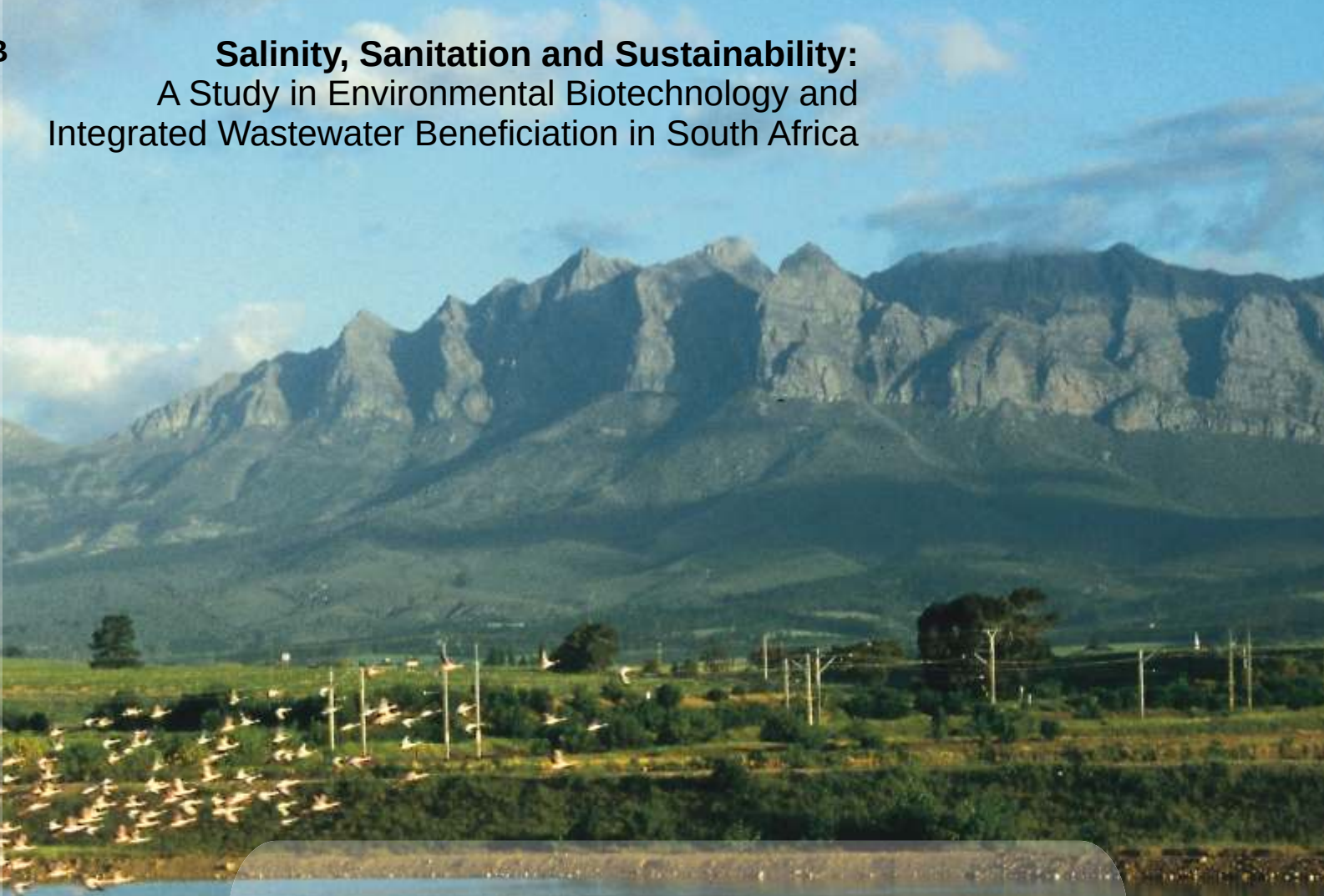
Salinity, Sanitation and Sustainability:
A Study in Environmental Biotechnology and
Integrated Wastewater Beneficiation in South Africa



Volume 3

Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters

Part 3B: Sulphate Saline Systems: Development of the ASPAM Process

C Wells, G Enongene, M Bowker, D Render,
H Joubert and PD Rose

WRC Report No TT 402/09

6B

Water Research Commission

SALINITY, SANITATION and SUSTAINABILITY
A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa

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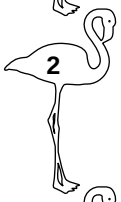

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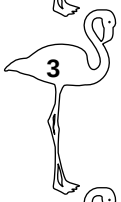

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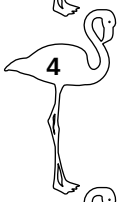

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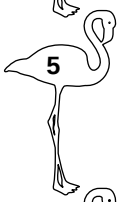

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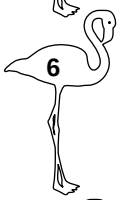

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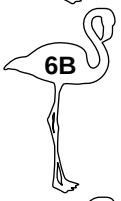

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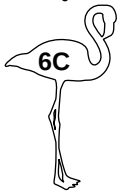

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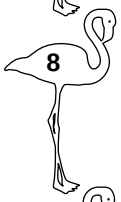

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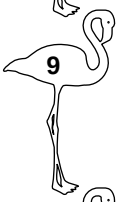

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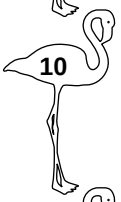

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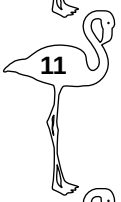

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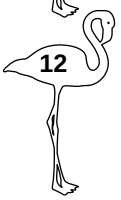

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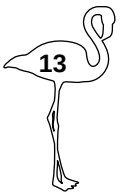

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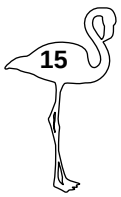

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Cover Photograph:

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 3

INTEGRATED ALGAL PONDING SYSTEMS AND THE TREATMENT OF DOMESTIC AND INDUSTRIAL WASTEWATERS

Part 3 B: Sulphate Saline Systems: Development of the ASPAM Process

Report to the
Water Research Commission on

By

C Wells, G Enongene, M Bowker, D Render, H Joubert, PD Rose

on behalf of

Environmental Biotechnology Research Unit
Rhodes University, Grahamstown.

WRC REPORT No. TT 402/09

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The publication of this report emanates from a project entitled: *Development of sustainable low-cost management for saline sewage and saline mine drainage wastewaters using integrated algal ponding systems* (WRC project No. K5/1621).

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PREFACE

The threat of rising salinity to the public water system in South Africa has been a cause of concern for many years and was a motivating factor in the establishment and early development of the Water Research Commission (WRC) in the 1970s. Since then the WRC has made a substantial investment in expanding an understanding of the nature of the problem and in the development of innovative remedial responses to it.

The investigation of a biotechnology-based response to the problem led to a research programme on saline wastewaters undertaken, over a 20-year period, by the Environmental Biotechnology Research Unit (EBRU) at Rhodes University. These findings have been published by the WRC in the series "Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa" – also known informally as the 'Flamingo Series'. A number of recommendations for follow-up actions emerged from these studies and included further development of the long-range sustainability potential available in linking the management of saline and sewage wastewaters in co-treatment operations.

A number of WRC follow-up studies have targeted aspects of these recommendations including the development of biological sulphide oxidation systems for the production of elemental sulphur from treated saline mine minewaters, the mathematical modeling of biological sulphate reducing systems (a major collaborative study led by University of Cape Town Civil Engineering Department), and the recovery and reuse of treated minewaters in agricultural production and thereby establishing conditions for sustainable economic, environmental and social mine closure. Among several other outcomes, the approach led to the development of the Rhodes BioSURE® Process which links the disposal of sewage sludges, and other complex organic wastes, in the treatment of saline acidic mine drainage wastewaters.

Two further follow-up recommendations of the "Salinity, Sanitation and Sustainability" study were tackled in Project K5/1621 and are dealt with in the current two-volume report series titled "Biotechnology of Saline Wastewater Treatment". Volume 1 (Flamingo 6: Part 3B) deals with further studies on the development of the WRC patented ASPAM Process which utilizes

Integrated Algal Ponding Systems in the treatment of sulphate salinity, acidity and heavy metal contamination in mine drainage wastewaters. The initial work on this process had been detailed in WRC Report TT 192/02 (Volume 6 in the Flamingo Series). Volume 2 (Flamingo 6: Part 3C) reports a preliminary investigation on the practical use of chloride saline wastewaters for the reticulation and treatment of domestic and industrial effluents. Aspects of the saline water reticulation study were also investigated in WRC Project K5/1456 and these findings are reported together in the Volume 2 report.

EXECUTIVE SUMMARY

INTRODUCTION

The increasing salinisation of the public water system in South Africa, and the needs of the mining industry for mine wastewater treatment technologies that are sustainable over the long term, has focused interest on biological treatment operations. One of these involved the investigation of Integrated Algal Ponding Systems (IAPS) as a low-cost approach in the active treatment of high volume mine drainage wastewater flows. These studies resulted in the development of the Algal Sulphate Reducing Ponding Process for Acidic Metal Wastewater Treatment (ASPAM). This work has been published as WRC Report TT 192/02 titled "Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 3: Mine Drainage Wastewaters – The ASPAM Model" (Rose *et al*., 2002).

The mining industry identified the importance of the general approach undertaken in the ASPAM development study and requested process technology evaluation to progress to pilot-scale investigations. This would need to be done in order to consider further implementation development and application of the system, possibly at technical- and full-scale operation. The project reported here has undertaken the evaluation of the ASPAM system at pilot-scale at the Environmental Biotechnology Research Unit (EBRU) at Rhodes University in Grahamstown, and using synthetic wastewater feeds. The principal objective was to provide information inputs to enable a decision to proceed to pilot or technical-scale studies on-site at the mines and using actual mine drainage wastewater feeds.

The initial studies and development of the ASPAM concept had been undertaken as bench-top investigations (Rose *et al*., 2002) and the current project provided the first opportunity to investigate aspects of the system at pilot scale.

THE ASPAM PROCESS

The ASPAM process composes a number of separate unit operations in the treatment of acidic mine waters which involve the use of IAPS for the breakdown of complex organic carbon substrates, the reduction of sulphate salinity, precipitation of metal sulphides, and the generation of alkalinity and of algal biomass for metal adsorption. The process flow diagram is shown in Figure 1 and details of the process unit operations are described below.

1. Metal Precipitation Unit Operation

The acidic metal-contaminated wastewater enters the system at (1) and, together with a sulphide-rich stream pass from (8) and an alkaline stream from the High Rate Algal Pond (HRAP) through (5) passes to the metal precipitation unit operation (2). Here in mixing with the influent stream, a combination of metal complex formation reactions occurs. Effective precipitation of heavy metals in the sulphidogenic tannery wastewater AFP, had been reported by Boshoff (1998) and Dunn (1998), and the relative advantages of metal precipitation as the sulphide, compared to metal hydroxide forms, has been widely noted (Peters and Ku, 1985; Singh, 1992 and Hammack *et al*., 1994). Dunn (1998) reported preliminary observations on both the *ex situ* precipitation of metals utilising the sulphide-rich AFP liquors in the tannery IAPS, and also their removal in the presence of algae in the HRAP. Both systems demonstrated strong alkalising properties with the influent pH elevated to values between 9 and 10, at the end of the ponding cascade.

2. High Rate Algal Pond 1

Following the removal of metal sludges in settler (3), the partly neutralised flow passes to HRAP 1 (4). Here photosynthetically-driven alkalisation takes place and final metal removal may be effected by micro-algal biosorption.

The observations of the alkalising and metal binding properties of micro-algae in tannery IAPS had been investigated by Boshoff (1998) and Dunn (1998). These findings indicated the need for the inclusion of HRAP 1, following preliminary metal removal and prior to the stream entering the AFP. The relatively high pH minima of the bio-sulphidogenic system in the AFP make the neutralisation of the acid stream essential to the operation of this unit. Neutralisation may be

handled by both feeding HRAP 1 liquors forward to the precipitation unit (5), and through *in situ* alkalisation in the raceway itself. Physiological stress in this unit may depress micro-algal growth rates. In this regard it would be important to maintain biomass against washout by replacement with algal biomass recovered from HRAP 2 (10).

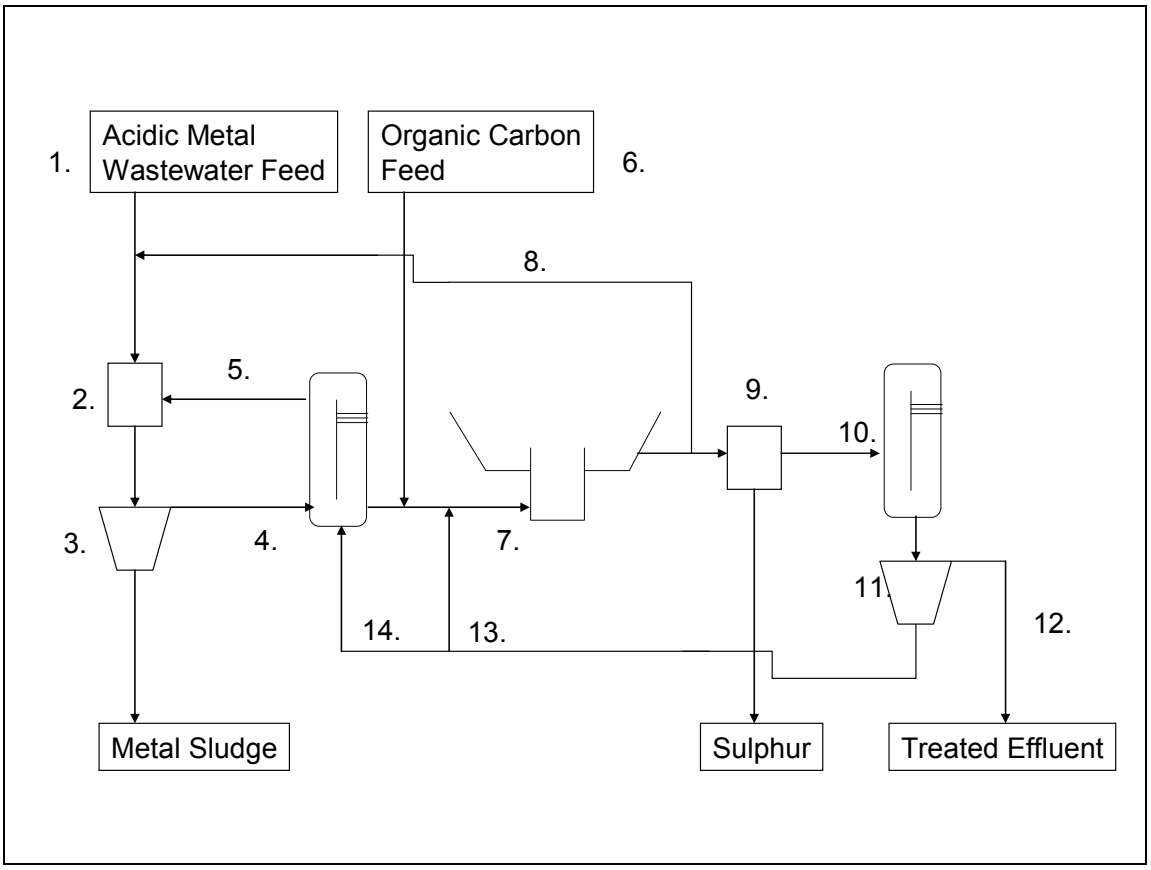

Figure 1.1. Flow diagram of the individual unit operations of the Integrated Algal Sulphate Reducing Ponding Process for Acid Metal Wastewater Treatment (ASPAM).

3. The Advanced Facultative Pond

The Advanced Facultative Pond (AFP) (7) provides an anaerobic pit located within the base of the pond where sulphate reduction takes place. In Figure 1 the partly treated flow enters at (7), together with the carbon source (6), which may utilise a range of organic wastes including tannery wastewaters, sewage sludges and agro-industrial organic waste products. Boshoff (1998) showed that algal biomass from HRAP 2 and returned through (13) would also provide an

effective electron donor source for sulphate reduction. Sulphide produced in this unit passes forward to the metal precipitation unit operation through (8).

The AFP described by Oswald (1998 a&b), provides for the inclusion of one or more Upflow Digesters (UD) in the base of the pond, enabling optimum anaerobic digestion function. Tannery pond studies in Wellington, South Africa, showed that sulphidogenic anaerobic digestion will perform efficiently utilising tannery waste as an electron donor source. High rates of sulphate reduction were observed to be associated with an accelerated hydrolysis and solubilisation of particulate organic matter fed to the system. An associated precipitation of heavy metals was also observed in this unit with low residual metal levels in the effluent (Dunn, 1998).

The maintenance of an aerobic compartment above the anaerobic layer was shown in the tannery IAPS not only to provide for odour control with the scrubbing of released gasses, but also ensured that the sulphur cycle was completed above the oxypause, with a nearly full oxidation of sulphide back to sulphate (Rose *et al*., 1996).

4. Sulphur Recovery and Biodesalination

A portion of the total sulphur load entering the system would be removed with the metal sulphide sludge. Where a more complete sulphate removal is required a biodesalination of the flow may be achieved by passing the reduced AFP waters through a sulphur recovery unit (9). Here elemental sulphur (S^o) could be a final by-product of the process. Sulphide oxidation and sulphur recovery is the subject of separate WRC Projects reported as WRC Report No TT 197/07 (Flamingo report series 11).

5. High Rate Algal Pond 2

At (10) the flow circulates through HRAP 2 where final polishing occurs and residual chemical oxygen demand (COD) and nutrients are removed. A high concentration of algal biomass is maintained and may be used for constant biomass replenishment to HRAP 1, via settler (11) and (14), in which a reduction of growth rates may be anticipated to occur due to acid stress. Harvested algal biomass may also pass to the AFP through (13).

Where necessary oxygenated water from the HRAP 2 may be recycled to the AFP to control sulphide emissions and for odour control. Pond capping operation has been investigated in the tannery IAPS (Rose *et al*., 2002a) and the Grahamstown AIWPS studies (Rose *et al*., 2002b).

6. Treated Water

Treated water leaves the system at (12).

PROCESS OPERATION

The operation of the anaerobic unit of the system functioned well, producing sulphide through the sulphate reduction reaction process. This was then oxidized through the various oxidation states of sulphur and reported to the various downstream compartments. Approximately 10% of the total influent sulphur could not be accounted for as either $H_2S(g)$, HS^- or SO_4^{2-} between the feed and the Advanced Facultative Pond (AFP). Evidence of elemental sulphur in the settled sludge suggests this may be where the residual sulphur is located. Losses to atmosphere were low.

The performance of the floating sulphur biofilm, and of photosynthetic bacteria present in the AFP water column, as an effective sulphide oxidation and sulphur removal operation, was an unexpected observation in the study. Although its operation was not a principal objective of the study, and was thus not the subject of separate optimization, it is evident that this could offer important advantages in the overall treatment of sulphate wastewaters in the ASPAM system. Given the substantial advances made in the operation of the Floating Sulphur Biofilm Reactor developed in WRC Project 1545, it is evident that a basis has been demonstrated here for potentially dealing with the technological bottleneck relating to sulphur removal following the sulphate reduction reaction in the biological treatment of mine wastewaters. This should be the subject of energetic follow-up.

Feeding of sewage sludge in small volumes to the pilot plant proved to be a severe constraint on the successful operation of the system and in future process designs adequate budgetary provision will need to be made to enable this operation. This problem impacted on a number of

other areas of the study including the successful operation of the HRAP and the use of algal-alkalinity in the metal removal operation studies.

The metal removal studies reported here demonstrated, not only the removal of metals in a synthetic influent stream, but showed that metal hydroxide precipitates may be used to remove sulphides from the final stream of a biological acid mine drainage (AMD) treatment operation. This work was the subject of scale-up evaluation to full-scale operation as a unit process in the Rhodes BioSURE® Process industrial-scale plant at the Grootvlei Mine/ERWAT Ancor Works plant where 10 Ml/day mine wastewaters are treated.

The molecular microbial ecology study of the AFP has provided a valuable insight into the system in operation, and the principle organisms involved. These included *Thiocapsa, Thiorhodococcus, Chromatiaceae* and *Rhodobacter* spp. This will provide useful information where more accurate kinetic studies are to be undertaken and indicating the various processes at work in this unit operation.

RECOMMENDATIONS

A number of recommendations can be made on the basis of the outcomes achieved in the studies reported here:

1. The basic concept of using algal ponding systems in general, and the IAPS design in particular, as a process design basis for the extensive, and possibly low-cost, treatment of mine drainage wastewaters, has been demonstrated. In the light of the urgent need for technological intervention in this area, as evidenced by environmental requirements for mining companies to achieve sustainable mine closure, it is recommended that the system be the subject of further scale-up evaluation;
2. The metal removal study reported here has already been the subject of up-scale evaluation to a full-scale process at the Rhodes BioSURE® Process 10 Ml/day plant in operation at Grootvlei Mine/ERWAT Ancor Works. It is recommended that studies on the linkage of this system with algal alkalinity generated in the HRAP be continued given

the need for extensive low-cost applications in this area;

3. The potential for sulphur removal in the system was unexpected and may link well with the parallel development of the Floating Sulphur Biofilm Reactor undertaken in WRC Project K5/1545. The problem of sulphur removal presents a severe technological constraint on the further development of passive and extensive active biological AMD treatment operations. It is recommended that this potential be followed up energetically in follow-up development studies.

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ABBREVIATIONS

AFP	Advanced Facultative Pond
AIWPS	Advanced Integrated Wastewater Ponding Systems
AMD	Acid Mine Drainage Wastewaters
ASP	Algal Settling Pond
ASPAM	Algal Sulphate Reducing Ponding Process for Acidic Metal Wastewater Treatment
BLAST	Basic Local Alignment Search Tool
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic acid
dNTP	Deoxyucleosidetriphosphate
EBRU	Environmental Biotechnology Research Unit
EPA	Environmental Protection Agency
HDS	High Density Sludge
HPLC	High Pressure Liquid Chromatography
HRAP	High Rate Algal Pond
HRT	Hydraulic Retention Time
IAPS	Integrated Algal Ponding Systems
IPTG	Isopropylthio-β-o-galactiside
LB	Luria Broth
NCBI	National Centre for Biotechnology Information
N-J	Near Neighbour Joining Algorithm
OSHA	Occupational Safety and Health Administration
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic acid
S	Sulphur
SDS	Sodium Dodecyl Sulphate
TE	TRIS/EDTA
UD	Upflow Digester
UV	Ultra Violet
WRC	Water Research Commission

ACKNOWLEGEMENTS

The authors wish to acknowledge the Water Research Commission for finance and support throughout the project. They would also like to thank the Reference Committee for critical comment and constructive suggestions.

Dr H Snyman
Ms H Greben
Mrs W Moolman
Mrs B Nel
Mr H Joubert
Mr L du Preez
Mr J Harrison

1. INTRODUCTION

1.1 BACKGROUND

The Water Research Commission (WRC) has made a substantial investment in the development of a knowledge base for the sustainable management of saline wastewaters. This has been done against the background of a limited water resource and an increasing salinisation of the public water system in South Africa. Aspects of this work have been published in the WRC report series "Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa", produced by the Environmental Biotechnology Research Unit at Rhodes University. This research initiative has resulted in the development of a number of novel technologies for dealing with these wastewaters, including WRC patented Integrated Algal Ponding Systems (IAPS) applications and the Rhodes BioSURE Process® for the treatment of saline mine drainage wastewaters.

One of the above studies involved the investigation of IAPS as a low-cost approach in the active treatment of high volume mine drainage wastewater flows. These studies resulted in the development of the Algal Sulphate Reducing Ponding Process for Acidic Metal Wastewater Treatment (ASPAM). This work has been published as WRC Report TT 192/02 titled 'Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 3: Mine Drainage Wastewaters – The ASPAM Model' (Rose *et al*., 2002). This work was undertaken at bench-scale as a preliminary process conceptualisation study.

The mining industry identified the importance of the general approach undertaken in the ASPAM development study and requested process technology evaluation to progress to pilot-scale investigations. This would need to be done in order to consider further implementation development and application of the system, possibly at technical- and full-scale operation. The project reported here has undertaken the evaluation of the ASPAM system at pilot-scale at the Environmental Biotechnology Research Unit (EBRU) in Grahamstown, and using synthetic wastewater feeds. The principal objective

was to provide information inputs to enable a decision to proceed to pilot or technical-scale studies on-site at the mines and using actual mine drainage wastewater feeds.

The production of treated saline waters in the ASPAM process raised the potential of synergies with a concurrent investigation, also being undertaken for the WRC by EBRU, on the productive use of saline wastewaters for the reticulation of domestic and industrial sewage streams. The widespread need, internationally, to initiate saline water use as a means of extending the fresh water resource was identified. Apart from dual reticulation system requirements, the availability of suitable technologies for the treatment of saline sewage wastewaters would be required. The use of IAPS for saline sewage treatment was identified as a potential process in this regard.

1.2 THE ASPAM PROCESS

The ASPAM process composes a number of separate unit operations in the treatment of acidic mine waters which involve the use of IAPS for the breakdown of complex organic carbon substrates, the reduction of sulphate salinity, precipitation of metal sulphides, and the generation of alkalinity and of algal biomass for metal adsorption. The process flow diagram is shown in Figure 1 and details of the process unit operations are described below.

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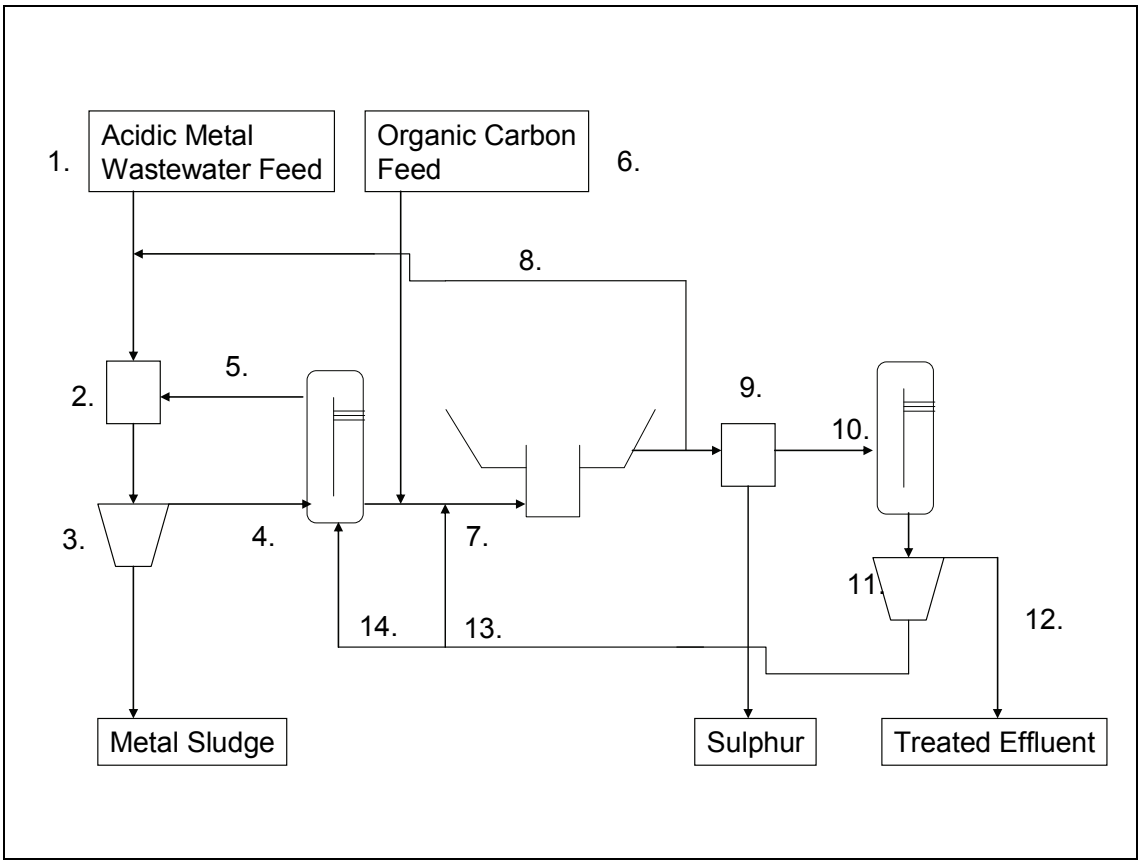

Figure 1.1: Flow diagram of the individual unit operations of the Integrated Algal Sulphate Reducing Ponding Process for Acid Metal Wastewater Treatment (ASPAM)

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Following the removal of metal sludges in settler (3), the partly neutralised flow passes to HRAP 1 (4). Here photosynthetically-driven alkalisation takes place and final metal removal may be effected by micro-algal biosorption.

The observations of the alkalising and metal binding properties of micro-algae in tannery IAPS had been investigated by Boshoff (1998) and Dunn (1998). These findings indicated the need for the inclusion of HRAP 1, following preliminary metal removal and prior to the stream entering the AFP. The relatively high pH minima of the bio-

sulphidogenic system in the AFP make the neutralisation of the acid stream essential to the operation of this unit. Neutralisation may be handled by both feeding HRAP 1 liquors forward to the precipitation unit (5), and through *in situ* alkalisation in the raceway itself. Physiological stress in this unit may depress micro-algal growth rates. In this regard it would be important to maintain biomass against washout by replacement with algal biomass recovered from HRAP 2 (10).

1.2.3 The Advanced Facultative Pond

The Advanced Facultative Pond (AFP) (7) provides an anaerobic pit located within the base of the pond where sulphate reduction takes place. In Figure 1 the partly treated flow enters at (7), together with the carbon source (6), which may utilise a range of organic wastes including tannery wastewaters, sewage sludges and agro-industrial organic waste products. Boshoff (1998) showed that algal biomass from HRAP 2 and returned through (13) would also provide an effective electron donor source for sulphate reduction. Sulphide produced in this unit passes forward to the metal precipitation unit operation through (8).

The AFP described by Oswald (1998 a&b), provides for the inclusion of one or more Upflow Digesters (UD) in the base of the pond, enabling optimum anaerobic digestion function. Tannery pond studies in Wellington, South Africa, showed that sulphidogenic anaerobic digestion will perform efficiently utilising tannery waste as an electron donor source. High rates of sulphate reduction were observed to be associated with an accelerated hydrolysis and solubilisation of particulate organic matter fed to the system. An associated precipitation of heavy metals was also observed in this unit with low residual metal levels in the effluent (Dunn, 1998).

The maintenance of an aerobic compartment above the anaerobic layer was shown in the tannery IAPS not only to provide for odour control, with the scrubbing of released gasses, but also ensured that the sulphur cycle was completed above the oxypause, with a nearly full oxidation of sulphide back to sulphate (Rose *et al*., 1996).

1.2.4 Sulphur Recovery and Biodesalination

A portion of the total sulphur load entering the system would be removed with the metal sulphide sludge. Where a more complete sulphate removal is required a biodesalination of the flow may be achieved by passing the reduced AFP waters through a sulphur recovery unit (9). Here elemental sulphur (S^o) could be a final by-product of the process. Sulphide oxidation and sulphur recovery is the subject of separate WRC Projects reported as WRC Report No TT 197/07 (Flamingo report series 11).

1.2.5 High Rate Algal Pond 2

At (10) the flow circulates through HRAP 2 where final polishing occurs and residual chemical oxygen demand (COD) and nutrients are removed. A high concentration of algal biomass is maintained and may be used for constant biomass replenishment to HRAP 1, via settler (11) and (14), in which a reduction of growth rates may be anticipated to occur due to acid stress. Harvested algal biomass may also pass to the AFP through (13).

Where necessary oxygenated water from the HRAP 2 may be recycled to the AFP to control sulphide emissions and for odour control. Pond capping operation has been investigated in the tannery IAPS (Rose *et al*., 2002a) and the Grahamstown AIWPS studies (Rose *et al*., 2002b).

1.2.6 Treated Water

Treated water leaves the system at (12).

2 AIMS

Based on the outcomes of the preliminary studies on the ASPAM system reported in WRC Report No. TT 196/02 (Rose *et al*.2002), the following aims were identified for the current project:

1. Undertake process development, at pilot-scale, of the WRC-patented ASPAM system using algal ponding for low-cost sustainable treatment of metal-contaminated acidic sulphate saline wastewaters
2. Investigate factors relating to the linkage and integration of the various unit operations of the process
3. Determine kinetic values and design parameters required for the full-scale implementation of the process
4. Undertake a fundamental investigation of the algal proton absorption capacity of the ASPAM system underpinning the metal precipitation and neutralisation unit, and also the sulphur biofilm operation as applied in the pond environment. Microbial populations operating in each unit will be identified and a descriptive model for system performance will be proposed.
5. Undertake process development, at pilot-scale, of saline sewage treatment in algal ponding systems
6. Determine kinetic values and design parameters required for the full-scale implementation of the process
7. Investigate factors underpinning the optimal performance of the bacterial and algal components of the microbial system. Microbial populations in the unit will be identified and a descriptive model for system performance will be proposed.

An additional aim was subsequently appended to the project and required the refurbishment of the technical-scale HRAP at the EBRU Field Station site. This was to enable ongoing ponding studies in other WRC projects and to provide for possible scale-up investigation of the systems to be studied in this project.

3 METHODOLOGY

The research program was undertaken at the EBRU laboratories and Experimental Field Station in Grahamstown using its facilities and infrastructure for the study of ponding systems applications. Simulated wastewater make-up was prepared in a plant established on site. Both defined chloride and sulphate streams were formulated.

An experimental IAPS pilot plant was constructed and operated on-site. Operational problems and potential process bottlenecks were to be identified, conditions determined and feasibility assessed for progressing the development of the technology to full-scale application.

In parallel with the pilot plant investigations, basic studies were undertaken to expand an understanding of the factors underpinning the performance of the ASPAM system. Questions to be addressed included the performance of the floating sulphur biofilm in the pond environment and establishing a molecular microbial model accounting for events occurring in the system.

Given the wide range of the original aims, limitations on resources, and operational constraints in the pilot plant design, and the need to operationalise individual unit operations, it was decided to limit the empirical studies to the operation of the pilot plant and focus in greater detail on the sulphur and metal removal unit operations where basic inputs were required.

3.1 Experimental Plant Design and Construction

Due to the restricted size of the pilot-scale plant, low flow rates need to be managed. The plant to be used in this study (process flow diagram shown in Figure 3.1), was designed to handle a flow of 35l.h^{-1}. Continuous feed of sewage at low flow rates led to ongoing problems with incessant blockages of pipes and pumps. The positive displacement pump was, therefore, replaced by a peristaltic pump (Watson Marlow), which reduced feed

interruptions and thus allowed a more stable operation of the system. In order to further reduce blockages, the recirculation of the sewage in the holding tank was discontinued since, while this supplied a more homogenous wastewater supply, it also prevented the settling of larger particles that obstructed the small diameter pipes. Ultimately, the continuous feeding of sewage sludge was discontinued and it was fed directly into the Pit on a batch-feed basis.

Preliminary data on the performance of the fermentation pit and AFP indicates these unit operations function under similar loading conditions to the non-saline IAPS, also investigated by EBRU. The HRAP, however, seem to require a slightly longer hydraulic retention time (HRT) to produce comparable results. As this was not allowed for in the original pilot plant design, splitter boxes had to be designed and installed to facilitate variable flow rates and thus variable HRT in the HRAPs (Figure 3.2).

A bridge, illustrated in Figure 3.2, was also constructed over the AFP to gain access to the fermentation pit for sampling and experimental purposes.

The as built plant is shown in Figure 3.3.

Pumps, valves and piping have been fitted as indicated. The make-up and storage tanks for the simulated minewater feed to the pilot plant are shown in Figure 3.4.

The pilot plant process flow, shown in Figure 3.1, indicates the supply of raw sewage and saline sulphate mine water entering the site to storage tanks. These components were then blended in the appropriate proportions to simulate the COD:SO_4 ratios required in process operation. The flow was then fed to the base of the anaerobic pit in the AFP (Figure 3.5). The fermentation pit operates as an upflow digester. The anaerobic conditions in the fermentation pit favour the reduction of sulphate to sulphide by sulphate reducing bacteria (SRB). This sulphide-rich water forms the source of the sulphide utilised for the metal precipitation mentioned earlier. Settled sewage provides the organic carbon source, used as an electron donor for the sulphate reduction reaction. High rates of

sulphate reduction are associated with an accelerated hydrolysis and solubilisation of particulate organic matter fed to the system (Rose *et. al*. 2002).

The fermentation pit is situated within the AFP, constructed from a 5 m diameter porta-pool, shown in Figure 3.6. The AFP maintains an aerobic compartment above the anaerobic fermentation pit. Odours are controlled by the scrubbing of generated gases in this aerobic zone.

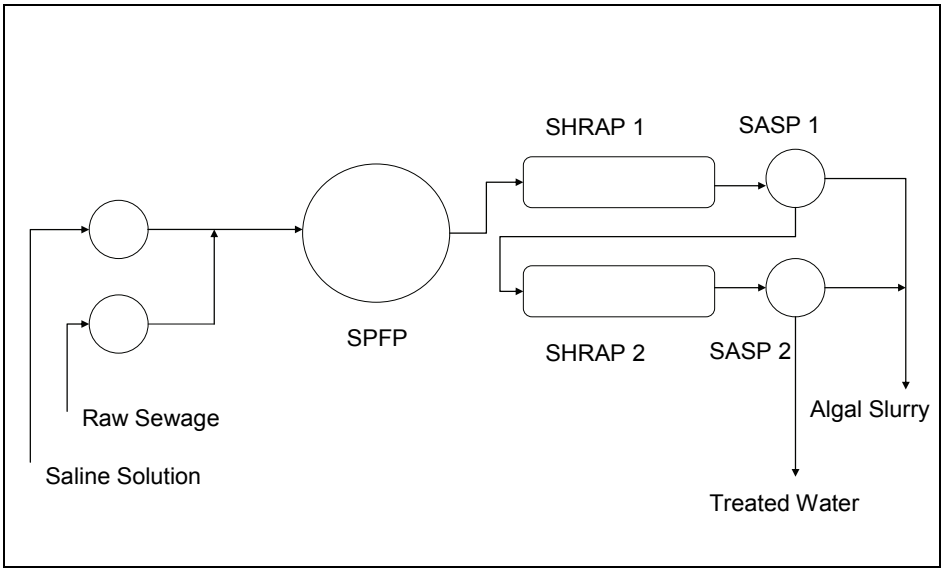


Figure 3.1: Plan diagram of the Integrated Algal Ponding Systems pilot plant constructed at the EBRU Field Station in Grahamstown. Raw sewage was blended into the saline solution in order to simulate the reticulation of sewage in a saline stream. (SPFP = saline primary facultative pond; SHRAP = saline high rate algal pond; SASP = saline algal settling pond).

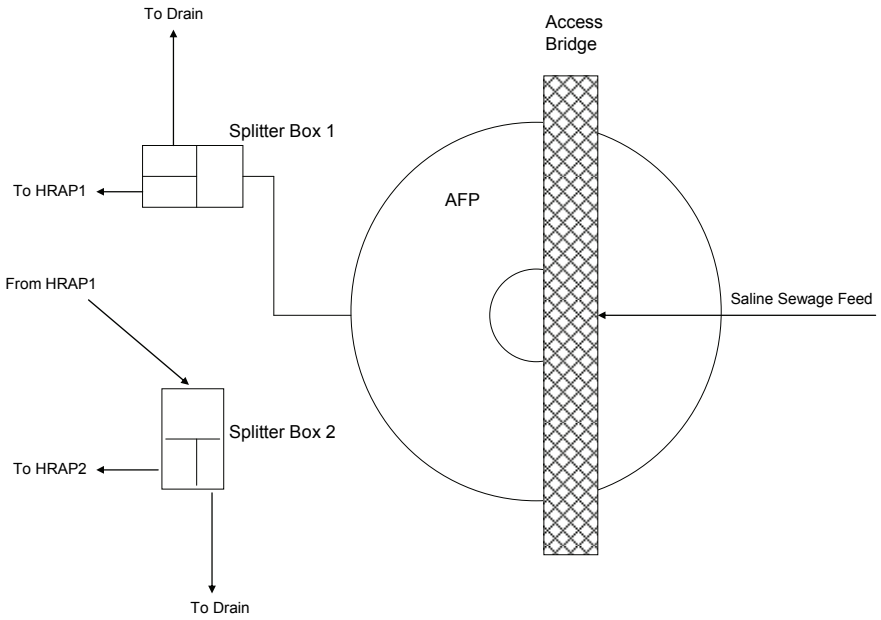


Figure 3.2: Splitter boxes were installed to control flow to the HRAPs and a sampling bridge was fitted over the Advanced Facultative Pond of the ASPAM pilot plant

Figure 3.3: Installation of High Rate Algal Ponds as components of the new ASPAM pilot plant

Figure 3.4: Make-up and storage tanks for synthetic acid mine drainage wastewater formulation. The influent sewage was macerated in the unit shown in the foreground before feeding to the plant.

Figure 3.5: Installation of the Fermentation Pit component of the Facultative Pond. This is shown prior to the installation of the outer pond works**.**

Figure 3.6: Advanced facultative pond constructed using a 5 m diameter porta-pool**.** The fermentation pit is located in the base of the pond.

Two HRAP were operated in the ASPAM pilot plant. The partly constructed HRAP are shown in Figure 3.3. One of these was fed with effluent from the AFP, initially under relatively light loading in order to maximise algal growth and thus alkalinity production for use in adjusting the pH of the incoming AMD stream. The second HRAP was operated with feed coming directly from the fermentation pit and therefore high in sulphide produced by the sulphate reduction occurring in this unit.

Algal biomass was removed in Algal Settling Tanks of the types shown in Figure 3.7 for downstream utilisation or as an additional carbon source to supplement the sewage feed in the sulphate reducing component of the reactor. Once the algal biomass has been removed, the treated water exits the system.

Figure 3.7: Algal settling tanks for removal of algal biomass

The splitter box is shown in Figure 3.8. The location of the saline IAPS and the ASPAM pilot plants at the EBRU campus is shown in Figure 3.9.

Figure 3.8: Splitter box for controlling flow to the high rate algal ponds

Figure 3.9: The retrofitted saline IAPS (right) and the newly constructed ASPAM (left) pilot plants located at the EBRU campus, Rhodes University, Grahamstown

3.2 Repair and Refurbishment of High Rate Algal Ponds

The algal ponding pilot plant at Grahamstown Sewage Works, constructed some 10 years ago by WRC, has provided the core facility in a number of WRC-funded projects for researching and developing various biotechnological processes which have led *inter alia* to the commercialised Rhodes BioSURE® Process as well as other algal technologies which have potential for full-scale implementation and commercialisation.

Over this 10-year period no major repair works were undertaken and the plant stood up very well to continuous use. However, in late 2004 it became apparent that settling of the ground under the High Rate Algal Ponding (HRAP) units resulted in cracking of the retaining walls, so severely as to put the ponds out of operation. Patch jobs attempted with water-resistant concrete screeds were unsuccessful and professional advice was that a pond liner needed to be fitted. A number of other minor repairs including the paddle wheels were also required. This repair and refurbishment was essential also for carrying out the experimental work in WRC projects 1362, 1619, 1621 and 1637

Figures 3.10 to 3.12 show aspects of the refurbishment work undertaken on the High Rate Algal Ponds.

Figure 3.10: The walls of the high rate algal ponds were raised with breeze blocks and then plastered

Figure 3.11: Fitting of the liner

Figure 3.12: Securing of the liner

4 OPERATION OF THE ASPAM PILOT PLANT

Where the earlier report on the development of the ASPAM concept had been based on laboratory and bench-top studies (Rose *et al.*, 2002), the objective of this component of the current study was to evaluate, at pilot-scale, the operation of the IAPS configuration as a sulphate reducing system. Here the principal focus was to investigate the operation and performance of the AFP fed a synthetic mine water feed made up as 1500 mg/l SO_4. Settled sewage sludge was blended with the influent stream to establish a COD:SO_4 ratio of 2:1 (m/m). This was fed directly to the anaerobic pit and from there, rising through the anaerobic sludge blanket, passes into the Facultative Pond.

Although no provision as made in this project to investigate the downstream sulphide oxidation stage of the ASPAM process train, it became apparent that sulphur biofilm formation on the surface of the AFP showed important correlation with the study of these systems being undertaken concurrently at that time at EBRU in WRC Project K5/1545, 'Investigation and Development of the Biotechnology of Sulphur Biofilms in the Beneficiation and Treatment of Wastewater.' The nature of the sulphur biofilm on the pilot plant AFP was investigated in the molecular microbial ecology study of the AFP reported in the following chapter.

The partly treated stream then passed to the HRAP where the sulphides produced in the AFP were completely oxidized to sulphate.

Given the synthetic nature of the mine water feed, and practical problems in maintaining appropriately reduced conditions for addition of metals to the feed water make-up, it was decided to investigate the metal removal unit operation at bench-scale in this study. For this reason a metals removal unit operation was not attached to the pilot plant at this stage. The bench-scale studies are reported in Chapter 6.

4.1 Commissioning and Operation

The construction of the ASPAM pilot plant was completed in August 2005 as described above and commissioning of the plant commenced in September 2005. In order to establish a viable community of sulphate reducing bacteria (SRB), 800L of primary sewage sludge was placed in the ASPAM anaerobic pit and inoculated with 80L of sludge from an active sulphate reducing reactor operating at EBRU. This was then made up to 2.5 m^3 with 1500 $mg.l^{-1}$ sulphate feed water and left to stabilise for 72hours. Following this period, feeding of the synthetic sulphate wastewater commenced and was fed thereafter on a continuous basis at a feed rate of 40 l/hour. The primary sewage sludge was sourced from the primary sedimentation tanks at the Grahamstown Disposal Works. This was pumped as required from a transport tank into a funnel (Figure 4.1) connected to the bottom of the pit by means of a 65 mm PVC pipe and delivered on a batch-fed basis. Continuous feeding had been attempted but it was found that delivery of the small volumes required by the size of the pilot plant system was inaccurate and that stable feeding rates could not be maintained over time.

Figure 4.1: Funnel for feeding primary sewage sludge which was implemented following problems encountered with the continuous feeding system

4.2 Analytical Methods

The following analytical methods were used in this study:

4.2.1 Sulphide

The Merck® spectroquant system was used for sulphide determination (Merck®, South Africa). Samples were collected in test tubes containing 100 µl of 0.1 m zinc acetate solution. Photometric readings were made using the SQ 118 spectrophotometer (Merck®).

4.2.2 Sulphate and Thiosulphate

Ion chromatography was used for the determination of sulphate with a model 600 Waters high pressure liquid chromatography (HPLC) and model 432 Waters conductivity detector (Waters, South Africa) fitted with an IC-Pak™ anion 4.6X50 mm column (Waters, South Africa). Samples were prepared using a ten-fold dilution of sample in milli-Q water and then filtered through a 0.45 µm nylon filter before passing it through two Waters Sep-Pak® light C_{18} cartridges (Waters, South Africa) to remove contaminating organic compounds. The samples were then injected and run at 1 ml/min and analysed using the EMPOWER software programme (Waters, South Africa). A Borate/Gluconate buffer concentrate was used for eluent preparation. All chemicals and filters were from Merk®, South Africa. A standard concentrate containing Fe^-, Cl^-, NO^{3-}, Br^-, HPO_4^{2-} and SO_4^{2-} in milliQ water was prepared. The injected standard was prepared weekly by diluting 100µl of the concentrate standard in 100 ml of milliQ water.

4.2.3 pH

pH was measured using a WTW pH330 meter (Merck ®, South Africa).

4.2.4 Sulphur

A modified Mockel (1984), reverse HPLC method was used for sulphur determination. Three 1 ml samples were collected, centrifuged for 5 min at 10 000 g on an Eppendorf, centrifuge 5415D (Merck®, South Africa), and air dried. After drying 1 ml of acetone was added to the pellet, and left to stand for 1 hour with vigorous shaking every ten minutes. The samples were filtered through a 0.45 μm nylon filter (Merck, South Africa) and analysed using the EMPOWER software on a 600 model Waters HPLC and a 2487 model dual λ absorbance detector fitted with a Nova-Pak® C^{18} 3.9X150 mm column (Waters, South Africa). The samples were injected into the HPLC and run at 2 ml.min-1 using a 5:95 of water: methanol (Hypersolv for HPLC, BDH from Merck®, South Africa) as the eluent. A 20 mg/l standard of elemental sulphur in acetone was prepared and injected with samples for standardisation.

4.2.5 Chemical Oxygen Demand

Chemical oxygen demand (COD) was measured using the method outlined in Standard Methods (APHA, 1998). Samples were acidified to remove sulphide before analysis.

4.2.6 Mass Balance Calculation

The sulphur mass balance for the ASPAM system was calculated as follows:

Total sulphur species IN = Total sulphur species OUT + Total sulphur recovered.

$$\mathbf{S^0 + SO_4^{2-} + HS^- + S_2O_3^- = (S^0 + SO_4^{2-} + HS^- + S_2O_3)_{OUT} + (S^0 + SO_4^{2-} + HS^- + S_2O_3)\ recovered}$$

Mass balance loss (%) = $[(S_{IN} - S_{OUT} + S\ recovered) / S_{IN}] * 100$

Mass balance recovery (%) = 100 – Mass balance loss

Sulphide removal (%) = $[(Sulphide_{IN} - Sulphide_{OUT})/Sulphide_{IN}]*100$

Sulphur recovery (%) = $[(Sulphur_{IN} - Sulphur_{OUT})/Sulphide_{IN}]*100$

4.2.7 Nitrate

Nitrate analysis was undertaken using the Merck Spectroquant nitrate analysis test kit system.

4.2.8 Phosphate

Phosphate analysis was undertaken using the Merck Spectroquant phosphate analysis test kit system.

4.2.9 Statistical Analysis

Statistical validation of the data was undertaken using the Statistica software package Version 7.1 (StatSoft, Inc. 2005). A 95% degree of confidence was adopted whereby the level of significance was accepted at $p<0.05$.

4.3 Results and Discussion

The pilot plant was operated over the 17 month period from September 2005 to February 2007. Over this period, samples were drawn on 172 sampling days and analysed as described above. The results of the analyses are reported in the following figures.

The change in sulphate concentration across the system for the 17 month study period is shown in Figure 4.2 and the mean of averages, shown as box & whisker plots, is reported in Figure 4.3. The most significant change here, occurs between the sulphate concentration in the feed and the sampling point immediately above the Pit which records the total sulphate reduction occurring in the system ($p <0.001$). The elevation of sulphate levels in the AFP and the HRAP are to be expected given the increasingly aerobic status of these units. This indicates complete oxidation of the sulphide remaining in the stream in these components of the system. However, the shortfall between the influent and effluent sulphate concentrations (effluent sample drawn after the HRAP unit), indicates a

substantial loss of sulphur through the system. This would likely be in the form of either sulphide gas or as elemental sulphur formed in the floating sulphur biofilm on the surface of the AFP.

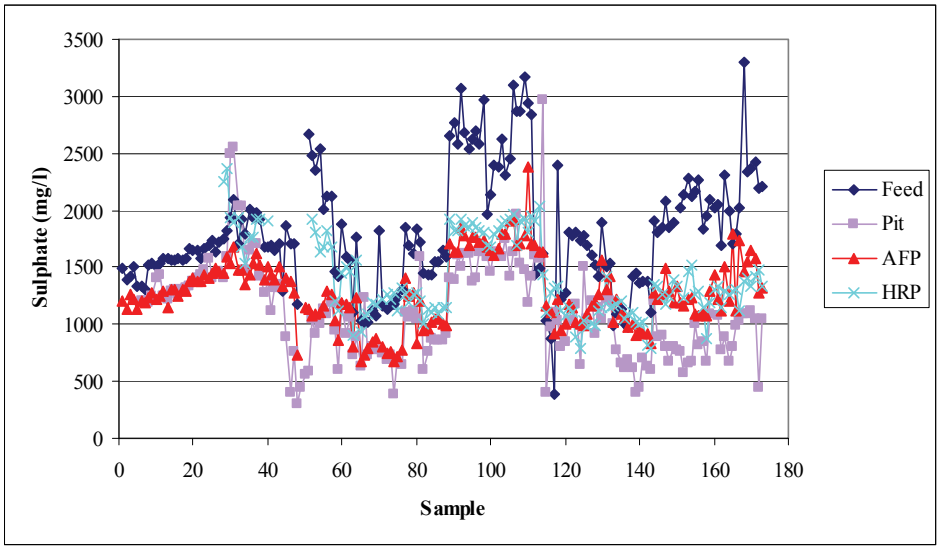


Figure 4.2: Sulphate concentration in the various units of the ASPAM pilot plant measured on the 172 sample days over the 18 month period from September 2005 to February 2007

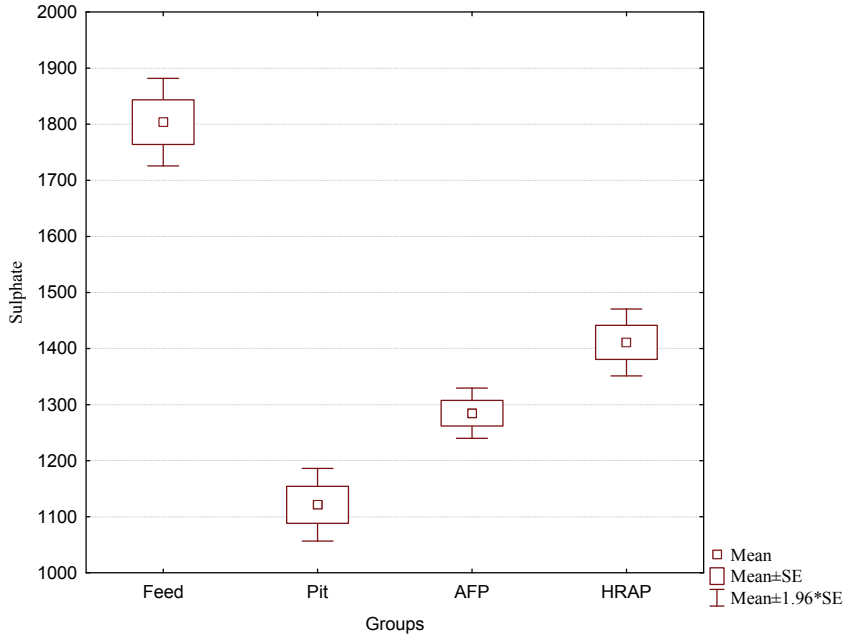


Figure 4.3: Sulphate concentration in the various units of the ASPAM pilot plant shown as the mean of averages with standard error. The differences between values in each of the units are significant with $p<0.05$ in each case (Scheffe Test).

The analyses for sulphide concentration are shown in Figure 4.4 and mean of averages box & whisker plots in Figure 4.5. These results indicate that oxidation reactions occurred to some degree in the AFP, but to completion in the HRAP. With one or two exceptions, sulphide was fully oxidized in the HRAP.

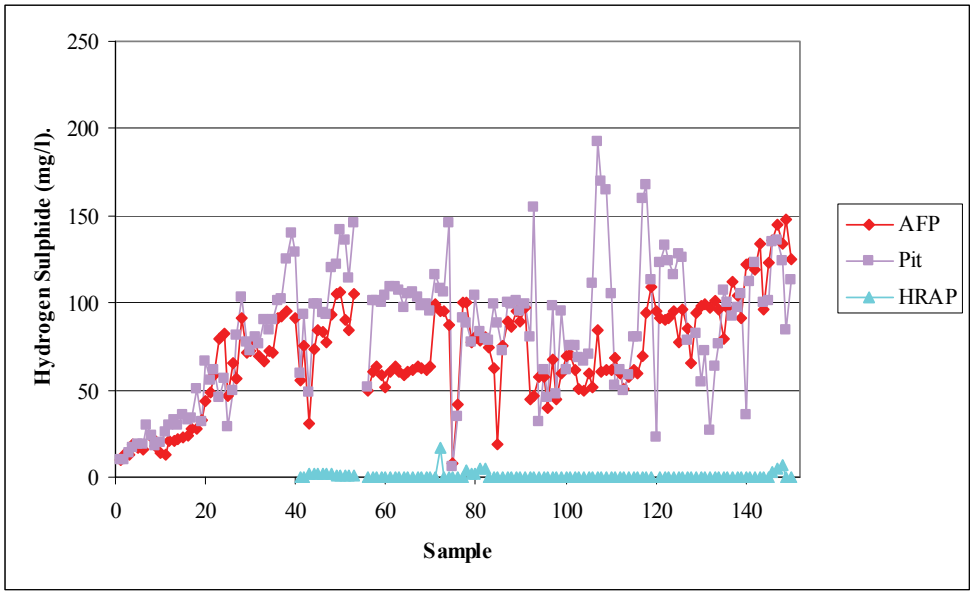


Figure 4.4: Sulphide concentration in the various units of the ASPAM pilot plant measured on the 150 sample days over the 18 month period from September 2005 to February 2007

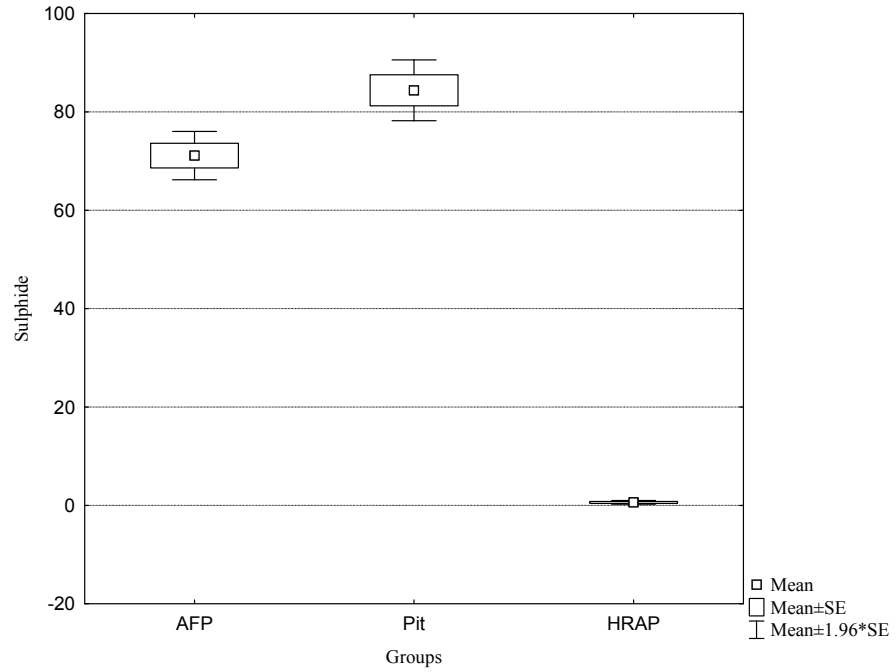


Figure 4.5. Sulphide concentration in the various units of the ASPAM pilot plant shown as the mean of averages with standard error. The differences between values in each of the units are significant with $p<0.05$ in each case (Scheffe Test).

Figure 4.6 reports the COD values for the various units in the ASPAM pilot plant and shows the change introduced around sampling day 40 when the continuous sewage sludge dosing system was abandoned and the batch feeding directly into the Pit of the AFP commenced. The mean of averages for the COD measurements is shown in the box & whisker plots in Figure 4.7. This change was to have a number of knock-on effects which will be outlined below. Although the COD feed, measured at the top of the Pit, was observed to fluctuate widely, due to the system in use, after this point, the total COD reduction was highly significant ($p<0.001$), and in the AFP and HRAP remained both effective and consistent. However, as the soluble COD (COD_s) results indicate (Figure 4.8), the final discharge COD was not able to approach the 75 mg/l General Standards limit for discharge into a public water course. Given the un-optimised status of this unit operation over the course of the study, it is felt that a much better final result could be achieved where this was an issue of primary focus in the study.

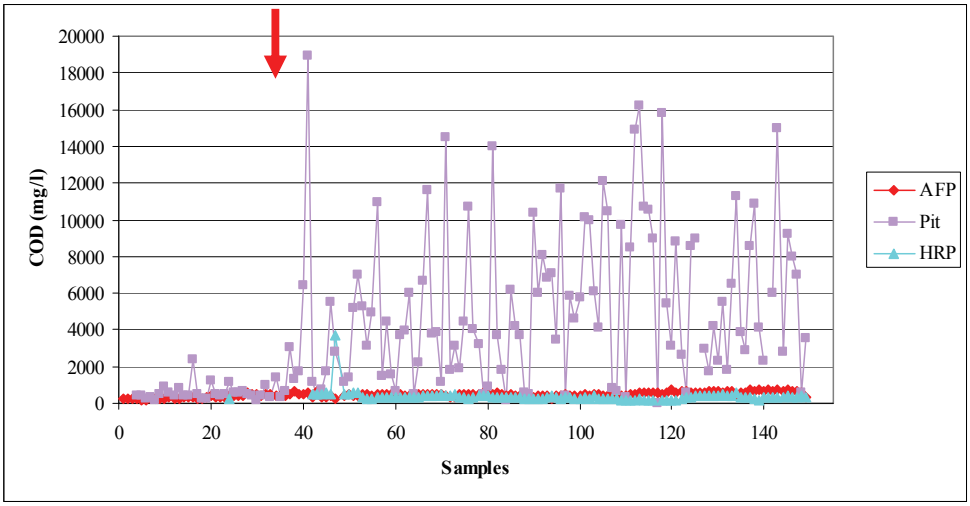


Figure 4.6: COD measured in the various units of the ASPAM pilot plant measured on the 150 sample days over the 18 month period from September 2005 to February 2007. The sludge feeding system was changed around sampling day 40 (arrow).

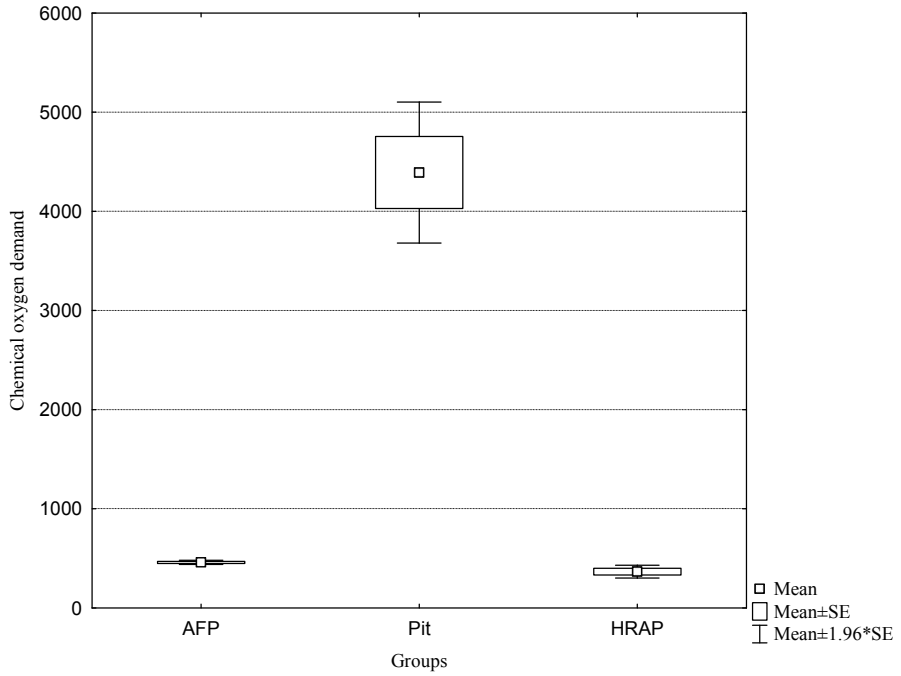


Figure 4.7: COD measured in the various units of the ASPAM pilot plant shown as the mean of averages with standard error. The differences between values in Pit and the AFP and HRAP units are highly significant ($p<0.001$, Scheffe Test). The differences between the AFP and HRAP measurements were not significant. ($p<0.05$).

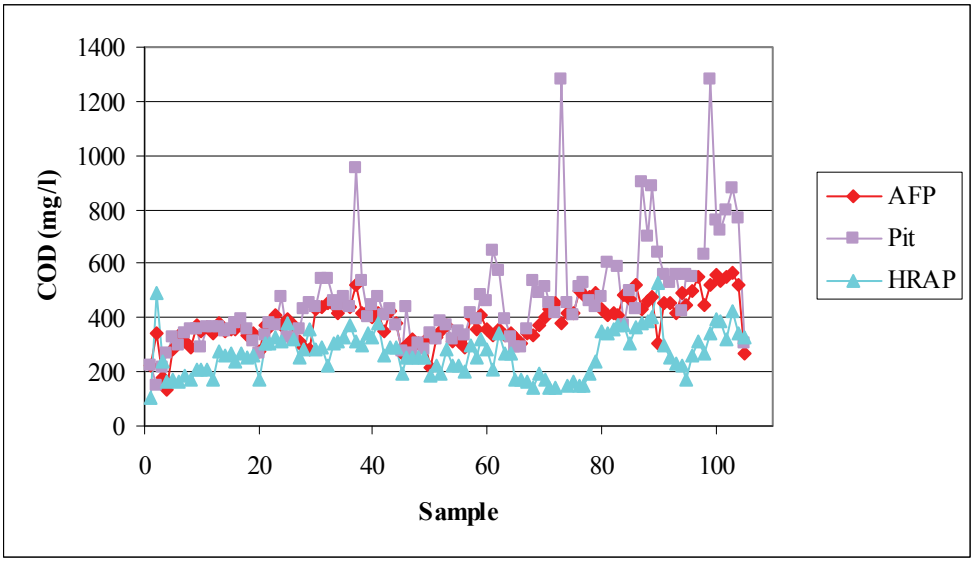


Figure 4.8: Soluble COD measured in the various units of the ASPAM pilot plant measured on the 120 sample days over the 18 month period from September 2005 to February 2007

The measurement of pH across the system is shown in Figure 4.9. Although pH measurements for the Feed, Pit and AFP range quite narrowly between pH 7 and 8 over the sampling period, the elevation of pH in the HRAP during the first part of the study is quite marked. Alkalinity production may be related to the active photosynthetic functioning of this system which is well described for HRAP operations (Wells, 2005). The result here correlates with limited organic loading during the initial period followed by an organic overload where the dosing of the system had been changed to the batch fed method. Apart from compromising the operation of the HRAP, the loss of alkalinity production in the system resulted in the subsequent metal studies being undertaken using alkalinity supplied by lime addition.

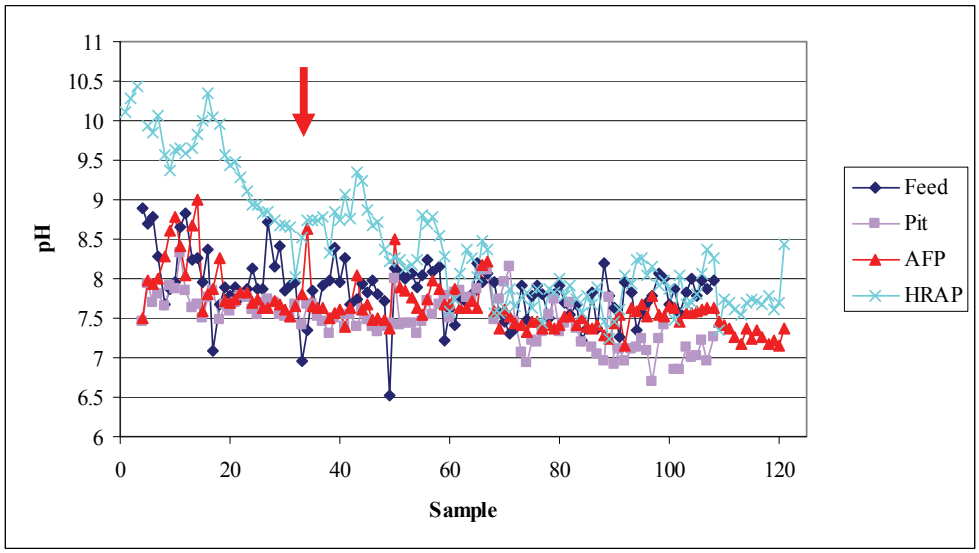


Figure 4.9. Measurement of pH across the ASPAM pilot plant measured on the 120 sample days over the 18 month period from September 2005 to February 2007. The sludge feeding system was changed around sampling day 40 (arrow).

Measurement of nitrate and phosphate concentrations in the various units of the ASPAM pilot plant are shown in Figure 4.10 and 4.11 Nitrate concentrations remain well within the General Standard for discharge (15 mg/l), which would be anticipated for a functioning facultative system providing both aerobic and anaerobic environments. However, phosphate levels are high and, with the exception of the early part of the study, indicate a poorly functioning HRAP. At elevated pH levels (>pH 9.5) in a properly functioning HRAP, phosphates precipitate as calcium phosphate and hydroxyapatite (Wells, 2005).

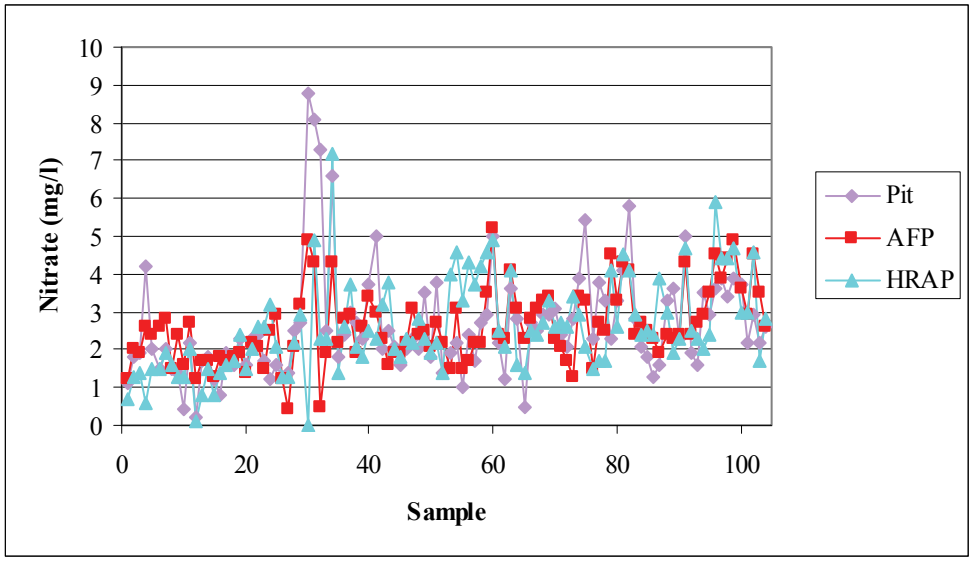


Figure 4.10: Nitrate concentration measured across the ASPAM pilot plant for the study period

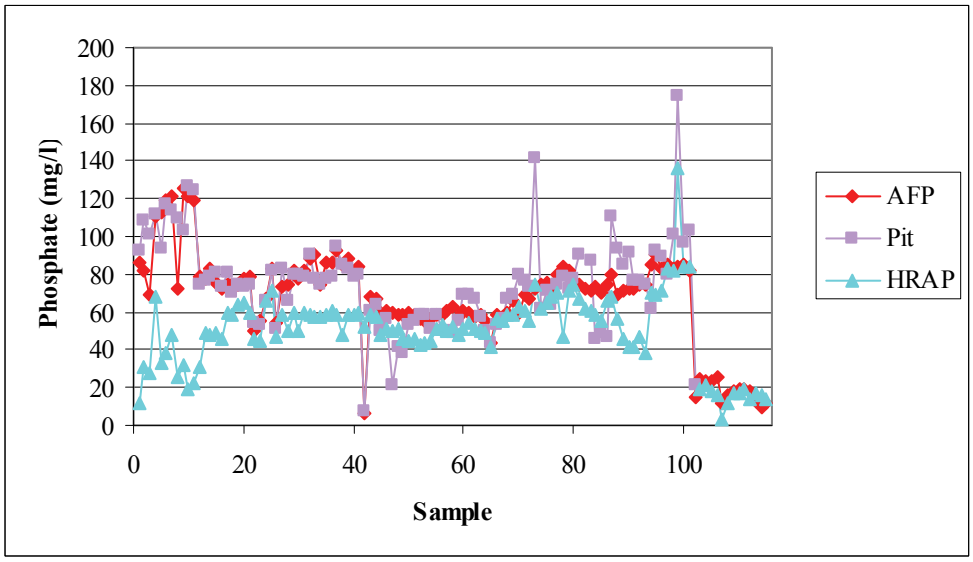


Figure 4.11: Phosphate concentration measured across the ASPAM pilot plant for the study period

4.3.1 Sulphur Balance

A sulphur balance was constructed to account for the passage of sulphur in its various oxidation states through the system. All the sulphur present in the system was introduced

in the feed in the form of sodium sulphate, and the average sulphate concentration of the feed over the monitoring period was 1 684 mg.l^{-1}. Sulphate was reduced in the Pit of the AFP and then partly reoxidised in the AFP and the residual completely oxidized in the HRAP.

The sulphur species analysed were sulphate, sulphide (dissolved and gaseous), thiosulphate and elemental sulphur. It was found that thiosulphate levels in the system were negligible. The amount of sulphide lost as H_2S to the atmosphere over the surface of the PFP amounted to 0.04% (87.5 mg.day^{-1}) of the total sulphide production. This was determined by gas capture in a head-space device inverted over the surface of the pond. The elemental sulphur present in the water column biomass accounted for 0.15% (362 mg.day^{-1}) of the total feed to the system calculated as S. It was estimated that approximately 8 306 mg elemental sulphur was present in the sludge on the floor of the AFP and settled from the floating sulphur biofilm. It was, however, difficult to determine the retention time and thus the deposition rate of the sulphur in this sludge. This sulphur could, therefore provide the remainder of the 10% sulphur missing in the balance between the feed and the AFP effluent. The sulphur balance for the ASPAM pilot plant study is shown in Figure 4.12.

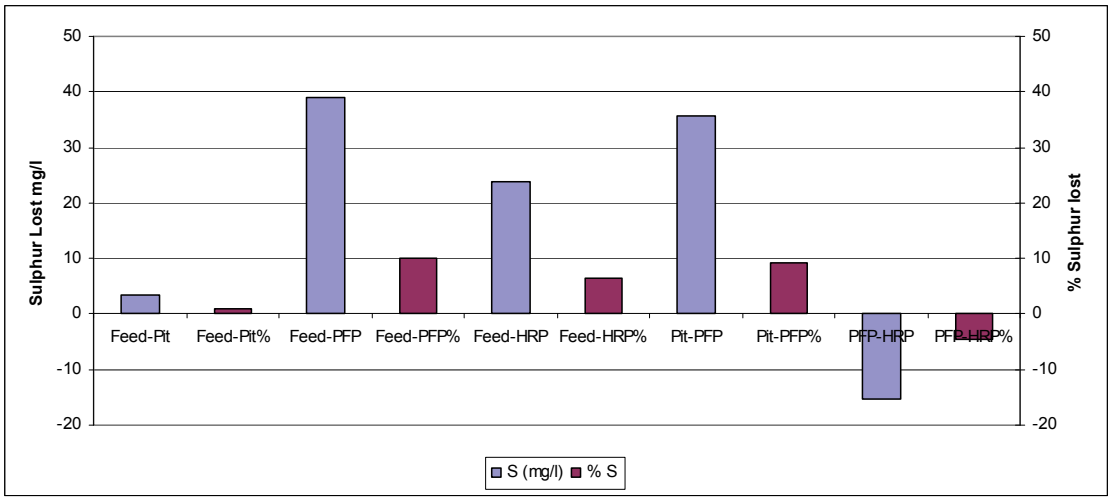


Figure 4.12: Report of the sulphur balance across the ASPAM system

It can also be seen from Figure 4.12 that most of sulphur unaccounted for between the feed and the AFP is lost between the fermentation pit and the facultative compartment, where conversion to elemental sulphur in the floating sulphur biofilm and by photosynthetic bacteria is most likely to occur. At the sampling point at the top of the Pit before these processes intervene, 99.1% of the influent sulphur (as S) is accounted for. The apparent increase in sulphur between the AFP and HRP may be accounted for by evaporative concentration of the stream in the HRAP.

4.4 CONCLUSION

The operation of the anaerobic unit of the system functioned well, producing sulphide through the sulphate reduction reaction process. This was then oxidized through the various oxidation states of sulphur and reported to the various downstream compartments. Approximately 10% of the total influent sulphur could not be accounted for as either $H_2S(g)$, HS^- or SO_4^{2-} between the feed and the AFP. Evidence of elemental sulphur in the settled sludge suggests this may be where the residual sulphur is located. Losses to atmosphere were low.

The performance of the floating sulphur biofilm, and of photosynthetic bacteria present in the AFP water column, as an effective sulphide oxidation and sulphur removal operation, was an unexpected observation in the study. Although its operation was not a principal objective of the study, and was thus not the subject of separate optimization, it is evident that this could offer important advantages in the overall treatment of sulphate wastewaters in the ASPAM system. Given the substantial advances made in the operation of the Floating Sulphur Biofilm Reactor developed in WRC Project 1545, it is evident that a basis has been demonstrated here for potentially dealing with the technological bottleneck relating to sulphur removal following the sulphate reduction reaction in the biological treatment of mine wastewaters. It is recommended that this should be the subject of follow-up investigation.

Feeding of sewage sludge in small volumes to the pilot plant proved to be a severe constraint on the successful operation of the system and in future process designs adequate budgetary provision will need to be made to enable this operation. This problem impacted on a number of other areas of the study including the successful operation of the HRAP and the use of algal-alkalinity in the metal removal operation studies.

Although not investigated in this study, previous work (Boshoff, 1998) showed that algal biomass could be used as a carbon source for the sulphate reducing compartment of the system. Depending on surface areas available, this could provide a measure of independence of the process from an external carbon source.

5 MICROBIAL ECOLOGY OF THE ADVANCED FACULTATIVE POND IN THE ASPAM SYSTEM

5.1 Background

The identification and quantification of members of a particular microbial community, and a clearer understanding of the functional relationship between its members, is required to fully appreciate, and possibly manage, the complex processes that these communities perform (Davey and O'Toole, 2000).

Reports show that up to 99% of all microorganisms in nature cannot be isolated in pure culture, since their culture requirements are not known (Wagner *et al.,* 1993; Amman *et al.,* 1995). The application of molecular methods has revolutionized the routine identification of bacteria from environmental and industrial sources (Bowker, 2002). Techniques based on the analysis of genetic material complement the conventional microbiological approach and are routinely used to determine the presence and distribution of individual bacterial species (Santegoeds *et al.,* 1998).

The quantitative recovery of nucleic acids from environmental samples imposes major limitations on the molecular approach. The initial extraction of nucleic acids is a crucial step because not all microorganisms can be fractured equally well (Amann *et al.*, 1995). It has been demonstrated that a combination of physical and chemical treatments such as freeze thawing, lysis with detergents and bead beating were shown to lyse approximately 96% of soil bacteria, smaller cells (0.3-1.2 μm) seem to be more resistant to lysis (Head *et al.,* 1998). It was also found that up to 99.8% lysis could be achieved by merely using extended lysis incubations and up to 6 freeze thaw cycles (Head *et al.,* 1998).

The application of 16S rRNA sequence analysis has revolutionized the study of both microbial ecology and phylogeny (Goebel & Stackebrandt, 1994). Several rRNA-based methods have been developed to identify and quantify microorganisms in complex environments. These methods can be used without isolation and cultivation.

The rRNA approach, together with other molecular techniques holds great potential for an analysis of microbial diversity which is unbiased by the limits of pure-culture techniques (Amann *et al.*, 1995).

rRNA has particular advantages in that it is present in all organisms, it has conserved and also variable regions (this enables the selection of general and specific target sequences), it contains enough sequence information to be used as a phylogenetic marker, the genes are not transferred horizontally between species and there are large data-bases of sequences available (Muyzer & Ramsing, 1995). With the aid of Polymerase Chain Reaction (PCR) a target rRNA sequence can be amplified.

Selectivity and errors in PCR amplification of rRNA genes are however a source of bias that can affect the results of molecular biological measures of diversity (Head *et al.,* 1998). Small differences in the sequence of universally conserved regions may result in selective amplification of some sequences, particularly when primer annealing is at high stringency and errors may occur during amplification (Head *et al.,* 1998).

Investigations of microbial community structure and diversity in environmental samples generally include cloning and sequence determination of 16S rRNA genes (Heuer *et al.,* 1999). The detection of minor members in environmental samples generally requires the analysis of many cloned sequences (Heuer *et al.,* 1999).

Although sequencing of clones provides insight into the community structure through phylogenetic affiliations of community members, the information about their physiological and ecological traits derived from the partial sequences is rather limited. Molecular and chemical results were therefore combined to provide an overall picture of how the different systems functioned.

In this study, the samples collected were subjected to molecular typing. This investigation was carried out to try to identify the bacterial species observed in the system. With this in mind, the trophic and metabolic functions of the species identified could be determined

and thus provide inputs into an interpretation of the role they play in the ASPAM system.

5.2 Materials and Methods

5.2.1 Sample collection

Samples were taken in the ASP, were placed in sterile microfuge tubes (Eppendorf, Merck®) and frozen at -20°C.

5.2.2 Molecular typing of samples

5.2.2.1 DNA Extraction

Total DNA extraction method followed that of Sambrook *et al.* (1989). Glass beads were added to the samples before vigorous shaking on a vortex Genie-2 (Scientific Industries). The samples were then collected in 2.5 ml microfuge tubes (Eppendorf, Merck®), concentrated by centrifugation at 13 000 g for five minutes in an Eppendorf 5415D desktop centrifuge.

The pellet was washed with 500 µl of 2 x TE (Tris/EDTA) buffer pH 8.0. The Tris /EDTA buffer was made up of 10 mM Tris/HCl, 1 mM EDTA and one part 50% glycerol (Bond *et al.,* 2000). The pellet was re-suspended in 500 µl of 2 x TE buffer. The sample was taken through further lysis by adding 6 µl of a 50 µl/ml lysozyme enzyme, the sample was then incubated shaking in a Labcon shaking incubator at 37^{o}C for three hours, followed by five cycles of one minute freeze and one minute thaw in liquid nitrogen and boiling water respectively.

The 250 µl samples had an equal volume of 10% sodium dodecyl sulphate (SDS) added to them. Then the cell lysate was extracted with an equal volume phenol, vortexed and centrifuged at 10 000 g for two minutes. The upper aqueous layer was collected and extracted in an equal volume of phenol: chloroform: isoamyl alcohol (24:24:1), vortexed,

centrifuged at 10 000 g for two minutes. This was repeated until the pink colour was removed from the aqueous layer.

Nucleic acids were precipitated with 2.5 volumes of ice-cold 96% rectified ethanol overnight at -20°C. The DNA was concentrated by centrifugation in an Eppendorf model 5810R desktop centrifuge at 4°C for 25 minutes at 13 000 g and re-suspended in 20 µl TE buffer. A 10 µl aliquot of each DNA was stored at 4ºC for immediate use and short-term storage, while the remainder was stored at -20°C. The DNA was electrophoresed on a 0.8% agarose gel (0.8 g agarose in 100 mL of 1 x TBE buffer). The TBE buffer comprised 10.78 g Tris base, 2.5 g, 4 mL EDTA 0.5 M pH 8.0. 100 µl of ethidium bromide (0.5 g of ethidium bromide in 1 ml of milliQ water) was added to the cooled agarose before casting the gel. A λ *Pst*1 molecular weight marker was used to check the molecular weight of the product. It was prepared by digesting 200 µl λDNA (0.25 µl/ml) with 24 µl of 10 x buffer H and 10 µl of *Pst* 1 enzyme for three hours at 37°C, before adding 550 µl of 10 mM TE buffer (pH 8.0) and 150 µl of 6 x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol).

5.2.2.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) enables the rapid and efficient analysis of specific DNA sequences (Mullis and Faloona, 1987). It was important to first optimize the PCR reaction in order to amplify the DNA. The universal 16S primer is GM5F (5' - cct acg gga gca gcag - 3') and 907R (5' - cgc ccg ccg cgc ccc gcg ccc gtc ccg ccg ccc ccg ccc gcc gtc aat tcc ttt gag ttt - 3') a gc clamped primer (Inqaba Biotec) were used in this study. The enzyme used was *Taq* DNA polymerase (Promega) at a concentration of 0.5 µl per 25 µl reaction. A 2.5 µl aliquot of buffer containing magnesium chloride ($MgCl_2$) was added per 25 µl reaction. In cases where the $MgCl_2$ concentration was adjusted, 2.5 µl of buffer without $MgCl_2$ was added per 25 µl reaction and the volume of water added adjusted accordingly to give a final volume of 25 µl. Each of the four deoxynucleoside triphosphates (dNTPs) was added to a final dNTP concentration of 1 µl per 25 µl reaction. The dNTPs were from Inqaba, Biotec. The reaction was made up to 25 µl with a

calculated volume of autoclaved pure water (Sigma).

Amplification was performed in a Hybaid PCR Sprint thermocycler using a touchdown PCR procedure (Table 5.1).

Table 5.1: Touchdown programme used for PCR amplification

Reaction	Temperature	Duration	No of cycles
Initial Denaturation	95ºC	2 minutes	1 cycle
Denaturation	94ºC	30 seconds	4 cycles
Annealing	68ºC	45 seconds	
Extension	72ºC	2 minutes	
Denaturation	94ºC	30 seconds	4 cycles
Annealing	66ºC	45 seconds	
Extension	72ºC	2 minutes	
Denaturation	94ºC	30 seconds	4 cycles
Annealing	64ºC	45 seconds	
Extension	72ºC	2 minutes	
Denaturation	94ºC	30 seconds	4 cycles
Annealing	62ºC	45 seconds	
Extension	72ºC	2 minutes	
Denaturation	94ºC	30 seconds	12 cycles
Annealing	60ºC	45 seconds	
Extension	72ºC	2 minutes	
Final extension	72ºC	5 minutes	1 cycle

The PCR product was analyzed on 1% agarose gel containing ethidium bromide and visualized on a UV transiluminator (UVP BioDoc-It^{TM} system) fitted with a digital camera.

5.2.2.3 Transformation and Cloning

The PCR product was cloned into the pGEM®-T Easy Vector system (Promega, USA) as per manufacturer's instruction, (Table 5.2) and transformed into high efficiency E. *coli* JM 109 competent cells (Sambrook *et al.,* 1989).

Table 5.2: Ligation Reactions for pGEM-T Easy Vector

	Standard Reaction	**Positive Control**	**Background Control**
2 x Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM-T Easy Vector	1 µl	1 µl	1 µl
PCR product	1 µl	-	-
Control Insert	-	2 µl	-
T4 DNA Ligase 3	1 µl	1 µl	1 µl
dddH_2O	2 µl	1 µl	3 µl

The ligations were incubated at 4°C overnight and then 2.5ul of each ligation reaction was added to 150 µl of thawed competent *E.coli* cells, the tubes were mixed and left on ice for 20 minutes. The cells were heat-shocked at 42ºC for 45 seconds and then cooled on ice for five minutes. 1 ml of SOC medium (Sigma) was added and the tubes were placed at 37ºC for 1 hour to allow the cells to recover.

The transformants were screened on Luria Bertani (LB) agar (30 g LB agar/ 1l milliQ) plates containing 100 µg/ml ampicillin (Amp). Before plating, the LB/Amp plates were spread with IPTG (1 m Isopropylthio-β-o-galactiside) and X-Gal (20 mg of 5-bromo-4-chloro-3-indolyl-β-o-galactoside in 1 mL dimethylformamide). The plates were incubated overnight at 37°C. Transformants with an insert in the β-glycosidase gene appeared white on the X-Gal plates as opposed to blue colonies which have a plasmid but no insert in the β-glycosidase gene. The white colonies were picked with a sterile toothpick, inoculated into 5 ml LB broth and incubated shaking overnight at 37°C.

The plasmids were extracted from the cells using the QIAprep Spin Miniprep Kit from QIAGEN®. The white colonies tooth-picked into 5 ml LB test tubes were grown at 37ºC overnight. 1 ml of each culture was placed into a sterile Eppendorf, the cells were pelleted by centrifuging them at 13 000 rpm for 1 minute. The supernatant was discarded and another 1 ml volume of culture was added, the centrifugation step was repeated.
The pelleted cells were resuspended in 250 µl of Buffer P2. 250 µl of Buffer P2 was added and the tube was inverted 5 times in order to mix. 350 µl of Buffer N3 was added and the tube was inverted immediately but gently 5 times. The sample was centrifuged at 10 000 g for 10 minutes, the supernatant was then applied to a QIAprep spin column. The sample was centrifuged for 1 minute and the flow-through was discarded. The QIAprep spin column was washed using 750 µl of Buffer PE and then centrifuged at 10 000 g for 1 minute. The flow-through was discarded and the samples were centrifuged for an additional 1 minute in order to remove any residual wash buffer.

The QIAprep column was placed into a sterile 2.5 ml Eppendorf tube and 50 µl of Buffer EB was added to the centre of the column in order to elute the DNA. The samples were left to stand for 1 minute and then centrifuged at 10 000 g for 1 minute. The DNA was placed at 4°C for short-term storage and at -20°C for long-term storage.

5.2.2.4 EcoRI Digest

The plasmid extraction had to be digested with *Eco*RI in order to check whether the insert was correct. The *Eco*RI enzyme cuts on either side of the inserted fragments and in some cases in the centre of the fragment. The correct result would be a 586 bp fragment or two smaller fragments whose combined size added up to 586 bp.

10 µl of each QIAprep plasmid extraction was used for the digest. The digest consisted of 0.2 µl of *Eco*RI (10 U/µl), 2 µl Buffer H, 10 µl plasmid and 7.8 µl of $dddH_2O$. The plasmid digest was mixed and placed at 37°C for 3 hours. The restriction endonuclease digestion products were resolved on a 1% agarose gel.

5.2.2.5 Thermocycling using the BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems)

The Thermocycling reaction sample consisted of 200-500 ng of plasmid DNA, 1 µl 10 µM SP6 primer stock solution, 4 µl BigDye, 2 µl 5 x Dilution Buffer and distilled water (to volume of 20 µl). The reaction samples were placed in the GeneAmp® PCR system 9700 thermocycler and the cycle sequencing method in Table 5.3 was followed.

Table 5.3: Cycle Sequencing on the 9700 PCR System

Step	Action
1	**Place the tubes in the thermal cycler and set the volume to 20µl**
2	**Repeat the following for 25 cycles**: • Rapid thermal ramp to 96°C. • 96°C for 10 seconds. • Rapid thermal ramp to 50°C • 50°C for 5 seconds. • Rapid thermal ramp to 60°C. • 60°C for 4 minutes.
3	**Rapid thermal ramp to 4°C and hold.**
4	**Spin down the contents of the tube in a microcentrifuge**
5	**Purify the extension products**

5.2.2.6 Purification of the Extension Products using the ZYMO Research DNA Clean & Concentrator™-5

100 µl of DNA binding buffer was added to each sample once cycle sequencing was complete. The samples were then loaded onto a Zymo-Spin column that was placed in a 2 ml collection tube. The samples were centrifuged at 10 000 g for 10 seconds and the flow-through was discarded. 200 µl of wash buffer was added to the column and then the samples were centrifuged for 10 seconds at maximum speed. The flow-through was

discarded and another 200 µl was applied to the column. The samples were spun for 30 seconds at 10 000 g and the flow-through was discarded.

To elute the DNA 8 µl of dddH_2O was applied directly to the column matrix. The Zymo-Spin column was placed in a sterile Eppendorf and centrifuged for 10 seconds at 10 000 g.

The tubes containing the DNA were left open and placed at 37°C in order to dry the DNA. The dry DNA pellet was stored at –20°C for sequencing.

Each ZYMO purified DNA sample was resuspended in 10 µl of deionised formamide, the samples were vortexed and spun down. The samples were then heated to 95°C for 2 minutes in order to denature them and then chilled on ice until ready to use.

Refer to the ABI PRISM 310 Genetic Analyser User's Manual (P/N 903565) for information on the sequencing process.

5.2.3 Analysis of the Sequencing Results

The sequencing data was converted to text format using Chromas software and then analyzed using the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database (Altschul *et al.,* 1997). To obtain the phylogenetic relationship between the clones, the data was analyzed using the Neighbour Joining (N-J) algorithm.

5.2 Results

5.3.1 Molecular typing

The extracted DNA was visualized on 0.8% agarose gel and showed a successful extraction with the high molecular weight DNA at the top of the gel (Figure 5.1).

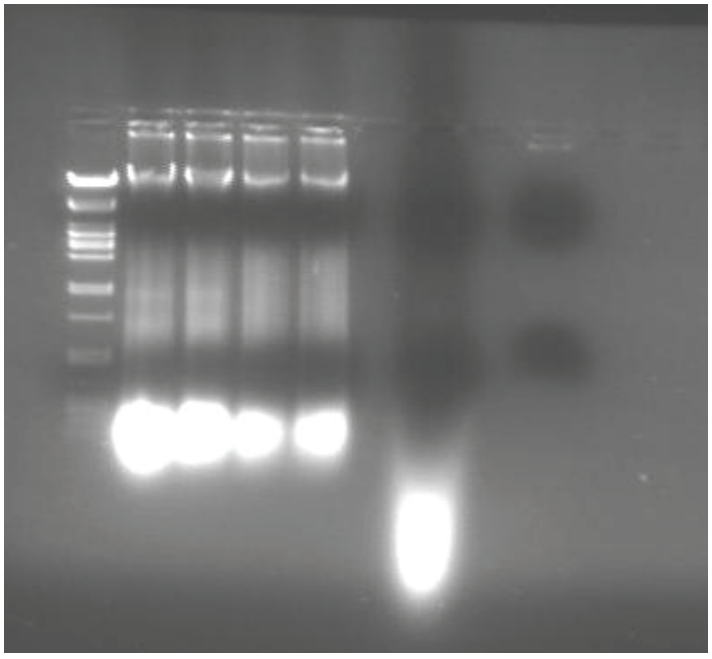


Figure 5.1: A 0.8% agarose gel showing high molecular weight DNA. The left lane contains a standard molecular weight marker, the other lanes contain DNA.

The 16S rDNA gene was amplified using the PCR primers GM5F and 907R yielded a 586 bp amplification product (Figure 5.2).

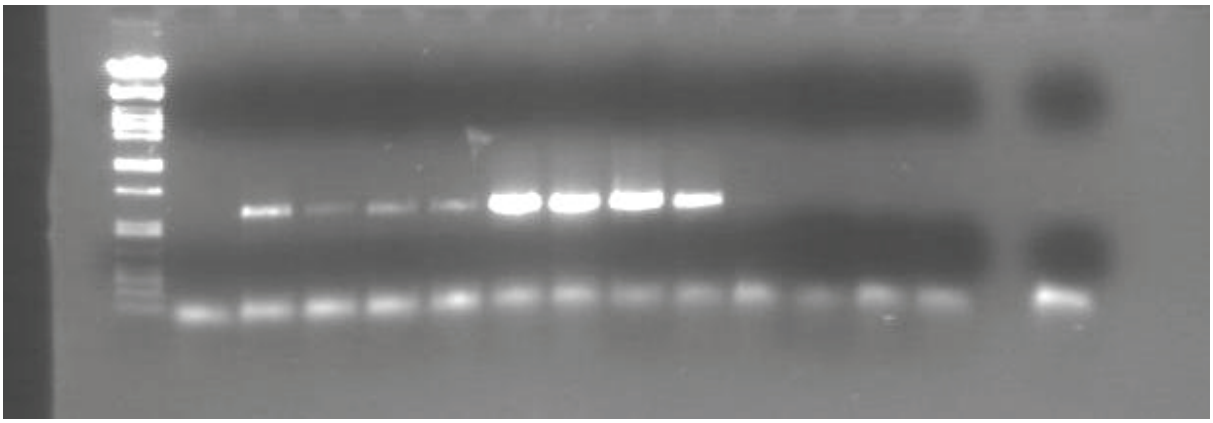


Figure 5.2: A 1% agarose gel showing 586 bp amplified PCR products. The left lane contains a standard molecular weight marker, the other lanes contain DNA.

The amplified DNA was transformed into high fidelity competent *E. coli* JM109 cells using a pGEM^{TM}-T Easy Vector as described previously. *Eco*R1 digest of the extracted plasmids was performed (Figure 5.3)

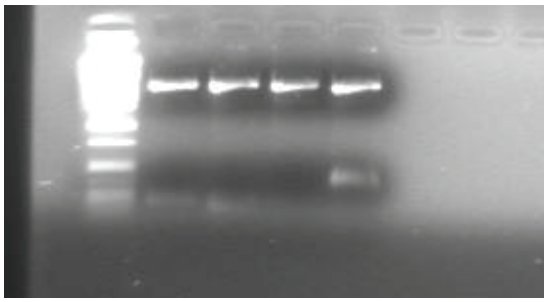


Figure 5.3: A 1% agarose gel showing the plasmid fragment and insert after digestion with EcoR1. The Top band is the plasmid band and the lower band is the cloned PCR product. The left lane contains a standard molecular weight marker, the other lanes contain DNA.

The samples with correct inserts were prepared for sequencing. The data obtained from the NCBI was used to obtain the description of the sequenced sample based on the percentage relation of the bacteria on the database to the sample as summarized in Table 5.4 and was used to formulate phylogenetic trees and incorporates the various clones sequenced (Figure 5.4).

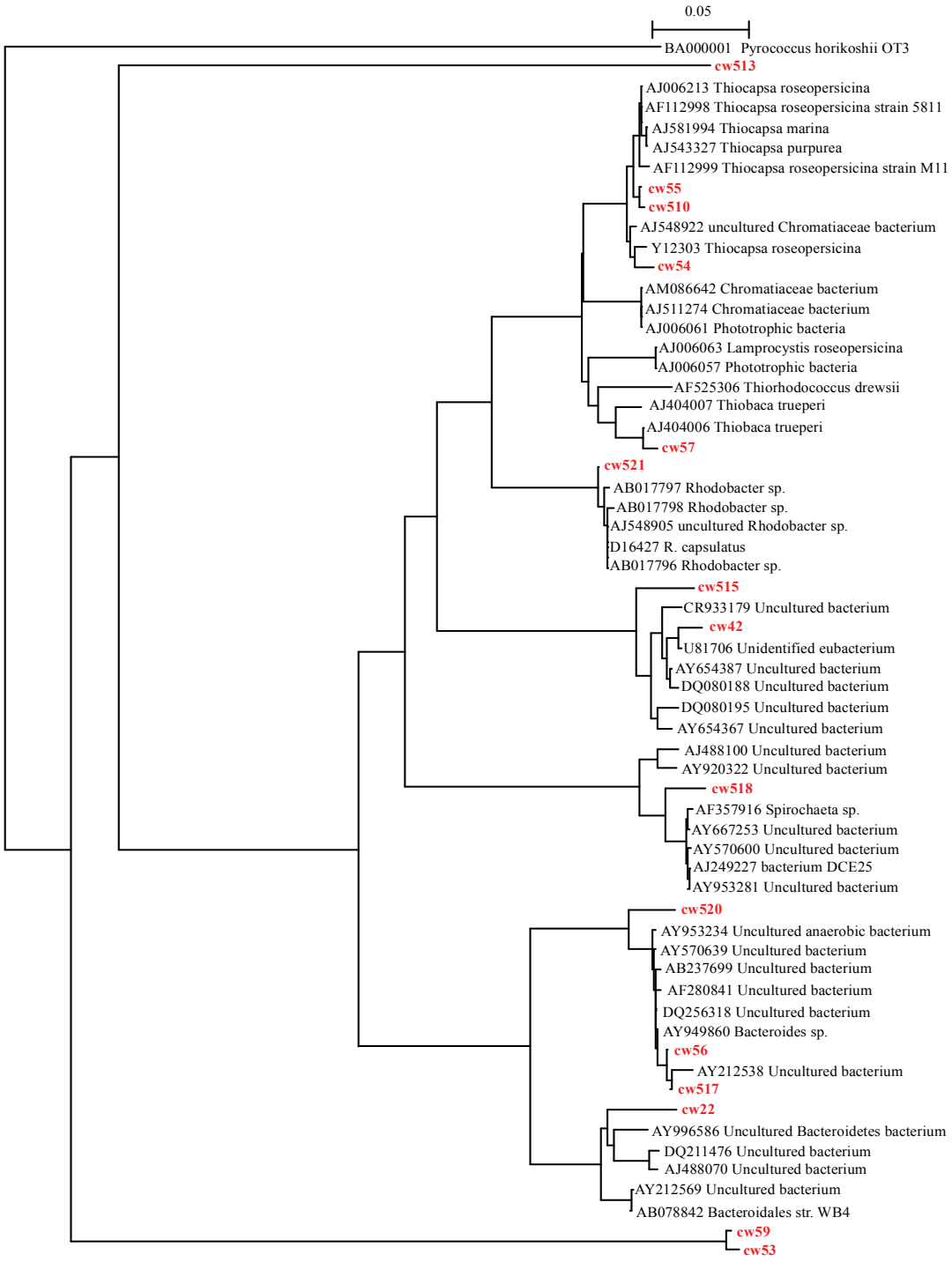


Figure 5.4: Phylogenetic tree used to relate the ASPAM samples to known species from the NCBI database.

Table 5.4: Summary of the species identified in the phylogenetic tree

BA000001 Pyrococcus	Archae	Outgroup
Cw513	Clone	
AJ006213 Thiocapsa roseopersicina	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiocapsa.	Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment
AF112998 Thiocapsa roseopersicina strain 5811	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiocapsa.	Aerobic turnover of dimethyl sulfide (DMS) by the anoxygenic phototrophic bacterium *Thiocapsa roseopersicina* M11
AJ581994 Thiocapsa marina	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiocapsa.	Thiocapsa marina: a new member of the genus Thiocapsa
AJ543327 Thiocapsa purpurea	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiocapsa.	*Thiocapsa purpurea*, sp. nov., a new purple sulfur bacterium containing okenon and isolated from several brackish and marine environments
AF112999 Thiocapsa roseopersicina strain M11	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiocapsa.	Aerobic turnover of dimethyl sulfide (DMS) by the anoxygenic phototrophic bacterium *Thiocapsa roseopersicina* M11
Cw55	Clone	
Cw510	Clone	
AJ548922 Uncultured Chromatiaceae bacterium	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; environmental samples.	High-diversity biofilm for the oxidation of sulfide-containing effluents
Y12303 Thiocapsa roseopersicina	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiocapsa.	Taxonomic rearrangements of the genera Thiocapsa and Amoebobacteria on the basis of 16S rDNA sequence analyses, and description of

		Thiolamprovum gen. nov
Cw54	Clone	
AM086642 Chromatiaceae bacterium F8	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae.	Isolation and polyphasic characterization of phototrophic sulfur bacteria from the meromictic Lake Cadagno
AJ511274 Chromatiaceae bacterium Cad16	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae.	Isolation and characterization of aggregate-forming sulfate-reducing and purple sulfur bacteria from the chemocline of meromictic Lake Cadagno, Switzerland
AJ006061 Phototrophic bacteria	Bacteria.	In situ analysis of phototrophic sulfur bacteria in the chemocline of meromictic Lake Cadagno (Switzerland)
AJ006063 Lamprocystis roseopersicina	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Lamprocystis.	In situ analysis of phototrophic sulfur bacteria in the chemocline of meromictic Lake Cadagno (Switzerland)
AJ006057 Phototrophic bacteria	Bacteria.	In situ analysis of phototrophic sulfur bacteria in the chemocline of meromictic Lake Cadagno (Switzerland)
AF525306 Thiorhodococcus drewsii	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiorhodococcus.	A new purple sulfur bacterium isolated from a littoral microbial mat, *Thiorhodococcus drewsii* sp. nov
Cw57	Clone	
Cw521	Clone	
AJ404007 Thiobaca trueperi	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiobaca.	*Thiobaca trueperi* gen. nov., sp. nov., a phototrophic purple sulphur bacterium isolated from freshwater

		lake sediment
AJ404006 Thiobaca trueperi	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Chromatiaceae; Thiobaca.	*Thiobaca trueperi* gen. nov., sp. nov., a phototrophic purple sulphur bacterium isolated from freshwater lake sediment
AB017797 Rhodobacter sp. TCRI 5	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter.	Direct Submission
AB017798 Rhodobacter sp. TCRI2	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter.	Naturally isolated *Rhodobacter* sp
AJ548905 Uncultured Rhodobacter sp.	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter.	High-diversity biofilm for the oxidation of sulfide-containing effluents
D16427 Rhodobacter capsulatus	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter.	Intragenetic structure of the genus Rhodobacter: Transfer of *Rhodobacter sulfidophilus* and related marine species to the genus Rhodovulum gen. nov
AB017796 Rhodobacter sp. TCRI 3	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter.	Naturally isolated *Rhodobacter* sp
Cw515	Clone	
CR933179 Uncultured bacterium	Bacteria; environmental samples	Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester
Cw42	Clone	
U81706 Unidentified eubacterium	Bacteria; environmental samples.	Molecular microbial diversity of an anaerobic digestor as determined by small-

		subunit rDNA sequence analysis
AY654387 Uncultured bacterium	Bacteria; environmental samples.	Rarity associated with specific ecological niches in the bacterial world: the 'Synergistes' example
DQ080188 Uncultured bacterium	Bacteria; environmental samples.	The reductive dechlorination of 2,3,4,5-tetrachlorobiphenyl in three different sediment cultures: evidence for the involvement of phylogenetically similar Dehalococcoides-like bacterial populations
DQ080195 Uncultured bacterium	Bacteria; environmental samples.	The reductive dechlorination of 2,3,4,5-tetrachlorobiphenyl in three different sediment cultures: evidence for the involvement of phylogenetically similar Dehalococcoides-like bacterial populations
AY654367 Uncultured bacterium	Bacteria; environmental samples.	Rarity associated with specific ecological niches in the bacterial world: the 'Synergistes' example
AJ488100 Uncultured bacterium	Bacteria; environmental samples.	Development and initial population analysis of stable bacterial consortia removing predominantly singly flanked chlorine substituents from chlorobenzenes
AY920322 Uncultured bacterium	Bacteria; environmental samples.	Characterization of Microbial Communities Removing Nitrogen Oxides from Flue Gas: the BioDeNOx Process
Cw518	Clone	
AF357916 Spirochaeta sp. Buddy	Bacteria; Spirochaetes; Spirochaetales; Spirochaetaceae;	Isolation of *Spirochaeta* sp. (strain Buddy) from a trichloroethene-

	Spirochaeta.	dechlorinating culture derived from freshwater river sediment
AY667253 Uncultured bacterium clone TANB18	Bacteria; environmental samples.	Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture
AY570600 Uncultured bacterium	Bacteria; environmental samples.	Microbial diversity in production waters of a low-temperature biodegraded oil reservoir
AJ249227 bacterium DCE25	Bacteria.	Anaerobic reductive dechlorination of chlorinated ethenes with an enriched mixed culture – physiological characterization and community analysis
AY953281 Uncultured bacterium	Bacteria; environmental samples	Evidence for microbial involvement in arsenic precipitation in Northern Chile salt deposits
Cw520	Clone	
AY953234 Uncultured anaerobic bacterium clone A-3D	Bacteria; environmental samples.	Unique Microbial Diversity of Anaerobic Swine Lagoons
AY570639 Uncultured bacterium clone PL-7B7	Bacteria; environmental samples.	Microbial diversity in production waters of a low-temperature biodegraded oil reservoir
AB237699 Uncultured bacterium	Bacteria; environmental samples	16S rRNA of uncultured microorganisms from deep subsurface groundwater
AF280841 Uncultured bacterium mle1-2	Bacteria; Bacteroidetes; environmental samples.	Phylogenetic analysis of bacterial communities in mesophilic and

		thermophilic bioreactors treating pharmaceutical wastewater
DQ256318 Uncultured bacterium clone EV818BHEB5102502SAR27F86	Bacteria; environmental samples.	The distribution of microbial taxa in the subsurface water of the Kalahari Shield, South Africa
AY949860 Bacteroides sp. strain Z4	Bacteria; Bacteroidetes; Bacteroidetes Bacteroidales; Bacteroidaceae; Bacteroides.	Bacteroides sp. strain Z4, from paper mill waste water
cw56	Clone	
AY212538 Uncultured bacterium clone 5.35	Bacteria; environmental samples.	Assessment of equine fecal contamination: the search for alternative bacterial source-tracking targets
Cw517	Clone	
Cw22	Clone	
AY996586 Uncultured Bacteroidetes bacterium clone KM8	Bacteria; Bacteroidetes; environmental samples.	Indicators of petroleum hydrocarbon biodegradation in anaerobic granitic groundwater
DQ211476 Uncultured bacterium clone nsc126	Bacteria; environmental samples	Bacterial Community Structure in Natural Circulation System (Shimanto River System) by Using Clone Library Analysis
AJ488070 Uncultured bacterium	Bacteria; environmental samples.	Development and initial population analysis of stable bacterial consortia removing predominantly singly flanked chlorine substituents from chlorobenzenes
AY212569 Uncultured bacterium clone 118ds10	Bacteria; environmental samples.	Assessment of equine fecal contamination: the search for alternative bacterial source-tracking targets
AB078842 Paludibacter	Bacteria; Bacteroidetes;	Phylogeny of

propionicigenes	Bacteroidetes (class); Bacteroidales; Porphyromonadaceae; Paludibacter.	numerically abundant culturable anaerobic bacteria associated with degradation of rice plant residue in Japanese paddy field soil
Cw59	Clone	
Cw53	Clone	

5.4 Discussion and Conclusions

The phylogenetic tree shown in Figure 5.4 confirms the presence of purple sulphur bacteria such as *Thiocapsa roseopersicina* and *Thiorhodococcus drewsii*. *Thiocapsa roseopersicina* has been documented to grow in brackish and marine environments as well as fresh water, these bacteria grow under anoxygenic conditions.

Many clones were found to be related to the *Chromatiaceae*, the purple sulphur bacteria, e.g. *Chromatium*. These are short, Gram-negative rods, ~1 µm in diameter and 3-4 µm long. They are able to use sulphur and sulphide as the sole photosynthetic electron donor and sulphur can be oxidized to sulphate. These bacteria use an inorganic sulphur compound, such as hydrogen sulfide as an electron donor. Purple sulphur bacteria must fix CO_2 to live, whereas non-sulphur purple bacteria can grow aerobically in the dark by respiration on an organic carbon source. Sulphur granules are stored inside *Chromatium* cells, they are visible in light microscopic examination as bright crystals and are deposited as a by-product of photosynthesis.

A clone similar to *Rhodobacter sp* was identified. *Rhodobacter sp.* are purple nonsulfur photosynthetic bacteria, belong to the a-subdivision of the *Proteobacteria*. This group of bacteria are among the most metabolically diverse organisms known, being capable of growing in a wide variety of growth conditions. They possess an extensive range of energy acquiring mechanisms including photosynthesis, lithotrophy, aerobic and anaerobic respiration. They are also known to fix molecular nitrogen, synthesize important tetrapyrroles, chlorophylls, heme, and vitamin B12. *Rhodobacter* sp. is a

gram-negative organism with ovoid cells that are 0.65 to 0.73 μm wide and 0.95 to 1.4 μm long and have a single subpolar flagellum and an intracytoplasmic membrane arrangement typical of *Rhodobacter* species. The *Rhodobacter* sp. cells contain both light-harvesting I and light-harvesting II photocomplexes and the B800-850-type antenna complex, as well as carotenoids, all of which are characteristic of purple non-sulfur photosynthetic bacteria.

Many of the clones were found to be related to uncultured bacteria, these bacteria were all originally isolated from anaerobic environments.

The molecular results indicate the presence of a dominant population of purple sulphur bacteria together with other photosynthetic bacteria present in the pond. Also a number of other anaerobic species were present.

The results of the molecular microbial ecology study accord well with observations of the establishment of a floating sulphur biofilm on the surface of the AFP, and the production of elemental sulphur which settled into the sludge on the bottom of the pond. The population groups of the micro-organisms identified in this system also correspond quite closely in their trophic relationships to those identified in a detailed structural/functional study of floating sulphur biofilms undertaken by Molwantwa *et al.* (2007).

6. METAL PRECIPITATION STUDIES

6.1 INTRODUCTION

The aim of this study was to evaluate the use of the sulphide stream generated in the AFP reactor for the precipitation of metals in the AMD influent to the ASPAM process. Given the complexity of the system, and the range of possible variables to be controlled, it was decided to focus the study on the formation of metal sulphide precipitates and the removal specifically of iron as the major heavy metal ion most commonly present in AMD. This would enable its removal directly in the ferrous form, without having to first oxidise the ferrous to the ferric iron form. This would offer important process advantages in AMD treatment. For ease of study a synthetic mine water was used and the alkalinity available in the HRAP was assumed. Also due to the large number of initial optimization studies that were required, it was decided to undertake this work at bench-scale rather than as a metal removal unit operation as a component of the pilot plant.

Developments here could have a wider generic application potential in other sulphate reducing process, such as the Rhodes BioSURE® Process, where sulphide removal was required following the reduction reaction in order to linearise the sulphate removal operation. The opportunity to evaluate metal sulphide formation as a means of treating these wastewaters arose with the Rhodes BioSURE® Process pilot plant treating wastewaters from the Grootvlei Mine in Springs, Gauteng Province. Here iron hydroxide precipitates were recovered from the High Density Sludge process in operation at the mine and applied in these studies.

6.2 Materials and Methods

6.2.1 Reagents

All the reagents and the standards used were of analytical grade and included: ferrous sulphate anhydrous ($FeSO_4$), calcium hydroxide ($Ca(OH)_2$), sodium sulfide nanohydrate

($Na_2S.9H_2O$) and nitric acid (HNO_3) from Merck Chemicals (Pty) Ltd. Sulfide test kit # (1.14779.0001), sulfate test kit # (1.14791.0001), iron cell test # (1.14896.0001) and iron test # (1.00796.0001) used for the determination of ferrous and ferric iron were obtained from Merck KGaA, Darmstadt, Germany. The concentrations of the reagents were initially modified throughout the course of the experiment until the optimum removal was achieved. Both the ferrous solution and the final effluent were continuously monitored for pH as well as the flow rates of the ferrous solution and the lime-sulfide mix. All necessary precautions were taken to maintain the reduced iron in its ferrous form. The mine water was sparged continuously with nitrogen throughout the course of the experiment, as this helped minimise oxidation of ferrous to ferric iron. Sample solutions were taken from a valve inserted just before the influent discharged into the settler (Figure 6.1) and analysed for pH, total iron and sulfide.

Alkalinity and pH were measured manually according to Standard Methods (APHA 1998). Alkalinity was measured by titrating the sample solution to pH 4.3 using 0.1 N HNO_3. The pH was measured using a WTW pH-Electrode Sentix 41, probe temperature transducer attached to a WTW pH 330, pH meter.

Sulphate and sulfide were analysed with Merck photometric test kits # 1.14791.0001 and # 1.14779.0001 (Merck KGaA, Darmstadt, Germany) respectively, and the absorbance was read with Merck Nova 60 Spectroquant® at a wavelength of 665 nm.

Ferric iron and ferrous iron were analysed using Merck test kits # 1.14779.0001 (1.0-50.0 mg/l Fe) and # 1.00796.0001 (0.001-5.00 mg/l Fe) with a bench-top Aquamate ThermoSpectronic and the total iron was determined with a Varian SpectraAA atomic absorption spectrophotometer.

6.2.2 Experimental set-up and system configuration

The laboratory-scale settler was constructed from Perspex with a total volume of 20 litres and had a cone shape to facilitate even settling and recycling of sludge (Figure 6.1). A ferrous solution made up from ferrous sulphate anhydrous, which represented the acid

mine water, was introduced into the settler through silicone tubing (length 5.90 m) into which was dosed a calcium hydroxide-sodium sulfide mix. The ferrous solution was fed at a flow rate of 214 ml/min and the calcium hydroxide-sulfide mix at a flow rate of 15 ml/min using a digital peristaltic pump (Watson Marlow, 505S). Nitrogen gas was sparged through rigid silicone gas tubing with an adjustable AFROX multi-stage series 9500 regulator, to prevent the oxidation of ferrous iron to ferric iron. A valve was fitted just before the reaction mix and the precipitate entered the settler which allowed samples to be drawn for analysis.

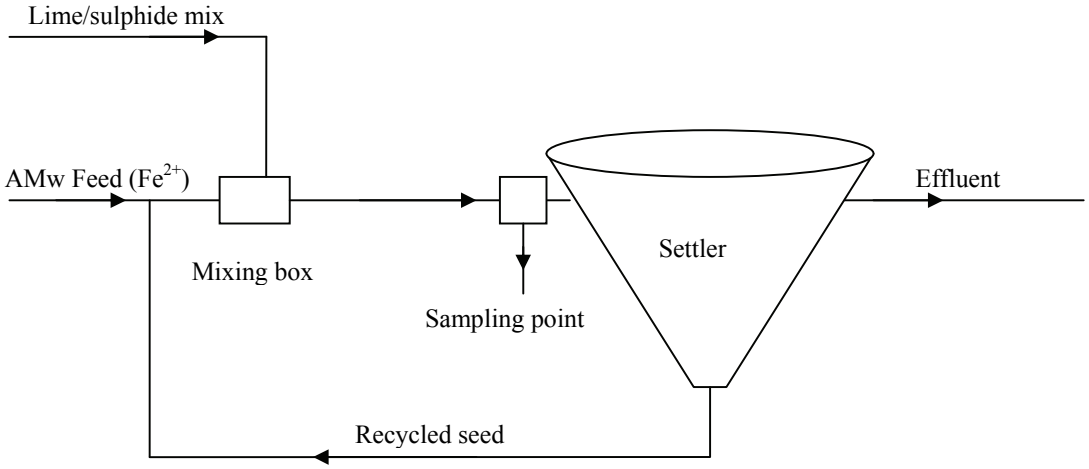


Figure 6.1: Schematic diagram of the experimental setup and reactor configuration

Samples used for the determination of sulfate, sulfide and alkalinity were allowed to stand for approximately 5-10 minutes and the supernatant solution was filtered through Whatman® GF/A glass microfibre filters (110 mm Ø, Cat no. 1820110, Int Ltd England) and the filtrate refiltered through a 0.45 µ membrane filters (Magna, Nylon, Supported, Plain, 0.45 µ, 25 mm, cat no. R04SP02500, Osmonics Inc.). The prefiltration through the Glass Microfibre Filters was necessary to reduce binding of the 0.45 µ filters. The filtrates were acidified with GR ISO nitric acid (65%) to pH < 2 and analysed for total iron.

In the studies on the removal of sulphide from the BioSURE ® Process, iron hydroxide from the High Density Sludge process at Grootvlei mine was sourced.

6.3 Results and Discussions

6.3.1 Batch iron removal in the presence of calcium hydroxide and sulfide

These experiments were carried out to determine the optimal amount of lime and sulfide needed to precipitate the ferrous iron from solution. The initial ferrous iron concentration was 1000 mg/l and the sulfide varied from 1.0-2.0 g/l. The $Ca(OH)_2$ was however, kept constant at a concentration of 100 mg/l. The results are presented in Figure 6.2.

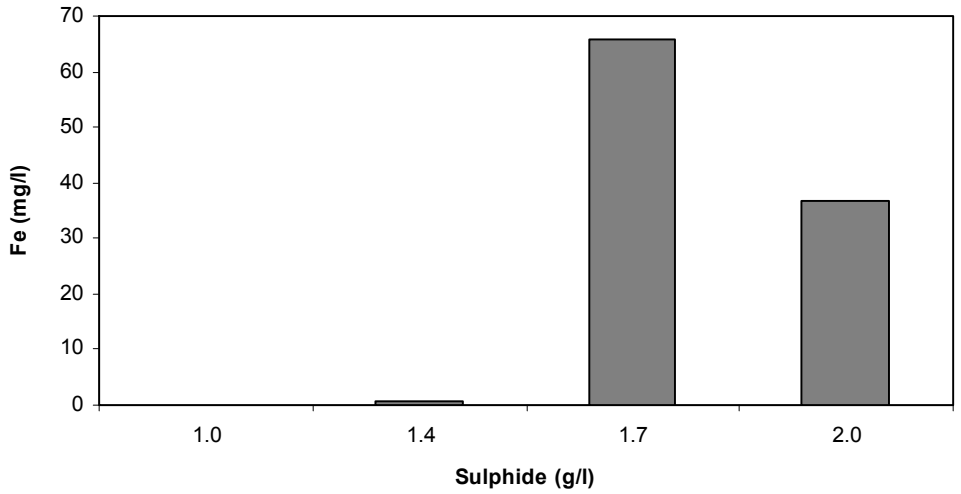


Figure 6.2: Precipitation of iron with 100 mg/l calcium hydroxide and varying concentrations of sulphide

Iron was completely precipitated at a sulfide concentration of 1.0 g/l and $Ca(OH)_2$ of 100 mg/l. Less iron was, however, removed with increasing sulfide concentration, with the least removal observed at a sulfide to iron ratio of 1.7:1.0 g/l. Further experiments were carried out to investigate the effect of $Ca(OH)_2$ only in the absence of sulfide on the precipitation of iron and these results are presented in Figure 6.3.

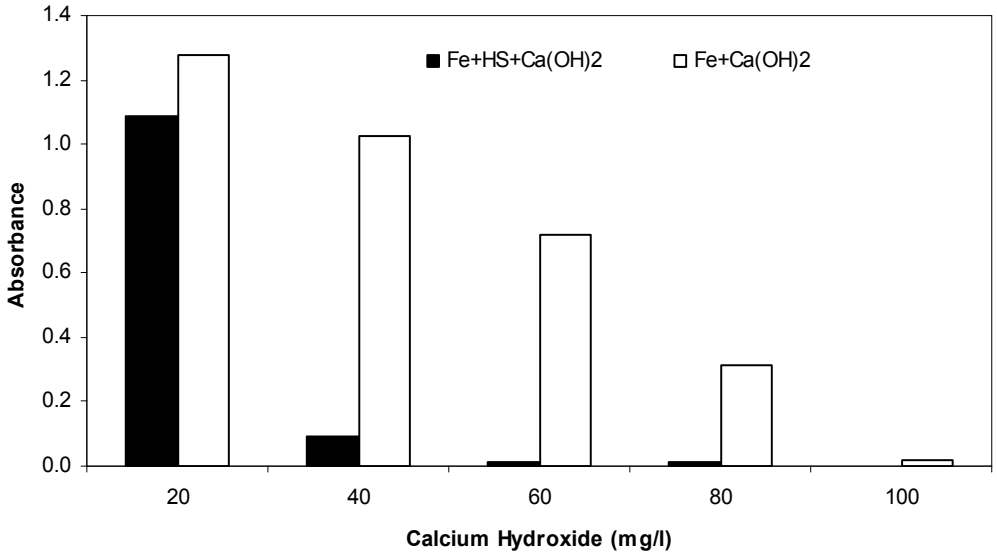


Figure 6.3: Effect of sulfide and calcium hydroxide mix and calcium hydroxide only on the precipitation of iron

The results indicated that improved iron removal was achieved in the presence of sulphide and $Ca(OH)_2$ as compared to $Ca(OH)_2$ alone. Further experiments carried out with increasing and varying concentrations of both $Ca(OH)_2$ (20-60 mg/l) and sulfide (0.6-1.6 g/l) indicated that best iron removal was achieved at a lime concentration of 60 mg/l and sulfide concentration of 0.6 g/l (Figure 6.3 and Figure 6.4).

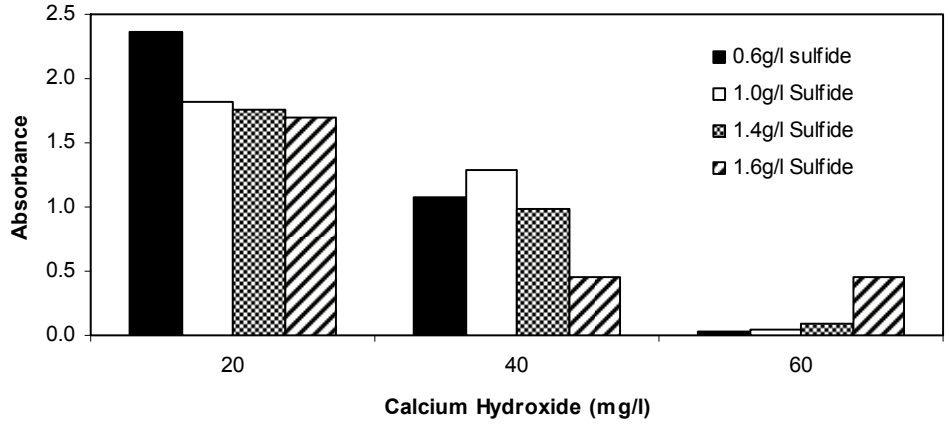


Figure 6.4: Effect of varying concentrations of calcium hydroxide and sulfide

Results shown in Figure 6.5, illustrates that at a $Ca(OH)_2$ concentration of 50 mg/l the best metal removal was achieved with a dosing regime of sulfide and $Ca(OH)_2$ (L/S) mix, compared to a dosing order of $Ca(OH)_2$ followed by sulfide (LS) and of sulfide followed by $Ca(OH)_2$ (SL).

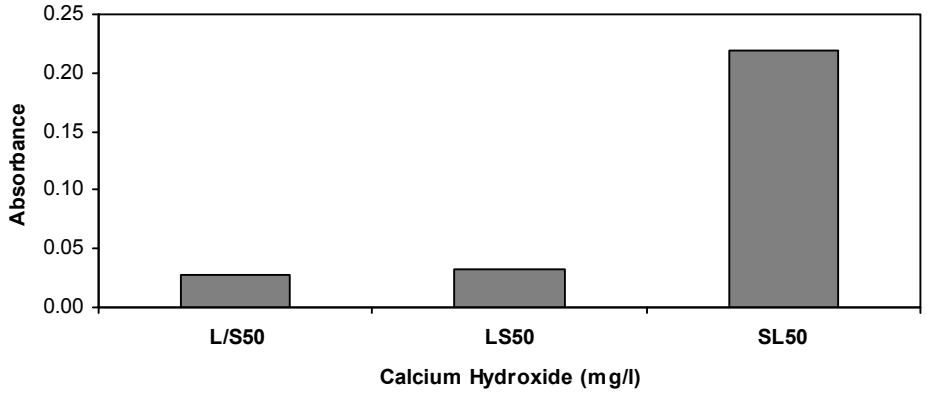


Figure 6.5: Effect of dosing order on metal precipitation

For optimum precipitation to be achieved it is, however, imperative that the order of dosing be taken into consideration as this significantly influences the metal precipitation.

6.3.2 Continuous precipitation of iron using calcium hydroxide and sulfide

Removal of iron was achieved in the continuous experiment by dosing the lime/sulfide mix into a stream of ferrous iron solution and it was allowed to react in a 5.9 m length of silicone tubing for a few seconds before reaching the settler. Samples were collected and filtered through the GF/A glass microfibre filters and the filtrate refiltered through a 0.45 µ membrane filters. The final filtrate was used for the determination of total iron, sulfide and pH and is presented in Figure 6.6 and Figure 6.7. Less than 1.0 mg/l of total iron was detected at lime concentrations of 1500-5000 mg/l. The pH was high in the effluent and was in the range of 7.65-11.79. This is. however, a suitable pH at which most of the iron will be expected to be precipitated from the solution. The amount of sulfide detected in the effluent was less than 1.0 mg/l throughout the course of the experiment and indicated the formation of iron sulfide (FeS). These results were true for both Figure 6.7 and

Figure 6.8 as illustrated below.

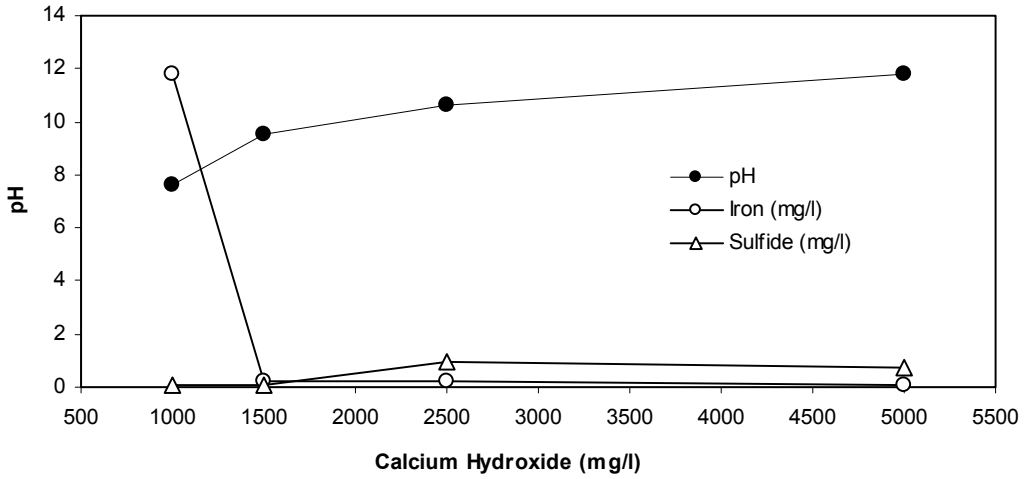


Figure 6.6: Precipitation of iron with 57 mg/l sulfide at varying calcium hydroxide concentration

Sulfide and $Ca(OH)_2$ precipitation of metals works under similar fundamental principles. In sulfide precipitation of metals, the precipitation process essentially converts soluble metal compounds into relatively insoluble sulfide compounds through the addition of precipitating agents such as sodium sulfide (Na_2S), sodium hydrosulfide (NaHS), ferrous sulfide (FeS) and calcium sulfide (CaS). This is an effective alternative technology to hydroxide precipitation for metal removal (Bhattacharyya *et al*., 1979; EPA, 1980; Ku and Peters, 1986). Sulphides (S^{2-}, HS^-) are very reactive with heavy metal ions over a wide range of pH, which makes sulfide precipitation suitable for the removal of lead, copper, chromium, silver, cadmium, zinc, mercury, nickel, thallium, antimony and vanadium from wastewaters (EPA, 1987). Increasing the sulfide ion concentration in the solution will directly result in an increase in the metals precipitated (EPA, 1987).

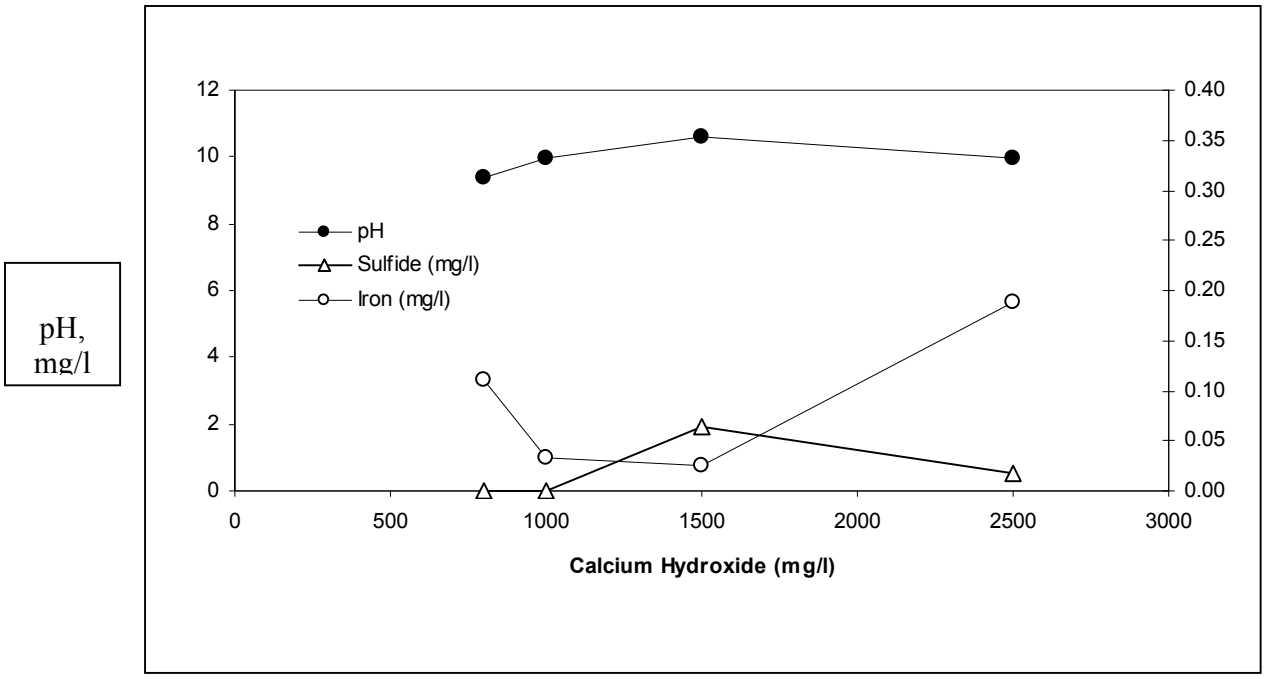


Figure 6.7: Precipitation of iron with 114 mg/l sulfide at varying calcium hydroxide concentrations

These precipitation reactions are generally induced over a pH range of 7.0-9.0. Nonetheless, metal sulfide precipitates most often require physical removal from the solution through the use of coagulants, flocculants or by means of filtration or clarification, leaving metal-sulfide sludge for subsequent disposal. The separation of metal sulphides from effluents can however, be achieved by the use of thickeners or clarifiers or a combination of both. The oxidation of the excess sulfide ions in the supernatants is achieved by employing aeration or by the addition of hydrogen peroxide. Theoretically, sulfide will precipitate metals in preferential order, that is, from lower K_{sp} to higher K_{sp} (Talbot, 1984), therefore, copper and lead with K_{sp} CuS = 1.2 x 10^{-37} and K_{sp} PbS = 7.0 x 10^{-29} respectively, will be precipitated easily compared to manganese and iron (II), with K_{sp} MnS = 7.0 x 10^{-16} and K_{sp} FeS = 4.0 x 10^{-19} respectively (Talbot, 1984).

The process of metal sulfide precipitation can be evaluated in its soluble and insoluble sulfide precipitation products. The difference between these processes lies in the manner in which the sulfide ions are introduced into the treatment process. Soluble sulfide precipitation uses water-soluble reagents (for example, NaHS or Na_2S) which crystallize

from aqueous $Na_2S.9H_2O$. High sulfide concentration in the soluble sulfide process characteristically leads to rapid precipitation of metal sulfides, which in turn results in small fine particulates and colloidal particles that have poor filterability and settling characteristics (EPA, 1987). This problem can, however, be solved with the effective use of coagulants and/or coagulant aids which result in the formation of large flocs with fast settling properties. On the other hand, insoluble sulfide precipitation process uses a slightly water-soluble FeS or CaS. This process was first patented as the "Sulfex Process" (Scott, 1993). The process removes dissolved metals by mixing the wastewater with the ferrous sulfide slurry in a liquid/solid contact chamber. A sulfide ion concentration of approximately 2 mg/l is thus maintained by dissolving the FeS in the solution (EPA, 1977). When the pH of the solution is maintained in the range of 8.5-9.0, the liberated ion will form a hydroxide as well as a precipitate. The unreacted or excess ions are then settled and/or filtered with the metal sulfide precipitate, leaving the final effluent virtually sulfide free (EPA, 1987). Ferrous sulfide has to be generated on-site from both Na_2S and $FeSO_4$ due to its instability. The following reactions (equations (1) and (2)) occur when FeS is added to a solution containing dissolved metals and metal hydroxide:

$$FeS \rightarrow Fe^{2+} + S^{2-} \quad (1)$$

$$Fe^{2+} + 2(OH)^{-} \rightarrow Fe(OH)_2 \quad (2)$$

The insoluble sulfide process, however, requires about two to four times the stoichiometric amount of FeS (EPA, 1980). The use of theses excess amounts of FeS could be advantageous since it stops the formation of biotoxic hydrogen sulfide (H_2S) due to its very low solubility. Despite its low solubility, residual sulfide levels could still be in the range of 1 to 10 µg/l. This in turns adds significantly to the overall chemical cost of the entire process as well as the sludge volumes of up to three times that of lime precipitation (Cushine, 1984).

Calcium sulfide can, however, serve as a potential alternative to FeS as a precipitating agent (Kim, 1981; Kim and Amodeo, 1983). Some of the problems associated with both the soluble and insoluble sulfide precipitation processes (such as the evolution of H_2S and

excess chemical reagents requirements), can be minimized by using CaS as the sulfide source. Solid CaS is often slurried before being used and it also produces easily settable metal precipitates and does not produce any significant volumes of sludge. Also, the calcium sulfide requirement is close to stoichiometric unlike FeS (EPA, 1977). Calcium sulfide is only stable in the solid form and reacts in aqueous solutions with water to produce calcium hydrogen sulfide ($Ca(HS)_2$) and Ca(OH) as illustrated in equation (3).

$$2CaS + 2H_2O \rightarrow Ca(HS)_2 + Ca(OH)_2 \quad (3)$$

After the addition of calcium sulfide, the main reactions that precipitate the metals (M) are thus as follows (equation (4)-(6)):

$$M^{2+} + HS^- \rightarrow MS + H^+ \quad (4)$$

$$H^+ + OH^- \rightarrow H_2O \quad (5)$$

$$M^{2+} + S^{2-} \rightarrow MS \quad (6)$$

The precipitation of metals using sulfide precipitation offers numerous advantages over hydroxide precipitation and includes:

- Low metal-sulfide solubilities can be obtained.
- Good heavy metal removal is possible even with weak chelating agents. On the other hand, however, strong chelating agents such as EDTA will hinder the sulfide precipitation process although the metals will still be removed (Ku and Peters, 1985; Peters *et al*., 1985).
- Sulfide precipitation can be operated over a broad pH range (pH 2 to 12). Therefore, since metal sulfide precipitates are less amphoteric than corresponding metal-hydroxides, they are less likely to resolubilized due to changes in pH.
- The sludge produced using sulfide precipitation has been reported to dewater easily and is less subject to leaching compared to the sludges from metal-hydroxide (Peters *et al*., 1984).

There are also a few disadvantages associated with sulfide precipitation of metals. The pH of the system must be kept high enough to prevent the release of toxic and hazardous H_2S gas. The H_2S gas concentration or emission in the workplace must not exceed the 10 ppm mark set by the Occupational Safety and Health Administration (OSHA), beyond which it is considered potentially dangerous. The rate of H_2S gas evolution from sulfide solution per unit of water/air interface nevertheless, depends on the temperature of the solution, which also determines the H_2S solubility, the concentration of dissolved sulfide and the pH of the solution (EPA, 1980). The major disadvantage of sulfide metal precipitation is the generation of biotoxic and hazardous metal-sulfide sludge, which also poses an odour problem. Also, insoluble sulfide precipitation produces about three times as much sludge compared to hydroxide precipitation.

6.3.3 Continuous precipitation of iron from the ferrous solution using calcium hydroxide and sulfide

In order to apply findings to a non-synthetic mine water, a stream of ferrous solution from the Grootvlei gold mine as used and dosed continuously with a mixture of sulfide and NaOH. The sulphide stream was sourced from the Rhodes BioSURE® Process reactor located at the Ancor Works. Both the ferrous solution and the sulfide-$Ca(OH)_2$ mix were dosed at varying rates to determine the accurate stoichiometric ratio of sulfide and iron in the molar ratio of 1:1. It was acknowledged that sulfide alone is not able to precipitate the metals from the solution and this therefore necessitated the use of $Ca(OH)_2$. It was, however, imperative to keep the $Ca(OH)_2$ concentration at the lowest possible level in order to minimize costs. It was not, however, possible to determine the amount of Fe^{2+} and Fe^{3+} that was present independently. The results presented, thus, reported the total iron concentration in the system and not individual species (Fe^{2+} and Fe^{3+}). The results obtained in these series of runs are presented in Appendix 1; 1-6.

The total iron concentration varied from 75.5-85 mg/l. The pH of the raw mine water ranged from 6.34-6.53. This was relatively high compared to typical mine waters whose pH is normally <3. This high pH can be attributed to aqueous phase weak acid/base equilibrium relationships which indicates the alkalinity and the acidity of the of the blend

as well as the carbonate system depending on the total dissolved CO_2^{3-} and OH^- species. The precipitation of the iron from the solution was achieved with a blend of varying concentrations of calcium hydroxide and sulfide. This was quite high with a removal efficiency of 88.4-99.7% recorded.

The effluent blend pH was in the range of 6.7-8.9. The pH of the effluent is governed by the sulfide system species (H_2S, HS^- and S^{2-}), the carbonate system species (H_2CO_3, HCO_3^- and CO_3^{2-}) and the water system species (H^+ and OH^-). Iron hydroxide, sulfide and/or carbonate precipitate when the sulfide-rich/calcium hydroxide mix is blended with the incoming mine water rich in iron. The iron precipitated in the set pH region is controlled by the $Fe(OH)_3$ or the sulfated ferric hydroxide (Schwertman and Fetcher, 1994; Drissi *et al*., 1995).

6.3.4 Sulfide removal from sulfide-enriched BioSURE® Process effluents using HDS iron slurry

The effect of the HDS iron slurry on the efficiency of sulfide removal from the biological effluent from the reactor was examined at different concentration. The results are presented in Tables 6.1 to 6.3 Varying concentrations of HDS slurry from Grootvlei Mine was added to the biological sulfide effluent from Reactor 2 of the BioSURE Process (East Rand Water Care Company (ERWAT-ANCOR)). The mixture was stirred gently for 4 minutes in a beaker to allow for complete reaction. This was then poured into a 250 ml volumetric flask and allowed to stand and settle for 1 minute. Samples were then taken, filtered thorough GF/A microfibre filters and the filtrate was used for analysis of alkalinity, pH and sulphide (Table 6.1 and Figure 6.8).

Table 6.1: Effect of iron dosage on sulfide removal and pH

Sample	HDS sludge dosage (ml)	Sulfide (mg/l)	pH	Observation
1	1	32	8.48	Clear green
2	2	37	8.50	Clear green
3	3	26	8.56	Clear green
4	4	17	8.61	Green turbid
5	5	6	8.65	Fair green turbid
6	10	3	8.30	Turbid white
7	15	3	8.20	Turbid white

NB: Samples 1-5 filtrate formed a white precipitate at the air liquid interface, whereas, sample 6-7, formed a white precipitate throughout the column of the measuring cylinder.

The presence of a white colloidal suspension over a pH range of 8.2-8.65 indicates the presence of calcium either from the carbonates, bicarbonates or hydroxides as depicted by high alkalinity, resulting from either the underground mine water from the Grootvlei Mine or the biological effluent from the BioSURE Process reactor.

As shown in Figure 6.9 the pH of the effluent remained quite high throughout the experimental procedure and was in the range pH 8.5-8.65. This is a result of increased surface charge which raises the effluent pH thus resulting in the precipitation of iron hydroxide.

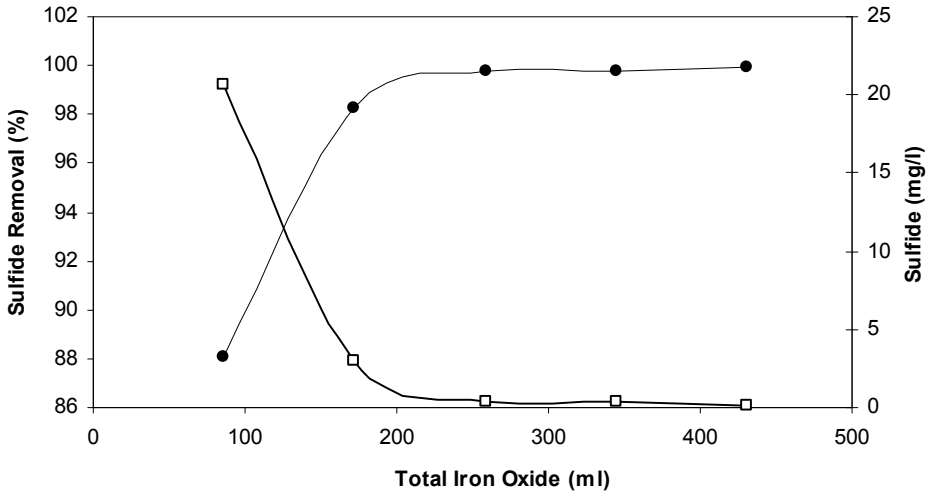


Figure 6.8: Sulfide removal with HDS sludge and iron as a 80 mg/l solution

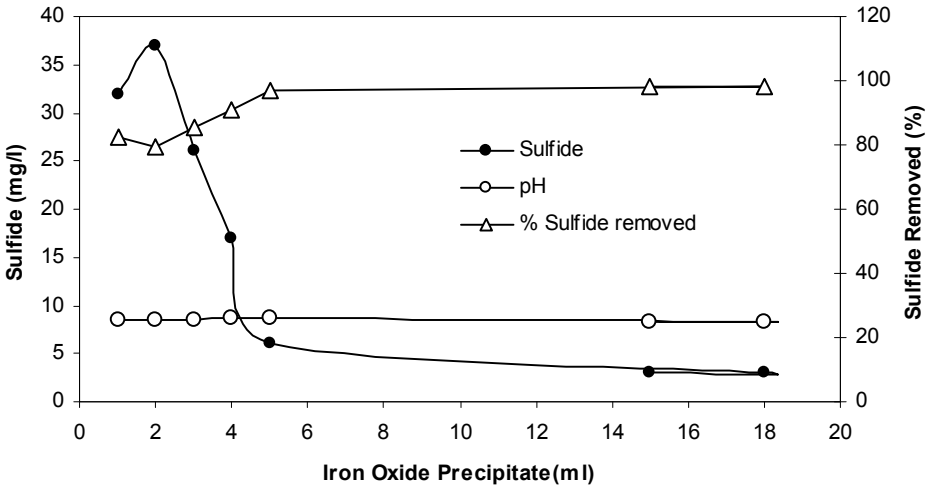


Figure 6.9: Variation of sulfide removed with the corresponding pH and iron as a 80 mg/l solution

The sulfide removal rate from the biological effluent was excellent and this was in the range of 79.6-98.3% (Figure 6.9). The concentration of the initial sulfide in the reactor effluent was reduced from the initial 182 mg/l to as little 3.0 mg/l. This was achieved using Fe_2O_3. This therefore illustrates an efficient and novel method for discarding or removing the excess sulfide that is produced by the BioSURE Process. The pH of the

effluent was in the range of pH 8.2-8.65, well above the minimal pH required for the optimal iron removal (Table 6.2).

Table 6.2: Effect of HDS sludge dosage on sulfide removal, pH and alkalinity

HDS sludge (ml)	Sulfide (mg/l)	pH	Alkalinity mg $CaCO_3$ /L)	Colour
0	183	7.73	1312	Clear green
4.8	132	8.18	1237	Green
7.2	98	8.22	1145	Green
9.6	85	8.32	1076	Green
12.0	65.25	8.33	1059	Green
14.4	42.5	8.30	1014	Green
16.8	23.25	8.30	971.4	White precipitate
19.2	15	8.29	914	White
21.6	4.25	8.16	852.8	White

Preparation of flocculent solution

Five grams (5 g) of Yang floc (synthetic polymer) was dissolved in 1000 ml of water (0.5%) and allowed to stand for one hour to enable full maturation of the floc. The solution was then diluted ten times to obtain a 0.05%, solution which was the desired final concentration. The results obtained after the addition of the flocculant are presented in Table 6.10. It was quite evident at this stage that the floc formed had good aggregating and settling properties compared to the runs with no addition of flocculants. The total iron concentration in the final effluent was 1.21 mg/l (Table 6.3) representing a removal efficiency of 99.96%. It was evident that the flocculant contributed significantly to improving the settleability of the FeS precipitates.

Table 6.3: Effect of flocculant total iron removal

HDS Sludge (ml)	pH	Alkalinity mg $CaCO_3$ /L)	Sulfide (mg/l)	Fe (mg/l)	Observation
0	7.64	430	207	104	No biological effluent
12.0	6.62	554.2	6.62	1.21	Settles well
14.4	6.53	550.11	6.53	8.9	Settles well
16.8	6.62	488.76	6.52	27.6	Settles poorly
19.2	6.69	429.45	6.69	46.3	Settles very poorly
21.6	7.29	930.5	7.29	4.3	Settles poorly

NB: 0.3 ml polyacrylamide flocculant added to 300 ml biological effluent

The pH of the reaction mixture remained constant throughout the course of this run and generally above pH 6.0, and is attributable to the high alkalinity. However, results reported in Table 6.3 showed a slight decrease in alkalinity due to a demand in alkalinity by the dosing of HDS sludge. The high alkalinity is due to the supplemental influent alkalinity from the sulphide reactor and reflects a well buffered system. The results obtained in the current study also indicate that increasing the amount of HDS sludge used also increases the removal efficiency of sulfide from the biological effluent. It can thus be deduced that the amount of sulfide removed from the reactor effluent is directly proportional to the amount of the HDS sludge used. However, a general decrease in alkalinity was observed. According to Loewenthal *et al*. (1986), the oxidation of ferrous to ferric ions involves a gain in alkalinity with a theoretical value of 0.89 mg as $CaCO_3$/mg Fe and this also explains the high alkalinity obtained in these studies. In Table 6.3, a decrease in alkalinity with increasing concentration of HDS sludge was observed. This is because the precipitation of ferric hydroxide ($Fe(OH)_3$ and iron sulfide placed a net demand on alkalinity, consequently resulting in a net loss of alkalinity with a theoretical value of -0.92 mg as $CaCO_3$/mg $FeCl_3$ or -2.67 mg as $CaCO_3$/mg Fe

(Loewenthal *et al*., 1986). The data obtained in the current studies is therefore in agreement with the theoretical loss of alkalinity. This high alkalinity observed in the current study could also be due to excess $Ca(OH)_2$ in the Grootvlei water which helps to neutralize the AMD.

Figure 6.10 shows the effect of varying $Ca(OH)_2$ on the total iron precipitated and the pH (Figure 6.11). A black precipitate was obtained reflecting the presence of FeS. The precipitate was, however, very unstable and reverted to the formation of a reddish-brown compound often after exposure to air for about two hours, and suggesting the presence of pyrite (iron II sulfide) and magnetite. This has severe environmental consequences as the FeS undergoes a series of geochemical reactions which results to the contribution of toxic heavy metals ions into mine waste waters. In these series of experiments, the results obtained in terms of iron removal was very poor with just about 50% of the iron removed in the solution. The results indicate that not all the iron was precipitated either as FeS or $Fe(OH)_3$.

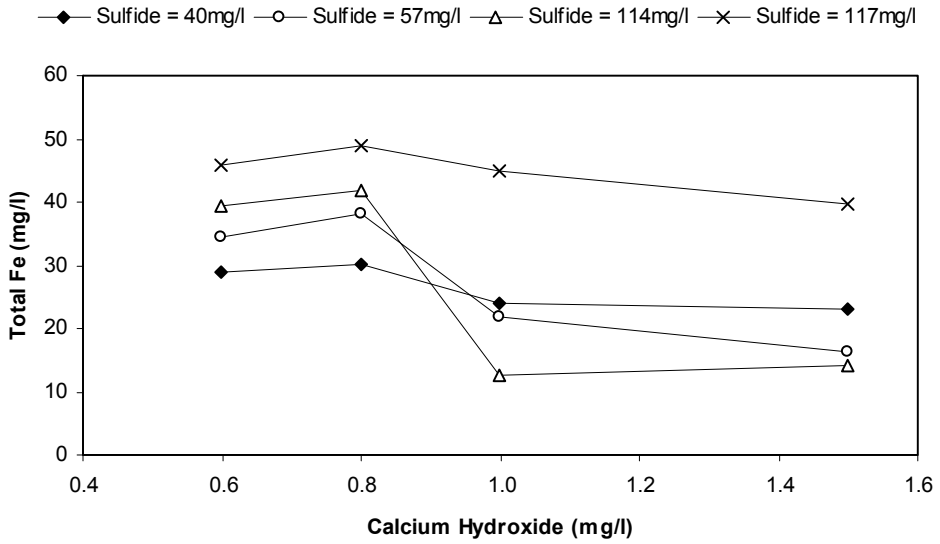


Figure 6.10: Effect of varying concentrations of sulfide and calcium hydroxide on iron removal

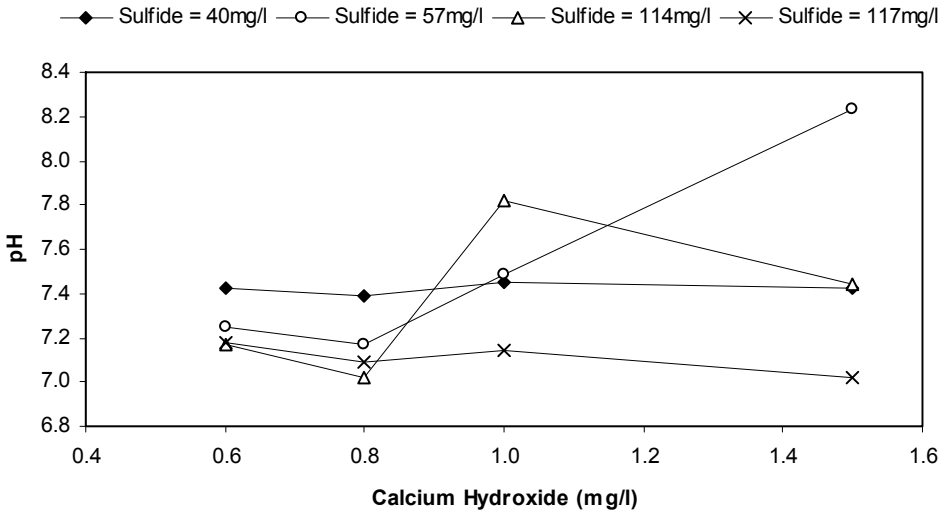


Figure 6.11: Effect of varying concentrations of sulfide and calcium hydroxide on pH

Magnetite can be used to effect the removal of ferrous metal and subsequent stabilisation through cation substitution with the major advantage being low oxygen requirements. The properties of magnetite have been exploited in recent years as a means to remove metals ions from waste streams. In the presence of heavy metals and with limited oxidation the stoichiometric reactions for ferrite formation from ferrous at pH > 10 are shown in equation (7) (Barrado *et al*., 1998):

$$xMe^{+n} + 3Fe(OH)_2 + \tfrac{1}{2}O_2 \rightarrow Me_xFe_{3-x}O_4 + xFe^{+n} \qquad (7)$$

where Fe^{+n} is the total concentration of iron replaced by heavy metal cations.
However, in the presence of excess oxidizing agents, goethite is formed as a replacement for magnetite as depicted by equation (8).

$$2Fe^{2+}\ \tfrac{1}{2}O_2 + 4OH^- \rightarrow 2\alpha - FeOOH + H_2O \qquad (8)$$

The formation from ferrous solutions of reasonably pure precipitates of magnetite is thought to be only possible in temperature ranges >90^{o}C (Cornell and Schwertmann, 1996).

While calcium hydroxide is presumed to be the cheapest pH elevating agent, and was used in the current study, it is believed to interfere with ferrite formation (Kampies *et al*., 1996; Wang *et al*., 1996; McKinnon *et al*., 2000). The incorporation of calcium into the crystal lattice of magnetite has been reported (Cornell and Schwertmann, 1996) and thought to have an inhibitory effect since the calcium ions disrupts the ferrite formation (Wang *et al*., 1996; Perez *et al*., 1998). Nevertheless, this argument is not definitive and should be taken with a lot of caution since the mechanism of ferrite formation has not yet been properly explained (Radenkovick *et al*., 1995; Cornell and Schwertmann, 1996).

The precipitation of iron using calcium hydroxide at pH greater than 7.9 resulted in the formation of a green precipitate. This occurs when the ferric and total iron are in the region of 10-20% and more than 3 mg/l dissolved oxygen in solution. However, further studies are needed to determine the exact nature of the constituents and characteristics of the floc formed. The literature, however, indicates that this precipitate is probably green rust, a Fe^{2+}/Fe^{3+} sulphated hydroxide ($Fe_2^{III}SO_4(OH)_{12}$) (Olowe *et al*., 1988). The co-precipitation of $Fe(OH)_3$ and $Fe(OH)_2$, a portion of which is transformed to green rust, would be expected. Green rust has been prepared synthetically in the laboratory from ferrous species and sulphate ions (Olowe and Génin, 1991; Génin *et al*., 1996) and from ferrous species and carbonate ions (Taylor, 1980; Hansen, 1989; Drissi *et al*., 1995).

In conclusion, it was clearly established that iron precipitation was dependent on pH and, at pH greater than 8, iron precipitation achieved was 99%+. However, it was also established that very minimal precipitation was achieved with sulfide addition alone and the use of lime was imperative to achieve any substantial precipitation. Flocs formed after dosing the blend of HDS sludge and the sulphide effluent with a flocculant (Yang floc) showed excellent aggregation, agglomeration and settling characteristics. These flocs were visually better than those obtained with previous experiments and excluded the formation of pin flocs. The alkalinity of the system was very high indicating a well buffered system. Further investigation, however, needs to be carried out to gain a better understanding of the biochemical and chemical processes taking place in this system as well as to accurately characterise the floc formed.

7. CONCLUSIONS

The initial studies and development of the ASPAM concept had been undertaken as bench-top investigations (Rose *et al*., 2002) and the current project provided the first opportunity to investigate aspects of the system at pilot scale.

The operation of the anaerobic unit of the system functioned well, producing sulphide through the sulphate reduction reaction process. This was then oxidized through the various oxidation states of sulphur and reported to the various downstream compartments. Approximately 10% of the total influent sulphur could not be accounted for as either $H_2S(g)$, HS^- or SO_4^{2-} between the feed and the AFP. Evidence of elemental sulphur in the settled sludge suggests this may be where the residual sulphur is located. Losses to atmosphere were low.

The performance of the floating sulphur biofilm, and of photosynthetic bacteria present in the AFP water column, as an effective sulphide oxidation and sulphur removal operation, was an unexpected observation in the study. Although its operation was not a principal objective of the study, and was thus not the subject of separate optimization, it is evident that this could offer important advantages in the overall treatment of sulphate wastewaters in the ASPAM system. Given the substantial advances made in the operation of the Floating Sulphur Biofilm Reactor developed in WRC Project 1545, it is evident that a basis has been demonstrated here for potentially dealing with the technological bottleneck relating to sulphur removal following the sulphate reduction reaction in the biological treatment of mine wastewaters. This should be the subject of energetic follow-up.

Feeding of sewage sludge in small volumes to the pilot plant proved to be a severe constraint on the successful operation of the system and in future process designs adequate budgetary provision will need to be made to enable this operation. This problem impacted on a number of other areas of the study including the successful operation of the HRAP and the use of algal-alkalinity in the metal removal operation studies.

The metal removal studies reported here demonstrated, not only the removal of metals in a synthetic influent stream, but showed that metal hydroxide precipitates may be used to remove sulphides from the final stream of a biological AMD treatment operation. This work was the subject of scale-up evaluation to full-scale operation as a unit process in the Rhodes BioSURE® Process industrial-scale plant at the Grootvlei Mine/ERWAT Ancor Works plant where 10 Ml/day mine wastewaters are treated.

The molecular microbial ecology study of the AFP has provided a valuable insight into the system in operation here, and the principle organisms involved. This will provide useful information where more accurate kinetic studies are undertaken to indicate the various processes at work in this unit operation.

7.1 RECOMMENDATIONS

A number of recommendations can be made on the basis of the outcomes achieved in the studies reported here:

1. The basic concept of using algal ponding systems in general, and the IAPS design in particular, as a process design basis for the extensive, and possibly low-cost, treatment of mine drainage wastewaters, has been demonstrated. In the light of the urgent need for technological intervention in this area, as evidenced by environmental requirements for mining companies to achieve sustainable mine closure, it is recommended that the system be the subject of further scale-up evaluation;
2. The metal removal study reported here has already been the subject of up-scale evaluation to a full-scale process at the Rhodes BioSURE® Process 10 Ml/day plant in operation at Grootvlei Mine/ERWAT Ancor Works. It is recommended that studies on the linkage of this system with algal alkalinity generated in the HRAP be continued given the need for extensive low-cost applications in this area;
3. The potential for sulphur removal in the system was unexpected and may link

well with the parallel development of the Floating Sulphur Biofilm Reactor undertaken in WRC Project K5/1545. This presents a severe technological constraint on the further development of passive and extensive active biological AMD treatment operations. It is recommended that this potential be followed up energetically in follow-up development studies.

8. REFERENCES

ALTSCHUL, STEPHEN F., THOMAS L. MADDEN, ALEJANDRO A. SCHÄFFER, JINGHUI ZHANG, ZHENG ZHANG, WEBB MILLER, AND DAVID J. LIPMAN(1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

AMANN, R. I.; LUDWIG, W. AND SCHLEIFER, K.H. 1995. Phylogenetic Identification and *in situ* Detection of Individual Microbial Cells Without Cultivation. *Microbiol. Rev*. **59**: 143-169.

ANDERSON, W.C. (1994). (ed.), Innovative Site Remediation Technology, *Chemical Treatment*. American Academy of Environmental Engineers.

APHA, AWWA, and WEF (1998). Standard Methods for the Examination of Water and Wastewater, 20th ed. *American Public Health Organisation*, Washington DC, USA.

BARNES, I. AND CLARKE, F.E. (1964). Geochemistry of ground water in mine drainage problems. *Geological Survey Professional* Paper 473-A, 6p.

BARRDO, E., PRIETO, F., VEGA, M. AND FERNANDEZ-POLANCO, F. (1998). Optimisation of the operational variables of a medium scale reactor for metal containing wastewater purification by ferrite formation. *Water Research*, 32(10):3055-2061.

BHATTACHARYYA, D., A.B. JUMAWAN, AND R.B.GRIEVES, (1979). Separation of toxic heavy metals by sulfide precipitation. *Sep. Sci. Technol*., **14**:441-452.

BLESA M.A. AND MATIJEVIC, E. (1989). Phase transformation of iron oxides, oxo-hydroxydes and hydrous oxides in aqueous media. *Advances in colloid and interface Science*, **29**:173-221.

BLOWES, D.W. AND PTACEK, C.J. (1994). Acid-neutralization mechanisms in inactive mine tailings. In:Blowes, D.W., Jambor, J.L. (Eds.)., Environmental

geochemistry of sulphide-mine wastes. In: Short Course Handbook, vol. 22. *Mineral Association of Canada, Waterloo*, pp. 271-292.

BOND, P.L.; SMIRGA, S.P. & BANFIELD, J.F. (2000). Comparison of Acid Mine Drainage Microbial Communities in Physically and Geochemically Distinct Ecosystems. *Appl. Envir. Microbiol*. **66**: 3842-3842.

BOSHOFF, G.A. 1998. Development of Integrated Biological Processing for the Biodesalination of Sulphate- and Metal-rich Wastewaters. PhD Thesis, Rhodes University, Grahamstown.

BOWKER, M. 2002. The Microbial Ecology of Floating Sulphur Biofilms. *Rhodes MSc. Thesis.*

CORNELL R.M. AND SCHWERTMANN, U. (1996). The iron oxides structure, properties, reactions occurrences and uses. VCH Germany.

CUSHNIE, G.C. (1984). Removal of metals from wastewater: Neutralization and precipitation. *Pollution Technology Review*, No. 107, Noyes Publications, Park Ridge N.J.

DAVEY, M.E. AND O'TOOLE, G.A., 2000. Microbial Biofilms: From Ecology to Molecular Genetics. *Microb. Molecular Biol Rev.* 64(4): 847-867.

DRISSI, S.H., REFAIT, P.H., ABDELMOULA, M. AND GENIN, J.M.R. (1995). The precipitation and thermodynamic properties of Fe(II) and Fe(III) hydroxide-carbonate (green rust (II)), Poubaix diagram of iron carbonate containing aqueous media. *Corrosion Science*. **37**(12):22025-2041.

ENONGENE, G.N. (2004). A novel method for the precipitation of ferrous iron and the removal of sulphide from the Rhodes BioSURE effluents. *EBRU internal report*.

EPA (1987). Technical Resource Document, Treatment Technologies for Metal/Cyanide-Containing Wastes. *Hazardous Waste Engineering Research Laboratory*, NTIS Order Number PB 38-143896. Washington, D.C.

EPA 600/2-77-049 (1977). Treatment of metal finishing wastes by sulfide precipitation. Washington, D.C.

EPA 625/8-80-003 (1980). Summary Report: Control and Treatment Technology for the Metal Finishing Industry; Sulfide Precipitation, Technology Transfer Division, Washington, D.C., 1980.

FENG, D., ALDRICH, C. AND TAN, H. (2000). Treatment of acid mine water by use of heavy metal precipitation and ion exchange. *Minerals Engineering*, **13**(6), 623-642.

GARLICK, S., OREN, A, PADAN, E. (1977). Occurrence of facultative anoxygenic photosynthesis among filamentous and unicellular cyanobacteria. *J. Bacteriol*. 129: 623-629.

GÉNIN, J.M.R., OLOWE, A.A., REFAIT, PH., AND SIMON, L. (1996). On the stoichiometry and Pourbaix diagram of Fe(II)-Fe(III) hydroxy-sulphate-containing green rust 2: an electrochemical and Mössbauer spectroscopy study. *Corrosion Sci*., **38**:1751-1762.

GOEBEL, B M. AND STACKEBRANDT, E. 1994. The biotechnological importance of molecular biodiversity studies for metal bioleaching. In: Priest F G, Ramos-Cormenzana A, Tindall B J. , editors; Priest F G, Ramos-Cormenzana A, Tindall B J. , editors. Bacterial diversity and systematics. New York, N.Y: Plenum Press; pp. 259-273.

GRAY, N.F. (1997). Environmental impact and remediation of acid mine drainage: a management problem. Environmental Geology 30, 62-71.

GRAY, N.F. (1998). Acid mine drainage composition and the implications for its impact on lotic systems. *Water Research*. **32**(7):2122-2134.

HANSEN, H.C.B. (1989). Composition, stabilisation and light absorption of Fe(II)Fe(III) hydroxyl-carbonate ("green rust"). *Clay Miner*, **24**:663-669.

HEAD, I.M.; SAUNDERS, J.R. AND PICKUP, R.W. **(**1998). Microbial Evaluation, Diversity and Ecology: a Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. *Microb. Ecol.* **35:** 1-21.

HEUER, H.; KRSEK, M.; BAKER, P.; SMALLA, K. AND WELLINGTON, E.M.H. **(**1999). Analysis of *Actinomycete* communities by Specific Amplification of Genes Encoding 16S rRNA and Gel-Electrophoretic Separation in Denaturing Gradients. *Appl. Environ. Microbiol.* **63:** 3233-3241.

JOHNSON, D.B. (2000). Biological removal of sulphurous compounds from inorganic wastewaters. In: Lens, P.N.L. Pol, L.H. (Eds.), Environmental Technologies to Treat Sulfur Pollution: Principles and Engineering. *IWA Publishing, London, UK*. Pp. 175-205.

KAMPIES, P., FRANZREB, M. AND EBERLE, S.H. (1996). Conditions of the formation of zinc-bearing ferrites in regard of heavy metal removal from wastewater by magnetic separation. *Acta. Hydrochim. Hydrobiol*. **24**(2), 61-67.

KIM, B.M. (1981). Treatment of metal containing wastewater with calcium sulfide. In *AIChE Symposium Series, Water*, **77**(209): 39-48.

KIM, B.M. AND P.A. AMADEO, (1983). Calcium sulfide process for treatment of metal-containing wastes. *Environ. Prog*., **2**(3): 175-180.

KU, Y., AND R.W. PETERS (1986). The effect of weak chelating agents on the removal of heavy metals by precipitation processes. *Environ. Prog*., **5**(3): 147-153.

LAHAV O, MORGAN, B.E., HEARNE, G. AND LOEWNTHAL, R.E. (2003). One-step ambient temperature ferrite process for the treatment of acid mine drainage waters. *Journal of Environ. Eng.* **129**(2), 155-161.

LOEWENTHAL, R.E., WIECHERS, H.N.S. AND MARAIS, GvR (1986). Softening and Stabilization of Municipal waters. *Water Research Commission*, Pretoria, June 1986.

LOWSON, R.T. (1982). Aqueous oxidation of pyrite by molecular oxygen. *Chem. Rev*. **82**, 461-497.

MARA, D.D., PEARSON, H.W., SILVA, S.A. (1996). Waste Stabilisation Ponds: technology and applications. *Wat. Sci. Tech*. 33 pp 1-262.

MAS, J., VAN GEMERDEN, H. (1987). Influence of sulphur accumulation and composition of sulphur globule on cell volume and buoyant density of *Chromatium vinosum*. *Arch. Microbiol*. 146: 362-369.

MCKINNON, W., CHOUNG, XU, Z. AND FINCH, J.A. (2000). Magnetic seed in ambient temperature ferrite process applied to acid mine drainage treatment. *Env. Sci. Technol*. **34**, 2676-2581.

MOLWANTWA, J.B., COETSER, S.E., HEATH, R., PULLES, W. AND ROSE, P.D. (2007). Salinity, Sanitation and Sustainability. Volume 4. The Rhodes BioSURE Process®. Part 3: Sulphur Production Unit Operations. WRC Report No TT 193/07. Water Research Commission, Pretoria.

MORGAN, B.E., LOEWENTHAL, R.E. AND LAHAV, O. (2001). Fundamental study of a one-step ambient temperature ferrite process for the treatment of acid mine drainage waters. *Water SA*, **27**(2), 277-282.

MULLIS, R.B. AND FALOONA, F A. (1987). Specific Synthesis of DNA *in vitro* via a Polymerase-Catalyzed Chain Reaction. *Methods in Enzymol.* **155:** 335-350.

MUYZER, G. AND RAMSIG, N.B. (1995). Molecular Methods to Study the Organisation of Microbial Communities. *Wat.Sci. Tech.* **32**: 1-9.

MUYZER, G.; DE WAAL, E.C. AND UITTERLINDEN, A.G. (1993). Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. *Appl. Environ. Microbiol.* **59:** 695-700.

OLOWE, A.A. AND GÉNIN, J.M.R. (1991). The mechanism of oxidation of Fe(II) hydroxide in sulphated aqueous media: importance of the initial ratio of the reactants. *Corros. Sci*. **14**, 131-149.

OLOWE, A.A., GENIN, J.M.R. AND BAUER, P.H. (1988). Hyperfine interactions and structures of ferrous hydroxide and green rust II in sulfated aqueous media. *Hyperfine Interactions*, **41**:850-856.

OSWALD, W.J. (1991). Introduction to Advanced Integrated Wastewater Ponding Systems. *Wat. Sci. Tech*. 24:5 pp 1-7.

PEREZ O.P., UMETSU, Y. AND SASAKI, H. (1998). Precipitation and densification of magnetic iron compounds from aqueous solutions at room temperature. *Hydrometallugy* **50**:223-242.

PETERS, R.W., Y.KU, AND D. BHATTACHARYYA (1985). Evaluation of recent treatment techniques for removal of heavy metals from industrial wastewaters. *AICHE Symposium Series, Separation of Heavy Metals and Other Contaminants,* **81**(243): 165-203.

RADENKOVICK, V.M, SHUTO, A.P. AND GOMELYA, N.D. (1995). Water treatment using magnetic fields. *Journal of Water Chemistry and Technology*, **17**(5):23-40.

ROSE P.D. (2002). Salinity, Sanitation and Sustainability; a Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa. Vol. 1 Overview. *WRC Report No: TT 187/02*.

ROSE, P.D., BOSHOFF, G.E., MOLIPANE, N.P. (2002). Integrated Algal Ponding Systems and the Treatment of Domestic and Industrial Wastewaters. Part 3: Mine Drainage Wastewaters, The ASPAM Model. *WRC Report No: TT 192/02.*

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 Patent 99/4585. US patent pending.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Springs Harbour Laboratory: Cold Spring Harbour, New York, USA.

SANTEGOEDS, C. M.; FERDELMAN T.G.; MUYZER, G. AND DE BEER, D. 1998. Structural and functional dynamics of sulphate-reducing populations in bacterial biofilms. *Appl. Environ. Microbiol*. **64** (10): 3731-3739.

SCHAUB, B.E.M., VAN GEMERDEN, H. (1994). Simultaneous phototrophic and Chemotrophic growth in the purple sulphur bacterium *Thiocapsa roseopersicinia* M1. *FEMS Mircro. Ecol*. 13; 185-196.

SCHWERTMAN, U. AND FETCHER, H. (1994). The formation of green rust and its transformation to Lepidocrocite. *Clay Minerals*, **29**:87-92.

SCOTT, J.R. (1993). Catchment water quality deterioration as a result of water level recovery in abandoned gold mines on the Eastern and Central Witwatersrand. *Provisional interim report covering the far East Rand investigation area*. Volume 1. Institute for Groundwater Studies, University of Orange Free State, Bloemfontein.

SINGER, P.C. AND STUMM, W. (1970). Acidic mine drainage: the rate-determining step. *Science*, **167**:1121-1123.

SKOUSEN, J., POLITAN, K., HILTON, T. AND MEEK, A. (1990). Acid mine drainage treatment systems; chemicals and cost. *Green Lands*, **20**(4):31-37.

TALBOT, R.S. (1984). Co-precipitation of heavy metals with soluble sulfides using statistics for process control. *In Proc. 16th Mid-Atlantic Indust. Waste Conf*., **16**: 279-88.

TAYLOR, R.M. (1980). Formation and properties of Fe(II)-Fe(III) hydroxycarbonate and its possible significance in soil formation. *Clay Miner*. **15**:369-382.

TRÜPER, H.G., (1983). Sulphur Metabolism in: Clayton, R.K., Sistrom, W.R (Eds.). *The Photosynthetic Bacteria*. Plenum Press, New York: 677-687.

U.S. Environmental Protection Agency, (1983). Neutralization of acid mine drainage, Design manual. USEPA-600/2-83-001, Cincinnati, OH. USA.

VAN GEMERDEN, H. (1986). Production of elemental sulphur by green and purple sulphur bacteria. *Arch. Microbiol*. 146: 52-56.

VAN HILLE, R.P. (2001). Biological Generation of Reactive Alkaline Species and their Application in a Sustainable Bioprocess for the Remediation of Acid and Metal Contaminated Wastewaters. PhD Thesis, Rhodes University, Grahamstown.

WAGNER, M.; FLAX, L.; BRUSSEAU, G.A. AND STAHL, D.A. 1998. Phylogeny of Dissimilatory Sulphite Reductases Supports an Early Origin of Sulphate Respiration. *J. Bacteriol.* **180:** 2975-2982.

WANG, W., XU, Z. AND FINCH (1996). Fundamental study of an ambient temperature ferrite process in the treatment of acid mine drainage. *Env. Sci. Technol*., **30**:2604-2608.

WELLS, C. (2005) Tertiary Treatment in Integrated Algal Ponding Systems. MSc Thesis, Rhodes University, Grahamstown.

WERDMULLER, V.W. (1986). The central rand. In: Antrobus, E.S.A. (Ed.), Witwatersrand Gold – 100 Years. *Geological Society of South Africa*, Johannesburg, p. 748.

WILLIAMSON, M.A. AND RIMSTIDT, J.D. (1994). The kinetic and electrochemical rate-determining step of aqueous pyrite oxidation. *Geochim. Cosmochin. Acta*, **58**:5443-5454.

ZINCK, J.M. AND GRIFFITH, W.F. (2000). *5th International Conference on Acid Rock Drainage*. May21-24, Denver, Colorado, vol. 2, 1027-1034, Society for mining, metallurgy and Exploration, Inc.

9. APPENDIX 1

Table A.1

Parameters	**First Run**	**Second Run**
Lime concentration Sodium sulfide concentration Sulfide concentration	0.5% $Ca(OH)_2$ 3.0% $Na_2S.9H_2O$ 3.99 g H_2S	0.5% $Ca(OH)_2$ 3.0% $Na_2S.9H_2O$ 3.99 g H_2S
Mine water flow rate Lime/sulfide flow rate Retention time	7.88 ml/sec 0.08 ml/sec 41.88 min	7.88 ml/sec 0.08 ml/sec 41.88 min
Mine water pH Effluent pH	6.39 6.97	6.43 7.02
Total iron mine water Total iron effluent Percentage removal	85 mg/l 8.1 mg/l 90.5%	84 mg/l 3.5 mg/l 96%
Comments	Couldn't continue with this procedure due to the overflow of the black precipitate just too soon	

Table A.2

Parameters	**First Run**	**Second Run**
Lime concentration Sodium sulfide concentration Sulfide concentration	0.5% $Ca(OH)_2$ 3.0% $Na_2S.9H_2O$ 3.99 g H_2S	0.5% $Ca(OH)_2$ 3.0% $Na_2S.9H_2O$ 3.99 g H_2S
Mine water flow rate Lime/sulfide flow rate Retention time	6.75 ml/sec 0.14 ml/sec 48.34 min	6.75 ml/sec 0.14 ml/sec 48.34 min
Mine water pH Effluent pH	6.49 8.43	6.51 8.24
Total Iron mine water Total Iron effluent Percentage removal	83 mg/l 0.19 mg/l 99.77%	84 mg/l 0.38 mg/l 99.55%
Comments	Overflow of the black precipitate just too soon despite the high percentage iron removal	

Table A.3

Parameters	**First Run**	**Second Run**
Lime concentration Sodium sulfide concentration Sulfide concentration	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l
Mine water flow rate Lime/Sulfide flow rate Retention time	1.67 ml/sec (30 RPM) 0.08 ml/sec 190.48 min	1.67 ml/sec (30 RPM) 0.08 ml/sec 190.48 min
Mine water pH Effluent pH	6.50 7.73	6.51 7.88
Total Iron mine water Total Iron effluent Percentage removal	83 mg/l 4.54 mg/l 94.53%	83 mg/l 4.35 mg/l 94.76%
Comment	This was carried out at a very low flow rate and the iron residual was >4.54 mg/l which is more than the legal discharge limit of <1.0 mg/l.	

Table A.4

Parameters	**First Run**	**Second Run**
Lime concentration Sodium sulfide concentration Sulfide concentration	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l
Mine water flow rate Lime/sulfide flow rate Retention time	2.78 ml/sec (50 RPM) 0.10 ml/sec 115.74 min	2.78 ml/sec (50 RPM) 0.10 ml/sec 115.74 min
Mine water pH Effluent pH	6.52 8.33	6.53 8.90
Total Iron mine water Total Iron effluent Percentage removal	83 mg/l 8.4 mg/l 89.88%	83 mg/l 7.4 mg/l 89.88%
Comment	The iron removal was >1.0 mg/l which is more than the legal discharge limit of <1.0 mg/l.	

Table A.5

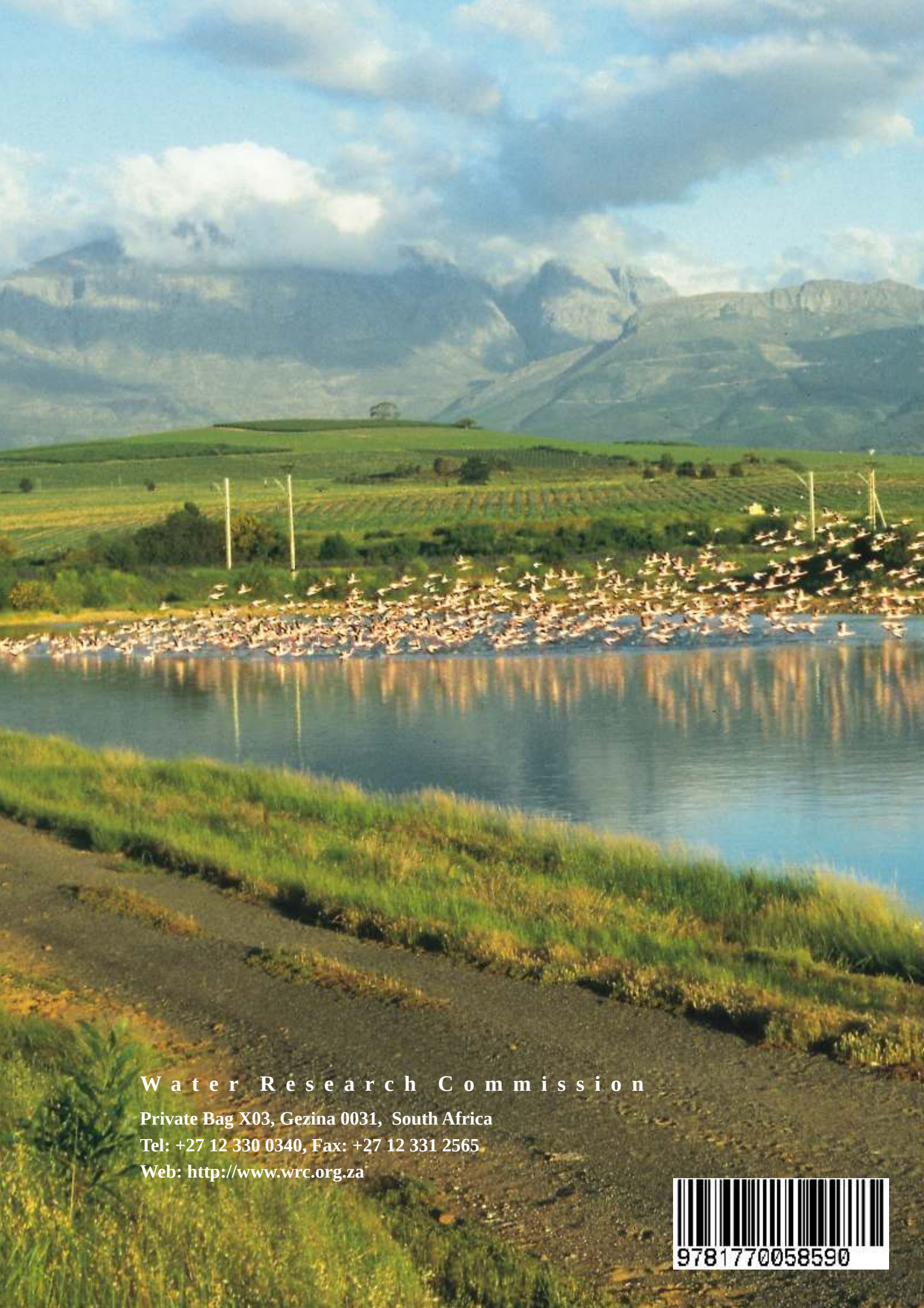
Parameters	**First Run**	**Second Run**
Lime concentration Sodium sulfide concentration Sulfide concentration	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l
Mine water flow rate Lime/sulfide flow rate Retention time	1.95 ml/sec (35 RPM) 0.10 ml/sec 162.60 min	1.95 ml/sec (35 RPM) 0.10 ml/sec 162.60 min
Mine water pH Effluent pH	6.42 8.69	6.43 8.09
Total Iron mine water Total Iron effluent Percentage removal	84 mg/l 0.9 mg/l 98.93%	84 mg/l 1.02 mg/l 98.79%
Comment	The iron removal was <1.0 mg/l which falls within the acceptable legal discharge limit of <1.0 mg/l.	

Table A.6

Parameters	**First Run**	**Second Run**
Lime concentration Sodium sulfide concentration Sulfide concentration	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l
Mine water flow rate Lime/Sulfide flow rate Retention time	2.51 ml/sec (45 RPM) 0.08 ml/sec 128.70 min	2.51 ml/sec (45 RPM) 0.08 ml/sec 128.70 min
Mine water pH Effluent pH	6.37 7.35	6.39 7.87
Total Iron mine water Total Iron effluent Percentage removal	79.2 mg/l 2.09 mg/l 97.36%	77.8 mg/l 2.91 mg/l 96.26%
Comment	The iron removal was >1.0 mg/l which is more than the legal discharge limit of <1.0 mg/l.	

Table 6.7

Parameters	**First Run**	**Second Run**
Lime concentration Sodium sulfide concentration Sulfide concentration	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l	0.5% $Ca(OH)_2$ 1.0% $Na_2S.9H_2O$ 1330 mg/l
Mine water Flow rate Lime/Sulfide Flow rate Retention time	2.51 ml/sec (45 RPM) 0.08 ml/sec 128.70 min	2.51 ml/sec (45 RPM) 0.08 ml/sec 128.70 min
Mine water pH Effluent pH	6.34 7.86	6.39 7.87
Total Iron Mine water Total Iron Effluent Percentage removal	75.4 mg/l 3.3 mg/l 95.62%	77.8 mg/l 2.01 mg/l 97.42%
Comment	The iron removal was >1.0 mg/l which is more than the legal discharge limit of <1.0 mg/l.	



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