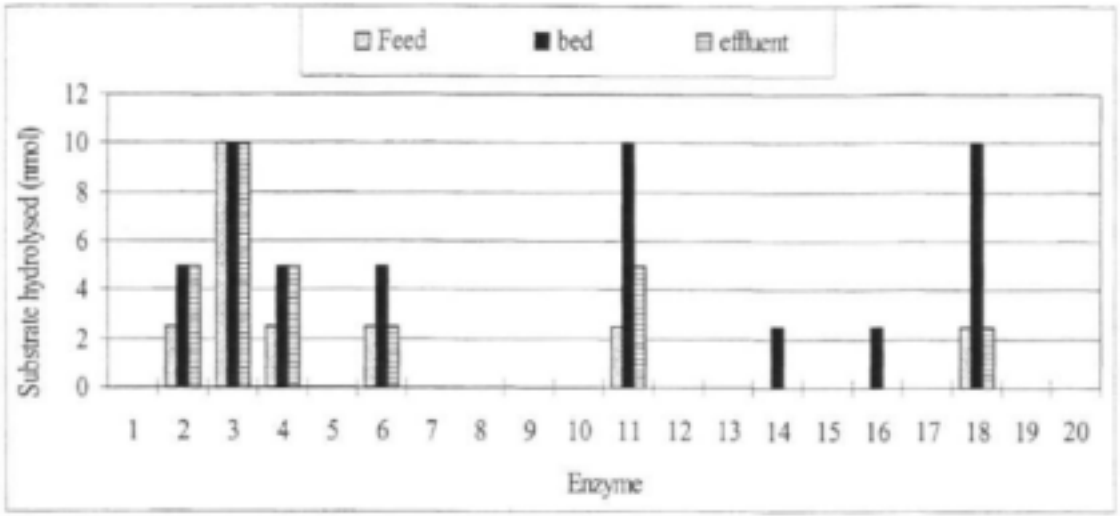


Figure 2.7. *Results of the API-ZYM analysis showing the activity of 19 enzymes in the multiple-stage lab- scale reactors. a) + sulphate system; b) – sulphate system. The key to the numbers is given in Table 2.1.*

Although enzyme activity was detected in the effluent of the single-stage + sulphate reactor, the activity of the enzymes in the bed were higher than in the feed (Figure 2.8a). A similar pattern was true for the – sulphate single-stage reactor (Figure 2.8b), but not for either of the multiple-stage systems (Figure 2.7). This suggested that the single-stage systems acted as accumulators of enzymes, and may have offered some advantage over the multiple-stage systems in this regard.

a)



b)

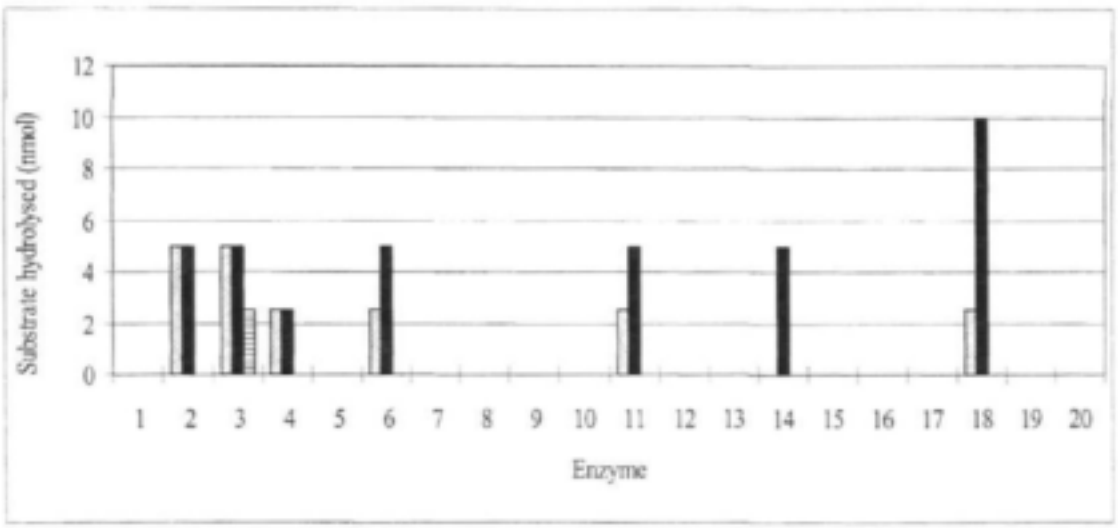


Figure 2.8. Results of the API-ZYM analysis showing the activity of 19 enzymes in the single-stage lab-scale reactors. a) + sulphate system; b) – sulphate system. The key to the numbers is given in Table 2.1.

It is widely accepted that while anaerobic digestion is adversely affected by the presence of sulphide, this is limited to the gasification step. The initial hydrolytic step is not considered to be affected by sulphide in concentrations up to 300mg.L^{-1} (Rudolfs and Amberg, 1952; Aulenbach and Heukelekian, 1955; Lawrence *et al.*, 1966; Maillacheruvu *et al.*, 1993). In light of the present findings, while this might describe the effect of sulphide on the overall performance of the hydrolytic process, it appears to be an over-simplification when considering enzymological effects. The enzyme profiles of the lab-scale reactors indicated that two enzymes, valine arylamidase and β-glucosidase, were only active in the sulphidogenic reactors. As the production of extracellular hydrolytic enzymes is closely linked to the available substrates

(Nybroe *et al.*, 1992), it is proposed that the presence of sulphide enhances the availability of the substrate for these enzymes. The precise mechanism involved is not understood and will be the subject of future studies.

2.5 CONCLUSIONS

The current study supported previous findings that a complex carbon source could be used as a electron donor source to drive biological sulphate reduction (Rose *et al.*, 1998). Furthermore, the study supported the observation by Dunn (1988) that the hydrolysis of complex carbon was enhanced in the presence of biological sulphate reduction. The exact mechanism by which sulphate reduction enhances the hydrolysis of complex substrates is not known at this stage. However, the reduction of floc size is though to be critical. The findings of the current study were that:

1. A complex carbon source such as PSS could be used to sustain biological sulphate reduction, although the percentage sulphate reduced was lower than could be expected in a fully optimised system;

2. The solubilisation of particulate organic matter in PSS was enhanced in the presence of biological sulphate reduction;

3. A RSBR, offering a linear flow settler design above a sulphidogenic recycling sludge bed appears to enhance the solubilisation of PSS. Biological sulphate reduction was critical to the success of the process;

4. A multiple-stage reactor system offered advantages over a single-stage reactor in terms of total sulphate reduced, and resulted in the conceptualization of a dual-stage process. The initial stage would involve primarily the hydrolysis of the complex substrate while the second stage would be optimised for maximum reduction of sulphate using soluble products from the first stage;

5. Flocs in the sulphate reducing environment were significantly smaller than those in the non-sulphidogenic reactors, and this is thought to be related to a sulphide-dependant reduction in floc strength;

6. Washout of hydrolytic enzymes from sulphidogenic systems was higher than from the non-methanogenic systems. Reactor design and retention times are therefore considered critical if washout of bio-catalyst and hydrolytic enzymes from sulphidogenic hydrolysis reactors is to be minimized;

7. The presence of sulphide appears to enhance the availability of certain macromolecular substrates, resulting in increased enzyme activity.

3 DEVELOPMENT OF A DESCRIPTIVE MODEL TO EXPLAIN THE ENHANCED HYDROLYSIS OF PSS UNDER BIOSULPHIDOGENIC CONDITIONS

3.1 BACKGROUND

In the previous chapter, it was demonstrated that not only could biological sulphate reduction be driven by a complex carbon source such as PSS, but that the solubilisation of PSS was enhanced under these conditions in a reactor that incorporated a settling and recycling sludge bed. This has important implications for the use of complex carbon sources as an electron donor for other biological reactions as well as for sludge disposal. However, before the process can be optimised, it is necessary to elucidate the mechanism underlying the process. It was thought that the best way to pursue this would be to investigate the effect of sulphate reduction and sulphide on the hydrolysis of the two most significant organic fractions in PSS, proteins and carbohydrates.

Carbohydrates and lignin make up a significant, but variable proportion of sewage. Estimates have ranged from about 25% of the settleable solids fraction of raw sewage (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965) to as high as 42% of the total solids in PSS (Elefsiniotis and Oldham, 1994). Despite the variable nature of PSS, it usually contains a considerable amount of lignocellulose. Reported concentrations suggest that up to 60% of the total carbohydrates in PSS may be in the form of cellulose (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965), with lignin contributing up to 6% of the settleable solids fraction of sewage (Heukelekian and Balmat, 1959). Common sources of this complex organic matter may include paper, rags and vegetable matter (Knapp and Howell, 1978). Cellulose is a homopolymer composed of D-glucose units linked by β (1-4)-glucosidic bonds (Bisaria and Ghose, 1981). While the amorphous region of cellulose is easily degraded, the crystalline region is structurally resistant to enzymatic attack (Gharpuray *et al.*, 1983). Cellulose never occurs naturally in its pure form, and is always closely associated with a variety of other polysaccharides, in particular lignin and hemicellulose (Janes, 1969; Bisaria and Ghose, 1981). As the susceptibility of cellulose to biological hydrolysis is dependent on its accessibility to cellulase enzymes, it is expected that the presence of associated polysaccharides will reduce the rate of cellulose hydrolysis. Lignin is not a carbohydrate, but a polymer of phenylpropane units linked together by ether and carbon-carbon bonds, and is highly resistant to biological breakdown. During the commercial Alkaline Pulping process, also referred to as the Kraft process or sulphate pulping, lignin is solubilised in the presence of alkalinity (Clayton, 1969). Recently, researchers have examined the effects of sulphate addition on the rates of degradation of lignocellulosic solid wastes, and initial results indicate that rates are enhanced (Kim *et al.*,

1997; Pareek *et al.*, 1998). There was, however, no evidence to suggest that this was due to removal of lignin.

Estimates of the protein content of municipal PSS varies from 17.2 to 29% (Heukelekian and Balmat, 1959; Eastman and Ferguson, 1981; Levine *et al.*, 1985; Pavlostathis and Giraldo-Gomez, 1991). Although a significant proportion of PSS is in the form of insoluble protein, very little is known about the hydrolysis of this class of organic compound under anaerobic conditions. Factors such as solubility, tertiary protein structure and type of end-groups are considered to influence the rate of protein hydrolysis (McInerney, 1988). As with all enzymatic processes, hydrolysis of protein is affected by pH, although there is no consensus with regards to an optimum pH range for proteolytic activity. To date, no studies have investigated the fate of proteins in PSS under sulphate reducing conditions. Pipes (1961) proposed that one possible advantage of digesting municipal waste under sulphate reducing conditions was the degradation of recalcitrant proteins in the presence of high sulphide concentrations. Although polypeptides themselves cannot be used as electron donors by SRB (Widdel, 1988), the sulphide produced during biological sulphate reduction is a strong reducing agent, and is capable of cleaving the disulphide linkages that are essential for maintaining the conformity of large proteins. This may allow for improved access of proteolytic enzymes to active sites within the protein matrix.

Considering the above, it is hypothesized that the enhanced solubilisation of the particulate fraction of PSS observed in laboratory-scale sulphidogenic reactors (Section 2) was due to accelerated hydrolysis of sludge proteins and/or the carbohydrate fraction, and that it would be possible to demonstrate this.

3.2 OBJECTIVES

1. Confirm that the hydrolysis of the particulate fraction of PSS is enhanced in the presence of sulphide;

2. Determine the effect of sulphide and sulphate reduction on the solubilisation of the carbohydrate and protein fractions of PSS;

3. To combine these findings, with those on the effects of sulphide on floc size, to develop a descriptive model to explain enhanced hydrolysis of PSS under sulphate reducing conditions in a settling sludge bed.

3.3 MATERIALS AND METHODS

3.3.1 The Effect of Sulphate Reduction on Particulate Organic Matter

The design of the experiment used to investigate the effect of elevated sulphide concentrations and pH, on the degradation of COD_p, is shown in Table 3.1. The experiment was conducted, in triplicate, in 1000mL flasks, and the contents stirred using magnetic beads. The pH of the flasks was adjusted,

after sulphide addition, with 32% HCl or 50g.L^{-1}NaOH. Sulphide was added to experimental flasks in the form of a concentrated solution (1g.L^{-1}) of $Na_2S.9H_2O$. An equal volume of distilled water was added to the control flasks. The PSS was collected from GDW approximately 1 hour before use, and passed through a sieve (2mm mesh size) to remove any large particulates. The COD concentration was adjusted to 2000mg.L^{-1} with distilled water. The bacterial inoculum was obtained from a 10L fed-batch stirred tank reactor (STR) that had been operating under sulphate reducing conditions for approximately 1 year. PSS was used as the sole carbon source for the system (2000mg.L^{-1} COD), and the COD:SO_4 ratio was maintained at approximately 1:1. In an attempt to minimise the quantity of sulphide added to the control flasks, nitrogen gas was bubbled through the inoculum for 15 minutes prior to inoculation. At the start of the experiment, and after each sampling event, the headspace of each flask was flushed with nitrogen before sealing.

Based on data from the preliminary experiments, it was decided that any change in the solubilisation rate of particulate COD would be evident within the first 48 hours of incubation. Thus, samples were analysed at the start of the experiment (t=0) and after termination of the experiment, at 48 hours. All flasks were incubated at 25°C (± 3) in the dark. Sulphide was removed from all samples prior to determination of COD concentrations, as described in Section 2. Sulphide concentrations were determined using NN'-Diethyl-*p*-phenylenediamine (Rees *et al.*, 1971). Alkalinity (mg.L^{-1} $CaCO_3$) was determined by titrating samples to pH 3.7 with 0.1M HCl (APHA, 1989). All other analytical methods used are described in Chapter 2. All percentage data were transformed (arcsin$\sqrt{}$proportion) prior to statistical analysis.

Table 3.1. Summary of the experimental design used to determine the effect of elevated sulphide concentrations on the hydrolysis of particulate COD.

Experiment	Sulphide (mg.L^{-1})	COD (mg.L^{-1})	Inoculum (mL)
-sulphide (pH7)	0	2000	100
+sulphide (pH7)	100	2000	100
-sulphide (pH10)	0	2000	100
+sulphide (pH10)	100	2000	100

3.3.2 The Effect of Sulphate Reduction on Hydrolysis of Complex Polysaccharides

Solubilisation of the carbohydrate fraction of PSS under methanogenic conditions was compared to solubilisation under conditions of elevated sulphide and sulphate. All experiments were conducted in sealed 500mL volumetric flasks, with a working volume of 400mL. Triplicate experimental and control flasks were inoculated with sieved (2mm mesh size) sludge (10% v/v) from the methanogenic anaerobic digester at GDW. Fresh PSS was used as the sole carbon source, and was prepared as described above, prior to incubation. The composition of the control (methanogenic) and experimental

flasks is detailed in Table 3.2. Sulphide and sulphate were added as concentrated solutions of $Na_2S.9H_2O$ and Na_2SO_4, respectively. Before sealing the flasks, the pH of the flasks was adjusted to pH 7 and the headspace of each was flushed with nitrogen gas. All flasks were incubated on a shaker (100 rpm) at room temperature (25°C ± 3) in the dark.

The hydrolysis of insoluble complex carbohydrates, was followed by monitoring the changes in the concentration of soluble carbohydrates. The carbohydrate standard curve was prepared using glucose, as this is released during the hydrolysis of cellulose. Microbial uptake of the soluble carbohydrates was inhibited by the addition of toluene, without affecting the extracellular hydrolysis of the polysaccharides (Boschker *et al.*, 1995). Flasks were incubated for 7 days, at which time 3% (v/v) toluene was added to each flask (t=0). The headspace of the flasks was flushed with nitrogen, the flasks re-sealed, and incubated under the same conditions for a further 6 hours (t=6). Samples of 50mL were collected at t=0 and t=6.

Table 3.2. Composition of the control and experimental flasks used to assess the impact of sulphate and sulphide on the solubilisation of complex carbohydrates.

Treatment	Sulphide ($mg.L^{-1}$)	Sulphate ($mg.L^{-1}$)	Final Volume (mL)
Control	0	0	400
Elevated sulphide	100	0	400
Elevated sulphate	0	2000	400

These were centrifuged at low speed (Universal 16A, 3000 rpm) for 10 minutes, and the supernatant passed through a GF/A glass microfibre filter (Whatman). The concentration of carbohydrate in the filtrate (i.e. soluble carbohydrate) was determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Preliminary studies indicated that this method was sensitive to sulphide. Thus, all samples were acidified to <pH 2 with concentrated H_2SO_4 and sparged with nitrogen for 15 minutes, prior to carbohydrate determination. Preliminary investigations showed that this was sufficient to remove all free sulphide from samples. Again, all data were transformed ($arcsin\sqrt{proportion}$) prior to statistical analysis.

3.3.3 The Effect of Sulphate Reduction and Sulphide on Delignification

The effect of sulphate reduction and sulphide on the rate of delignification was examined in flask experiments. Details of the experimental setup are shown in Table 3.3. All contained 500mL of sieved PSS (prepared as above) and were inoculated with 200mL of sludge from the municipal anaerobic digester at GDW. The final concentration of sulphide in the + sulphide treatment was elevated to $500mg.L^{-1}$ with a concentrated sulphide solution ($Na_2S.9H_2O$). The initial sulphate concentration in the two + sulphate treatments was $2000mg.L^{-1}$, also added as a concentrated stock solution. The final liquid volume of each flask was made to 2L with water. A pellet of known SRB was

added to the + sulphate + SRB treatment while the only SRB present in the + sulphate – SRB treatment were those present in the inoculum or PSS. The pellet of SRB was prepared by isolating bacteria from the technical-scale RSBR (Section 4) on modified Postgate medium B (Hiligsmann *et al.*, 1998). A single colony was then allowed to multiply in liquid Postgate medium. After 3 days, 100mL of the medium was centrifuged (Universal 16A, 3000 rpm) and the bacterial pellet placed into the experimental flask.

Before sealing the flasks, the pH of each was adjusted to pH 7 as described previously, and the headspace of each flask was flushed with nitrogen gas. The flasks were then incubated on a shaker (100 rpm) at room temperature (25°C ± 3) for the duration of the experiment.

Table 3.3. Composition of control and experimental flasks used to assess the impact of sulphate reduction and sulphide on the hydrolysis of lignin in PSS. All volumes in mL.

Treatment	Sludge inoculum	Sulphide Stock	Sulphate Stock	Water	PSS
Control	200	0	0	1300	500
+ sulphide	200	500	0	800	500
+ sulphate – SRB	200	0	500	800	500
+ sulphate + SRB	200	0	500	800	500

Samples (100mL) were collected from each flask at 0 (30 minutes after initial setup), 24-, 48- and 72-hours, and the pH of each flask adjusted to the desired value before resealing. 10mL of each sample was filtered through a GF/A filter (Whatman), the filtrate acidified and then sparged with nitrogen gas for 10 minutes to remove all residual sulphide. A control, containing sulphide but no phenol, was treated in the same way to ensure that the method removed all sulphide that may have interfered with the colorimetric assay. The concentration of total phenolic material in each sample would give an estimate of the degradation products of lignin, and was determined using the method described by Box (1983). The standard curve was constructed using laboratory grade phenol (Merck) and each assay was conducted in triplicate.

3.3.4 The Effect of Sulphide, Sulphate Reduction and pH on Protein Hydrolysis

The experiment was conducted in 250mL flasks. All flasks contained 175mL of fresh macerated PSS, and were inoculated with 25mL of sludge from the anaerobic digester at GDW. The experimental design used to test for the effect of sulphide and sulphate reduction on the hydrolysis of protein is shown in Table 3.4. Two flasks were stored at 4°C for 7 days, and were used as undigested controls. Preliminary studies (van Jaarsveld, pers. comm., 1999) showed that no protease activity occurred in PSS at 4°C. In order to test if sulphide cleaved sludge proteins in the absence of biological activity, control flasks were incubated with and without sulphide. Although the concentration of free sulphide within the pilot-scale reactor rarely exceeded 150mg.L^{-1}, the

sulphide concentration in the control flask was elevated to 500mg.L^{-1}. This was to ensure enough sulphide was present to react with the protein. The remaining flasks were incubated on a shaker at 25°C for 7 days. One flask was maintained under methanogenic conditions, as a secondary control. 100mg.L^{-1} and 500mg.L^{-1} sulphide was added to two of the flasks (as $Na_2S.9H_2O$) to test for the effect of elevated sulphide concentrations, in the presence of normal biological activity, on the hydrolysis of sludge proteins.

Table 3.4. Design of experiment to examine the effect of sulphide and sulphate reduction on the hydrolysis of sludge protein at pH 7.

Flask	Treatment	Incubation temperature	Sulphide (mg.L^{-1})	Sulphate (mg.L^{-1})	MoO_4 (mM)
1	Control – sulphide	4°C	0	0	0
2	Control + sulphide	4°C	500	0	0
3	Digested – sulphide	25°C	0	0	0
4	Digested + sulphide	25°C	100	0	0
5	Digested + sulphide	25°C	500	0	0
6	Digested + sulphate reduction	25°C	0	2000	0
7	Digested – sulphate reduction	25°C	0	2000	30

The effect of sulphate reduction and sulphate alone was also tested. Two treatments contained 2000mg.L^{-1} sulphate (added as Na_2SO_4). 30mM molybdate was added to one of the flasks to inhibit sulphate reduction (Lens *et al.*, 1995). The pH of all flasks was adjusted to 7 with 32% HCl or 50g.L^{-1} NaOH. The volume of liquid within all flasks was equalised with distilled water (final volume = 250mL), so as to ensure approximately equal concentrations of protein in all treatments. The original PSS was not diluted to a COD of 2000mg.L^{-1} so as to maximize the recovery of protein for SDS-PAGE. The headspaces of all flasks were flushed with oxygen-free nitrogen gas and the flasks sealed. After 7 days, the proteins were extracted and concentrated using the acetone method (Cantor, 1982), and then analyzed by SDS-PAGE (5-30% gradient, Hoefer SE 600 gel system, 200V, 35A, 100W). After staining the gels with Coomassie Brilliant Blue, the bands were analyzed using Kodak Digital Science 1D image analysis software. The concentration of protein in each band was calculated against a band containing 5µg BSA (Böehringer Mannheim). As this was the first time that SDS-PAGE had been used to visualize the degradation of proteins in primary sludge, the procedure had to be optimised. The optimisation procedure is reported in detail elsewhere (Whittington-Jones, 2000).

3.4 RESULTS AND DISCUSSION

3.4.1 Effect of Sulphate Reduction, Sulphide and pH on Particulate Organic Matter, Complex Carbohydrates and Lignocellulose

An increase in pH and sulphide concentration had a positive effect on the solubilisation of particulate matter in PSS. Both variables might have influenced the solubilisation step individually, but as maximum reduction of particulate organics was achieved at pH 10, and at an elevated sulphide concentration, it is suspected that a synergistic effect was involved.

The mean percentage decrease in COD_p in the four treatments were significantly different (ANOVA, df = 3,8; $P<0.05$). The highest solubilisation of particulate COD was achieved at pH 10 and at elevated sulphide concentrations (Table 3.5). At a neutral pH, addition of sulphide resulted in a mean difference of 375% while at pH 10, the increase was only 53%. The addition of sulphide combined with an elevation of the pH resulted in more than a 500% increase in the percentage removal of COD_p, relative to the control (i.e. –sulphide at pH7).

For one of the repeats, the particulate concentration was calculated at both t=24 and t=48. Results showed that in both the experiments and controls, solubilisation of particulates did not only occur in the first 24 hours of incubation (Figure 3.1). Under conditions of elevated sulphide concentrations, at both pH 7 and pH 10, the percentage of particulates solubilised within the first and second 24-hour periods were approximately equal. Thus, the effect of the elevated sulphide concentration in this system was cumulative over time, rather than instantaneous. Purging of the inoculum sludge with nitrogen prior to innoculation failed to remove all dissolved sulphide and sulphide in the controls was higher than 20mg.L^{-1} at the start of the experiment (Table 3.5).

Table 3.5. The effect of elevated concentrations of sulphide, and pH, on the hydrolysis of particulate COD in batch studies. Standard deviations are indicated in brackets.

Treatment	Mean % decrease in CODp	Sulphide t=0 (mg.L^{-1})	Sulphide t=48 (mg.L^{-1})	% change in alkalinity
-sulphide (pH7)	3.1 (5)	23.8 (8)	21 (8)	11.9
+sulphide (pH7)	10.4 (9)	77 (10)	68 (20)	20.0
-sulphide (pH10)	16.8 (2)	32 (13)	27 (12)	9.1
+sulphide (pH10)	24.0 (4)	108 (20)	91 (30)	9.2

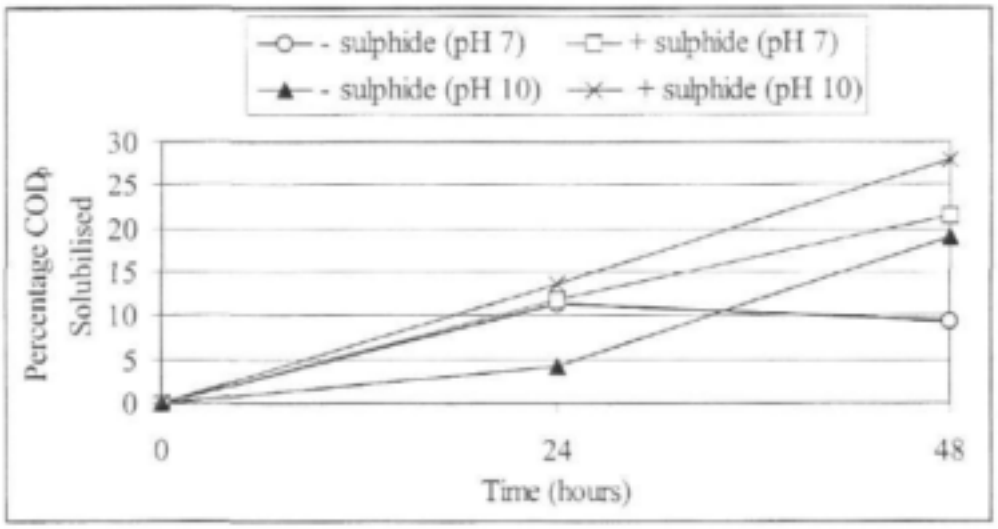


Figure 3.1. Percentage solubilisation of PSS particulates after 24- and 48-hours.

As predicted, the addition of sulphide, either chemically or biogenically through sulphate reduction, resulted in significantly enhanced hydrolysis of complex carbohydrates (ANOVA, df = 2,6; P<0.01). The concentration of soluble carbohydrate in the methanogenic systems decreased by a mean of 1.5% in the 6-hour period after addition of toluene. In the same time period, the concentration of soluble carbohydrate in the sulphate reducing and sulphide-addition systems increased by 39.3% and 25.4% respectively (Table 3.6). The reason for the higher efficiency of the sulphate reducing system is not understood but may be related to the continuous production of alkalinity or removal of the small phenolic end-products of lignin solubilisation.

The mean concentration of glucose in the +sulphide treatment at t=0 was higher than that in the control and +sulphate treatment. The increase in the concentration of sulphide within the sulphate-rich systems, from 0.3mg.L^{-1} at the start of the experiment to 25mg.L^{-1} at t=0 (7 days later), indicated that sulphate reduction had occurred. A sulphide concentration of as low as 25mg.L^{-1} in the sulphate reducing system was enough to enhance the solubilisation of the complex carbohydrates within the PSS.

The pH of all three treatments had decreased by the end of the digestion period (7 days) indicating that hydrolysis and acidogenesis had occurred (Table 3.7). The pH of the + sulphide treatment showed the largest decrease, and dropped from pH 7 at the start of the experiment to pH 5.8 on day 7. The pH of the control and sulphate reducing treatments on day 7 were pH 6.1 and pH 6.4, respectively. The decrease in the + sulphate system was surprising considering the alkalinity produced as a product of sulphate reduction.

Table 3.6. The effect of sulphate and sulphide addition on the hydrolysis of complex carbohydrates. Standard deviations are indicated in brackets. n = 3. t = 0 hours indicates the time of toluene addition.

Treatment	Sulphide ($mg.L^{-1}$)		Soluble Carbohydrate ($mg.L^{-1}$)		% Increase in Soluble Carbohydrate
	Start	t=0	t=0	t=6	
Control	0.8	0 (0)	33 (1)	32 (8)	-1.5
+ sulphate	0.3	25 (2)	28 (0.3)	39 (17)	39.3
+sulphide	68.5	31 (9)	41 (5)	52 (15)	25.4

Table 3.7. The effect of sulphide and sulphate addition on the pH of the digester contents, after digesting anaerobically for 7 days. Standard deviations are indicated in brackets.

Treatment	pH (start)	pH (t=0)
Control	7	6.1 (0.3)
+ Sulphate	7	6.4 (0.1)
+ Sulphide	7	5.8 (0.1)

Sulphate reduction and high concentrations of sulphide had a significant impact on the concentration of soluble phenolic compounds in the 2L batch reactors, and provided indirect evidence that solubilisation of lignin was enhanced under these conditions (Figure 3.2). This might explain the enhanced hydrolysis of lignocellulosic wastes under sulphate reducing conditions in landfills reported by other authors (Kim *et al.*, 1997; Pareek *et al.*, 1998) and of PSS in the current study. The total concentration of phenolic material in the + sulphate – SRB and + sulphate + SRB treatments increased by 107% and 86% respectively ($12.7mg.L^{-1}$ to 26.3 and $23.7mg.L^{-1}$), over the experimental period. The increase in the + sulphide treatment was 27% if calculated as the difference between the calculated concentration at t = 0 ($63.5mg.L^{-1}$) and t = 3 ($81.2mg.L^{-1}$). However, if one considers that the initial concentration of soluble phenolic material immediately before introduction of sulphide (although not determined) was probably around $11mg.L^{-1}$ (Figure 3.2), then the increase was approximately 638%.

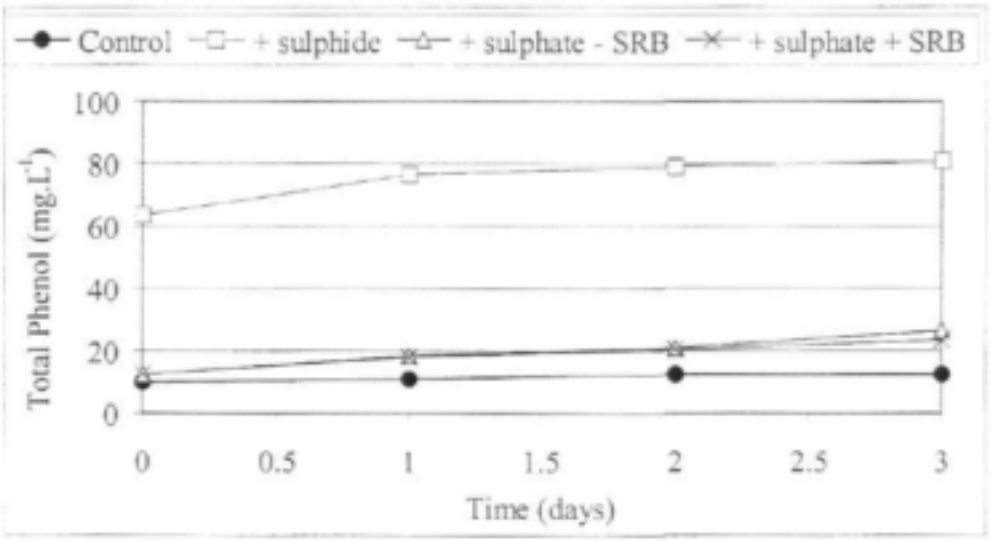


Figure 3.2. The effect of sulphide and sulphate reduction on the concentration of total soluble phenolic material in PSS when digested anaerobically (n=1).

Table 3.8. Change in sulphide concentrations in batch experiments used to determine the effect of sulphide and sulphate reduction on the concentration of soluble phenolic compounds in PSS. All concentrations in $mg.L^{-1}$.

	Time (days)			
Treatment	**0**	**1**	**2**	**3**
Control	1.6	5.5	3	7.1
+ sulphide	447	446	468	451
+ sulphate – SRB	1.0	13	29	45
+ sulphate + SRB	0	13	30	45

The addition of SRB, to ensure a strong population of these bacteria, had no noticeable effect on the concentration of soluble phenolic material or sulphide (Table 3.8) over the experimental period. The rate of sulphate reduction, determined from the increase in the concentration of sulphide in both of the + sulphate flasks, was similar. The increase in soluble lignin was closely correlated to the sulphide concentration in the treatments. Thus, although it has been reported that SRB are capable of using phenol-like compounds as their sole carbon source (Fang and Zhou, 1997), this would not explain enhanced hydrolysis of PSS in the + sulphide treatments in the current study.

3.4.2 Hydrolysis of PSS Proteins under Sulphate Reducing Conditions

Hydrolysis of the protein fraction of PSS was successfully visualized using an optimised SDS-PAGE procedure, and banding patterns corresponded to proteins in the 45 – 26kDa size-range. The banding pattern was consistently

repeated and appeared to be representative of local sludge proteins, with approximately 4 major breakdown products (Figure 3.3).

Four distinct bands were present in the sulphide-free control (lane 2), at 41, 37, 29 and 26kDa. The two larger protein bands (41 and 37kDa) were absent from the control that contained 500mg.L^{-1} sulphide (lane 3). The quantity of protein in the remaining two bands was only slightly less than in the respective bands in the sulphide-free control (Figure 3.3).

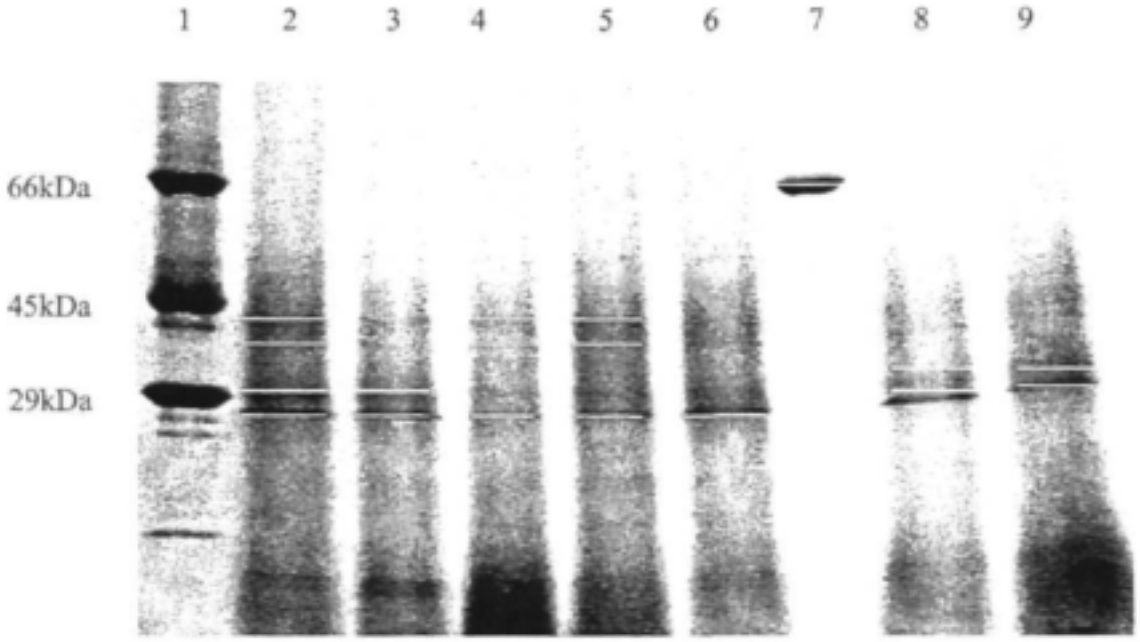
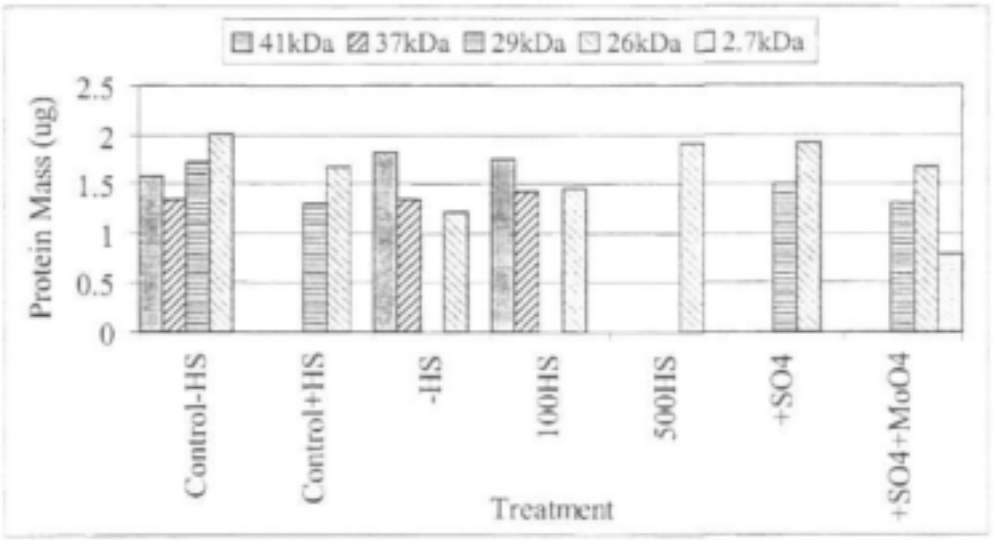


Figure 3.3. The effect of sulphide and sulphate reduction on the hydrolysis of proteins in primary sludge. The molecular weights of the marker proteins (lane 1) are indicated. Lanes: 2 = sulphide-free control, 3 = control + 500mg.L^{-1} sulphide, 4 = digested – sulphide, 5 = digested + 100mg.L^{-1} sulphide, 6 = 500mg.L^{-1}sulphide, 7 = BSA, 8 = digested + sulphate + sulphate reduction, 9 = digested + sulphate – sulphate reduction. Lines indicate protein bands detected by Kodak 1D with the sensitivity level set at –2.

When the PSS was digested in the presence of 500mg.L^{-1} sulphide (lane 6), only one protein band was seen at 26kDa. The quantity of protein in that band was slightly greater than in the undigested sulphide-rich sample (lane 3), but lower than in the sulphide-free control (lane 2) (Figure 3.4a). The quantity of 41kDa protein in the – sulphide treatment increased slightly, while the quantity of 26kDa protein decreased, relative to the sulphide-free control. The addition of 100mg.L^{-1} sulphide before digestion appeared to make no difference to the hydrolysis of sludge protein. The position and intensity of the banding patterns in lanes 4 and 5 are almost identical after 7 days incubation.

a)



b)

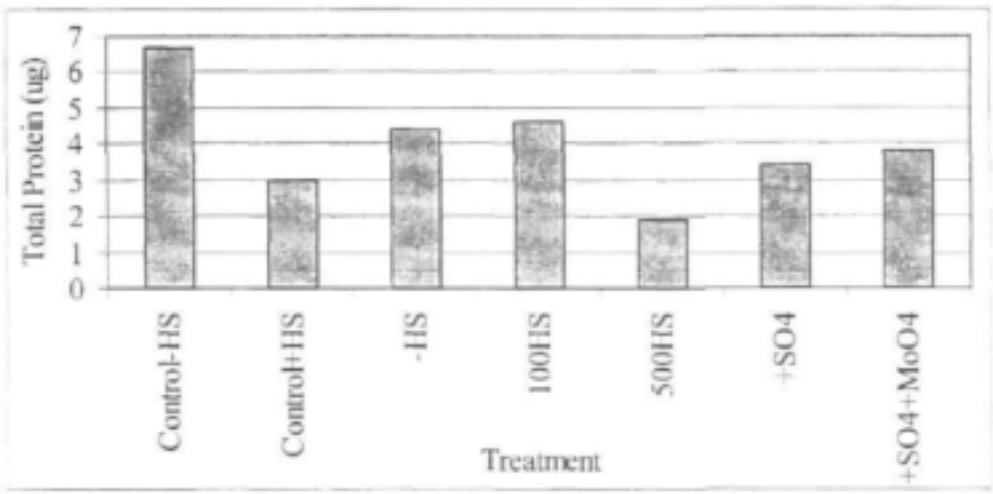


Figure 3.4. The effect of sulphide and sulphate reduction on the hydrolysis of sludge proteins. a) the quantity of protein in each of the bands after SDS-PAGE; b) the sum of protein in all bands. –HS = no added sulphide; +HS = elevated sulphide; +MoO_4 indicates addition of molybdate.

After 7 days, the concentration of sulphide in the sulphate-rich sample (lane 8) had increased from 1.5 to 68.4mg.L^{-1}, and indicated that active sulphate reduction had occurred. The total quantity of protein in each of the distinct bands was calculated, and the results shown in Figure 3.4b. The quantity of protein in the sulphide-free control was higher than in any of the other treatments. The total protein in the + sulphide control was approximately 50% of that in the – sulphide control (Figure 3.4b). This could be explained either by sulphide preventing the migration of protein onto the gel or that the presence of sulphide resulted in cleavage of proteins to small polypeptides that moved off the gel. The appearance of the small 2.7kDa band when sulphate reduction was inhibited by the addition of molybdate, suggests that the latter was more likely. The lowest quantity of protein was found in those samples that contained 500mg.L^{-1} sulphide, with the quantity of protein in the digested

samples being lower than in the control. The addition of 100mg.L^{-1} sulphide to the digested samples resulted in a decrease of nearly 30% in the total quantity of protein. The quantity of protein was also not affected by the presence or absence of active sulphate reduction, possibly because the concentration of sulphide in these treatments remained low.

This study provided evidence that the hydrolysis of proteins in PSS was influenced by sulphide. The fact that some of the bands were present even in the undigested controls suggested that at least some of the digestion of sludge proteins occurred prior to collection i.e. within the sewer. Alternatively, the proteins in the bands were not only hydrolysis products, but also included bacterial hydrolytic enzymes that have been shown to accumulate within the matrix of sludge flocs (Frølund *et al.*, 1995; Goel *et al.*, 1998). The consistent bands present even in the controls, may have been produced by these enzymes that were released from the sludge flocs upon treatment with SDS. An unknown proportion of the protein contained in PSS was almost certainly too large to penetrate the gel matrix. After each run, wells still contained organic material, although the quantity was reduced if the sludge had been macerated before extraction of the protein. The appearance of bands corresponding to proteins of slightly higher molecular weights, on days 3 to 5 of the time series study, proved that at least some of the proteins on the gels must have been hydrolysis products (data not shown). This is supported further by the increase in the total quantity of small proteins within all treatments over the 5-day digestion period.

The hydrolysis of the proteins was enhanced in the presence of sulphide, with a reduction in the total quantity of protein at higher sulphide concentrations. The number of visible bands was reduced when sludge was digested in the presence of 500mg.L^{-1} sulphide. The total concentration of protein was reduced by approximately 30% upon addition of 100mg.L^{-1} sulphide, and the banding pattern was similar to that of the sulphide-free control. Only two concentrations of sulphide were used in the study, and the threshold for the enhanced hydrolysis of sludge proteins by sulphide may be lower than 500mg.L^{-1}.

The role of SRB in the enhanced hydrolysis of sludge protein is probably limited to the production of sufficiently high concentrations of sulphide. The concentration of sulphide in the sulphidogenic treatment was less than 100mg.L^{-1}, and should have been insufficient to result in enhanced protein hydrolysis. Considering that the presence of SRB themselves was inconsequential, and that the sulphide concentration was below the estimated threshold, it was surprising that the banding pattern of the sulphidogenic system was more similar to that of the control with 500mg.L^{-1} sulphide than the sulphide-free control. No explanation can be determined for this observation. The alkalinities of the sulphidogenic treatment and sulphide-rich control were identical (1866 mg.L^{-1}) and higher than that of the sulphide-free control (1416mg.L^{-1}). The higher alkalinity was, however, not the reason for the reduced banding. The banding pattern of the non–sulphidogenic sulphate-rich treatment was similar to that of the sulphidogenic treatment and sulphide-

rich control, although the alkalinity was significantly higher (3866 $mg.L^{-1}$ $CaCO_3$). Due to time constraints, the fate of those proteins too large to move onto the gel, and of those small polypeptides that may have passed off the gel, was not studied. This information is vital to understanding the mechanism behind sulphide-enhanced hydrolysis of sludge proteins, and is part of an ongoing investigation.

3.4.3 Proposed Model Describing the Enhanced Hydrolysis of PSS under Sulphate Reducing Conditions

The empirical data from this study was used to develop a model describing the process of enhanced solubilisation of PSS under sulphate reducing conditions. The rate and extent of hydrolysis of complex substrates, such as PSS, is dependent on the enzyme concentration and contact between enzymes and the substrate (Jain *et al.*, 1992), as well as colonisation of the surface of particulates by hydrolytic bacteria (Vavilin *et al.*, 1996). It is proposed that the products of biological sulphate reduction both directly and indirectly facilitate contact between enzyme and substrate, while simultaneously increasing the surface area of particulate substrates available to colonisation by hydrolytic bacteria.

The effect of sulphate reduction on the hydrolysis of both the protein and carbohydrate fractions of PSS was examined. The soluble proteins in digested and undigested PSS samples were small size, probably as a result of digestion within the sewer. Sulphide was shown to enhance the hydrolysis of proteins in PSS, and was thought to have been the major contributing factor to the process of enhanced solubilisation of the PSS. Based on the profiles obtained by SDS-PAGE, it is proposed that initial hydrolysis of sludge proteins was slow, and that sulphide was responsible for the cleavage of smaller polypeptides in the 20 – 70 kDa size-range. The concentration of sulphide required for enhanced hydrolysis of proteins may have been less than the $500mg.L^{-1}$ measured in this study and it is possible that concentrations were sufficiently high in localised areas of high sulphate reduction, within the sulphidogenic bioreactors. A concentration of less than $100mg.L^{-1}$ sulphide was shown to enhance the solubilisation of lignocellulose. Experimental results indicated that the Alkaline Pulping process (Clayton, 1969) could provide a useful model for understanding the underlying mechanism. Lignin is solubilised in the presence of alkalinity and is prevented from re-polymerising by HS^- ions. The increase in soluble phenolic compounds upon digestion of PSS in the presence of biological sulphate reduction or sulphide, indicated that this reaction was probably occurring in this system. Cellulose is closely associated with lignin in plant tissues, and is protected from contact with cellulase enzymes by a lignin shield. After solubilisation of lignin by alkalinity and sulphide ions, the underlying cellulose is exposed and may be hydrolysed enzymatically. As lignocellulose is known to contribute significantly to the total mass of PSS (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965; Elefsiniotis and Oldham, 1994), enhanced solubilisation of this fraction will improve the overall mineralisation of PSS significantly. Furthermore, this mechanism explains the improved rates and degree of solubilisation of lignocellulosic

wastes in landfills when operated under sulphate reducing conditions (Kim *et al.*, 1997; Pareek *et al.*, 1998). Various authors have shown that certain species of SRB can utilise phenolic compounds as their sole carbon source (Holliger *et al.*, 1988; Widdel, 1988; Schink *et al.*, 1992; Drzyzga *et al.*, 1993; Kuever *et al.*, 1993; Pavlostathis, 1994). Thus, the possibility that SRB may directly degrade lignin cannot be excluded and requires further investigation.

The absence of any enhanced β-glucosidase activity under sulphate reducing conditions was unexpected. Upon exposure of the substrate, cellulose, induction of enzyme production was expected. The accumulation of glucose observed could have been due to hydrolysis of starch, particularly as α-glucosidase was detected in the flask and laboratory-scale studies. Starch is, however, rapidly degradable (San Pedro *et al.*, 1994) and it is unlikely that high concentrations were present in the flasks at the time when the accumulation of glucose was recorded. Thus, the soluble carbohydrates must have been derived from the hydrolysis of cellulose. The API ZYM kits used in this study are qualitative but only semi-quantitative, and may not have been suitable for the detection of cellulase in anaerobic systems. Further studies into the effect of sulphate reduction and sulphide on the enzymology of PSS digestion are required, and should employ more rigorous enzymological techniques. Based on the results of the semi-quantitative enzyme kits, sulphide did not appear to inhibit or enhance enzyme activity. The reduction in enzyme activity seen in the flask studies was explained in terms of inhibition of enzyme production by hydrolytic bacteria, rather than inhibition of the enzymes themselves. The inhibition was removed if a period of acclimation was introduced i.e. in the continuous reactor system.

The average floc size within a digester is dependent on the relative rates of flocculation and floc fracture. Constant floc growth is offset by enzymatic (Nielsen *et al.*, 1996) and shear-induced (Spicer and Pratsinis, 1996) deflocculation. The smaller average floc size in the sulphidogenic lab-scale reactors indicated that deflocculation was accelerated under those conditions. The mechanism of enhanced floc fracture in the settling sludge bed is poorly understood, but is likely to involve enhanced solubilisation of carbohydrates and proteins, in the presence of biological sulphate reduction. Little is known about the composition and formation of flocs in non-granulating anaerobic digesters. If it is assumed that the composition and structure of these flocs is similar to those in non-bulking activated sludge systems, then the composition is likely to reflect that of the feed to the digester. As such, a significant proportion of the flocs will be proteins and carbohydrates, held together by non-covalent bonds (Forster, 1982; Eriksson and Alm, 1991; Bruus *et al.*, 1992; Urbain *et al.*, 1993). It has already been shown that the solubilisation of carbohydrates, lignin and proteins are enhanced under sulphate reducing conditions. The increased rate of floc fracture is most likely due to a loss of integrity of the floc due to enhanced hydrolysis of important structural components such as lignin, cellulose and proteins. Cinq-Mars and Howell (1977) provided further evidence for the importance of cellulose in maintaining the integrity of flocs in PSS. When they treated PSS with fungal

cellulase, the gel-like characteristic of the raw sludge changed to that of a slurry of fine particles in less than 2 hours.

A reduction in floc size may offer further advantages to the solubilisation of organic wastes. Particulate organic matter of varying size would also be incorporated into the flocs, and would be enveloped in non-particulate organic molecules, or EPS. Li and Ganczarczyk (1990) suggested that the significant quantity of EPS in activated sludge flocs provided diffusional resistance to the movement of substrates and products to and from the flocs. Likewise, EPS and other macromolecular components of the flocs in anaerobic digesters may offer steric resistance to the passage of bacteria and hydrolytic enzymes. Although bacteria would also be incorporated into the growing flocs, the majority of degradation of the floc components is likely to be from the outer surface where the concentration of bacteria will be high. As such, the macromolecules within the floc will be protected from enzymatic degradation. As the flocs disintegrate, macromolecules that were previously protected from enzyme attack are exposed, and may be degraded by hydrolytic enzymes. By increasing the frequency of floc cleavage, it is possible to facilitate hydrolysis by increasing the contact between enzyme and substrate. Furthermore, deflocculation will allow hydrolytic bacteria and their associated enzymes to penetrate into the floc matrix, where hydrolysis of previously unexposed macromolecules will take place. The rate at which freshly exposed particulate organics are degraded is related to the size of the particles, with small particles degraded more rapidly than larger ones (Torrijos *et al.*, 1993; Wentzel *et al.*, 1995; Vavilin *et al.*, 1996).

Figure 3.5 summarizes the proposed mechanism for the accelerated hydrolysis of PSS under sulphate reducing conditions. Te sequence of the most important events involved in the process are shown in the order in which they are thought to occur, while the indirect and direct effects of the various components of the reaction on each other are shown in Figure 3.6. The design of the settling sludge bed reactor is thought to facilitate the proposed process, resulting in enhanced hydrolysis of the PSS.

The disadvantage of smaller flocs is their increased susceptibility to washout from reactors. As hydrolytic bacteria and hydrolytic enzymes are closely associated with the floc matrix (Boczar *et al.*, 1992; Frølund *et al.*, 1995; Confer and Logan, 1998; Goel *et al.*, 1998) they will also be removed from the reactor. The study of the enzymology of the lab-scale reactors revealed that the loss of hydrolytic enzymes from the sulphidogenic reactors did exceed that from the sulphate-free systems. Improved retention of small sludge flocs and associated hydrolytic enzymes would need to be considered during the design phase of the pilot-scale reactor system. This phase of the project is reported in Part 1 of this report (Rose *et al.*, 2002b).

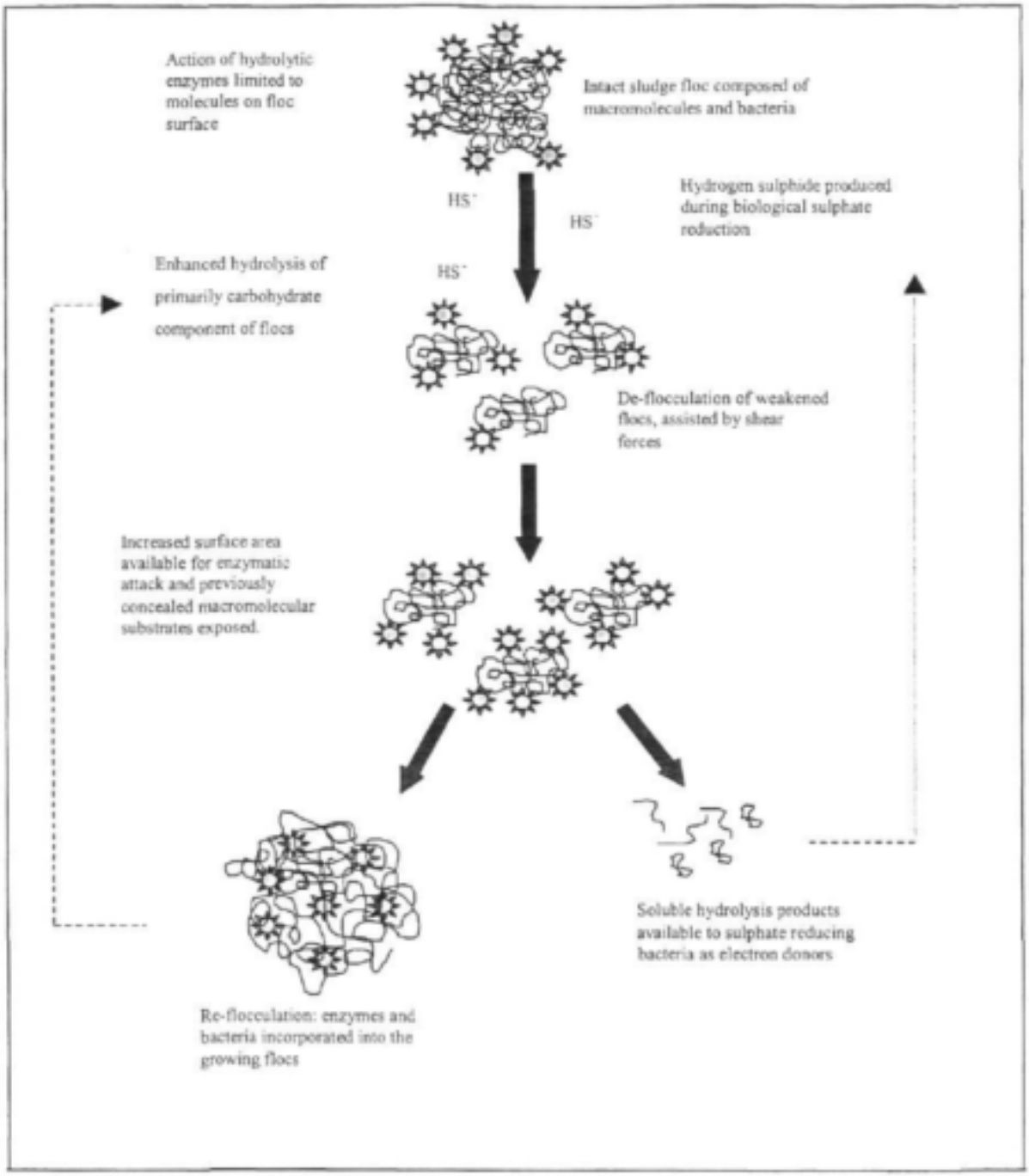


Figure 3.5. Flow diagram summarising the factors involved in the process of enhanced hydrolysis of PSS under sulphate reducing conditions.

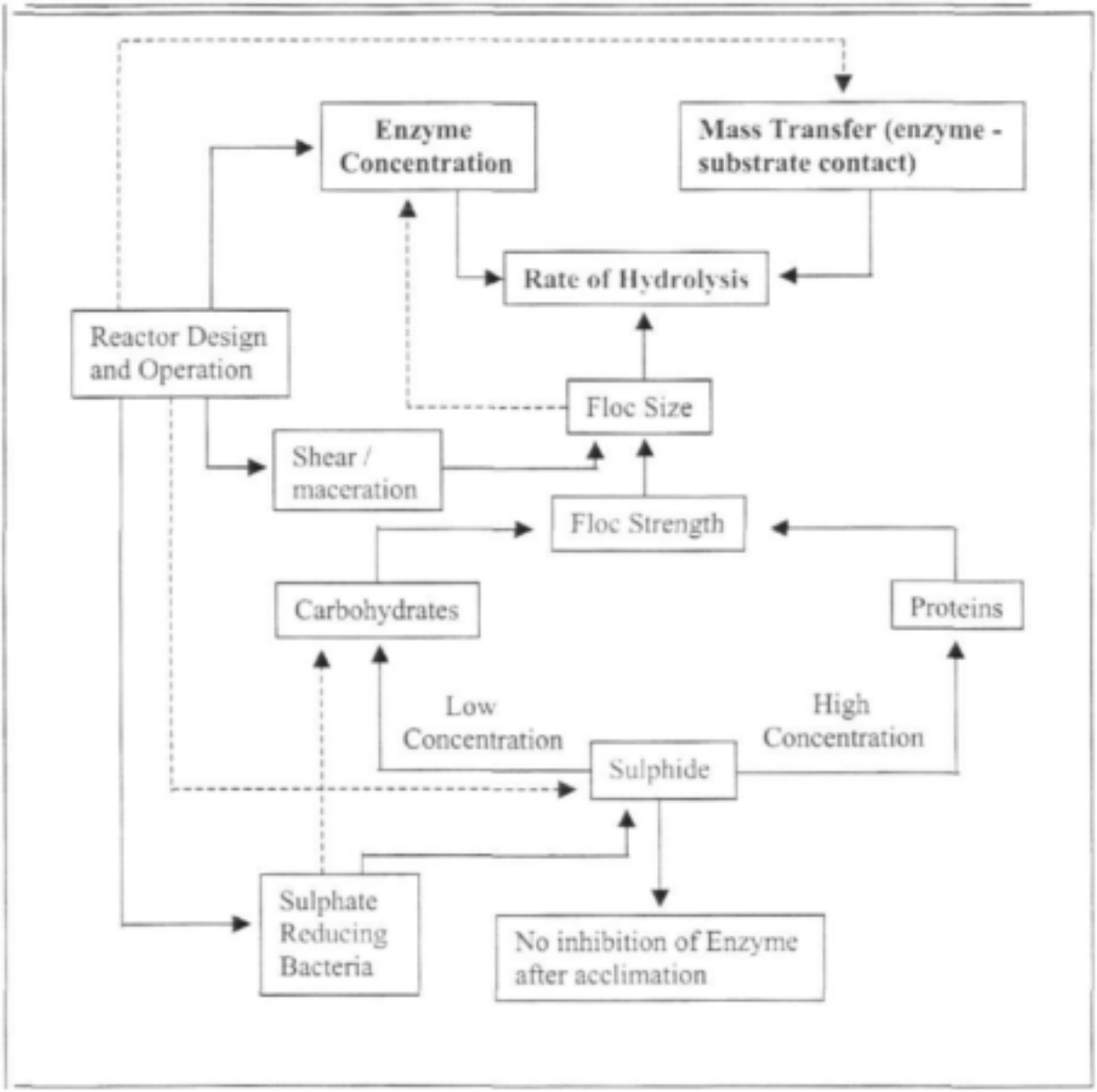


Figure 3.6. Flow diagram indicating the interrelationships of factors on the process of enhanced hydrolysis of PSS under sulphidogenic conditions. Dotted lines indicate possibly lower level impacts.

In summary, the results of this investigation indicate that enhanced hydrolysis of complex carbon sources relies on a reduction of floc stability in the presence of sulphide as a result of an increased rate of hydrolysis of lignin, carbohydrates and proteins. Deflocculation and mixing are essential for the exposure of previously inaccessible macromolecules to cleavage by hydrolytic enzymes as well as the release of trapped products thereby alleviating possible end-product inhibition. Tis will, in effect, reduce the effect of mass transfer limitations on the hydrolytic step. The reflocculation step, facilitated by the reciprocation of partially digested sludge, is also crucial and serves to increase contact between particle-bound enzymes, undigested substrates and biomass in

a sulphate-rich micro-environment within newly formed flocs. It is predicted that this model is an accurate reflection of the processes that occur in natural systems and explains the enhanced hydrolysis of complex carbon sources that was observed by Dunn (1998).

3.5 CONCLUSIONS

1. The solubilisation of COD_p was significantly enhanced in the presence of sulphide at both pH 7 and pH 10;

2. The rate of hydrolysis of complex carbohydrates is enhanced in the presence of sulphide. The rate of hydrolysis is higher in the presence of biological sulphate reduction than when sulphide was added chemically, possibly due to the production of alkalinity;

3. The rate of delignification is enhanced in the presence of sulphide or biological sulphate reduction;

4. The rate of degradation of intermediate size proteins was high, even in the absence of sulphide, and offered an explanation for the absence of bands on the gels corresponding to proteins of this size;

5. A sulphide concentration of 100mg.L^{-1} resulted in an increase in the rate of hydrolysis of sludge proteins, relative to that in the sulphide-free control. The rate was increased further at higher sulphide concentrations;

6. A descriptive model has been proposed to describe the enhanced hydrolysis of PSS in a novel RSBR, in the presence of biological sulphate reduction.

4 CONFIRMATION OF THE CONCEPT OF ENHANCED HYDROLYSIS OF PRIMARY SEWAGE SLUDGE IN A TECHNICAL-SCALE RECYCLING SLUDGE BED REACTOR

4.1 BACKGROUND

Results from the flask and laboratory-scale reactor studies provided support for the findings by Dunn (1998), whose investigations on the microbial ecology of tannery ponds, suggested enhanced hydrolysis of complex organic carbon substrates in sulphidogenic environments. Futhermore, evidence showed that the recycling sludge bed system was highly effective and that a dual-stage process would be required to maximise the use of soluble hydrolysis products for sulphate reduction. Based on these findings, it was decided to test the descriptive model in a technical-scale RSBR. In order to minimize loss of small sludge flocs and their associated hydrolytic enzymes, it was necessary to incorporate an upper region of linear flow and a lower region consisting of a sulphidogenic settling sludge bed. Recycling of the settled sludge from the base of the reactor was required to prevent compaction of the undigested sludge but more importantly, to ensure that the sequence of deflocculation and reflocculation events occurred as described in the model.

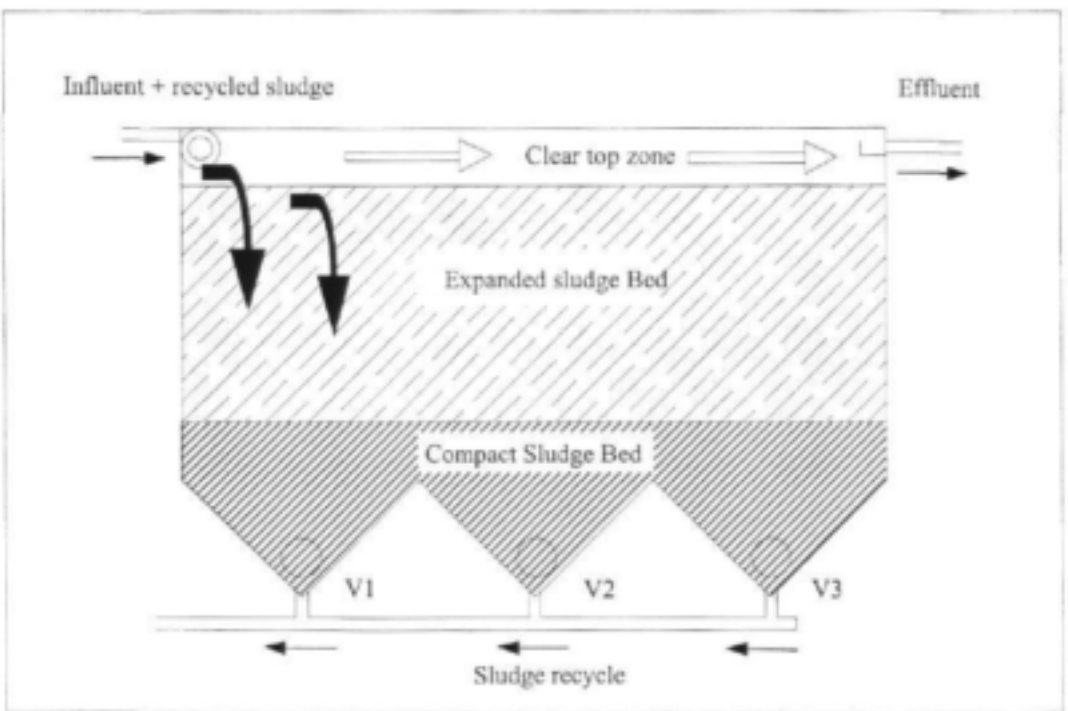


Figure 4.1. The Recycling Sludge Bed Reactor showing the segregation of settled sludge, the recycle of the sludge bed to blend with the incoming flow, and the supernatant liquid carrying the solubilised product to the second stage in the BioSURE Process®.

It would facilitate separation of soluble product from the undigested material, while encouraging flocculation of undigested substrate, bacteria and enzymes. In order to minimise cost of construction, while at the same time maximising performance, the technical-scale reactor was constructed as a modified trench-style settler (Figure 4.1). A single elongated reactor that would allow for a longer period of settling replaced the 3-stage system that was found to be effective in the laboratory-scale studies. Internally, the lower third of the trench was divided longitudinally into three valleys, while the upper region of the reactor was undivided (Rose *et al.*, 2002). It was proposed that this configuration, known as the RSBR, would allow for maximum settling of small flocs and controlled recycle of the settled sludge. Simpler horizontal-flow trench-type anaerobic digesters have proved highly successful in the digestion of hog manure in Taiwan (Hong, 1986).

The RSBR formed the first part of a multiple-unit Rhodes BioSURE Process® designed for the bioremediation of AMD and other sulphate-rich wastewaters. The process train consisted of three principal stages namely sludge solubilisation, sulphate removal, and polishing (Rose *et al.*, 2002b). Supplementary sulphate removal was carried out in an Anaerobic Baffle Reactor (ABR) and was followed by polishing in High Rate Algal Ponds (Figure 4.2). Provision was made for the removal of heavy metals from the AMD by sulphide precipitation prior to it entering the system.

The scale-up of the process proceeded from the 2L laboratory-scale reactors, through 10L, $1m^3$ to the $23m^3$ technical-scale pilot plant. Oxidation of sulphide in the perspex reactor was, however, found to be extremely rapid and resulted in the formation of a floating sulphur biofilm covering the entire surface of the reactor within 24 hours. The mechanism, rates and microbial ecology involved in the formation of these films have been studied and will be the subject of a future report (WRC Project K5/1073).

Towards the end of 1997, the EBG was invited to participate in the Grootvlei Desalination Technology Evaluation Exercise. A decision was made to proceed to the design and construction of the technical-scale BioSURE® pilot plant to enable participation in this exercise. At the time, Grootvlei Gold Mine, on the Far East Rand Mining Basin (FERMB), was discharging an estimated 130 $ML.d^{-1}$ highly saline AMD water into the nearby Blesbokspruit, an ecologically sensitive Ramsar wetland site (Van Wyk and Munnik, 1998). Prior to discharge, the concentration of iron in the water was reduced to the accepted discharge levels by lime addition in a High Density Sludge (HDS) Plant (Grootvlei Mines, 1997). Further details regarding the design, construction, operation and performance of the pilot-plant are the subject of Part 1 of this series (Rose *et al.*, 2002b).

4.2 OBJECTIVES

1. To determine if enhanced solubilisation of PSS could be achieved in a technical-scale RSBR reactor under sulphate reducing conditions;

2. To verify the accuracy of the hypothesis and descriptive model of enhanced hydrolysis of complex carbon sources;

3. To determine whether the RSBR was effective in retaining hydrolytic enzymes;

4. To validate the descriptive model of enhanced hydrolysis in a RSBR.

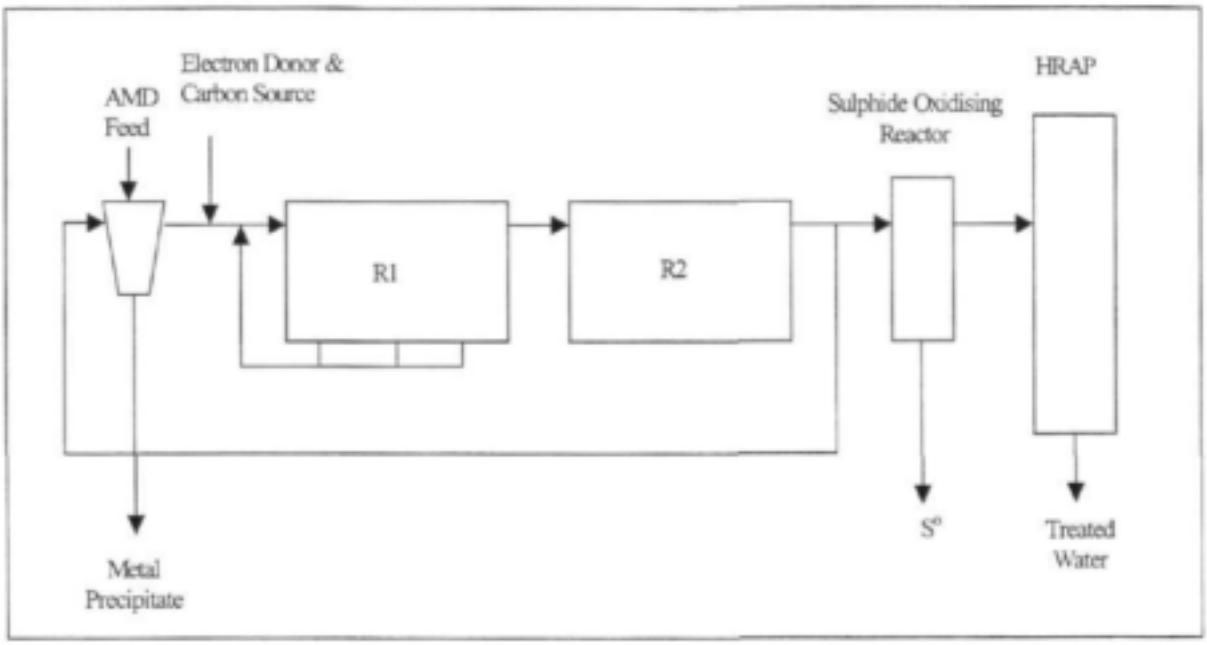


Figure 4.2. Process flow diagram of the Rhodes BioSURE Process® applied to the treatment of acid mine drainage wastewater. R1 = Recycling Sludge Bed Reactor; R2 = anerobic baffle reactor; HRAP = High Rate Algal Pond; PSS = primary sewage sludge.

4.3 MATERIALS AND METHODS

The methods including COD, sulphate, pH and the use of API ZYM kits for the determination of enzyme profiles, are described in Section 2. Suspended solids and settleable solids were calculated as described in Standard Methods (APHA, 1989). Sludge profiles were studied by inserting a 2.5m transparent perspex tube through roof of the reactor. Once the tube had reached the base of the reactor, the upper end was stoppered and the tube removed. The depth of the dense and expanded regions of the sludge bed was recorded for each of the three valleys. Loose sludge was characterised as consisting of distinct flocs, while the compact sludge was tar-like, and of a uniform consistency.

The concentrations of COD_t and sulphate in the influent and effluent of the hydrolysis unit were determined daily for 120 days. Sludge profiles were monitored on a regular basis. A complete sample profile of the reactor was carried out on three occasions, during September, November and December 1998. At these times, samples were drawn from the Blend Tank and effluent of the hydrolysis unit, as well as from three depths within each of the three

valleys of the reactor. Samples (500mL) were collected and analysed for COD_t, COD_f, COD_p, sulphate, pH and suspended solids.

Samples were collected for enzyme analysis during September 1998, after the pilot-plant had been running at steady state for 3.5 months. Samples (1L) were collected from the feed (Blend Tank) as well as from the top, middle and bottom of each of the three valleys in the hydrolysis unit. These were stored in airtight plastic bottles, and analysis of the enzyme activity was initiated within 30 minutes of collection. After inoculation of the API ZYM strips, they were incubated at room temperature (25°C) for 4 hours.

4.4 RESULTS AND DISCUSSION

4.4.1 Summary of Performance

The overall performance of the reactor during the study period, including the effect of the dual-stage system, is documented in Part 1 of this series (Rose *et al.*, 2002b) and only data relevant to the objectives set out in section 4.2 of this report will be discussed further.

Mean removal of COD and removal of sulphate, for the three audit periods, were 57.9 (± 6)% and 31.4 (± 14)%, respectively (Table 4.1). These values exclude removal in the ABR of the dual-stage process. Both parameters exhibited some variation within the sample periods. The mean pH of the effluent, pH 7.0, was slightly higher than that of the influent value of pH 6.4. Values for COD_p and COD_f were not calculated during the November audit, and, consequently, the mean values for these parameters were only calculated from two sample periods. A high percentage of influent COD_t (85.5%) was in the form of particulate organic matter, with a small percentage (14.5%) being in the soluble form.

The average volume of settleable solids in the PSS during September was 990mL. This dropped to 90mL in the Blend Tank (where PSS is blended with metal-free AMD prior to entering the RSBR) due to dilution, and was between 1 and 3mL in the effluent of the RSBR. This, together with the fact that accumulation of sludge within the RSBR was low, indicated that the rate of solubilisation of PSS within the RSBR was high. A detailed record of COD removal and sulphate removal during September is shown in Table 4.1. The COD: SO_4 ratio of the feed was maintained at 2:1 for the duration of the pilot plant study.

Table 4.1. Summary of the RSBR performance during the three audit periods. All values in $mg.L^{-1}$ unless otherwise stated. Standard deviations are indicated in brackets.

	Sample Times			
Parameter	**September**	**November**	**December**	**Mean**
COD_t in	3003	3207	3553	3254 (278)
COD_t out	1439	1362	1274	1358 (82)
COD_p in	2568	-	2779	2670 (154)
COD_p out	841	-	986	913 (102)
COD_f in	442	-	774	608 (234)
COD_f out	598	-	288	443 (219)
SO_4 in	1656	1673	1771	1700 (62)
SO_4 out	1407	956	1128	1163 (227)
COD_t removed	1564	1845	2279	1896 (360)
% COD_t removed	51.2	57.5	64.1	57.9 (6)
SO_4 removed	249	717	643	536 (251)
% SO_4 removed	15.0	42.8	36.3	31.4 (14)
pH in	6.5	6.6	6.0	6.4 (0.3)
pH out	6.6	7.5	7.1	7.0 (0.4)

Based on the data given in Tables 4.1 and a stoichiometric requirement of 2g COD required to reduce 1g sulphate (Isa *et al.*, 1986; Lens *et al.*, 1995), it is possible to calculate the minimum percentage of particulate COD hydrolysed. It must also be assumed that only soluble COD can be utilized by SRB, and that COD_p is unavailable to this group. Taking the mean data from the three audit periods (Table 4.1), $536mg.L^{-1}$ SO_4 was reduced, which would require $1072mg.L^{-1}$ of soluble COD (COD_f).

$$\text{Total } COD_{f\,required} = (COD_{f\,SO4\ removal} - COD_{f\,in}) + COD_{f\,out}$$
$$= (1072\text{-}608) + 443$$
$$\therefore\ COD_{f\,required} = 907mg.L^{-1}$$

The concentration of COD_p remaining in the reactor was $1757mg.L^{-1}$. Thus, a minimum of 51.6% of the particulate COD that remained in the reactor had to have been hydrolysed to produce enough soluble COD to have supported the amount of sulphate reduction that took place. Athough not yet fully optimised, this value exceeds published maximum yields of around 35% (Karlsson and Göransson, 1993; Hatziconstantinou *et al.*, 1996). The minimum percentage of COD_p hydrolysed on the day of the December audit was only 44%. The calculated values of the minimum percentage COD_p that was solubilised were, however, likely to have been underestimates. It is thought that the quantity of sulphate reduced, and consequently, the quantity of COD_f utilised, was actually higher than represented by the data. Re-oxidation of the sulphide gas on the surface layer of the reactor, may have resulted in a cycle of sulphate reduction and sulphide oxidation within the reactor. Thus, an estimation of the quantity of sulphate reduced based on comparison of influent and effluent sulphate concentrations, was probably conservative. The presence of elemental sulphur on the surface of the reactor contents is evidence of this oxidation, as too is the increase in the concentration of sulphate on the surface of the reactor

from the inlet to the outlet of the RSBR (Figure 4.4a). When the headspace of reactor was purged with nitrogen, the concentration of sulphate on the surface decreased along the length of the reactor (results not shown). The sulphate concentration in the effluent before and after purging was, however, similar. Any utilisation of COD_f by methanogenic bacteria would also have resulted in an underestimation of the quantity of COD_p solubilised. Under the conditions within the hydrolysis unit, i.e. potentially high concentrations of sulphide and a low COD_f : SO_4 ratio, methanogenesis would have been severely retarded. Methanogens are only expected to out-compete SRB at $COD:SO_4$ concentrations of above 2:1 (Li *et al.*, 1996).

With a mean $COD:SO_4$ ratio of 0.5 near the surface of the reactor, SRB would be expected to dominate, if they could resist being washed out. However, in the sludge bed, where the sulphate concentration is lower and the concentration of COD_f is high, the $COD:SO_4$ ratios are 15 and 30 for the middle and lower bed, respectively. Under these conditions, in the absence of inhibitory concentrations of sulphide, methanogens could dominate (Li *et al.*, 1996). The free sulphide in the sludge bed never exceeded 100mg.L^{-1} (data not shown), and would thus probably not severely inhibit methanogenesis. Nevertheless it appeared that methanogenesis remained low or inactive. Using similar calculations, it was possible to estimate what fraction of the COD_t retained in the reactor was used for sulphate reduction and, consequently, what percentage was likely to have been removed by settling. Values are based purely on COD_t of the influent and effluents and sulphate removed. Mass balance figures based on a 2gCOD/g sulphate removed showed shortfalls or surplus of COD, and values ranged from shortfalls of 52% to a surplus of 78.8%. On average for the month of September, there was a mean surplus of 36.8% (± 36). Thus, a certain proportion of the COD was being removed by the RSBR, but was not utilised for sulphate reduction. This may reflect that portion of the PSS that is highly recalcitrant.

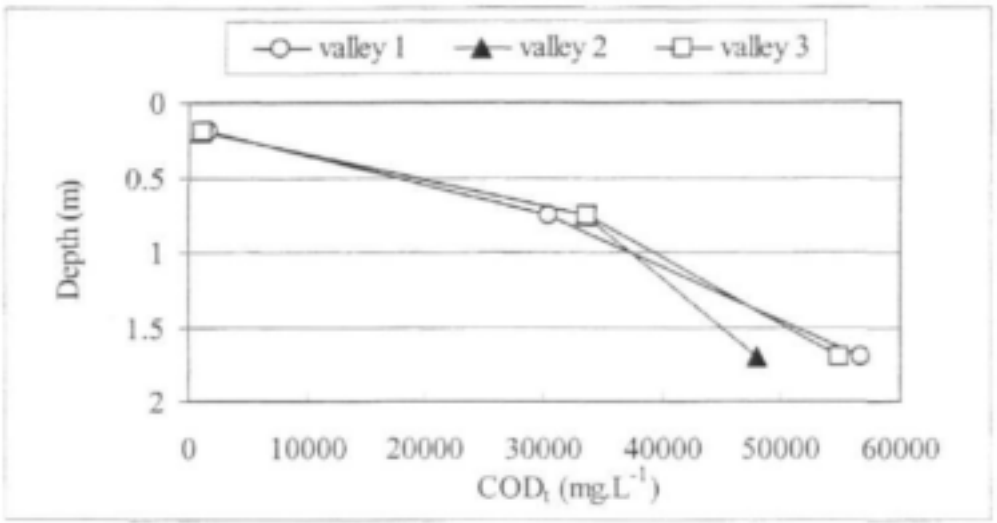
The mean concentrations of all COD fractions showed similar profiles in all three valleys (Figure 4.3). The COD concentration just below the surface of the reactor was low and increased significantly with depth. The difference between the upper and middle samples was far larger than that between the middle and lowest sample, with mean COD_t in the beds between 50 000 and 60 000mg.L^{-1} (Figure 4.3a). These values showed little variation over the entire experimental period. The mean concentration of COD_f in the middle of valley 3 was significantly higher than in the other two valleys, at the same depth, due to a high concentration of COD_f at that position during the September audit. The COD_f decreased to a relatively lower concentration in the bed of valley 3 (Figure 4.3c), possibly due to an accumulation of non- or slowly biodegradable COD in that region. The COD_f concentration in the bed of valley 1, where it was expected that a significant proportion of the particulate COD was fresh and easily biodegradable, was almost twice that of the other two valleys.

The mean sulphate concentration at the various depths exhibited a profile opposite to that of COD, with a dramatic decrease in sulphate concentration

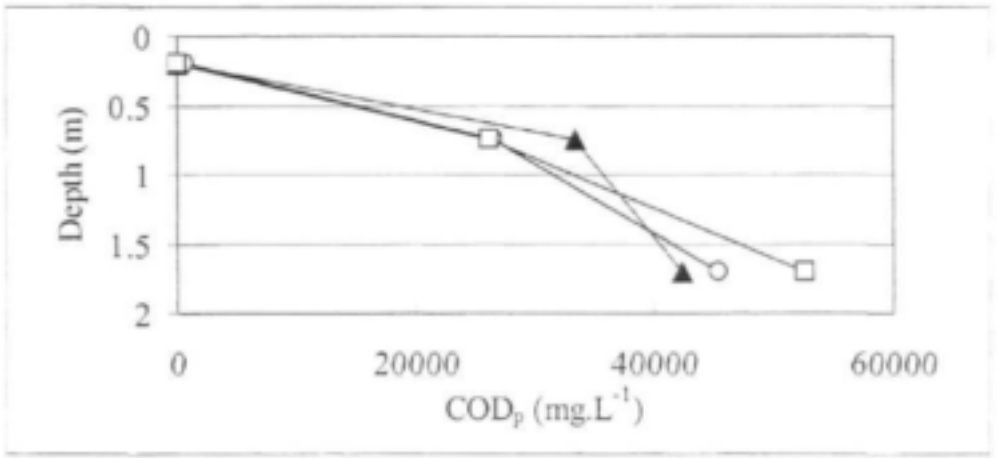
with depth (Figure 4.4a). The mean concentration near the surface of valley 1 was slightly lower than valley 2 and 3 (Figure 4.4a). The concentration of sulphate decreased from between 1000 and 1200mg.L^{-1} on the surface, to approximately 300mg.L^{-1} in the beds. This profile may be linked to the increased availability of hydrolysis products towards the base of the reactor or the washout of sulphate reducers from the upper zones. The presence of 200 – 400mg.L^{-1} sulphate in the base of the RSBR might have been as a result of inhibition of sulphate reduction by high sulphide concentrations, and indicated that the rate of recycle needed to increased. The accumulation of COD_f supports this idea and, as expected, the pH in the lower region of the RSBR was lower than at the surface. The increase in pH from the middle to bottom of the first two valleys was probably due to constant removal of soluble products from the lower region during the recycling of sludge. Sludge was drawn less frequently from the third valley and may have allowed for the accumulation of product, and a drop in pH, in this region. There was very little difference in the mean pH profiles of the valleys at different depths, with the largest difference being 0.2 pH points (Figure 4.4b).

Suspended solids were measured during the September audit. The profiles were similar to those of COD_t and COD_p, showing an increase in suspended solids concentration as a function of depth (Figure 4.4c). There was little difference in the top and middle samples from all valleys, but the concentration of suspended solids in the bed of valley 1 was almost double that in the beds of valleys 2 and 3. As can be seen in Figure 4.5, accumulation of sludge within the RSBR was minimal, although some accumulation did occur within valley 3 after day 113. The profiles also show that the entire sludge bed in valley 1 was dense, while the beds in valleys 2 and 3 consisted of a dense bed under a loose bed. This together with the theoretical COD requirements for sulphate reduction, provide evidence for the enhanced hydrolysis of PSS in the RSBR.

a)



b)



c)

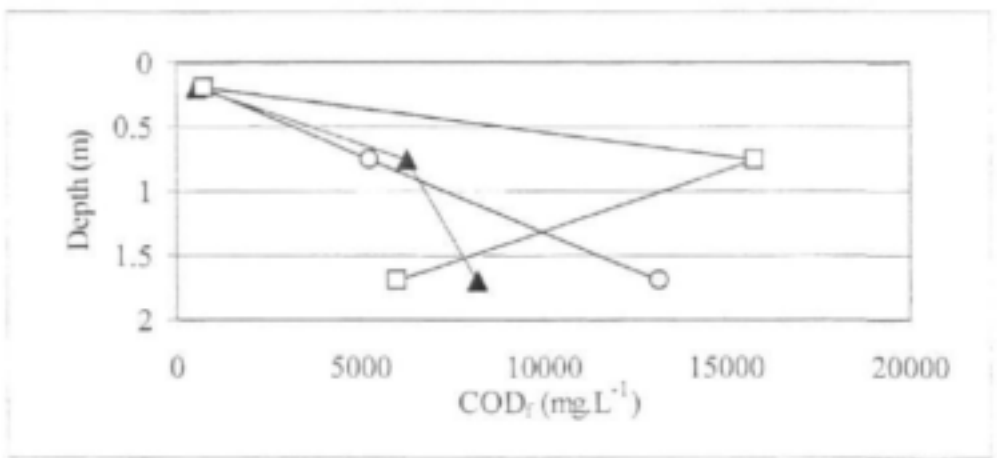
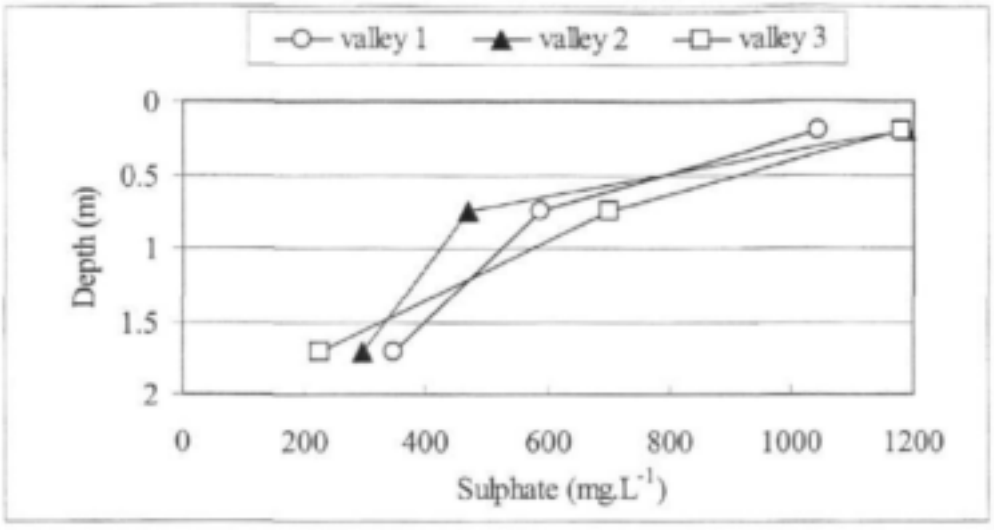
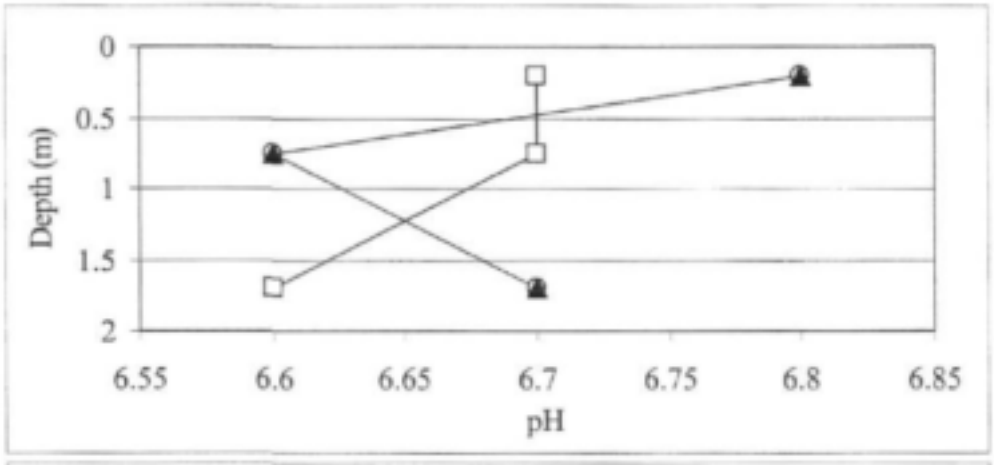


Figure 4.3. Mean COD concentrations at three depths in the RSBR for the three audit periods a) September; b) Novemebr; c) December. Standard deviations were omitted for clarity.

a)



b)



c)

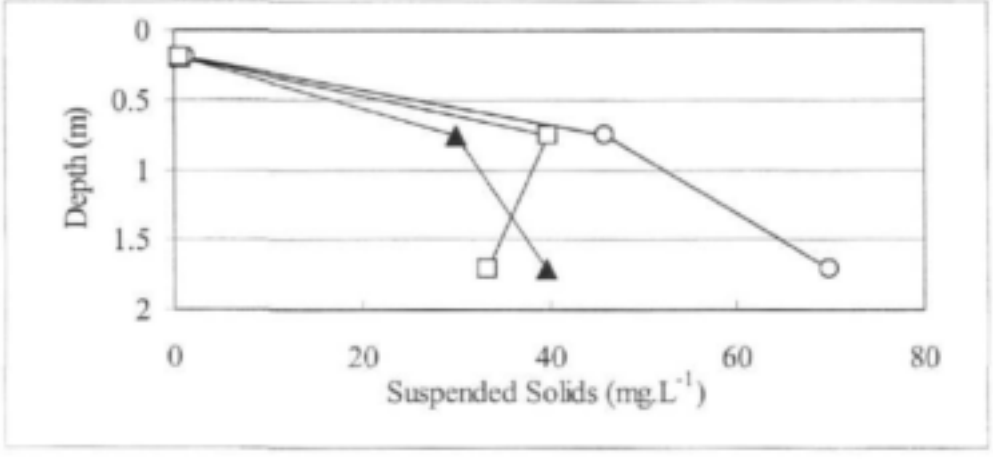


Figure 4.4. Mean sulphate concentrations, pH and suspended solids concentrations at three depths in the technical-scale RSBR for three audit periods (n = 1 for suspended solids). Standard deviations were omitted for clarity.

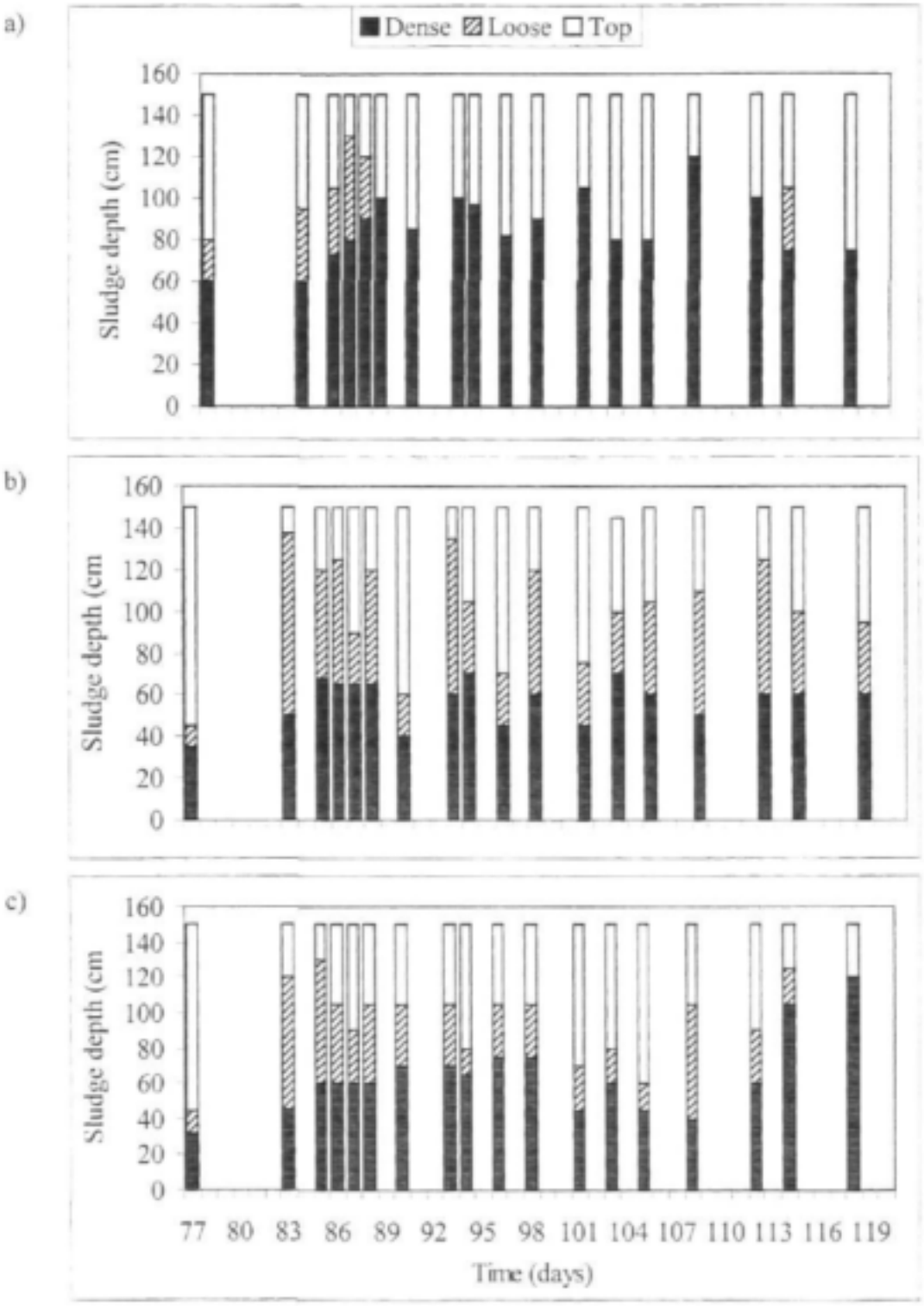


Figure 4.5. Analysis of sludge in three valleys within the technical-scale RSBR after steady state had been reached. a) valley 1, b) valley 2, c) valley 3. The depth of the loose and dense sludge is indicated.

4.4.2 Enzyme Profiles

The enzyme activity within the technical-scale hydrolysis unit was high when compared to the activity within the lab-scale reactors (Figure 4.6). However, any comparisons between the lab- and technical-scale systems must be made with caution, as the specific activity of the enzymes was not calculated. A number of the enzymes showed no activity, and included both proteases (9, *10), some of the glucosyl hydrolases (15, 19, 20) and lipase C14 (5). Cysteine* arylamidase (8) activity was not detected in any of the samples except for the top of valley 1, where its activity was very low (Figure 4.6a). The activity of β-galactosidase (14) and α-galactosidase (13) was only detected in the Blend Tank and in some of the samples taken from valley 1 (Figure 4.6).

The activity of enzymes in the samples taken from the Blend Tank and the top of the valleys was always low, and increased significantly towards the base of the reactor. The activity of most of the enzymes tested was higher in the base than in the middle region, although the activity of some of the enzymes did not conform to this pattern. In valley 1, esterase lipase C4 (3), esterase lipase C8 (4), phosphatase acid (11) and naphthol-AS-BI-phosphohydrolase (12) all exhibited higher activity in the middle region than in the base (Figure 4.6a).). This pattern of activity was also seen for esterase lipase C8 (4) and α-glucosidase (16) in valley 2 (Figure 4.6b). The decease in the activity of α-glucosidase from valley 1 to valley 3 was not surprising. The enzyme is responsible for the hydrolysis of starch and as this is rapidly biodegradable it is expected that starch would only be present in large quantities in valley 1. In valley 3, the activity of each of the enzymes was lower in the base of the reactor than in the middle (Figure 4.4c). Some enzymes, such as phosphatase alkaline (2), valine arylamidase (7) and α-glucosidase (16) showed activity in the top and middle regions of the reactor, but no activity in the base. In order to obtain a more accurate assessment of the regions of high and low within the technical-scale reactor, the total enzyme activity (as nmol substrate converted) was calculated for each of the samples (Table 4.2).

Table 4.2. Total enzyme activity (as nmol substrate converted) of samples from 9 regions in the technical-scale reactor. The total activity of the feed was 1980nmol.

Depth (m)	Valley 1	Valley 2	Valley 3
0.2	2100	997	892
0.75	10459	10822	9840
1.7	11480	12710	2870

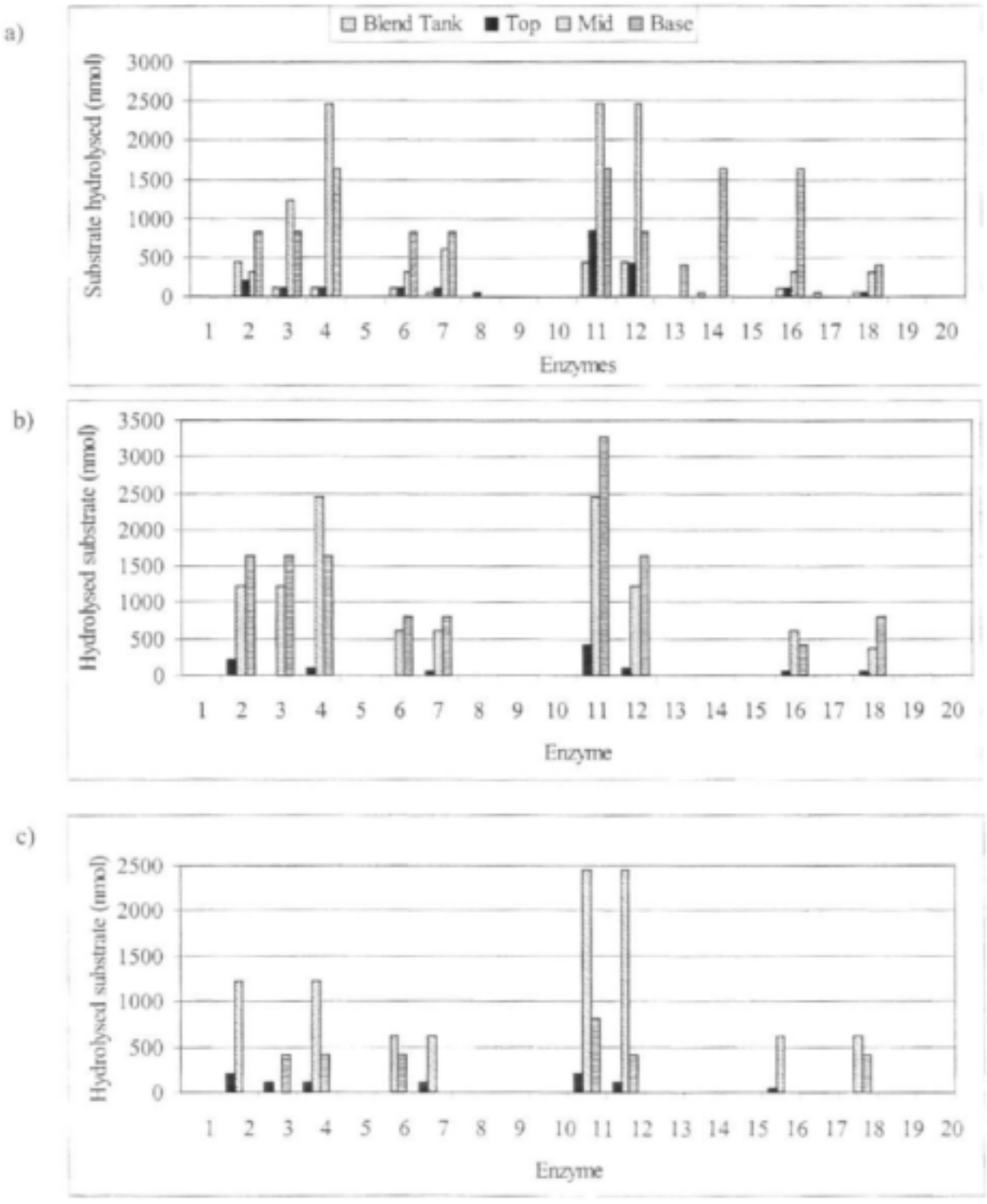


Figure 4.6. Enzyme activity in the technical-scale hydrolysis unit in a) Blend Tank and valley 1, b) valley 2 and c) valley 3. The list of enzymes is given in Section 2 of this report.

The total activity in the top of all valleys, and in the base of valley 3 were very low in comparison to the other 5 areas within the reactor. This is a significant finding, indicating that the design of the reactor was able to overcome the problem of enzyme washout that was seen in the laboratory-scale reactors. The

high levels of enzyme activity in most regions of the reactor relative to that in the effluent is indicative of an effective enzyme accumulator. This can be attributed to the improved retention of small flocs with which the enzymes are associated. The low enzyme activity in the base of valley 3 is most likely due to accumulation of non-biodegradable organic matter in this region that should probably be wasted on a regular basis.

4.5 CONCLUSIONS

1. An indirect but reliable estimate of COD solubilisation, based on levels of sulphate reduction, indicated that on average at least 52% of the particulate COD entering the RSBR had to have been completely solubilised. This value is significantly greater than any published value.

2. The above estimate was probably lower than the actual value, as some re-oxidation of the sulphide had taken place. This may also have accounted for the relatively low levels (31%) of sulphate reduction achieved. Sulphate remaining in the effluent of the RSBR passed to the ABR where the rate of sulphate reduction was high. A maximum removal of sulphate exceeded 80% over the dual-stage system.

3. The design of the RSBR was able to overcome the problem of enzyme washout as was seen in the laboratory-scale multiple-stage reactors;

4. Results supported the proposed model describing the process of enhanced hydrolysis of PSS in a RSBR under sulphate reducing conditions.

5 CONCLUSIONS AND RECOMMENDATIONS

Rose *et al.* (2002b) described the development of the dual-stage Rhodes BioSURE Process® for the treatment of AMD, where the process development had been based on prior studies in the microbial ecology of sulphidogenic ponding environments. This process incorporated a novel RSBR, for the hydrolysis of complex carbon sources, followed by an Anaerobic Baffle Reactor (ABR) where soluble hydrolysis products are consumed to drive biological sulphate reduction. The aim of the current study was to provide proof of the concept of the enhanced hydrolysis of complex carbon sources within a biosulphidogenic RSBR, to develop of descriptive model of the underlying mechanism and to evaluate the performance of a technical-scale pilot plant.

5.1 FINDINGS

Results from a laboratory-scale RSBR confirmed that a complex carbon source such as PSS could be used to drive biological sulphate reduction and that the hydrolysis of PSS was enhanced in a recycling biosulphidogenic environment. The sludge flocs in the sulphidogenic reactor were significantly smaller than those in the methanogenic control. Although it is thought that this plays a role in enhanced hydrolysis by reducing mass transfer limitations, it also increased the susceptibility of washout of associated bacteria and hydrolytic enzymes. A comparison of single- and multiple-stage reactors indicated that the total percentage of sulphate reduced was improved in the latter, and this was taken into account when finalising the design of the dual-stage BioSURE Process®.

The solubilisation of the particulate fraction of PSS was followed in flask studies, and was enhanced in the presence of sulphide at both pH 7 and pH 10. A more detailed examination of the mechanism of sulphide-enhanced hydrolysis confirmed that both the protein and carbohydrate fractions were affected. The extent of protein hydrolysis was followed using a new SDS-PAGE technique and showed that a sulphide concentration of 100mg.L^{-1} was required to achieve enhanced hydrolysis of this organic fraction. The exact mechanism by which sulphide enhanced the hydrolysis of the carbohydrate fraction is not understood at this stage, although it may involve removal of lignin either due to the action of sulphide and alkalinity or through direct degradation by SRB.

Based on the above findings, a descriptive model was developed to explain the phenomenon of enhanced hydrolysis in the RSBR. It is proposed that sulphide has a negative affect on the physical integrity of the sludge flocs, and during the reciprocation events, large flocs are fractured. This promotes the release of soluble hydrolysis products and sulphide from the floc. The smaller flocs are recycled to the inlet of the reactor where they come into contact with fresh substrate, and reflocculate in the upper settling zone of the RSBR. During reflocculation, fresh substrate, sulphate, bacteria and associated hydrolytic enzymes are incorporated into the center of the flocs. These then settle into the

base of the reactor and as hydrolysis proceeds, the products of the process stimulate further hydrolysis.

Scale-up of the RSBR proceeded through 2L, 10L, $1m^3$ to $23m^3$ reactor units, and based on the quantity of sulphate reduced, it was concluded that a minimum of 52% of the particulate carbon entering the RSBR had to have been solubilised to its readily available form. This value exceeded all previous published values by approximately 20% and demonstrated the efficacy of the recycling sludge bed concept.

5.2 RECOMMENDATIONS

A number of recommendations relating to the sulphidogenic enhanced hydrolysis concept emerged from the combined research programme undertaken in WRC Projects K5/869 and K5/972.

1. Where the descriptive model had provided evidence for the concept of sulphidogenic enhanced hydrolysis, process development to industrial-scale application would require a mathematical modeling approach to description of the relationship between physico-chemical and biological parameters within the reactor. The initiation of these studies was recommended.

2. While a provisional investigation of the enzymology in the RSBR was undertaken, it was apparent that more detailed quanitative studies were required to adequately describe the underlying events. Follow-up studies in this area were recommended.

3. Deflocculation events and the release of soluble products were found to be central to the enhanced hydrolysis reactions in the RSBR. Further studies on the solubilisation process would be necessary in system optimization studies and were proposed.

5.3 RESEARCH PRODUCTS

The combined investigation undertaken in WRC Projects K5/869 and K5/972 led to a number of follow-up studies based on the above recommendations. These are the subject of separate investigations and project reports, are noted below under follow-up actions and are listed in Appendices 1.

Student training associated with these projects has included 1 Post-Doctoral Fellow, 6 PhD and 8 MSc students. Publication of the results of these studies is ongoing but currently includes 7 patents, 9 journal articles and 2 reports. Publication in conference proceedings includes 5 plenary and key note lectures, 13 international and 20 local conference presentations. Student training and publication outputs are reported in Appendix 2.

Industrial technology transfer has been undertaken, involving the products of these studies, and is noted in Appendix 3. In addition, research spin-off

developments which have resulted in associated follow-up research projects have been noted below and in Appendix 4 of this report.

5.4 FOLLOW-UP ACTIONS

Although it has been proved over a range of scales, from laboratory experiments to a technical-scale RSBR pilot plant, that the hydrolysis of a complex carbon source such as PSS is enhanced significantly under biosulphidogenic conditions, a number of aspects relating to the Rhodes BioSURE Process® are still under investigation. The principles involved have also provided the basis for follow-up studies.

5.4.1 Enhanced Hydrolysis of Other Complex Carbon Sources

Although PSS is readily available in large quantities, in some circumstances it may be preferable to utilize other "cleaner" carbon sources to drive sulphate reduction. This would be particularly relevant when the effluent from the Process is required for the cultivation of products destined for human consumption eg. crop plants. Alternative carbon sources being studied at present include maize waste, wood chips and grass. The studies are being undertaken in collaboration with a DACST Innovation Fund project lead by Pulles Howard and De Lange and also with Eskom in active and passive approaches to coal mine wastewater treatment.

5.4.2 The Role of Sulphate Reducers and Sulphide

The results of the study supported the general mechanism for enhanced hydrolysis in the RSBR. However, the exact role of sulphide, sulphate and SRB in the destabilization of the floc integrity is not clearly understood. Current research is focusing on the effect of sulphide on hydrolytic enzymes in sewage sludge as well as the ability of sulphate reducers to directly enhance the hydrolysis of lignocellulosic material. This work is being undertaken as a component of the Innovation Fund Project and in WRC follow-up project K5 / 1170 by Prof. C. Whiteley's Environmental Enzymology Group in the Dept. Biochemistry, Microbiology and Biotechnology, Rhodes University.

5.4.3 Oxidation of Sulphide

Biodesalination implies the final removal of sulphate salinity from the waste stream. The concentrations of sulphide in the effluent of the BioSURE Process® can exceed 200mg.L^{-1} and the EBG is investigating the oxidation of this compound to elemental sulphur which may be precipitated and removed from the system.

5.4.4 Metal Precipitation

The mine wastewater used in the pilot study passed through a HDS process to remove the majority of the iron prior to it entering the RSBR. However, the Rhodes BioSURE Process® allows for metal removal by sulphide precipitation and the control of this step is still under investigation. It is envisaged that a small stream from the ABR will be mixed with the influent metal-rich and the metal-sulphide precipitate will be settled out of solution prior to it entering the RSBR. The chemistry of the process is under investigation in collaboration with Prof. R. Loewenthal of the Dept. Civil Engineering at the University of Cape Town.

5.4.5 Modeling of the Recycling Sludge Bed Reactor

Although a descriptive model has been proposed to describe enhanced hydrolysis within the RSBR, a mathematical model would improve understanding of the relationships between physico-chemical and biological parameters within the reactor. This approach will feed into process optimisation initiatives and was undertaken in collaboration with Prof. G. Hansford and Dr A. Lewis in the Dept. Chemical Engineering at the University of Cape Town (Ristow *et al.*, 2002).

5.4.6 Disposal of PSS and Biological Nutrient Removal (BNR)

The disposal of large quantities on undigested PSS is a global problem. Only a limited quantity may be disposed of on agricultural land and the disposal of the remainder, usually by incineration or dumping, is costly. The concept of enhanced hydrolysis in a RSBR offers a novel alternative and the soluble product may be used to drive BNR processes. This project involves collaboration with ERWAT and a full-scale reactor for the enhanced disposal of PSS is currently being constructed ANCOR sewage disposal works, Springs.

5.4.7. Interrogation of the Descriptive Model

Although enhanced hydrolysis of complex carbon sources under biosulphidogenic conditions has been demonstrated at both laboratory and technical-scale, and has resulted in the development of a descriptive model, certain aspects of the model require further examination. According to the model, deflocculation events are central to enhanced hydrolysis of complex carbon sources. Destabilization of the sludge flocs allow release of soluble products of the anaerobic digestion process, primarily volatile fatty acids and reducing sugars, which could otherwise inhibit hydrolysis through negative feedback on the hydrolytic enzymes.

Subsequent experimental work conducted by J. B. Molwantwa (2002) confirmed that the maximum percentage solubilisation of PSS was significantly greater under sulphidogenic (63%) than methanogenic conditions (31%). This suggests that a certain organic fraction that would be considered

recalcitrant under methanogenic conditions is susceptible to microbial degradation under sulphidogenic conditions. The rates of hydrolysis were investigated using highly selective metabolic inhibitors, and it was shown that the rate of hydrolysis, measured as the rate of production of reducing sugars, was three times greater under sulphidogenic than methanogenic conditions. The rate of utilization of soluble products was also more rapid under the former, and was thought to be as a direct result of the ability of SRB use a wider range of compounds as electron donors than methanogenic populations.

The results of this study confirmed that both the yield and rate of hydrolysis of PSS were enhanced under sulphidogenic conditions and that this was most likely as a result of reduced accumulation of soluble products. Within the Recycling Sludge Bed Reactor, enhanced hydrolysis through the alleviation of product accumulation is achieved through floc fracture and rapid utilization of released products by resident SRB.

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APPENDICES

APPENDIX 1

WRC STUDY 'SALINITY SANITATION AND SUSTAINABILITY' - PROJECT REPORTS

The WRC study which has been summarised here developed out of a number of closely interrelated studies, undertaken for the WRC by the Rhodes University Environmental Biotechnology Group, over a 10 year period. The detailed findings associated with this work will be published separately as individual project reports. The following lists the WRC reports which cover the various investigations dealt with in the programme. The individual WRC projects under which the various studies were undertaken are listed separately below:

Report 1

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 1. Overview

Report 2

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 2. Integrated Algal Ponding Systems and the Treatment of Saline Wastewaters.
Part1: Meso-saline Wastewaters - The *Spirulina* Model.

(Project K5/495: A Biotechnological approach to the removal of organics from saline effluents - Part 1.)

Report 3

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 2. Integrated Algal Ponding Systems and the Treatment of Saline Organic Wastewaters.
Part 2: Hyper-saline Wastewaters - The *Dunaliella* Model.

(Project K5/495: A biotechnological approach to the removal of organics from saline effluents - Part 2.)

Report 4

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.

Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part1: The AIWPS Model.

(Project K5/651: Appropriate low-cost sewage treatment using the integrated algal high rate oxidation ponding process.)

Report 5

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 2: Abattoir Wastewaters.

(Project K5/658: Algal high rate oxidation ponding for the treatment of abattoir effluents.)

Report 6

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters.
Part 3: Mine Drainage Wastewaters - The ASPAM Model.

(Project K5/656: Appropriate low-cost treatment of sewage reticulated in saline water using the algal high rate oxidation ponding system.)

Report 7

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters.
Part 4: System Performance and Tertiary Treatment Operations.

(Project K5/799: Development and monitoring of integrated algal high rate oxidation pond technology for low-cost treatment of sewage and industrial effluents;
Project K5/1073: Extension of applications and optimisation of operational performance of algal integrated ponding systems technology in appropriate low-cost treatment of industrial and domestic wastewaters.
Project K5/1362: Development and technology transfer of IAPS applications in upgrading water quality for small wastewater and drinking water treatment systems.)

Report 8

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.

Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters.
Part 5: Winery and Distillery Wastewaters.

(Project K5/1073: Extension of applications and optimisation of operational performance of algal integrated ponding systems technology in appropriate low-cost treatment of industrial and domestic wastewaters.)

Report 9

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®.
Part 1: Biodesalination of Mine Drainage Wastewaters.

(Project K5/869: Biological sulphate desalination and heavy metal precipitation in industrial and mining effluents using the IAPS.)

Report 10

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®.
Part 2: Enhanced Hydrolysis of Organic Carbon Substrates - Development of the Recycling Sludge Bed Reactor.

(Project K5/972: Process development and system optimisation of the integrated algal trench reactor process for sulphate biodesalination and heavy metal precipitation in mining and industrial effluents.)

Report 11

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®.
Part 3: Sulphur Production and Metal Removal Unit Operations.

(Project K5/1078: Development and piloting of the integrated biodesalination process for sulphate and heavy metal removal from mine drainage water incorporating co-disposal of industrial and domestic effluents;
Project K5/1336: Scale-UP development of the Rhodes BioSURE Process® for sewage sludge solubilisation and disposal.)

Report 12

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®.
Part 4: Treatment and Disposal of Sewage Sludges:

(Project K5/1169: Intermediate scale-up evaluation of the Rhodes Process for hydrolysis and solubilisation of sewage sludges in a sulphate reducing bacterial system.)

PROJECTS

The following lists the WRC Projects the findings of which have been detailed in the reports as outlined above:

Project K5/410

A Biotechnological approach to the removal of organics from saline effluents.

Report: 1. Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 1. Overview.

Project K5/495

A Biotechnological approach to the removal of organics from saline effluents.

Report: 2. Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 2. Integrated Algal Ponding Systems and the Treatment of Saline Wastewaters. Part1: Meso-saline Wastewaters - The *Spirulina* Model.

Report: 3. Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 2. Integrated Algal Ponding Systems and the Treatment of Saline Organic Wastewaters. Part 2: Hyper-saline Wastewaters - The *Dunaliella* Model.

Project K5/651

Appropriate low-cost sewage treatment using the integrated algal high rate oxidation ponding process.

Report 4: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part1: The AIWPS Model.

Project K5/656

Appropriate low-cost treatment of sewage reticulated in saline water using the algal high rate oxidation ponding system.

Report 6: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 3: Mine Drainage Wastewaters - The ASPAM Model.

Project K5/658

Algal high rate oxidation ponding for the treatment of abattoir effluents.

Report 5: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 2: Abattoir Wastewaters.

Project K5/799

Development and monitoring of integrated algal high rate oxidation pond technology for low-cost treatment of sewage and industrial effluents

Report 7: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 4: System Performance and Tertiary Treatment Operations.

Project K5/869

Biological sulphate desalination and heavy metal precipitation in industrial and mining effluents using the IAPS.

Report 9: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®. Part 1: Biodesalination of Mine Drainage Wastewaters.

Project K5/972

Process development and system optimisation of the integrated algal trench reactor process for sulphate biodesalination and heavy metal precipitation in mining and industrial effluents.

Report 10: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®. Part 2: Enhanced Hydrolysis of Organic Carbon Substrates - Development of the Recycling Sludge Bed Reactor.

Project K5/1073

Extension of applications and optimisation of operational performance of algal integrated ponding systems technology in appropriate low-cost treatment of industrial and domestic wastewaters.

Report 7: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 4: System Performance and Tertiary Treatment Operations.

Report 8: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 5: Winery and Distillery Watewaters

Project K5/1078

Development and piloting of the integrated biodesalination process for sulphate and heavy metal removal from mine drainage water incorporating co-disposal of industrial and domestic effluents.

Report 11: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®. Part 3: Sulphur Production and Metal Removal Unit Operations.

Project K5/1169

Intermediate scale-up evaluation of the Rhodes Process for hydrolysis and solubilisation of sewage sludges in a sulphate reducing bacterial system.

Report 12: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®. Part 4: Treatment and Disposal of Sewage Sludges.

Project K5/1336

Scale-up development of the Rhodes BioSURE Process® for sewage sludge solubilisation and disposal.

Report 11: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 4. The Rhodes BioSURE Process®. Part 3: Sulphur Production and Metal Removal Unit Operations.

Project K5/1362

Development and technology transfer of IAPS applications in upgrading water quality for small wastewater and drinking water treatment systems.

Report 7: Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa.
Volume 3. Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 4: System Performance and Tertiary Treatment Operations.

APPENDIX 2.

RESEARCH PRODUCTS

2.1. STUDENTS TRAINED

2.1.1 Post-Doctoral Fellow

Dr N. Nagabushana (2000) Carbon digestion in mine water treatment.

2.1.2 PhD Students

G. Boshoff (Graduated 1998) - Development of integrated biological processing for the biodesalination of sulphate and metal-rich wastewaters.

K. Whittington-Jones (Graduated 2000) - Sulpide-enhanced hydrolysis of primary sewage sludge: implications for the bioremediation of sulphate-enriched wastewaters.

A. Clarke (current) - Molecular microbial ecology of sulphate reducing environments degrading complex organic carbon substrates.

L. Dekker (current) - IAPS in the treatment of acidic and saline organic wastewaters.

C. Ehlers (current) - The degradation of aromatic compounds in sulphate reducing environments.

H. Roman (current) - Digestion of cellulose in sulphate reducing environments.

D. Sanyahumbi (current) - The manipulation of immobilised sulphate reducing bacterial systems.

2.1.3 MSc Students

C.J. Corbett (Graduated 2001) - The Rhodes BioSURE Process® in the Treatment of Acid Mine Drainage Wastewaters.

J. Gilfillan (Graduated 2000) - Biological sulphide oxidation and sulphur recovery from mine drainage wastewaters.

P. Molipane (Graduated 2000) - Sulphate reduction utilising hydrolysis of complex carbon sources.

M. Bowker (Graduated 2002) - The biology of loating sulphur biofilms.

G. Chauke (Graduated 2002) - The molecular microbial ecology of sulphate reducing bacteria.

M. Madikane (Graduated 2002) - The hydrolysis of lignin in sulphate reducing environments.

J. Molwantwa (Completed 2002) - The enhanced hydrolysis of sewage sludge in sulphidogenic environments.

N. Rein (Graduated 2002) - Sulphide oxidising biofilms and the biology of sulphur production.

2.2 PATENTS

Rose P.D., Boshoff, G.A., Hart, O.O., Barnard, J.P. 1997. The double deck trench reactor.
RSA 97/4165 (final)
Australia 711069(final)

Rose P.D., Duncan, J.R., van Hille, R.P., Boshoff, G.A. 1998. Alkalinity and biorefining.
RSA 98/3204 (Final).

Rose,P.D., and Hart, O.O. 1988. Treatment of Water-modification.
RSA 98/9429 (Final)

Rose,P.D. and Hart, O.O. 1988. Treatment of sewage.
RSA 98/9428 (Final).

Rose, P.D. 1998. Treatment of sulphate containing metaliferous wastewater.
RSA 98/3202 (Final)

Rose P.D., Duncan, J.R., van Hille, R.P., Boshoff, G.A. 1999. Use of ponds to treat sulphate solutions and ASPAM process.
RSA 99/4585 (Final). US patent pending.

Van Hille, R.P., Boshoff, G.A., Rose, P.D., Duncan, J.R. 1999. A continuous process for the biological treatment of heavy metal contaminated acid mine drainage water.
RSA 99/3867.

2.3 PAPERS

1. Rose,P.D., Boshoff, G.A., van Hille, R.P., Wallace, L.M.C., Dunn, K.M. and Duncan, J.R. 1998. An integrated algal sulphate reducing high rate ponding process for the treatment of acid mine drainage wastewaters. Biodegradation 9:247-257.

2. van Hille, R., Boshoff, G., Rose, P. and Duncan, J. 1999. A continuous process for the biological treatment of heavy metal contaminated acid mine water. Resource Conservation and Recycle, 27:157-167.

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APPENDIX 3. TECHNOLOGY TRANSFER ACTIVITIES

The current report is a component investigation of the WRC research programme in Salinity, Sanitation and Integrated Algal Ponding Systems, as noted in Appendix 2 above. This study was based on previous developments of IAPS in treatment of saline wastewaters (WRC Project K5/495), and in turn led to a number of follow-on spinoff developments of which the Rhode BioSURE Process® became the principal focus. Technology diffusion and technology transfer activities initiated during the course of the study involved interactions with industry partners and led to scaled-up evaluations of the technology under development.

3.1 OFFICIAL OPENING OF THE RHODES BIOSURE® PILOT PLANT

The investigation of sulphate saline wastewater treatment and the resulting scale-up developments of the Rhodes BioSURE Process® largely took place at sites remote from the EBG laboratories in Grahamstown. As a result it was decided to establish a BioSURE® pilot plant on-site at the EBFS to facilitate both fundamental and up-scale/down-scale investigations.

The BioSURE® and Sulphur Biology Pilot Plants, located at the Rhodes University Environmental Biotechnology Field Station, at the Grahamstown Works, was opened during the Mine Water Conference at the BIOY2K meeting in January, 2000, by the Executive Director of the Water Research Commission, Mr P Odendaal. The event was attended by around 150 people including conference delegates from academia, industry and government. The plant has since been visited by several hundred people.

Figure A 3.1. The Rhodes BioSURE® Pilot Plant at the Environmental Biotechnology Field Station opening by the Executive Director of the Water Research Commission, Mr P Odendaal. At left Dr D Woods, Vice Chancellor, Rhodes University.

APPENDIX 4

RESEARCH SPIN-OFF DEVELOPMENTS

4.1 TREATMENT FOR SEWAGE SLUDGE SOLIDS

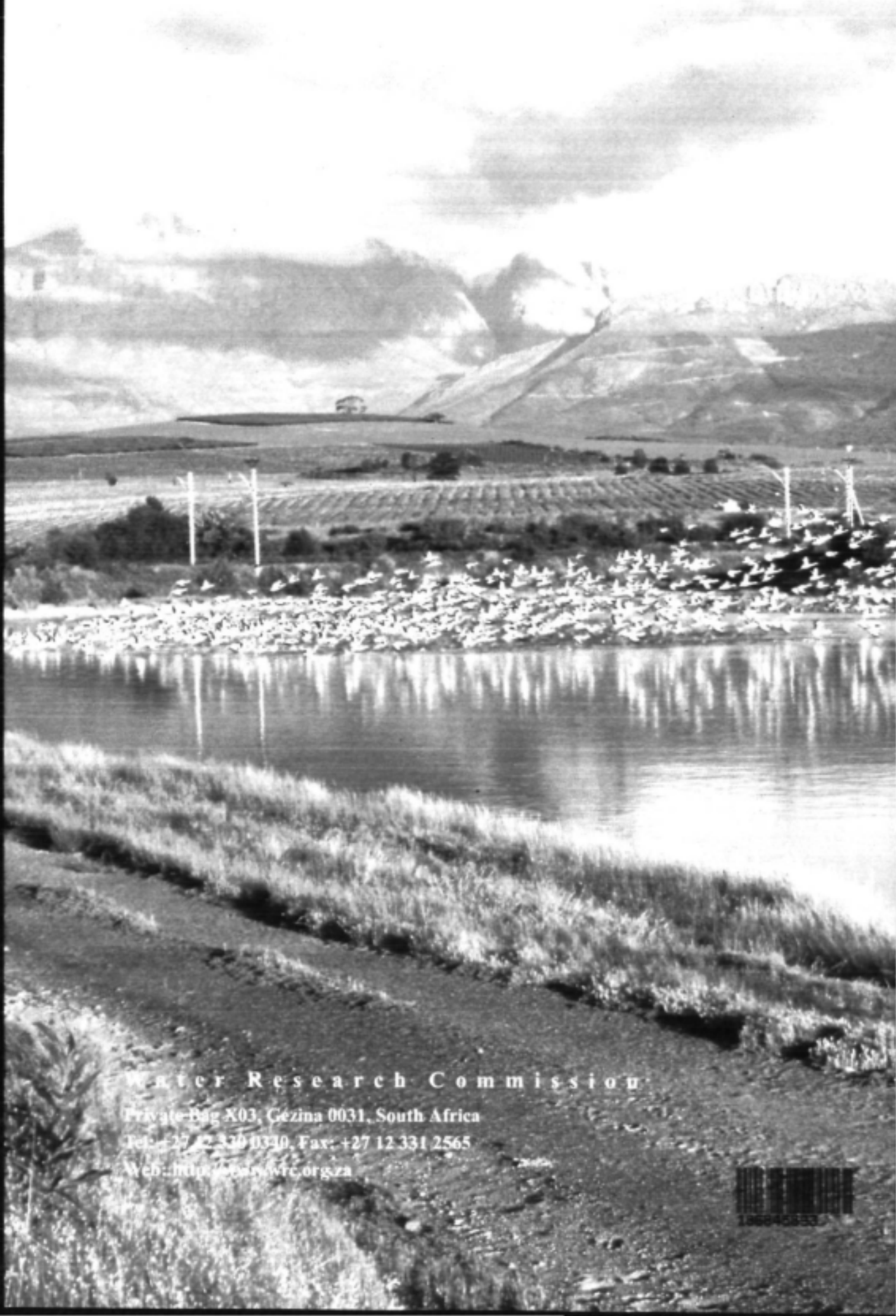
The use of sewage sludge as a carbon source for the Rhodes BioSURE Process® led to observations of accelerated hydrolysis of both primary and secondary sludges in sulphidogenic environments. In collaboration with the ERWAT a joint WRC/ERWAT/Rhodes University project (K5/1169) has commenced to develop an application of the process using AMD as a sulphate source for sewage sludge solubilisation and disposal. In addition to solids solubilisation and the removal of heavy metals, high levels of sludge disinfection are achieved. This study involves the scale-up of the RSBR to 2 ML reactors, shown under construction in Figure A 4.1.

Figure A 4.2. The 2 ML scaled-up Recycling Sludge Bed Reactor in the BioSURE® sewage sludge solubilisation technical-scale plant constructed at Erwat's Ancor Works in Springs. Surface struts provide supports for a covering membrane.

4.2 PASSIVE SYSTEMS AND THE INNOVATION FUND PROJECT

Passive treatment systems for the remediation of mine drainage wastewaters have been under investigation by William Pulles of PHD Co. The Rhodes EBG were invited to participate in the Department of Arts Culture Science and Technology (DACST) Innovation Fund award in which the commercialisation of sulphate removing passive treatment systems was to be investigated. Current research is investigating the application of a degrading bed reactor for

the solid state digestion of lignocellulosic wastes as a feedstock in the passive treatment operations. The sulphate reducing degrading packed bed reactor has been based on the RSBR development associated with the BioSURE Process®.



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