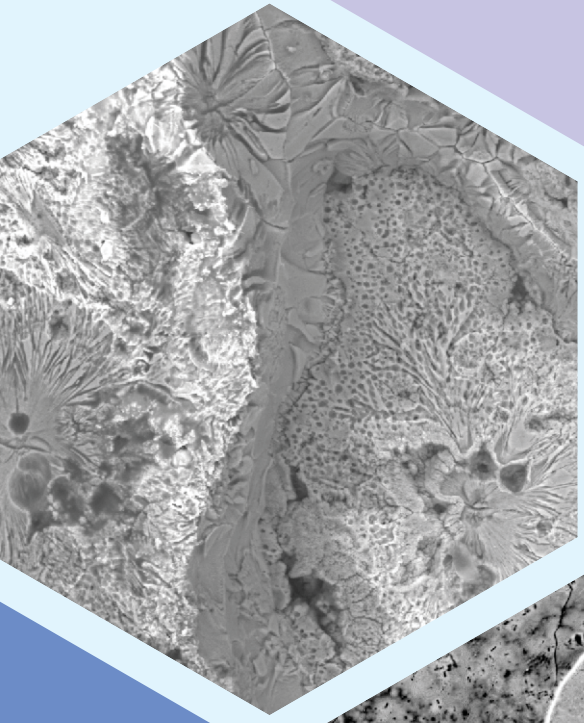
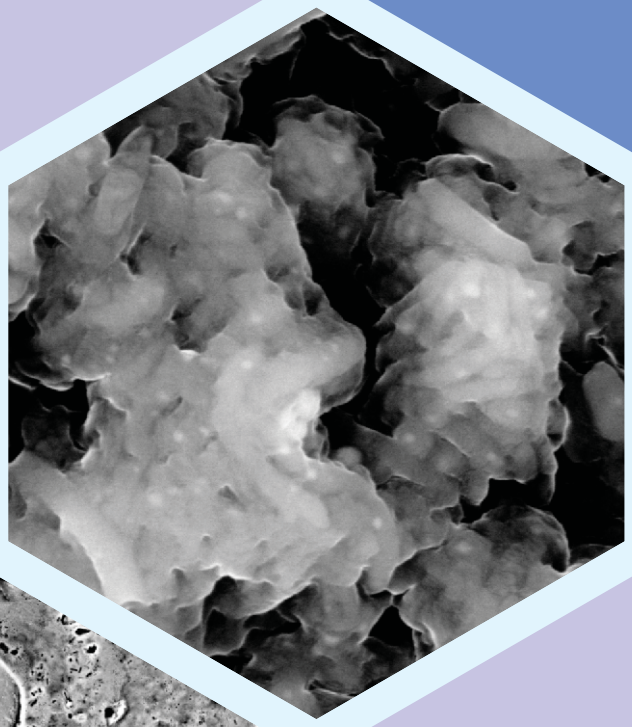
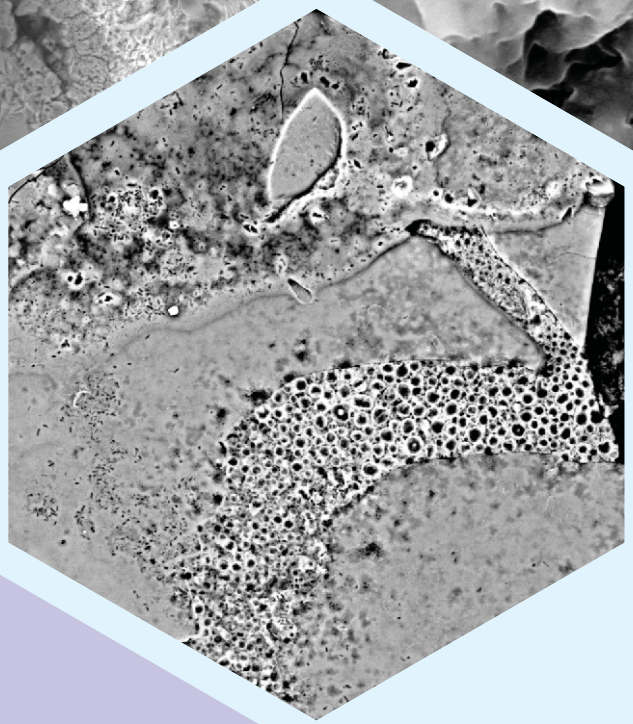
DEVELOPMENT OF A NOVEL SEMI-PASSIVE PROCESS FOR ENHANCED SULPHATE REMOVAL AND ELEMENTAL SULPHUR RECOVERY FOR REMEDIATION OF ACID ROCK DRAINAGE

Tynan S Marais, Nyasha T Tawodzera, Sharon Rademeyer, Mariette Smart, Sarah Fernandes and Susan T.L Harrison







WATER RESEARCH COMMISSION

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Development of a Novel Semi-Passive Process for Enhanced Sulphate Removal and Elemental Sulphur Recovery for Remediation of Acid Rock Drainage

Report to the
Water Research Commission

by

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Executive Summary

Acidic, sulphate-rich waters, termed acid rock drainage (ARD) or acid mine drainage (AMD) form a major source of pollution in regions mining sulphidic ores. AMD presents in two major ways: as high-volume streams from mine workings or as lower volume seepage and run-off from disturbed lands and mine waste. While treatment processes are in place for neutralisation and metal removal, processes for long-term sulphate removal remain a challenge. Biological sulphate reduction (BSR) has been proposed as a potential approach to the latter form of AMD and to treating process effluents rich in sulphide. To date, commercial applications are limited to active systems, such as developed by Paques which integrated biological sulphate reduction with partial sulphide oxidation to yield an elemental sulphur product and upgraded water; however, these seldom provide an economically viable solution for AMD.

A variety of approaches to passive BSR systems have been put forward. While promising in concept, the operation of these is typically inconsistent, lacking robust operation over time. To overcome this, the concept of a semi-passive BSR approach was put forward and has been built on by the team at the Centre for Bioprocess Engineering Research at the University of Cape Town. This has facilitated the integration of the BSR and partial sulphide oxidation steps into a single linear flow channel reactor (LFCR), resulting in upgraded water and a solid product comprising nutrients and elemental sulphur and with application as a fertiliser.

In optimising this process to enable techno-economically feasible application, three factors are highlighted: (1) a cost-effective and readily available carbon source and electron donor is required; (2) the reaction kinetics must be sufficiently swift; and (3) good management of sulphide is required through its separation out of the liquid phase, preferably into the solid phase for recovery – here as elemental sulphide. While the integration of the floating sulphur biofilm (FSB) generation into the LFCR addresses (3) and the inclusion of carbon microfibrils to enable biomass retention addresses (2), both previously reported, it was necessary to maximise the usage of the reactor volume to further address reaction kinetics (2), to identify a carbon source and electron donor with commercial potential to address (1) and to demonstrate the process using an actual AMD. These form the focus of this report.

To enhance the kinetics of BSR, it is important to retain an active SRB community in the reactor through biomass retention, use the full reactor volume through minimising dead spots and to create zoning in the reactor to create microbial niches suited to high and low sulphate concentrations as well as differing substrates for BSR, and regions for partial sulphide oxidation using sulphur-oxidising bacteria (SOB). Here we explore achieving this through the baffling of the LFCR to achieve a defined plug flow pattern from inlet to outlet. Polyurethane foam was introduced to enable retention of SRBs through entrapment in the full bulk volume of the LFCR, while partial sulphide oxidation occurred at the surface of the reactor. This modified reactor configuration was operated with a feed of both 1 g/L and 2 g/L sulphate, using lactate as carbon source and electron donor, to allow comparison with the previous reactor configuration. A second LFCR was added to enable additional partial sulphide oxidation. The BSR was improved in the baffled reactor with cell retention on foam over that in the absence of baffles and cell retention on carbon microfibrils. An average improvement of 20% in sulphate conversion efficiency was observed over hydraulic retention times (HRT) of 2 to 6 days with a feed of 1 g/L sulphate, with the best improvement of 23% at a 4-day HRT. Sulphide conversion and recovery was also improved. The addition of the secondary reactor further increased sulphide conversion to sulphur by 51% and 45% at a 6- and 2-day HRT respectively. Optimum performance with a 1 g/L sulphate feed was found at a 4-day HRT, yielding 91% sulphate conversion, 81% sulphide removal and 55% sulphur recovery.

While sulphate conversion lay in the range 78 to 91% across a 2- to 6-day HRT with a feed of 1 g/L sulphate (8.2 to 9,5 mmol/L converted), 66 to 72% sulphate conversion resulted across a 2- to 5-day HRT at a 2/L feed (12.7 to 14.9 mmol/L converted). Enhanced volumetric sulphate reduction rates were found in the baffled LFCR. At a 1 g/L feed, VSRR ranged from 0.079 mmol/L.h at a 5-day HRT to 0.169 mmol/L.h at a 2-day HRT. At a 2 g/L sulphate feed, these increased to 0.120 mmol/L.h at a 5-day HRT and 0.286 mmol/L.h at a 2-day HRT.

In seeking an appropriate carbon source and electron donor, the defined substrates lactate (as reference), ethanol and acetate were compared to complex substrates algal lysate, honey and molasses. Through batch

tests, molasses was clearly identified as the best performing complex substrate with maximum sulphate conversion of 89% in batch operation. Honey and algal lysate both performed poorly as substrates for BSR with conversions of 11 and 34% respectively, and were taken beyond batch experiments.

The performance of BSR on an AMD stream generated in a pilot study on coal discards was evaluated. Using batch tests, it was illustrated that pre-treatment to neutralise the AMD was required which this was carried out by lime addition. Pretreatment to remove metals through precipitation as metal sulphides did not result in significant performance difference.

The performance of the baffled LFCR packed with polyurethane foam for biomass retention was assessed on AMD pre-treated with lime. Lactate with synthetic AMD at a 3-day HRT was used as a base case. Following this, the partially treated AMD with lactate, after which lactate was switched out for molasses as a carbon source. Despite the removal of all extra media components from the feed, the BSR performance on true AMD exceeded that of synthetic AMD, with an average sulphate conversion of 87%, compared to 72% on synthetic AMD. On true AMD and molasses, the system maintained performance with the base case, with a sulphate conversion of 72%. However, with both carbon sources, partial oxidation of sulphide was compromised, limiting the formation of the floating sulphur biofilm. This may result from the inhibition of the SOB or from the SOB being more nutritionally fastidious and thereby compromised from the removal of nutrients.

This study provides evidence of a resilient reactor system for BSR and partial sulphide oxidation. The inclusion of baffles to enable plug flow and zoning in the reactor and the use of a foam matrix for biomass retention throughout the bulk of the reactor provided positive improvement to the reactor performance. Treatment of neutralised AMD was efficient and the replacement of lactate by molasses was demonstrated. We recommend that the outlet of the reactor is re-designed to minimise re-oxidation of sulphate. Further, we recommend that the performance of the reactor under increased loading is explored. Finally, we recommend studies into the factors limiting the formation of the FSB when treating AMD in the absence of replete nutrients.

The operation of an efficient semi-passive process for AMD treatment continues to be much sought, with the system described in this study showing much promise.

Acknowledgements

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Acronyms and abbreviations

ARD	Acid Rock Drainage
BSR	Biological sulphate reduction
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
FSB	Floating Sulphur Biofilm
HPLC	High Pressure Liquid Chromatography
HRT	Hydraulic residence time
LFCR	Linear flow channel reactor
SOB	Sulphur oxidising bacteria
SRB	Sulphate reducing bacteria
UPBR	Up-flow packed bed reactor
VFA	Volatile fatty acid

Glossary of Terms

Biofilm (sessile)	A structured community of microorganisms enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.
Chemical oxygen demand	A measure of the total amount of oxidisable organic material in solution.
Community dynamics	Change in relative abundance of microbial community in response to change in physicochemical conditions. This describes the ability of the microbial population to react and adapt to environmental perturbations.
Hydrodynamics	Refers to the motion and interaction of fluid in a reactor system
Inoculum	Microbial cell suspension added to start a culture or bioreactor
Metabolism	The physicochemical transformations through which simple substrates are either synthesised into complex compounds or complex compounds are converted to simple ones through which energy is obtained to maintain microbial activity and growth.
Planktonic cells	Free living microbial cells in suspension.

1 Introduction

Acid Rock Drainage (ARD) is a major environmental issue facing South Africa and is a growing concern in many countries worldwide. The generation of ARD can be defined as the accelerated oxidation of sulphide minerals, predominantly pyrite, resulting from exposure to oxygenated water because of the mining and processing of metal ores and coal (Johnson & Hallberg, 2005; Kaksonen & Puhakka, 2007). Historically, the potential impact of ARD has been underestimated by both the mining companies and the government, resulting in the need for the emergency measures currently being implemented to deal with ARD in the Witwatersrand basins. Despite efforts to mitigate the situation, the coal mining legacy in the Witbank coalfield of South Africa has led to the extensive contamination of ground water and surface water.

The negative environmental impact associated with ARD discharge may vary widely, depending on the climate, geomorphology, distribution of ARD generating deposits, and the severity of pH change (McCarthy, 2011). This impact has contributed to the destruction of both terrestrial and aquatic ecosystems, food chains and loss of biodiversity. The effects of ARD are cumulative and will be a concerning problem for many decades or even on the order of centuries, after mining activity has ceased (Johnson & Hallberg, 2005). Therefore, the development and implementation of an economically sustainable method for remediation of ARD pollution is critical for the preservation of the country's natural environment, freshwater resources, and agricultural land, as well as to ensure human safety (McCarthy, 2011). While it is desirable to avoid or inhibit ARD formation at the source, this has not been achieved historically and has left a long-term ARD legacy which remains a challenge for full compliance. Hence remediation of ARD by minimising the negative impact on receiving water bodies and the environment is of key importance. While progress has been made with localised active treatment processes for treating large water streams to potable or fit-for-purpose water, these require ongoing investment. These active processes are not suitable for long-term, lower flow, distributed sources of ARD which nevertheless have widespread impact on the environment and communities in those areas where it occurs. These situations require passive or semi-passive solutions.

There are several physicochemical and biological approaches that can be implemented to achieve treatment objectives. These may be divided into two broad categories, active and passive treatment processes. Active treatments are based on the continuous replenishment of alkaline materials to neutralise ARD and precipitate metals. Active processes typically involve the use of agitated reactor units which are dependent on maintenance and control of operational conditions (temperature, pH, pressure) as well as the addition of alkaline chemicals or substrates (Johnson & Hallberg, 2005). These processes are characterised by faster kinetics and enhanced control when compared to passive treatments. Many of these systems are unsustainable both from an environmental and economic perspective, given the timescale of the ARD challenge. Passive treatments refer to the use of natural or constructed wetland ecosystems. While these do have an advantage in that they require very little to no external additional maintenance (cost) once installed, the systems are dependent on processes that are kinetically slower than those involved in active systems and therefore require longer hydraulic retention times (HRTs) and larger areas to obtain similar results. The less defined conditions result in less control and predictability. Passive treatment options may include wetlands; anoxic limestone drains (ALDs); permeable reactive barriers; and engineered biological systems (Johnson & Hallberg, 2005).

South Africa faces two major sources of ARD. The first is associated with the groundwater rebound that passes through abandoned underground mine workings, primarily from the gold mining impacted basins of the Witwatersrand. This results in high volumes (several 100 ML/day) of heavily impacted water (Rose, 2013). The second type of ARD originates from diffuse sources, primarily associated with the coal industry in South Africa. These sources include waste rock dumps, spoil heaps and open pits. The volume of discharge is significantly lower than those from the underground basins but may vary substantially depending on the site. The long-term impact of these industries, especially the coal mining industry in South Africa is likely to affect a far greater area and may persist for a longer period, given the number of potential sites and the unique combination of climate, geography, distribution, and scale of the deposits. The nature of these discharges makes it more suitable for remediation by passive and semi-passive options.

Biological treatment approaches have the potential to be more cost effective and sustainable than physicochemical alternatives. Biological treatment of ARD is centred on the activity of sulphate reducing bacteria (SRB). SRB are anaerobic microorganisms that obtain energy through the utilisation of sulphate ions as terminal electron acceptors for metabolism of organic substrates. Sulphate reducing bacteria are classified within several different phylogenetic lines. By the year 2009, 60 genera containing over 220 species of sulphate reducing bacteria were known (Barton & Fauque, 2009). By understanding the parameters that affect SRB metabolic activities in terms of their ability to reduce sulphate in ARD contaminated wastewater; these processes can be optimised as a sustainable solution to address the ARD crisis.

A variety of reactor configurations such as stirred tank reactors (Moosa *et al.,* 2005a; Oyekola *et al.,* 2010), packed bed reactors (Jong & Parry, 2003; Hessler *et al.,* 2018) membrane reactors ((Nagpal *et al.,* 2000) and (Qian *et al.,* 2016) have been used to study anaerobic sulphate reduction and to treat ARD. The effects of various parameters on biological sulphate reduction (BSR) such as sulphate concentration (Moosa *et al.,* 2002; Oyekola *et al.,* 2010), temperature (Moosa *et al.,* 2005b), pH (Elliott *et al.,* 1998a), electron donor availability and type , inhibitory effects of metal (Utgikar *et al.,* 2003a) and sulphide concentration, as well as the use of carrier matrices (Baskaran & Nemati, 2006; Hessler *et al.,* 2018) have been investigated. The correct regulation and maintenance of these parameters within BSR system is essential for optimal process efficiency.

In biological wastewater treatment, freely suspended cells in a continuous system dictates a high HRT to avoid wash out of the cells, in immobilised cell systems, biomass retention time is uncoupled from the HRT. It then becomes possible to operate the system at short HRT, while maintaining high biomass concentration and enhanced reaction rates (Baskaran & Nemati, 2006). The choice of support material is a determining factor in the selection of the microbial population within a reactor where different supports can be incorporated for specific applications. There have been numerous studies based on immobilised cells on supports and it is a crucial component in wastewater treatment where decoupling of the biological and hydraulic residence time is key (Silva *et al.,* 2006). Numerous active commercial processes have been developed based on biological sulphate reduction. These systems, such as the Paques Thiopaq and the Rhodes BioSURE process (Rose, 2013), have been well described but have been limited to niche applications, mainly due to the relatively slow growth of sulphate reducers and the associated kinetic constraint, high cost of the electron donor (ethanol, methanol, volatile fatty acids) and challenges in managing the resulting sulphide product, which is significantly more toxic than sulphate. To ensure the sustainability of biological treatments as a long-term solution for ARD remediation, biomass retention to enhance kinetics, the use of an alternative electron donor and appropriate management of hydrogen sulphide is critical. The development of a partial sulphide oxidation process whereby large yields of elemental sulphur can be produced based on bacterial oxidation is a promising solution.

Biological sulphate reducing systems have been well described in literature. However, these have been primarily limited to batch, and up-flow anaerobic sludge blanket (UASB) reactors, while continuous stirred tank reactors (CSTR) have been used to explore microbial kinetics. Passive systems studied have been either traditional wetlands or packed bed reactors (PBR) of low permeability. The main drawbacks associated with these systems are the unpredictability of system performance (Zagury *et al.,* 2006). For biological sulphide oxidation systems, several studies have been conducted, but are largely confined to active treatment systems. These systems mainly make use of either membrane or gas-lift reactors (Henshaw & Zhu, 2001). However, a major drawback is the requirement for large amount of energy in the form of continuous supply of oxygen, light and a large surface area, as well as the difficulty in controlling the oxygen supply to ensure the elemental sulphur product.

Ideally, there is a need for the development of an ARD treatment that can be applied in remote regions where there is a lack of infrastructure and electricity. It is within this background that current passive and active strategies show system constraints and are typically not feasible solutions for long-term treatment of ARD. In this study, we expand our research to overcome the unpredictability of passive systems and the cost and energy requirements of active systems through further developing the linear flow channel reactor approach (LFCR) as a semi-passive system for biological sulphate reduction and partial sulphide oxidation to remove acid and sulphate and yield elemental sulphur as product.

2 Literature review

2.1 Acid Rock Drainage

Acid Rock Drainage (ARD) is a major environmental issue facing South Africa and is a growing worldwide concern, predominantly associated with the mining of sulphidic minerals. South Africa is renowned for its gold and coal mining industry (Feris & Kotze, 2014; McCarthy, 2011), both having associated sulphidic fractions. The long term legacy of theses mining activities has resulted in ARD pollution that threatens the environment and places increased pressure on the water security of the country. Historically, the potential impact of ARD has been underestimated by mining companies and the government, resulting in the need for the emergency measures currently being implemented to deal with ARD discharge from the underground workings within the Witwatersrand basins (Feris & Kotze, 2014) as well as ongoing environmental degradation associated with diffuse acidic leachates from legacy sulphidic waste rock dumps and tailings facilities.

2.1.1 Generation of ARD

The generation of ARD can be defined as the accelerated oxidation of sulphidic minerals, predominantly iron pyrite (FeS_2) resulting from exposure to oxygenated water as a consequence of their liberation through mining and processing of sulphidic mineral ores and pyritic coal (Johnson & Hallberg, 2005; Kaksonen & Puhakka, 2007). The complete process of pyrite oxidation may be summarised by Reaction 2.1 as follows:

$$4\,FeS_2 + 15\,O_2 + 14\,H_2O \rightarrow 4\,Fe(OH)_3 + 8\,SO_4^{2-} + 16\,H^+ \qquad \text{Reaction 2-1}$$

During mining operations, overburden rock is removed in order to gain access to a valuable ore body, creating a network of well-ventilated underground workings as well as exposed waste rock. These exposed mineral rocks may contain sulphide minerals such as FeS_2, the most abundant sulphide mineral on the planet. Naturally, sulphide minerals have developed under anaerobic conditions found deep underground. Once exposed to aerobic conditions during mining activities, sulphide minerals (including FeS_2) are oxidised, this oxidation process is accelerated in the presence of iron- and sulphur-oxidising microorganisms (Sánchez-Andrea *et al.,* 2014) which catalyse the regeneration of the leach agents Fe^{3+} and H^+.

2.1.2 Environmental impact

As an example of the environmental impact of ARD in South Africa, mining of pyritic coal in the Witbank coalfield of South Africa has led to uncontrolled ARD seepage into the surrounding areas with extensive contamination of ground and surface water (McCarthy, 2011). The negative environmental impact associated with ARD discharge may vary widely depending on the climate, geomorphology, nature and distribution of ARD generating deposits as well as their relationship to acid neutralising minerals and the associated severity of pH change (McCarthy, 2011). The impact of ARD has contributed to the destruction of both terrestrial and aquatic ecosystems, food chains and ultimately the loss of biodiversity (Feris & Kotze, 2014).

Traditionally the treatment of mine water has focused on pH neutralisation and the removal of heavy metals. Less attention has been placed on the mitigation of dissolved sulphate levels due to their lower environmental risk and regulatory standards when compared to those for acidity and dissolved metals (Arnold *et al.,* 2016). However, regulatory agencies have become increasingly concerned over elevated sulphate concentrations. In some regions, industrial effluents have discharge limits as low as 10 mg/L, although typically this ranges between 250 and 1 000 mg/L (Table 2.1).

Table 2-1: Sulphate concentration discharge levels based on different country guidelines compared to the drinking water standard defined by the World Health Organisation (WHO) (adapted from Arnold *et al.,* 2016)

Authority	Sulphate concentration (mg/L)
South Africa	200-600
USA	10-500
Canada	500
Finland	2000
Australia	1 000
World Health Organisation	250 (drinking water standard)

Sulphate-rich waste streams are not only produced by mining operations but also as effluents from a variety of industrial operations, including galvanic processing, paper and pulp manufacturing, petrochemical industries, paint and chemical manufacturing, food processing (molasses, oil and seafood), pharmaceutical industries as well as the manufacturing of batteries and chemicals (Lens *et al.,* 2003; Brahmacharimayum *et al.,* 2019). These industrial effluents may contain a high concentration of sulphate, ranging from 100 to >20 000 mg/L. Sulphate concentrations in sewage are typically less than 500 mg/L. The composition of ARD, from leaching of sulphidic minerals, varies significantly, depending on site, environmental conditions, mineralogy and extent of oxidation (Brahmacharimayum et al., 2019) and result in the release of sulphate and hydrogen sulphide contaminated wastewater streams into the environment.

The acceptable sulphate limit for taste is <250 mg/L, while international water discharge legislation allows for a sulphate content that ranges between 250-500 mg/L (WHO, 2004). More than 600 mg/L of sulphate is known to cause disturbances in the human gastrointestinal tract often leading to symptoms of diarrhoea, nausea and dehydration. The sulphate concentrations in ARD generally far exceed the permissible discharge levels for human consumption. The excessive release of sulphate, if left unchecked, can lead to pollution of important freshwater resources (surface and ground) and arable agricultural land. This will ultimately have devastating consequences on the water and food security as well as the rich biodiversity of the country.

The effects of ARD are cumulative and present a concerning problem for decades after mining activity have ceased, owing to its ongoing generation. Therefore, the development and implementation of an economically sustainable method for the remediation of ARD pollution is critical in order to preserve the environment, freshwater resource, agricultural land and to ensure human safety (McCarthy, 2011). While it is preferable to prevent the formation of ARD in the first place, once underway, ARD generation can persist for 10s or even 100s of years. This highlights the importance of remediation approaches for legacy ARD sites.

2.1.3 **ARD management strategies**

Preventative control of ARD formation

Ideally, to address ARD, source control measures that minimise and prevent the formation of contaminated waters should be implemented (Johnson & Hallberg, 2005). There have been numerous efforts that have focussed on the predictive characterisation and prevention of ARD formation. Although this may be the most preferable solution to address the problem, it may not be a practical or feasible approach in cases where abandoned mines have already begun to decant large quantities of contaminated water. Most prevention strategies, also known as 'source control', are based on the fundamental principle that the formation of ARD is primarily mediated by the exposure of sulphidic minerals to oxygen and water (accelerated by microbial activity) and, therefore, by excluding these factors it may be possible to prevent or minimise ARD formation (Johnson &

Hallberg, 2005). Approaches include backfilling, flooding and sealing of abandoned deep mines where dissolved oxygen is consumed by microbial activity and the replenishment of oxygen is hindered by sealing (Johnson & Hallberg, 2005). Other approaches have investigated the role played by iron and sulphur oxidising bacteria in catalysing the generation of ARD, which has led to the use of biocides for the inhibition of their activity within mineral tailings and spoils. However, the application of biocides varies in effectiveness, requires regular application of chemicals and only provides a short-term control to the ARD problem (Johnson & Hallberg, 2005).

Migration control

Due to the practical challenges associated with preventing ARD formation at the source, remediation of ARD effluents prior to discharge is required in some cases to reduce its negative impact on receiving water bodies and the surrounding environment (Johnson & Hallberg, 2005). Ideally, the remediation of ARD should neutralise the acidity, decrease sulphate concentration and remove or recover heavy metal contamination (Gopi Kiran *et al.,* 2017). In addition, due to the time-frame of ARD formation and discharge, treatment should be economically sustainable in the long term. Strategies to achieve these objectives can be further subdivided into active and passive treatment processes.

2.1.4 **Sources of ARD**

The successful implementation of any remediation strategy is highly dependent on the chemical nature and source of ARD. ARD is characterised by the volume of the effluent, concentration and type of contaminants as well as the pH of the water (Gazea *et al.,* 1996). There are two major sources of ARD that can be distinguished in South Africa. The first is associated with the groundwater rebound from abandoned underground mine workings, primarily from the gold mining impacted basins of the Witwatersrand, Gauteng Province. ARD originating from underground basins are generally characterised by high volumes (several 100 Ml/day) of heavily impacted water containing high sulphate and heavy metal concentration (Rose, 2013). The most appropriate management strategy is to pump and treat, using conventional active processes such as the high density sludge (HDS) process, followed by reverse osmosis (RO). These are costly, require constant addition of alkaline chemicals and do not address the issue of sulphate salinity adequately, however, sustainable alternatives are not yet available to deal with such high volumes (Arnold *et al.,* 2016). As a result, the application of RO for treating ARD is still the most effective.

The second type of ARD originates from diffuse sources leaching from waste rock dumps, spoil heaps and open pits. In South Africa, much ARD generation is associated with the coal industry predominantly within the Mpumalanga province (Figure 2.1). The volume of discharge is significantly lower than that from the underground basins but may vary substantially depending on the site. It has been reported that the long term impact of ARD from diffuse sources, especially from the coal mining industry in South Africa, is likely to affect a far greater area and may persist for a long period of time, given the number of potential sites and the unique combination of climate, geography, distribution, and scale of the deposits. Despite the high risk potential, it has received far less attention from the media, government and mining companies (McCarthy, 2011). Considering the extent of existing and planned mining operations within the region, the management of these sources is of critical importance (Figure 2-1).

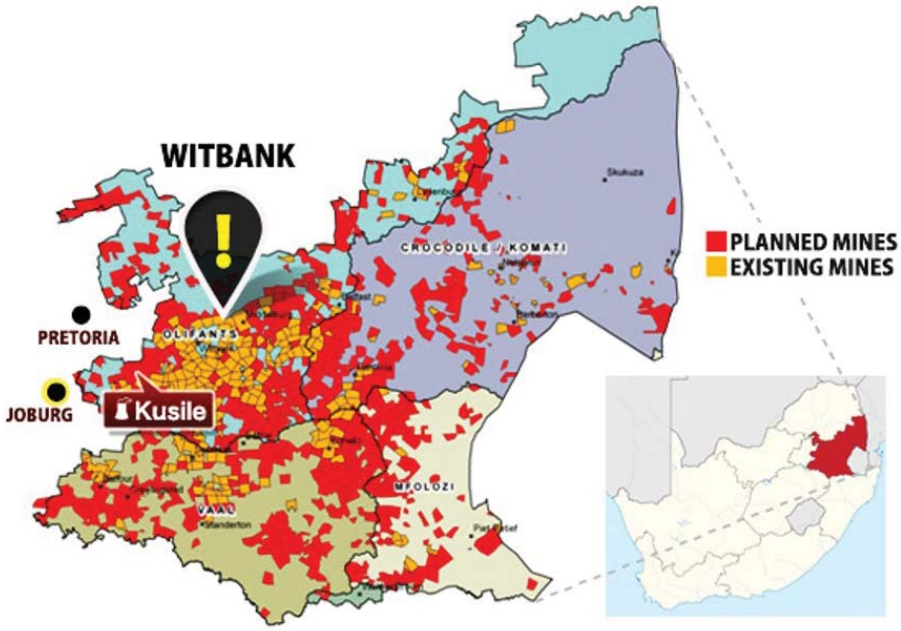


Figure 2-1: Geographic landscape of the coal mining industry in South Africa predominantly within the Mpumalanga province illustrating the extent of planned and existing mines. The vast number of sites will have a significant impact on the surrounding environments with a long term potential risk for the generation and pollution of ARD.

2.1.5 Active treatment technologies

Physicochemical treatment

Active chemical treatment processes using chemical neutralising agents are most widely used for the mitigation of acidic effluents (Johnson & Hallberg, 2005; Sheoran *et al.,* 2010). On addition of an alkaline chemical reagent to ARD contaminated wastewater, increased pH, acceleration in the rate of chemical oxidation of ferrous iron, and precipitation of many metals present in solution as carbonates and hydroxides results. The level at which sulphate is reduced are controlled by the solubility of gypsum which, depending on the ionic strength of the solution, ranges from 1500 to 2000 mg/L. The High Density Sludge (HDS) process is a two stage active process that is based on lime neutralisation, commonly used in the commercial-scale treatment of ARD. This process provides an advantage over conventional chemical neutralising processes in that it provides substantial reduction in sludge volume as well as increases sludge stability (chemically and physically). The HDS process is most widely associated with the refinery and mining industries, and currently it is being used at the Grootvlei Gold Mine on the East Rand. The drawbacks implicated with the HDS process include the special disposal of the resulting sludge, does not significantly reduce salinity levels associated with ARD and has a high chemical consumption suggesting that it is not a sustainable long term solution for ARD remediation. However, it may serve as an effective pre-treatment process prior to sulphate removal (INAP, 2014).

South Africa currently employs physicochemical active treatment systems to treat the voluminous quantities of acidified drainage effluent waters, particularly in the Witwatersrand basin due to ground water rebound from abandoned gold mines. The eMalahleni Mine Water Reclamation Plant in the Mpumalanga Province of South Africa is the result of a joint venture between mining companies Anglo American Thermal Coal and BECSA (Hutton *et al.,* 2009). This treatment facility makes use of active dewatering, followed by oxidation, neutralisation, metal precipitation and multi-stage ultrafiltration and reverse osmosis. The plant produces drinkable water and pure gypsum sludge as a by-product of the ARD treatment (Bezuidenhout, n.d.).

Biological Sulphate Reduction (Bioreactors)

Active systems are generally abiotic based on physicochemical methods. However, there are a number of active treatment technologies that rely on the activity of biological processes. The use of bioreactors is one approach to actively reduce sulphate concentration through exploiting reductive biological processes under defined operating conditions. Biological sulphate removal has the potential to be economically and environmentally favourable treatment option in comparison to current physicochemical processes ((Ayangbenro *et al.,* 2018; Nielsen *et al.,* 2018). The major advantages of biological sulphate reduction include (INAP, 2014):

1) Both sulphate and trace metals can be reduced to low concentration levels,
2) Minimal sludge production
3) Capital costs are relatively low and operating costs can be reduced using inexpensive carbon and electron donor sources, and
4) Trace metals can be selectively recovered, as metal sulphide precipitates, and sold for additional value (INAP, 2014).

Sulphate reducing bioreactors are ideally operated under strict anoxic conditions and rely on the activity of a consortium of specialised microorganisms called sulphate reducing bacteria (SRB) (Sánchez-Andrea *et al.,* 2014). These microorganisms are either autotrophic or heterotrophic organisms and reduce sulphate to sulphide via assimilatory and/or dissimilatory processes (Barton & Fauque, 2009). Biological sulphate reduction can be catalysed by a phylogenetically diverse group of bacteria and some taxonomic genera within archaea, where organic substrate acts as an electron donor while sulphate acts as an electron acceptor (Muyzer & Stams, 2008). These microorganisms are metabolically versatile and can degrade a range of electron donors including ethanol, hydrocarbons, volatile fatty acids, primary sewage sludge and lignocellulosic materials (Liamleam & Annachhatre, 2007; Muyzer & Stams, 2008). However, this is highly dependent on availability and cost of the substrate (Hao *et al.,* 2014). During biological sulphate reduction treating ARD, sulphate is reduced to sulphide in the presence of a suitable electron donor (Reaction 2.2). The process involves the reduction in acidity where the strong acid (H_2SO_4) is transformed into a weaker acid (H_2S) while alkalinity is produced in the form of bicarbonate (HCO_3) (Johnson & Hallberg, 2005).

$$2CH_2O + SO_4^{2-} \rightarrow 2HCO_3^{-} + H_2S \qquad \text{Reaction 2-2}$$

The sulphide generated can be co-precipitated with heavy metals in solution to form stable metal sulphides (Reaction 2.3) (Johnson & Hallberg, 2005). Under the correct pH conditions metal sulphides can be selectively precipitated and recovered and are less soluble than their hydroxide equivalents.

$$Me^{2+} + H_2S \rightarrow MeS + 2H^{+} \qquad \text{Reaction 2-3}$$

(Me^{2+} is a cationic metal such as Cd^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}).

The application of SRB to treat mine impacted water has been successfully demonstrated at industrial scale. The most recognised being the two patented technologies Biosulphide™ by BioteQ Environmental Technologies Inc., Canada, and Thiopaq™, by Paques, The Netherlands (Ayangbenro *et al.,* 2018). The Thiopaq™ technology incorporates biological sulphate reduction in a gas-lift bioreactor. The Thiopaq™ system consists of two primary stages: 1) An anaerobic stage in which sulphate is reduced to sulphide; and 2) An aerobic stage in which the sulphide, produced in step 1, is oxidised to elemental sulphur. In practice, many variants of the Thiopaq™ technology exist having been tailored to a host of applications treating sulphate- and sulphide-rich industrial waste streams, including the budel Zink (Budelco) refinery (Netherlands) and Kennecott Utah Copper mine (USA) (Hussain *et al.,* 2014).

2.1.6 **Passive treatment technologies**

Passive treatments refer to the use of natural or constructed wetland ecosystems and are advantageous in that they require very little to no external additions or maintenance (cost) once established (Kaksonen & Puhakka, 2007; Gopi Kiran *et al.,* 2017). These characteristics distinguishes passive from active processes which are highly dependent on maintenance and control of operational conditions, including temperature, pH, and pressure, as well as energy input and the addition of chemicals or substrates (Johnson & Hallberg, 2005). A range of passive

treatment options exist and have been applied in different environments, many occurring in various configurations in industry. Well established passive treatments, often used in ARD remediation, include aerobic and anaerobic wetlands and compost bioreactors as well as anoxic limestone drains (ALD).

Passive treatment technologies have advantages over active treatments in that they do not require regular human intervention, operation or maintenance (Johnson & Hallberg, 2005; Gopi Kiran *et al.,* 2017). The construction of passive treatment systems generally incorporates the use of natural materials and promotes the growth of natural vegetation (Skousen *et al.,* 2017). Passive treatment systems are based on the use of gravity flow for water movement instead of active mechanical pumping (Neculita *et al.,* 2008). Ideally a passive system functions without electrical power and can operate for long periods of time (> 5 years). There are several chemical, physical and biological processes that contribute to the amelioration of water quality in passive treatment. These may include adsorption and exchange by soil, plants and other biological materials; metal uptake into live roots and plants; abiotic or microbially-catalysed metal oxidation and hydrolysis reactions in aerobic zones; and microbially-mediated reduction processes in anaerobic zones (Kaksonen & Puhakka, 2007).

A well described method for neutralisation of acidic water is through direct contact with limestone within ALDs (INAP, 2014). Based on its chemical properties, limestone dissolves to deliver calcium and bicarbonate alkalinity; the latter neutralises acidity and buffers pH. The solubility of limestone is dependent on temperature, pH and CO_2 (Johnson & Hallberg, 2005). Mine waters that exhibit a net alkaline characteristic may be treated passively via constructed aerobic (oxidising) wetlands. The system incorporates the abiotic oxidation of ferrous iron and the hydrolysis of the ferric iron produced, resulting in a net acid-generating reaction. Natural vegetation such as macrophytes and Typha are planted for aesthetic reasons to regulate water flow (prevent channelling) and to filter accumulating ferric precipitate (ochre) (Kaksonen & Puhakka, 2007). Additionally, they provide surface area for precipitation of solid phase ferric iron compounds and are also capable of absorption/uptake of heavy metals (Gazea *et al.,* 1996).

In contrast to aerobic wetlands, compost bioreactors make use of anaerobic reactions to mitigate ARD (Johnson & Hallberg, 2005). These systems are enclosed entirely below ground level and do not support vegetation. These systems depend on microbially catalysed reactions that generate net alkalinity and biogenic sulphide. They can be used in the treatment of mine waters that exhibit net acidic and high metal concentrations such as ARD originating from abandoned metal mines. The reductive reactions that occur within compost bioreactors are dependent on electron donors derived from organic material (compost) (Gopi Kiran *et al.,* 2017). The choice of organic material varies based on local availability and effectiveness. Generally, the composts used consist of a mixture of relatively biodegradable materials (e.g. mushroom compost, horse or cow manure) with more recalcitrant materials (e.g. straw and sawdust) (Skousen *et al.,* 2017). In compost bioreactor systems, sulphate and iron reducing bacteria (SRB and FRB) are generally considered to play the major roles in ARD remediation (Johnson & Hallberg, 2005).

2.1.7 Summary of treatment technologies

In summary of Sections 2.1.5 and 2.1.6, the successful remediation strategy of ARD is dependent on accomplishing several objectives, namely the treatment should neutralise acidity, decrease sulphate concentration and remove or recover heavy metals. Furthermore, the ARD treatment adopted should be economically sustainable due to the long term nature of the ARD problem (Gopi Kiran *et al.,* 2017).

Active processes typically include mechanical operations highly dependent on maintenance and control of operational conditions (temperature, pH, pressure) as well as the addition of alkaline chemicals or substrates (Johnson & Hallberg, 2005). These processes are characterised by faster reaction kinetics and enhance control when compared to passive treatments. However, many active systems are unsustainable both from an environmental and economic perspective (Johnson & Hallberg, 2005; Rose, 2013). Alternatively, passive treatments require very little or no external additional maintenance (cost) once installed. However, these systems are dependent on kinetically slower sub-processes and therefore require longer hydraulic retention times (HRTs) and larger areas to obtain effective treatment. In addition, the less defined operating conditions reduce the level of control and predictability (Neculita *et al.,* 2008; Sheoran *et al.,* 2010).

In recent years there has been an increased development of passive bioreactor systems that require periodic active management, such as carbon source addition and/or temperature control, to sustain desired conditions and process performance. Under these conditions, these treatment technologies are referred to as semi-passive (G. Nielsen *et al.,* 2018). These systems provide an attractive approach over conventional active and passive treatments, with lower capital and operational costs as well as better process control and predictability (Harrison *et al.,* 2014; G. Nielsen *et al.,* 2018).

Table 2-2: Overview of ARD treatment technologies categorised as either active or passive, based upon a summary of system performance as well as associated advantages and disadvantages (adapted from INAP, 2014).

	Active Processes			Passive Processes	
	Limestone/ lime	RO	Bioreactor	ALD	Wetland
Pre-treatment	No	Yes	Yes	Yes	Yes
Feed water SO_4^{2-}	3000 mg/L	4920 mg/L	8342 mg/L	3034 mg/L	1700 mg/L
Product water SO_4^{2-}	1219 mg/L	113 mg/L	198 mg/L	1352 mg/L	1540 mg/L
SO_4^{2-} reduction rate	-	-	12-30 g/L.day	-	0.3-197 mg/L.day
Sludge production	Low-moderate	Low	Low-moderate	No	No
Maintenance	Low	High	Moderate	Low	Low
Operating costs	Moderate	High	Moderate	Unknown (low)	Unknown (low)
Advantages	Metal removal Low cost	Water quality	Metal removal	Gypsum product Metal removal	Metal removal Passive treatment
Disadvantages	SO_4^{2-} removal Sludge product	Scaling Membrane lifecycle	Cost of carbon source	Design requirement	SO_4^{2-} reduction

Based on this information, when implementing a remedial strategy, careful consideration must be taken based on the advantages and disadvantages associated with a given treatment technology (Table 2.3). Mine location, climate, water characteristics, available utilities and infrastructure, footprint, and disposal areas all preclude a "one-size fits all" solution (Arnold *et al.,* 2016). Active treatments are preferred for treating ARD characterised by high volume, low pH and high metal loading, while passive or semi-passive treatments are typically favoured for treating lower volume and less aggressive discharge of longevity. Therefore, due to the nature of diffuse sources, the application of active treatment is not economically viable, particularly in remote areas where minimal infrastructure is available and contaminated streams are located over large distances. Consequently, biological sulphate reduction has been identified as a promising approach for addressing low volume mine-impacted water through the application of passive and semi-passive treatment (Harrison *et al.,* 2014).

For the purpose of the current research, the subsequent sections of the literature review focus on the potential application of biological treatment approaches for treating sulphate-rich wastewater streams. The sections cover key concepts and develop the rationale for conducting the current work.

2.2 Biological sulphur cycle – treatment options

Sulphur conversions between different forms of reduced and oxidised sulphur compounds involve the metabolism of several specialised microbial communities (e.g. sulphate-reducing bacteria and sulphur oxidising-bacteria) (Sheoran *et al.,* 2010). These microorganisms possess unique physiological and metabolic traits; novel microorganisms isolated from extreme conditions (pH, temperature and salinity) are reported regularly. These microorganisms are well described throughout literature and have been exploited for industrial application in

pollution control of sulphate-rich and sulphide-rich waste streams. Depending on the desired treatment (sulphate reduction or sulphide oxidation), specific microorganisms associated with conversion of sulphur can be cultivated within bioreactors with high efficiency. Although these treatments are aimed at pollution control, new research have focused on the development of a circular economy with a focus on sulphur, metal and water recovery and reuse (Lens *et al.,* 2003).

The enhancement of sulphate reducing processes has become a major focus in ARD treatment in recent years. The advantages of biological treatment over conventional physicochemical treatment are driven by its potential for sustainability, low cost and minimal waste production. Furthermore, depending on the application, these processes can be performed at low temperature with minimal maintenance. An important parameter to the successful operation of biological processes is to understand the biocatalytic reactions that regulate sulphate concentration within these systems. The sulphur cycle is an important biogeochemical cycle (Figure 2-2) and comprises a collection of processes by which sulphur interchanges to and from minerals and living systems (Sheoran *et al.,* 2010; Gopi Kiran *et al.,* 2017). The sulphur cycle consists of four critical steps; 1) Mineralisation of organic sulphur into inorganic forms such as hydrogen sulphide, sulphide minerals and elemental sulphur; 2) Oxidation of hydrogen sulphide, and elemental sulphur to sulphate; 3) Reduction of sulphate to sulphide; and 4) Incorporation of sulphide and organic compounds into metal containing derivatives (Sheoran *et al.,* 2010).

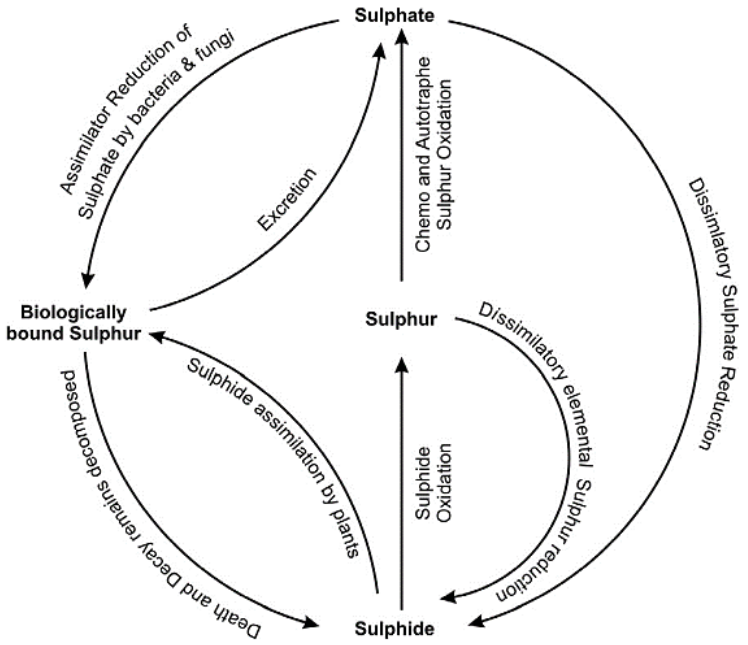


Figure 2-2: The biological sulphur cycle illustrating the intricate network of sulphur transformation that are performed by plants and microorganisms within natural environments such a marine sediments, thermal vents and soil. Many of these reactions are performed by autotrophic and heterotrophic organisms under a range of environmental conditions (Sheoran *et al.,* 2010).

The microbial communities of sulphate-reducing bacteria (SRB) and sulphide-oxidising bacteria (SOB) are responsible for the cycling of sulphur compounds. These microbial communities play a pivotal role in the development of sustainable biotechnological applications in order to restore the balance in the sulphur cycle such as in the treatment of sulphate-rich wastewaters (Muyzer & Stams, 2008; Syed *et al.,* 2006).

2.3 Biological sulphate reduction

In this section, SRB applied beneficially for treatment of sulphate-rich wastewater are reviewed. For this purpose, SRB are discussed in terms of biochemical pathways, microbial ecology, metabolic requirements and

current application in the bioremediation of ARD effluents. Subsequently, key parameters for successful SRB application are also addressed.

2.3.1 Sulphate reducing bacteria

SRB, including both bacteria and archaea, are a unique and highly diverse group of anaerobic microorganisms that obtain energy through the utilisation of sulphate ions (SO_4^{2-}) as a terminal electron acceptor for metabolism of organic substrates (Muyzer & Stams, 2008). Under anaerobic conditions these microorganisms use sulphate as a terminal electron acceptor and couples the oxidation of the substrate (inorganic or organic compound) to the reduction of sulphate. The energy produced is used by the SRB for cellular growth and metabolic maintenance. Although small amounts of reduced sulphur are used for assimilatory sulphate reduction through the synthesis of essential sulphur-containing cellular components, including amino acids and proteins, large amounts are released as free hydrogen sulphide as a generated waste through dissimilatory sulphate reduction for cellular energy (Postgate, 1984). In the context of ARD, as sulphate is reduced to sulphide, there is a reduction in acidity as a strong acid (H_2SO_4) is transformed into a weaker acid (H_2S), while alkalinity produced in the form of bicarbonate (HCO^{3-}) neutralises acidity. These reactions are summarised in Reaction 2.4-2.5 (Oyekola *et al.,* 2012; Nielsen *et al.,* 2018):

$$\text{Electron donor} + SO_4^{2-} \rightarrow 2HCO_3^{-} + H_2S \qquad \text{Reaction 2-4}$$

$$HCO_3^{-} + H^{+} \rightarrow H_2O + CO_2 \qquad \text{Reaction 2-5}$$

The sulphide may remain in solution, evolve as H_2S gas or be coupled to the precipitation of metal sulphides (Reaction 2.6; (Johnson & Hallberg, 2005). These precipitates are less soluble than their hydroxide equivalents allowing lower residual metal concentrations in solution.

$$Me^{2+} + HS^{-} \rightarrow MeS_{(s)} + H^{+} \qquad \text{Reaction 2-6}$$

There have been several studies that have investigated the selective precipitation and recovery of heavy metals in a range of different reactor configurations (Johnson & Hallberg, 2005; Kaksonen & Puhakka, 2007).

2.3.2 Bioreactor configurations

A variety of active and passive reactor configurations, have been applied to study anaerobic sulphate reduction or to treat ARD, are summarised in Figure 2-3. These include continuous stirred tank reactors (CSTR) (Moosa *et al.,* 2002; Oyekola *et al.,* 2012), up-flow packed bed reactors (Jong & Parry, 2003; Hessler *et al.,* 2018), membrane reactors (Nagpal *et al.,* 2000) and up-flow anaerobic sludge bed reactors (Sánchez-Andrea *et al.,* 2014). The selection of bioreactor configuration should consider cost, energy and maintenance requirement, efficiency of mixing and mass transfer, as well as efficient biomass retention to facilitate optimal process performance.

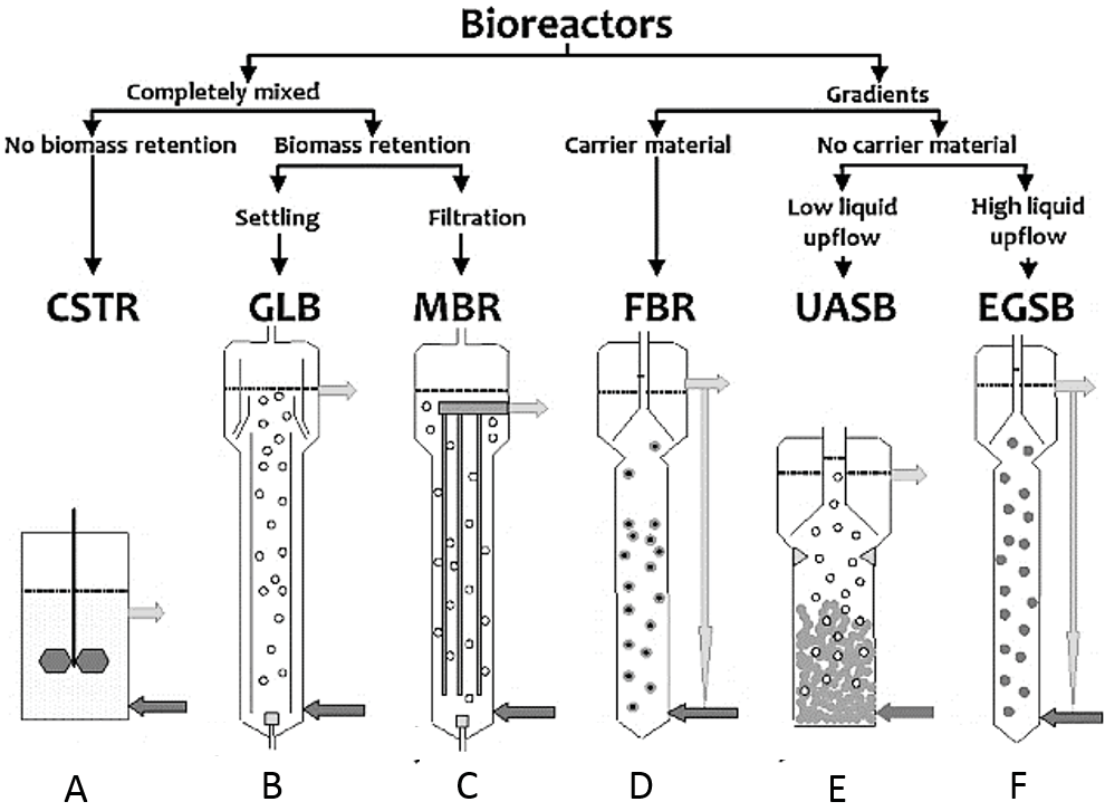


Figure 2-3: Reactor types used for sulphate reduction showing: A) continuous stirred tank reactor (CSTR), B) gas-lift bioreactor (GLB) with internal loop, C) submerged membrane bioreactor (MBR), D) fluidised bed reactor (FBR), E) upflow anaerobic granular sludge bed (UASB) bioreactor, and F) expanded granular sludge bed EGSB) reactor configurations. These systems can be further categorised by their mixing regime, ability to retain biomass, and use of carrier matrix (adapted from (Sánchez-Andrea *et al.,* 2014)).

Two main operational configurations have been broadly used for sulphidogenic treatment of ARD: 1) Two-stage reactors where sulphate reduction occurs in one reactor and the produced sulphide is recirculated to another reactor for metallic sulphide precipitation; and 2) A one stage reactor configuration where sulphate reduction and metal sulphide precipitation occur in a single operational unit (Sánchez-Andrea *et al.,* 2014). The one-stage reactor configuration is preferred for its simple design and reduced capital and operational costs. However, downstream separation and recovery of the metal sulphide can be complex (Sánchez-Andrea *et al.,* 2014). In most cases multiple reactor units are applied in series to enhance sulphate reduction and metal precipitation (Kaksonen & Puhakka, 2007).

2.3.3 Effect of operational parameters

Numerous studies have investigated the effects of various parameters on the BSR performance such as sulphate concentration, temperature, pH, electron donor availability and type, inhibitory metal and sulphide concentration, as well as the use of different solid support matrices (Elliott *et al.,* 1998; Moosa *et al.,* 2002, 2005b; Utgikar *et al.,* 2003; Baskaran & Nemati, 2006). The correct regulation and maintenance of these parameters within BSR systems is essential for optimal process efficiency.

Hydraulic residence time

The hydraulic residence time (HRT) influences the hydraulic conditions within the reactor, the retention of planktonic organisms and the contact between the incoming ARD stream and the microorganisms responsible for catalysing the reactions that govern the process, with consequent influence on the physicochemical conditions. Hence it is an important operating parameter for establishing optimal conditions within different sulphate reducing reactor configurations (Vasquez *et al.,* 2016, 2018) and enhancing efficiency (Greben &

Maree, 2000; Kaksonen & Puhakka, 2007). A short HRT may not allow adequate time for SRB activity to neutralise acidity and precipitate metals and, in the absence of biomass retention, may result in cell wash-out. Alternatively, a longer HRT may dictate the depletion of available carbon source or sulphate for SRB activity (Oyekola *et al.,* 2010). Previous studies have revealed that as HRT is decreased (flow rate increase) in a system without cell retention, volumetric sulphate reduction rate (VSRR) increased up until a threshold point after which a further decrease in HRT resulted in considerable loss in system performance. This was attributed to uncontrolled cell washout, proliferation of competitive microorganisms and reaction kinetic constraints associated with BSR (Greben & Maree, 2000; Moosa *et al.,* 2002; Oyekola *et al.,* 2012).

pH

The operating pH of a sulphate reducing bioreactor is a critical parameter for maintaining optimal microbial activity. Most known SRB have been reported as neutrophilic and grow optimally in the pH range of 7.5-8 (Brahmacharimayum *et al.,* 2019). Typically, SRB are inhibited at acidic (<6) and very alkaline (>9) pH ranges. Studies have reported on an optimal operating pH range between 5 and 8. Outside this range, the rate of microbial sulphate reduction significantly declines. Ideally, to ensure optimal biological sulphate reduction performance, a highly acidic waste stream would require pre-treatment neutralisation to limit SRB inhibition. BSR is typically conducted at neutral pH. However, several studies have detected the growth of acidophilic SRB in natural environments growing at pH<3. Several studies have successfully performed BSR treating ARD under acidic conditions (Elliott *et al.,* 1998a; Kolmert & Johnson, 2001). A study by Elliott et al. (1998) investigated the effects of acidic conditions on SRB activity. The study evaluated a range of pH conditions in a porous up-flow bioreactor. The system achieved 38% sulphate conversion at pH of 3.25 and 14.4% at pH of 3.0. SRB have been isolated from acidic environments where they exist within microhabitats of suitable pH conditions (Elliott *et al.,* 1998a). These microorganisms buffer their surrounding environment by consuming hydrogen as an electron acceptor.

Temperature

SRB can be classified as mesophiles, moderate thermophiles and extreme thermophiles based on their optimum growth temperature (Sheoran *et al.,* 2010). Operating temperatures affects microbial growth, kinetics of organic substrate decomposition, as well as hydrogen sulphide solubility (Sheoran *et al.,* 2010). Under psychrophilic conditions, biogenic alkalinity is hardly produced because of low activity and incomplete oxidation of the electron donor (lactate or ethanol) to acetate. At low temperatures sulphate reduction is kinetically slower, while at the higher temperatures, chemical and enzymatic reaction rates increase (Greben & Maree, 2000). Moosa et al. (2002) reported that sulphate reduction rate increased with increasing temperature from 20 to 35°C in a CSTR, employing a mesophilic SRB culture. Optimum temperature is dependent on the microbial consortium present. SRB thrive over a wide range of temperatures, exhibiting high flexibility to temperature fluctuations and can generally tolerate temperatures from -5 to 75°C (Postgate, 1984). Studies have reported SRB grow at temperatures as low as 5°C, while some have observed spore forming thermophilic SRB species grow well at temperature as high as 65 to 80°C (Sheoran *et al.,* 2010). BSR has been recorded at temperatures as high as 100°C (Teske *et al.,* 2014). Most SRB are predominantly mesophilic, metabolising optimally at a temperature of 25 to 40°C (Sheoran *et al.,* 2010). Temperature is a critical parameter on the operation of biological sulphate reducing systems particularly where temperature is not regulated within the system. Under these conditions, the key to success of a BSR process is its ability to perform at lower temperatures as well as its resilience to seasonal fluctuation in temperature (Sato *et al.,* 2017).

Solid support matrix

The performance of a sulphate reducing system is highly dependent on the microbial biofilm formation on a solid support matrix and its regeneration capacity (Gopi Kiran *et al.,* 2017). The use of solid support matrices in BSR systems facilitates the attachment of microorganisms and enhances biomass retention. An alternative or additional approach to biomass retention is the entrapment of biomass in porous matrices, such as foam structures (Hessler *et al.,* 2018). These retention approaches facilitate the decoupling of the biomass retention time and HRT, allowing operation of the system at high dilution rates, while maintaining high biomass concentration and enhanced reaction rates (Baskaran & Nemati, 2006). SRB do not granulate readily, hence require a solid support on which they can establish microenvironments within biofilms for their survival in the

presence of extreme conditions such as high oxygen concentrations and low pH (Gopi Kiran *et al.,* 2017). When SRB have access to a porous surface, higher sulphate reduction rates are observed compared to a free-cell suspension (Silva *et al.,* 2006). The choice of support material can be a determining factor in the selection of the nature and quantity of the active microbial community within a reactor. Solid supports are selected for incorporation into specific applications, hence numerous studies have reported the immobilisation of SRB on different support matrices (Silva *et al.,* 2006). It is essential that the choice of support material within field-bioreactors maintain a balance between surface area and pore size without significantly compromising reactor volume (Neculita *et al.,* 2008).

COD/Sulphate ratio

An important factor in anaerobic treatment of sulphate-rich wastewaters is the competitive interaction between sulphate reducing bacteria (SRB), methane producing archaea (MPA) and fermentative microorganisms. The COD:sulphate ratio regulates microbial competition. SRB predominate when sulphate is in excess, while under limiting sulphate concentration MPA and fermentative microorganisms dominate (Oyekola *et al.,* 2009), based on their relative affinities for the substrate. SRB have a higher affinity for acetate and hydrogen and outcompete MPA at low substrate concentrations. At a COD:sulphate ratio below 0.67 g/g (stoichiometric ratio for complete oxidation), sulphate reduction is favoured over methane production (Oyekola *et al.,* 2009). Since the treatment of ARD requires the supplementation of organics (carbon source), it is important to regulate organic loading ratio such that sulphate reduction is favoured over competing reactions, complete sulphate removal is achieved whilst minimal residual COD is released within the effluent stream.

2.3.4 Biological sulphate reduction treatment technologies

The application of BSR offers an attractive approach to achieving sustainable treatment of sulphate-rich industrial wastewaters. Several developmental studies, based on sulphate reduction have been conducted on laboratory simulated (synthetic) and raw wastewater effluents contaminated with a range of pollutants, particularly containing high concentration of sulphate. Most of these studies were performed at laboratory bench-scale, with limited data available on commercial-scale applications. Though many of these studies show promising results, most are either not economically feasible or failed at demonstration-scale (Hussain *et al.,* 2014). Despite the extensive research on BSR systems, their widespread application remains limited due to several challenges experienced at commercial scale.

2.4 Challenges facing biological sulphate reduction

From a technical perspective and review of literature, a study by Harrison et al. (2014) identified three major challenges that would need to be overcome in order to make sulphate reduction technologies more applicable, economically feasible and attractive as a treatment approach. These included: 1) the provision of a cost-effective electron donor, 2) the enhancement of reaction kinetics, and 3) improved management of the generated sulphide. These challenges are discussed in further detail in this section.

2.4.1 Selection of a suitable electron donor

ARD is typically characterised by a low organic carbon content <10 mg/L) and requires the supplementation of an electron donor to facilitate biological sulphate reduction activity (Kolmert & Johnson, 2001). The choice of substrate is governed by several criteria: (1) the ability of SRB to assimilate the substrate; 2) the suitability of the substrate for the application (active vs. passive, reactor configuration, etc.); (3) the amount of sulphate to be reduced and the cost of substrate per unit of sulphide produced; 4) local availability; and 5) potential secondary pollution generated from incomplete degradation of the substrate (Kaksonen & Puhakka, 2007). The type of carbon and electron donor used in BSR can be categorised into two broad groups namely, direct or simple organic substrates or indirect or complex organic substrates. The sequential degradation pathway of these sources for sulphate reduction is presented in Figure 2-4. Since SRB are characterised by their ability to degrade organic carbon, the selection of electron donor has significant implications on the active microbial community within a given bioreactor system and can affect the degree of performance.

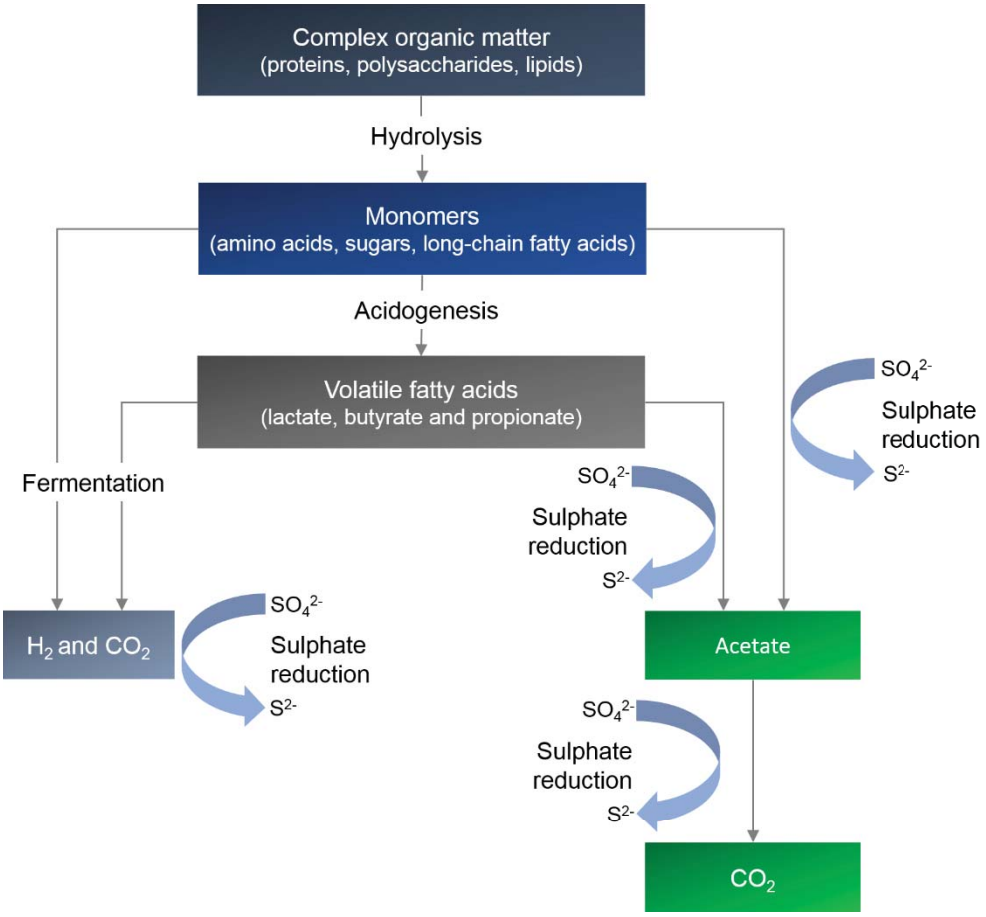


Figure 2-4: Sequential pattern of microbial degradation of complex organic matter in anoxic environment in the presence of sulphate. Macromolecules, such as proteins, polysaccharides, and lipids are hydrolysed by hydrolytic bacteria. Subsequently, the monomers (amino acids, sugars, fatty acids) are fermented by fermentative bacteria during acidogenesis resulting in a range of fermentation products such as acetate, propionate, butyrate, lactate as well as hydrogen and CO2. In the presence of sulphate, SRB couple the consumption of these products towards sulphate reduction (adapted from Muyzer & Stams (2008)).

Direct/simple organic substrate

Direct or simple organic substrates can be defined as easily degradable fraction of organic matter such as low molecular weight compounds with simple structures (Sheoran *et al.,* 2010). These carbon sources do not require decomposition by other microorganisms prior to utilisation by SRB, such substrates include: organic acids (e.g. acetate, butyrate, propionate and lactate); sugars (e.g. sucrose, fructose and glucose); alcohols (e.g. ethanol and methanol) (Liamleam & Annachhatre, 2007). Direct substrates are preferable in active treatments, it provides better predictability and provides consistent performance. Major drawbacks are associated with the cost and availability of simple substrates.

Indirect/complex organic substrate

Indirect or complex organic substrates are those requiring decomposition by a group of microorganisms to provide SRB with easily degradable carbon (Sheoran *et al.,* 2010). SRB are not capable of degrading biopolymers directly. Therefore, when a complex polymeric substrate is used, SRB rely on the activity of hydrolytic and fermentative bacteria. Several studies have investigated the potential of various complex substrates for achieving sulphate reduction. These substrates include lignocellulosic waste (Mooruth, 2013b), sewage sludge (Rose, 2013) micro-algal digestate (Harrison *et al.,* 2014) and rice bran (Sato *et al.,* 2017). The sustained release of organic acids from these substrates is an essential parameter when assessing the economic feasibility of a complex carbon source to support SRB over an extended period. A study by Mooruth (2013), determined that the rate limiting step in the performance of a degrading packed bed reactor, using lignocellulosic material for

sulphate reduction, was the hydrolysis and release of soluble organic carbon that can be assimilated by the SRB community.

Depending on the application, a solid, liquid or gaseous substrate can be preferential. For passive treatment, solid complex substrates comprising of plant or waste material are often required to allow passive operation without active pumping of the substrate. However, complex substrates are characterised by a limited lifetime and often require frequent replenishment or will need to be replaced overtime. In active bioreactor configurations, liquid and gaseous substrates are preferred for continuous and consistent process operation providing better control, predictability and stable performance (Sheoran *et al.,* 2010).

An important parameter in selecting a suitable substrate besides cost and availability, is its effectiveness for sulphate reduction. The chemical oxygen demand (COD)/sulphate ratio can be defined as the interaction of SRB with the carbon source available and the electron donor (Gopi Kiran *et al.,* 2017). The minimum COD/sulphate ratio of 0.67 is considered the ideal stoichiometric proportion required for complete sulphate reduction and degradation of the organic substrate. The applied COD/sulphate ratio can vary depending on the type and source of carbon and can range between 0.7-1.5. For simple carbon sources the optimum COD/sulphate ratio applied ranges between 0.55 and 0.84 while for organic waste products (activated sludge and municipal compost) the ratio can vary between 1.6 and 5 (Greben & Maree, 2000; Gopi Kiran *et al.,* 2017). The selection of an optimal COD/sulphate ratio is a key parameter in assuring the efficiency of any system designed for treating sulphate. It is important to consider that an effective complex carbon source successfully identified and tested at laboratory-scale may not necessarily be applicable at larger scale. This is largely determined by its availability, sourcing requirements and cost.

Lactate and acetate as a carbon source

Lactate based on energy and biomass production is a superior electron donor for SRB activity compared to alternative sources such as acetate, propionate and ethanol (Nagpal *et al.,* 2000). It also facilitates the growth of a diverse range of SRB species. For this reason, lactate has been regarded as the model substrate for studying sulphate reducing activity. It has been applied in numerous kinetic studies for modelling sulphate reduction in continuously stirred tank reactors (Bertolino *et al.,* 2012; Oyekola *et al.,* 2012) as well as a range of different reactor configurations and batch biokinetic tests. Despite the many advantages of lactate in BSR systems, its application has been restricted to laboratory bench-scale studies due to its cost and availability.

Lactate is particularly beneficial for evaluating novel bioreactors systems where the outcome of the study is not constrained by the electron donor to achieve effective sulphate reduction. It has also been implicated in the rapid start-up of BSR systems when supplemented during initial colonisation and biofilm formation (Celis *et al.,* 2013).

Table 2-3: Sulphidogenic degradation reaction of different electron donors and the respective Gibbs free energy change (adapted from Bertelino *et al.,* 2012).

Chemical reaction	ΔG° (kJ)		
$2\ Lactate + 3\ SO_4^{2-} \rightarrow 6\ HCO_3^- + 3\ HS^- + H^+$	-225.3	2-7	Reaction
$2\ Lactate^- + SO_4^{2+} \rightarrow HS^- + 2\ Acetate^- + 2\ HCO_3^- + H^+$	-160.1	2-8	Reaction
$2\ Ethanol + SO_4^{2-} \rightarrow 2\ Acetate^- + HS^- + 2\ H_2O + H^+$	-66.4	2-9	Reaction
$Acetate^- + SO_4^{2-} \rightarrow HS^- + 2\ HCO_3^-$	-47.8	2-10	Reaction

After lactate, ethanol and acetate are favoured; these have also been extensively studied. While ethanol has been successfully applied at industrial scale (Thiopaq™ process), its widespread application is constrained by local availability and cost. Acetate serves as an important intermediate in the degradation of complex substrates and is generated during hydrolysis and fermentation (Figure 2-4). In addition, the accumulation of acetate as a result of incomplete oxidation of substrates, such as lactate (Reaction 2.8) and ethanol (Reaction 2.9), is the rate limiting step within many high-rate sulphate reducing systems (Kaksonen & Puhakka, 2007). The major drawback associated with acetate can be attributed to the slow growth rate of complete oxidising SRB. Incomplete oxidising SRB associated with lactate metabolism have a doubling time of between 3-10 h while complete oxidisers associated with acetate metabolism have a doubling time between 16-20 h (Celis *et al.,* 2013; Postgate, 1984). Thermodynamically, SRB obtain more Gibbs free energy from the incomplete oxidation of substrates (lactate = -160.1 kJ and ethanol = 66.4 kJ) than from the complete oxidation of acetate= -47.8 kJ/mol (Bertolino *et al.,* 2012; Celis *et al.,* 2013). However, complete oxidation is important to deliver product water with the lowest levels of contamination.

2.4.2 Biological sulphate reduction kinetics

The second major challenge associated with BSR systems is the reaction kinetics. These are governed by several parameters that span metabolic requirements to operational parameters. As previously discussed, the correct regulation and control of these conditions is therefore important to ensure optimal process performance. The feasibility of a wastewater or ARD treatment process, besides capital and operational cost, is highly dependent on the quality and rate of treatment. This is largely dictated by the reaction kinetics of the system, as well as the extent of reaction readily achieved. Biological sulphate reduction can be accomplished with freely suspended cells or immobilised cells. In free cell suspended BSR systems, a low dilution rate is required to prevent cell wash-out. The wash-out of critical SRB species occurs when the dilution rate exceeds that of the specific growth rate of that species where no retention strategy is employed. Since SRB are generally characterised as slow growing microorganisms, in the absence of sufficient biomass retention, BSR processes are governed by long residence times and slow kinetics.

The use of solid support matrices is an important parameter in BSR systems and was briefly introduced in Section 2.3.3.6. The immobilisation of biomass onto a support matrix is preferred in BSR systems in order to decouple the biomass retention time from the hydraulic residence time, allowing operation at high flow rates without significant cell washout. The enhanced biomass retention in immobilised cell bioreactors improves sulphate reduction kinetics (Baskaran & Nemati, 2006). The performance of sulphidogenic bioreactor is highly dependent

on the microbial biofilm formation and its regeneration capacity (Gopi Kiran *et al.,* 2017). Besides overcoming kinetic constraints observed in freely suspended cell systems, the immobilised cells encapsulated within an EPS matrix tolerate higher concentrations of toxic compounds than freely suspended cells. The outer layers protect the inner layers of the biofilm from exposure to inhibitory concentrations due to mass transfer resistance. In addition, these studies have shown enhance resistance to metals as a result of EPS that protect cells by binding heavy metals and retarding their diffusion within the biofilm (Kaksonen & Puhakka, 2007).

Several solid support matrices have been evaluated for sulphate reduction. These include: polyurethane foam, vegetal carbon, low density polyethylene, alumina based ceramics, sand, glass beads and carbon microfibers (Jong & Parry, 2003; Baskaran & Nemati, 2006; Silva *et al.,* 2006; van Hille *et al.,* 2015; Hessler *et al.,* 2018). The type of reactor configuration (freely suspended cells or immobilisation) and support matrix can affect the ability of SRB to compete for certain substrates as studies have shown preferential immobilisation of non-SRB (Hessler *et al.,* 2018).

A key consideration when selecting a solid support matrix is to ensure minimal compromise on the working volume capacity of the system. Therefore, important factors that govern the effectiveness of a support matrix is whether the material is chemically inert and will not have an adverse (toxic) effect on the microorganisms, the pore size and total surface area, as well as the practical application within the reactor configuration.

2.4.3 **Sulphide management**

The third major challenged faced by BSR systems is the generation of sulphide during anaerobic treatment; this represents one of the bottlenecks associated with the application of these processes and is considered a "secondary pollution" (Pokorna & Zabranska, 2015). It is essential to partition the sulphide product out of the solution phase and, preferably, into a solid form for easy recovery. During the treatment of ARD, the generated sulphide may be co-precipitated with heavy metals to form stable metal sulphides that can be removed from solution, removing S and metals simultaneously. However, depending on the source of ARD, when there is a deficit in heavy metal concentrations and BSR is highly effective, the excess sulphide generated requires further management. In order to ensure the sustainability of BSR as a long-term solution to ARD remediation, the use of an appropriate management strategy of hydrogen sulphide is critical. One approach that has gained a lot of interest in recent years is the potential partial oxidation of sulphide to elemental sulphur. In the following sections a review of sulphide chemistry and application of partial sulphide oxidation for elemental sulphur recovery is discussed.

Sulphide chemistry

In addition to its unpleasant smell, hydrogen sulphide (H_2S) gas is highly toxic (Cai *et al.,* 2017). Upon inhalation, hydrogen sulphide reacts with enzymes in the bloodstream and inhibits cellular respiration resulting in pulmonary paralysis and death. The continuous exposure to hydrogen sulphide concentrations as low as 15-50 ppm results in irritation to mucous membranes and may cause headaches, dizziness and nausea. Higher concentrations of 200-300 ppm may result in respiratory arrest leading to coma and unconsciousness while exposures for more than 30 minutes at concentrations greater that 700 ppm have been fatal (Syed *et al.,* 2006).

Sulphide mainly undergoes oxidation by two reactions in the presence of oxygen shown in Reaction 2.11 and 2.12 (Cai *et al.,* 2017). The reactions represent the oxidation pathways whereby sulphide is converted to sulphate or elemental sulphur under chemical or biological conditions.

$$2\ HS^- + O_2 \rightarrow 2\ S_0 + 2\ OH^-$$ Reaction 2-11

$$2\ HS^- + 4\ O_2 \rightarrow 2\ SO_4^{2-} + 2\ H^+$$ Reaction 2-12

The Pourbaix diagram (Figure 2-5) provides an indication of the sulphur chemical system in terms of ionic activities as well as thermodynamic forces where equilibrium distribution of dominant sulphur-containing species are represented according to specific Eh (redox) and pH values.

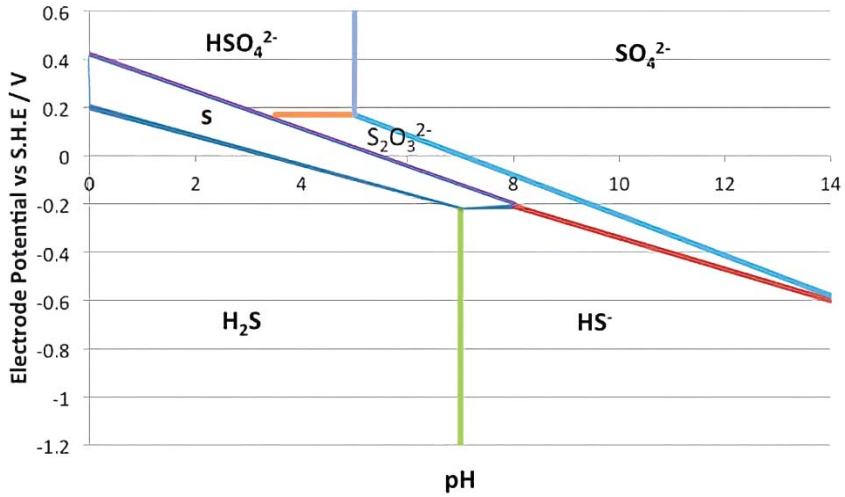


Figure 2-5: Pourbaix diagram Eh = f(pH) representing the predominant sulphur-containing species in equilibrium at different oxygen pressure (redox potential) and acidity (pH) with iron and sodium ions in an aqueous solution.

In comparison to the other dominant oxidised forms of sulphur, elemental sulphur is confined to a narrow window of redox potential and pH. A study by (Nielsen *et al.,* 2005) reported Arrhenius temperature dependency of chemical and biological sulphide oxidation. The rate of chemical and biological sulphide oxidation doubled with temperature increase of 9 and 7°C, respectively. The pH dependence on abiotic and biotic sulphide oxidation is a function of H_2S dissociation with H_2S being oxidised at a lower rate than HS^-. (Lewis *et al.,* 2000) suggested that equilibrium thermodynamics impact the major product of sulphide oxidation less than kinetic considerations in biological processes. Conditions present within the bulk chemical phase differ from the intracellular conditions in living systems. Therefore, further investigation into the microbial kinetics associated with the biological sulphur removal processes will provide insight into process optimisation and conditions that promote partial oxidation of sulphide to elemental sulphur over complete oxidation to sulphate.

Sulphide treatment options

The partial oxidation of sulphide to elemental sulphur is an approach that has gained considerable amount of attention. Both physicochemical and biological options have been investigated. Physicochemical processes involved in the removal of sulphide from solution include chemical precipitation and oxidation reactions which usually results in the production of metal sulphide sludge that requires special disposal of. For oxidative reactions, sulphide ions encounter oxygen under controlled redox potential and pH conditions that promote the production of S^0 (elemental sulphur) and hydroxide ions. The produced sulphur with an oxidation state of zero, consisting mainly of cyclic S_8 molecules that aggregate into larger crystals are then separated from solution by flotation or alternative separation techniques (Janssen *et al.,* 1999).

Industrialised physicochemical processes most widely recognized for the removal of sulphide include the Clause process (gas desulfurisation). This multi-step process is used in the petrochemical industry for the recovering of elemental sulphur by stripping gaseous hydrogen sulphide into a glycol or amine solution at high temperature and pressure and subsequently catalytically converting it to elemental sulphur.

The use of the abovementioned physicochemical method for treating sulphide-rich waste streams is highly effective; however, there are associated disadvantages, such as the high energy input, operational cost and the use of speciality chemicals (catalyst). Due to these requirements, physicochemical treatments are unsuitable for treating ARD (Cai *et al.,* 2017). An alternative approach to achieve sulphide removal with elemental sulphur recovery is the application of biological sulphide oxidation (Harrison *et al.,* 2014).

2.5 Biological sulphide oxidation

The management of hydrogen sulphide is essential for the development of a sustainable, linearised sulphur treatment such that a stable less toxic form of sulphur is removed and recoverable. Strategies reported for the removal of sulphide include metal sulphide precipitation (Lewis, 2010), chemical solvent extraction and oxidation to elemental sulphur (Janssen *et al.,* 1999; Molwantwa *et al.,* 2004). The potential application of partial sulphide oxidation to form elemental sulphur within wastewater treatment strategies would not only contribute significantly to the sustainability of the process in managing the sulphide generated during BSR but will also result in the recovery of a sulphur product.

2.5.1 Sulphur oxidising bacteria

The study of biological sulphide removal under aerobic conditions has been documented since the early 1990s. Studies by Buisman et al. (1990) reported the effect of dissolved oxygen and sulphide loading on the performance of biological sulphide oxidation. The study demonstrated that the final product of biological oxidation is highly dependent on the ratio of oxygen to sulphide as represented by Reactions 2.18-2.20 (Guerrero *et al.,* 2015):

$$H_2S + \frac{1}{2}\,O_2 \rightarrow S^0 + H_2O \qquad \text{Reaction 2-13}$$

$$S^0 + H_2O + \frac{3}{2}O_2 \rightarrow SO_4^{2-} + 2H^+ \qquad \text{Reaction 2-14}$$

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \qquad \text{Reaction 2-15}$$

These reactions indicate that partial oxidation of sulphide to elemental sulphur proceeds under oxygen-limiting conditions while complete sulphide oxidation toward sulphate occurs when oxygen is in excess.

2.5.2 Generation of biologically produced sulphur

Characteristics of biologically produced sulphur

Biologically produced sulphur is divided into two groups, based on whether they are produced by internal and external sulphur excretion mechanisms (Cai *et al.,* 2017). Internal sulphur is present within the periplasm SOB as invaginations that are often encapsulated by a protein envelope though to be purely structural in function. External sulphur exists as globules that are not enclosed in the cell membrane and is the preferred form for harvesting from wastewater treatment processes (Cai *et al.,* 2017; Pokorna & Zabranska, 2015).

According to previous reports, biological sulphur forms transparent globules which are deposited on the inside or outside of SOB. Biologically produced sulphur has a white or pale colour and a higher refractive index than water (Cai *et al.,* 2017). X-ray measurements of sulphur obtained from sulphide oxidising bioreactors revealed it is partly built up of orthorhombic sulphur crystals (S_8). Orthorhombic sulphur is usually highly insoluble in water (5 g/L), although it can be readily dissolved in a non-polar solvent such as hexadecane. However, solubility tests of biologically produced sulphur particles indicated that they were soluble in water, rather than in hexadecane, indicating that they are hydrophilic. The hydrophilic characteristic of biosulphur was attributed to the presence of amphiphilic compounds covering the hydrophobic S_8 nucleus. A study by Janssen et al. (2001) conducted electrophoretic mobility measurements and flocculation experiments on biosulphur and suggested that the sulphur particles are covered by an extended negatively charged polymeric layer, most likely made up of protein.

2.5.3 Biological sulphide oxidation treatment technologies

The study of sulphide oxidation for sulphur recovery has been studied in a variety of reactor configurations. However, like BSR systems its widespread application at industrial scale has been largely limited to few technologies applied under niche environments that enable their feasibility. The most successful commercialised systems are the THIOPAQ™ SULFATEQ™ and THIOPAQ™ O&G processes developed by Paques and Paqell (joint venture between Paques and Shell), respectively (Cline *et al.,* 2002).

The THIOPAQ (SULFATEQ) process operated at the Budelco zinc refinery in the Netherlands utilises the sulphide oxidising ability of SOB (Cline *et al.,* 2002). The overview of the process is presented in (Figure 2-6). The process treats zinc sulphate-containing process water. Sulphate reduction takes place in full-scale (500 m^3) sulphate-reducing gas-lift reactor. Synthesis gas is used as the electron donor by steam-reforming natural gas. Sulphide generated through sulphate reduction precipitates zinc which is collected in a settler and reused in the roasting process. Excess sulphide is directed toward the aerobic bioreactor under oxygen-limiting conditions, where partial sulphide oxidation occurs resulting in the recovery of elemental sulphur downstream (Muyzer & Stams, 2008).

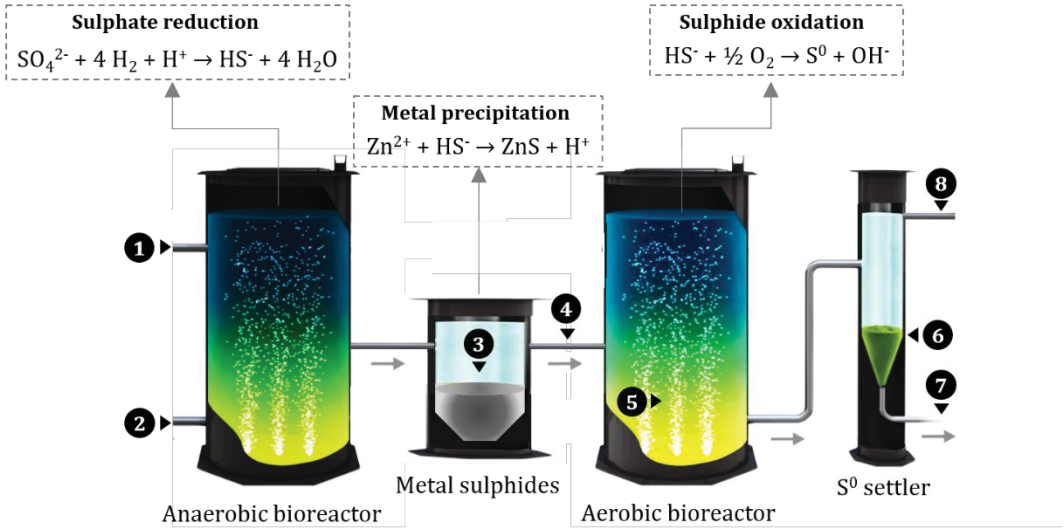


Figure 2-6: Schematic overview of the THIOPAQ™ SULFATEQ™ process to remove sulphate and heavy metals from waste water. 1) Sulphate and metal contaminated water enter the anaerobic bioreactor where sulphate-reducing bacteria reduce sulphate to sulphide using 2) hydrogen (H2-rich gas) as an electron donor. Subsequently, the sulphide generated is used to 3) precipitate the heavy metals. The 4) excess of sulphide is converted to elemental sulphur by sulphide oxidising bacteria in a 5) aerated bioreactor. The precipitated metal sulphides and elemental sulphur can be 6) separated and 7) recovered while the 8) purified effluent is safe for discharge.

Most chemical and biological sulphide oxidation (THIOPAQ™) processes are operated actively as a downstream process treating sulphide-rich waste streams generated at chemical refineries. The major drawback of these systems is the high process costs, maintenance and use of specialised equipment. This has significantly hindered its application in the treatment of ARD discharge. However, the discovery of floating sulphur biofilms (FSB) and their potential application in the treatment of ARD wastewater has provided a promising approach to achieving partial sulphide oxidation with the recovery of elemental sulphur, under passive operating conditions (Molwantwa, 2007; Mooruth, 2013b). The occurrence of FSB and its potential application for treating sulphide-rich waste streams will be discussed in the following sections.

2.6 Floating sulphur biofilm in wastewater treatment

The generation of elemental sulphur through biological sulphide oxidation occurring in several natural environments have been well documented. One biological structure of high biotechnological interest is floating sulphur biofilms (FSB), which were found forming naturally on the surface of highly sulphidic tannery wastewater ponds (Molwantwa, 2007). These sulphur-rich films were also observed adhering onto the glass walls of sulphide-rich reactors at the gas-liquid interface (Oyekola *et al.,* 2010). These floating films were later identified to consist of a diverse microbial community comprising of sulphide oxidising bacteria (SOB) which are involved in the conversion of sulphide to biological elemental sulphur. Through elemental analysis these biofilms were

predominantly comprised of elemental sulphur. This generated further interest into understanding the function and relevance of FSB occurring in sulphide-rich water bodies and its potential application for treating sulphide-rich wastewater (Molwantwa, 2007).

A biofilm is defined as an aggregation of microorganisms in which cells that are often enclosed within a self-produced extracellular polymeric substance (EPS) adhere to each other and/or to a surface or interface (Paytubi *et al.,* 2017). Biofilms that form at the air-liquid interface, are generally referred to as "pellicle". The air-liquid interface serves as a favourable niche environment for the growth of bacteria where nutrients are acquired from the liquid and oxygen from the surrounding environment. There has been a growing interest in the study of pellicle formation, with most studies conducted on pure bacterial cultures including *Bacillus subtilis* (Kobayashi, 2007), *Shewanella oneidensi*s (Armitano *et al.,* 2013), *Acinobacter baumannii* (Chabane *et al.,* 2014) and *Salmonella enterica* (Paytubi *et al.,* 2017).

The ability to colonise the air-liquid interface to form a floating structure requires high organisation due to the lack of a solid surface for initial attachment (Chabane *et al.,* 2014). The formation of floating biofilms or pellicle begins with the attachment of planktonic cells to the surface. Flagella and pili appendages of microorganisms have shown to play an important role in the migration and attachment of cells (Chabane *et al.,* 2014). After reaching the air-liquid interface, bacteria adapt to the environment and an increase in EPS synthesis is initiated. A homogenous layer forms after which the biofilm matures into a three-dimensional biofilm structure where bacterial cells are covered and connected by an intricate EPS network. A study by (Armitano *et al.,* 2013) reported the importance of aerotaxis (movement of motile cells, in the direction corresponding to an increasing gradient of oxygen) in floating biofilm development.

The formation of FSB, though more complex in microbial community dynamics and structure, exhibits similar developmental stages described in pellicle formation of pure cultures at the air-liquid interface. Studies by Molwantwa (2007) and Mooruth (2013), performed an extensive analysis on the formation and structure of FSB. The studies demonstrated the potential application of FSB to achieve partial sulphide oxidation with high elemental sulphur recovery.

2.7 Current state of research and development

The integrated semi-passive bioprocess developed at the Centre for Bioprocess Engineering Research (CeBER) at University of Cape Town has potential to address the current drawbacks associated with traditional passive and active treatments (WRC funded projects K5-2110, K5-2392 and K5-2393). The process facilitates simultaneous biological sulphate reduction and partial sulphide oxidation via a floating sulphur biofilm (FSB) within a single reactor unit. There have been several studies that have contributed to development of the integrated process particularly the initial preliminary studies around the Pulles Howard & de Lange IMPI process (van Hille *et al.,* 2011), fundamental information with regards to residence time, hydrodynamics, organic loading rate and sulphur speciation in the LFCR (Mooruth, 2013b) and insight into FSB with regards to microbial community and mass transport of oxygen through the biofilm (Molwantwa, 2007). Furthermore, a study by van Hille et al. (2015), which demonstrated that micro-carbon fibres can be used as an efficient support matrix for SRB attachment. However, due to the novelty of the system, there is still a need for further development to maximise processes efficiency within the hybrid LFCR, especially since the system has been reconfigured to accommodate both sulphate reduction and sulphide oxidation within one reactor unit. Recent studies conducted have confirmed the proof of concept whereby biological sulphate reduction through enhance biomass retention using carbon microfibers and partial sulphide oxidation via the formation of a floating sulphur biofilm can be achieved within a single reactor unit (Marais *et al.,* 2020a). Additionally, the hydrodynamics and effect of hydraulic residence time on system performance has been performed (Marais *et al.,* 2020b). The effect of HRT on system performance showed that as HRT decreased the rate of sulphate reduction (system performance) increased to a maximum at a 2-day HRT. Upon further decrease, a threshold point of 1 day was found where an additional increase in flow rate had a negative effect on the system leading to cell washout followed by a decrease in sulphate reduction and sulphide oxidation rates. Optimal HRT based on system performance (volume treated and volumetric sulphate reduction rates) was obtained at a 2-day HRT. Additional investigations have since been conducted into the effects of different operational parameters on the performance of the

process. This includes the effect of temperature, sulphate loading as well as the use of alternative carbon and electron donor sources on process performance and microbial community dynamics.

2.8 Purpose and Scope of the Evaluation

Although significant progress has been made towards the characterisation and development of the integrated semi-passive hybrid LFCR process, key aspects of the process require further investigation. These are based on the final recommendations concluded within previous work (WRC projects K5-2392 and K5-2393) which led to the identification of key focus areas that are the next crucial steps toward realising real world application of the process. The key areas to be considered in the current research include:

- Enhancement of overall sulphate conversion efficiency within the reactor in order to lead to higher sulphur recovery yields.
- Assessment of the integrated process on treating raw ARD.
- Identification and selection of suitable carbon sources that can be applied at large-scale for semi-passive treatment systems.

2.9 Project aim and objectives

The aim of this study is to alleviate the burden of persistent low flow ARD pollution through the development of a sustainable semi-passive treatment solution. The research is comprised of the re-design and improvement of integrated linear flow channel reactor (LFCR) process and its application to treating real world ARD as well as identification of a suitable carbon substrate.

The following objectives are investigated in this report:

1. Assess the effect of polyurethane foam as a support-matrices for enhanced sulphate reduction kinetics and its ability to preferentially retain and select for an SRB microbial community.

2. Investigate a selection of alternative carbon sources (simple and complex) such as lactate, acetate, ethanol, fructose, molasses, algal digestate, vermicompost and honey for biological sulphate reduction.

3. Evaluate the potential of the hybrid LFCR to treat a raw ARD stream sourced from mine site.

2.10 Innovation and new knowledge

The hybrid LFCR process is a novel process that incorporates simultaneous biological sulphate reduction (BSR) and partial sulphide oxidation within a single reactor unit. The process is operated under semi-passive conditions and requires minimal energy, thereby reducing associated operating costs. The design has been shown to achieve comparable performance to that obtained in biological active treatment alternatives.

In conventional treatments for sulphate these processes are generally separated into two reactor units under contrasting operating conditions to maintain biological sulphate reduction under anoxic conditions and sulphide oxidation under oxic conditions. These processes, such as the THIOPAQ process (Paques), are operated under active conditions and require extensive maintenance and operational intervention to ensure optimal performance. The re-designed linear flow channel reactor is envisaged to enhance overall process performance under semi-passive conditions, allowing recovery of both elemental sulphur and fit-for-purpose water through a process demonstrating techno-economic feasibility. Further development of the process may result in a sustainable solution that can be applied for treatment of ARD streams located in remote regions where limited infrastructure is available. The recovery of elemental sulphur is a highly attractive feature of the process as this is a valuable product that can be used in agriculture as a fertiliser, allowing for resource recovery.

3 Materials and Methods

3.1 Microbial cultures

The SRB mixed microbial community (stock culture), originally sourced from the laboratory of Prof Peter Rose (Department of Microbiology, Biochemistry and Biotechnology) at Rhodes University, South Africa, has been maintained at the University of Cape Town (UCT) over an extended period on modified Postgate B (MPB) medium since 2001. The culture was originally derived from an anaerobic compartment of a facultative pond at the Grahamstown sewage treatment works. The culture serves as the starting inoculum for continuous bioreactor and batch culture studies. The sulphide oxidising bacteria (SOB) consortium was developed at UCT using enrichments from SRB reactors (Mooruth, 2013b). The SOB culture is maintained as floating sulphur biofilm in a continuously operated LFCR. Depending on the experiments, the synthetic media was made up in 1 L (batch cultures) and 10 L (continuous bioreactor operation) Schott bottles. The synthetic feed was sterilised by autoclaving at 121°C, 103 kPa for 20 min.

Table 3-1: Modified Postgate B medium composition and quantities that will be used in the current study (Oyekola *et al.,* 2012).

Component	Amount (1 g/L SO_4^{2-})	Amount (2 g/L SO_4^{2-})
K_2HPO_4	0.46 g/L	0.46 g/L
NH_4Cl	1.00 g/L	1.00 g/L
$MgSO_4.7H_2O$	2.00 g/L	2.00 g/L
$NaSO_4$	0.30 g/L	1.80 g/L
yeast extract	1.00 g/L	0.20 g/L
sodium citrate	0.30 g/L	0.30 g/L

3.2 Analytical analysis

3.2.1 Chemical reagents

All chemical and reagents used throughout the research project will be analytical grade sourced from reputable suppliers such as Merck, Accsen Instrumental, Kimix, and Thermo Fisher Scientific. A range of analytical methods was employed to monitor process performance within the continuous bioreactor and batch culture studies.

3.2.2 pH and REDOX potential

The pH and redox potential were measured using a Cyberscan 2500 micro pH meter and a Metrohm pH lab 827 redox meter fitted with a Metrohm Redox platinum-ring electrode, respectively. The pH probe was calibrated daily using Accsen Instrumental standard buffering solutions (pH 4.0 and 7.0).

3.2.3 Bicarbonate alkalinity

Bicarbonate alkalinity was assayed by titrating 5 ml crude samples with 0.1 N H_2SO_4 to pH 4.5 according to the standard method by APHA, (1975) (APHA method number 2320). H_2SO_4 was continuously added using a burette while the pH was concurrently monitored. The volume of acid added was recorded at the pH end points, 4.5. Values of the volume of H_2SO_4 utilised in titration were used to calculate the concentration of bicarbonate alkalinity (Oyekola *et al.,* 2009).

3.2.4 Hydrogen sulphide analysis

Dissolved sulphide was quantified using the colorimetric methylene blue technique (APHA, 2012). Briefly, an appropriate volume (20 µL) of sample collected from the experimental system(s), as described above, was added to 200 µL of 1% (w/v) zinc acetate immediately after sample collection and made up to 5 mL using dH_2O. To this was added 500 µL N,N-dimethyl-p-phenylenediamine hydrochloride solution, followed by 500 µL of ferric chloride solution. This mixture was vortexed for 10 s and allowed to react for 15 min at room temperature before the absorbance was read at 670 nm (A_{670}). The final sulphide concentrations were determined by interpolation from a sulphide standard curve that ranged between 0.2-1.0 mg/L.

3.2.5 Sulphate analysis

The barium chloride method, based on the turbidimetric analysis of barium sulphate formation in solution, was also used to quantify the residual sulphate concentration (APHA, 2012). Analysis was conducted on samples prepared following the removal of dissolved sulphide, by addition of 40 µl 10% (w/v) $ZnCl_2$ solution to 2 mL sample to precipitate ZnS. The samples were vortexed vigorously for 5 s before centrifugation (13,000 rpm for 15 min at room temperature), to remove the ZnS precipitate, and the supernatant was filtered. These samples were appropriately diluted into 5 mL dH_2O, followed by the addition of 250 µL of conditioning reagent and an excess amount of finely ground $BaCl_2$ to facilitate the precipitation reaction. The conditioning reagent prevents the formation microcrystalline $BaSO_4$ and stabilises the suspension. This reaction mixture was vortexed for 60 s after which absorbance was measured at a wavelength of 420 nm (A_{420}) using a spectrophotometer (VWR® model: V-1200). Sulphate concentrations were determined from the absorbance readings using a sulphate standard curve ranging from 10-50 mg/L.

3.2.6 Volatile fatty acids

Volatile fatty acids (VFAs) analysis was conducted to quantify the concentration of lactic, acetic and propionic acids in the feed and reactor samples. VFA concentration was determined using high pressure liquid chromatography (HPLC) on a Waters Breeze 2 system equipped with a Bio-Rad organic acid column (Aminex HPX-87H, 30 cm x 7.8 mm, 9 µm) and a UV (210 nm wavelength) detector. Acidified deionised water (0.01 M H_2SO_4) was used as the mobile phase at a flow rate of 0.6 mL/min (Marais *et al.,* 2020a). Samples were prepared by appropriately diluting with dH_2O and filtering samples through a 0.22 µm Millex nylon syringe filter into HPLC vials. Standard solutions (0.1-0.6 g/L) were prepared by performing serial dilutions of acetate, propionate and lactate stock solutions (10 g/L), respectively, with dH_2O.

3.2.7 Chemical oxygen demand

All chemical oxygen demand (COD) measurements were carried out using the Merck reagent test protocols for low (100-1500 mg/L) concentrations. The method is based on the oxidation of the sample with a hot sulphuric acid solution containing potassium dichromate, with silver sulphate as the catalyst. The chloride is masked with mercury sulphate. The concentration of unconsumed yellow $Cr_2O_7^{2-}$ ions or green Cr_3^{+} ions is then determined photometrically and used to quantify oxygen demand. To quantify the COD concentrations, standard solutions (0, 250, 500, 750, 1000, 1250, 1500 mg/L COD (low range) were prepared using potassium hydrogen phthalate.

3.3 Management of the floating sulphur biofilm

The floating sulphur biofilm (FSB) develops at the air-liquid interface of the bulk fluid within the reactor. The FSB was harvested after defined periods as sulphide oxidation became limited (Section 4.2.1). Following biofilm harvesting, the biofilm re-formed at the surface and sulphide oxidation proceeded. The sulphur product was recovered by removing the mesh-screen and collecting the accumulated biofilm (termed harvesting). The biofilm was then dried at 60°C, weighed and stored for CHNOS elemental analysis. Elemental analysis of the harvested FSB was determined using an Elementar Vario EL Cube Elemental Analyser, for quantifying carbon, hydrogen, nitrogen and sulphur content (Central Analytical Facility (CAF), Stellenbosch University, South Africa).

3.4 Data handing and analysis

Reactor performance data and statistical analyses was conducted using Microsoft® excel® 365. The analytical measurements obtained from the experimental studies were analysed using a range of formulae in determining process kinetics and overall process performance. This involved assessing volumetric sulphate reduction rates, volumetric sulphide oxidation rates, substrate utilisation and sulphur recovery (Moosa *et al.,* 2002; Oyekola *et al.,* 2010; Marais *et al.,* 2020b).

Measurement and data collected during the experiments was recorded in hard cover laboratory books and consolidated into spreadsheets for data integration, analysis and monitoring overall process performance. Digital files containing raw and analysed data, recorded images, documents and presentations were uploaded and stored in Microsoft Teams and made accessible to all members of the research team. In addition, a copy of the master file was backed up onto a hard drive.

3.4.1 Kinetics calculations

Sulphate conversion and lactate conversion:
The sulphate conversion and lactate conversion were calculated using the general equation

$$\text{Conversion} = \frac{S_0\text{-}S}{S_0} \text{ x } 100 \qquad \text{Equation 3-1}$$

where S_0 and S represent the feed and residual substrate (sulphate or lactate) concentration (mmol/L), respectively. LC accounts for both oxidation and fermentation of lactate to acetate and propionate.

Expected sulphide (ES):
The expected sulphide was calculated theoretically, based on the amount of sulphate converted to sulphide.

$$\text{ES} = \frac{S_0 - S}{3} \qquad \text{Equation 3-2}$$

where S_0 and S represents the feed and residual sulphate concentration (mmol/L).

Volumetric sulphate loading rate (VSLR):
This is the product of the feed sulphate concentration and the dilution rate and represents the availability of sulphate for reaction:

$$\text{VSLR} = S_0 \text{ x } D \qquad \text{Equation 3-3}$$

where S_0 is the feed substrate (sulphate or lactate) concentration (mmol/L) and D the dilution rate (1/h).

Volumetric sulphate reduction rates and substrate utilisation and production rates:
The volumetric sulphate reduction (VSRR) and volumetric substrate utilisation rates (mmol/L.h), represented as r_s, were calculated as follows:

$$r_s = (S_0 - S)\, D \qquad \text{Equation 3-4}$$

where S_0 and S represent the feed and residual substrate (sulphate, lactate or acetate) concentration (mmol/L) respectively and D is the dilution rate (1/h), the inverse of hydraulic residence time calculated as F/V where F is the feed flow rate and V the reactor volume.

Sulphide conversion:
The conversion of sulphide to elemental sulphur was estimated by the difference between the expected sulphide and that measured:

$$HS^{-}_{removal} = \frac{ES - S}{ES} \times 100 \quad \text{Equation 3-5}$$

where ES and S represents the expected sulphide produced and residual effluent sulphide concentration (mmol/L) respectively.

Biofilm sulphur recovery:
Elemental sulphur recovery through the biofilm can be evaluated based on three parameters.

1) Sulphur recovery based on total sulphate-S load:

$$S^{0}_{recovery} = \frac{S_{FSB}}{SO_{4\,total}} \times 100 \quad \text{Equation 3-6}$$

where S_{FSB} represents the amount of sulphur recovered as elemental sulphur from the biofilm and S_{total} the total amount of sulphate-S load over the duration of the experimental run.

2) Sulphur recovery based on the expected (generated) sulphide-S load:

$$S^{0}_{recovery} = \frac{S_{FSB}}{ES_{total}} \times 100 \quad \text{Equation 3-7}$$

where S_{FSB} represents the amount of sulphur recovered as elemental sulphur from the biofilm and ES_{total} represents the cumulative amount of sulphide-S generated through sulphate reduction over the period operation, between collapsing and harvesting the biofilm.

3) Sulphur recovery based on sulphide removal:

$$S^{0}_{recovery} = \frac{S_{FSB}}{ES_{total} - S_{total}} \times 100 \quad \text{(Equation 3-8)}$$

where S_{FSB} represents the amount of sulphur recovered as elemental sulphur from the biofilm. ES_{total} and S_{total} represents the cumulative amount of expected sulphide and effluent sulphide over the period of operation between collapsing and harvesting the biofilm.

4 Demonstration of the modified hybrid baffled reactor

4.1 Introduction

The potential application of carbon microfibres to enhance the attachment of biomass and to achieve high volumetric sulphate reduction rates (VSRR) within a hybrid LFCR was successfully demonstrated by Marais et al. (2020a). However, one of the drawbacks associated with the carbon microfibers is the selection of non-SRB over SRB specific communities. This may result in differences in biomass concentration distribution across the reactor with low planktonic cell concentrations in the bulk volume of the reactor and higher cell concentrations in the sludge at the base of the reactor. Recent studies have shown the potential application of polyurethane foam as a support matrix for sulphate reduction within a packed bed reactor configuration (Hessler *et al.,* 2018). The structure of the pores in the polyurethane foam enables the biomass to be retained within the matrix without the considerable compromise on the working volume of the reactor. Another important aspect is the contact between active microbial biomass and the ARD stream to be treated. Fluid dynamics within the hybrid LFCR is largely dictated by passive mixing. In a previous investigation the rates were potentially limited by poor contact and diffusion of the inlet feed into the active biomass zone. This is due to the build-up and settling of biomass resulting in an uneven distribution of biomass across the reactor. During operation, the feed was hypothesised to by-pass dense biomass regions of the reactor where a differential in localised hydraulic residence time between the planktonic phase and biomass would lead to variation in measured concentration profiles.

With the addition of baffles, it is proposed to direct the flow throughout the reactor and achieve better contact with the active biomass, enhancing overall BSR kinetics. The configuration would also facilitate a gradient profile, with associated reactor zones of differing sulphate concentration, as sulphate is removed along the compartments of the reactor. Hessler *et al.* (2018, 2020, 2022) has demonstrated that such zoning enables enrichment for microbial communities tailored to rapid sulphate conversion at high concentrations, sulphate scavenging at low concentrations as well as tailoring to residual carbon source and electron donor. It is hypothesised that the use of polyurethane foam, which retains biomass in its matrix rather than needing active attachment, will result in a high retention of SRBs and enhanced system performance compared to the use of carbon microfibres. The study investigates the hydrodynamics and performance of polyurethane foam as a support matrix within the modified hybrid baffled reactor. The hydrodynamics are assessed to confirm satisfactory mixing of the bulk volume as well as the liquid transport to the air-liquid interfacial zone which is critical for the sulphide oxidation component and formation of the floating sulphur biofilm. The performance is compared directly to experimental results previously obtained during operation of the hybrid LFCR with carbon microfibers (Marais *et al.,* 2020a,b. The following diagram illustrates the experimental approach for the study.

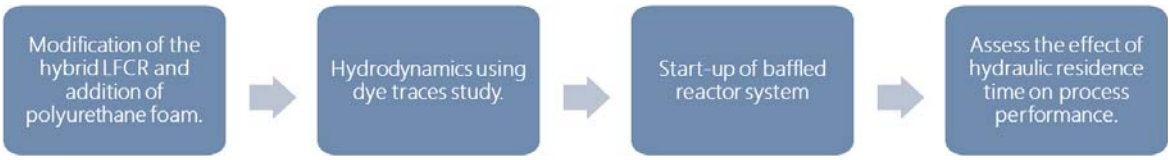


Figure 4-1: Flow chart illustrating the experimental approach for testing the modified hybrid baffled reactor.

4.2 Methods

4.2.1 Conceptual model of the baffled linear flow channel reactor

The modified linear flow channel reactor (LFCR) applied in previous studies (Marais *et al.,* 2020a, b, c, 2022) was designed for semi-passive operation requiring low maintenance. The bulk surface of the reactor is exposed to

the surrounding environment in which the air-liquid interface facilitates the formation of the FSB. In the bulk volume, anaerobic conditions promote the activity of biological sulphate reduction. The 8 L LFCR was constructed from Perspex (11 mm thickness) and had internal dimensions of 450 mm (l) x 200 mm (w) x 150 mm (h). The front facing side of the reactor is fitted with nine sampling ports, allowing the bulk reactor volume to be monitored across the length and at different heights of each compartment.

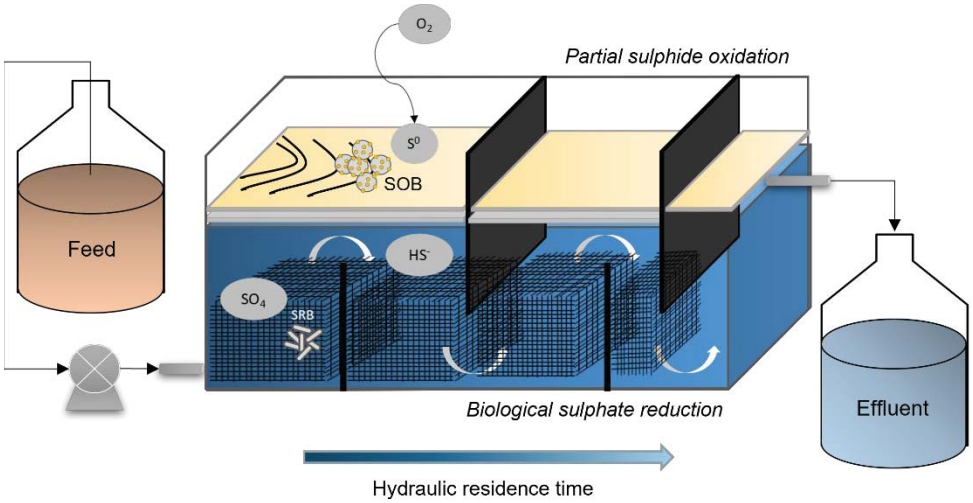


Figure 4-2: Conceptual model of the modified baffled reactor system incorporating sulphate reduction and sulphide oxidation.

Modification of the LFCR

The reactor was modified with four baffles inserted across the width of the reactor (Figure 4-2). This was done to ensure a defined fluid flow with enhanced hydrodynamics to favour sulphate reduction and sulphide oxidation at the air-liquid interface as well as to facilitate zoning within the reactor. Polyurethane foam was added to the bulk volume of the reactor as a support matrix for enhanced biomass retention. Harvesting screens, constructed out of plastic mesh fixed to an aluminium frame, were fitted into the two surface compartments. The screens were designed to lie 5 mm below the liquid surface to facilitate biofilm capture and harvesting.

Microbial cultures

An active SRB inoculum was cultured in a 10 L Schott bottle under a fed-batch feeding regime. Once the culture achieved high sulphate reducing activity and sufficient sulphide concentration was built up, the culture was used inoculate the hybrid baffled reactor. A fresh culture of active floating sulphur biofilm was taken from a stock LFCR that is continuously operated and maintained in the CeBER laboratory. The FSB sample was dispersed onto the bulk surface of the reactor. This provided an initial SOB inoculum for the development of the floating sulphur biofilm upon start-up of the baffled reactor.

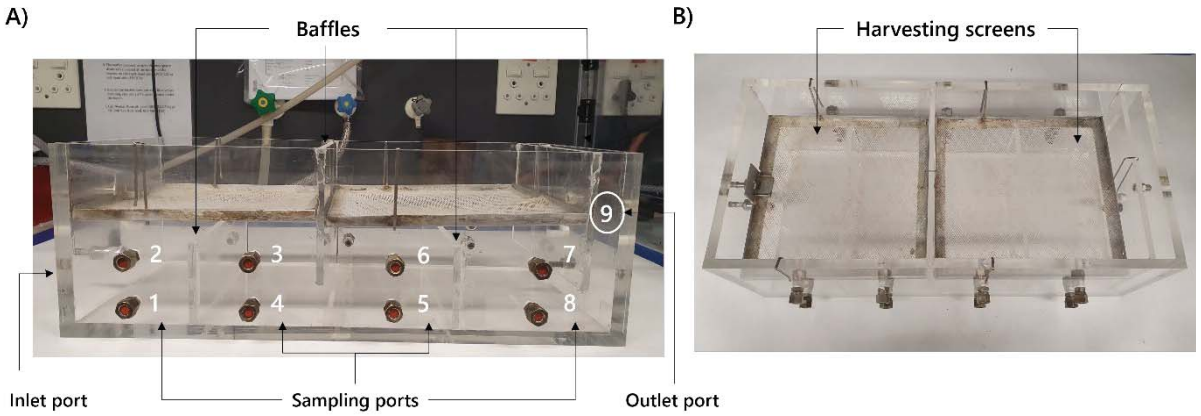


Figure 4-3: Modified hybrid baffled reactor showing A) baffles and sampling ports (1-9) positioned across the length and depth of the reactor as well as the positions of the inlet and outlet ports. B) top view of the reactor fitted with harvesting screens for FSB recovery

Sampling layout

The eight sampling ports were sampled regularly using a 19 gauge epidural needle (8 cm) to monitor the solute concentrations in the reactor fluid across the length and depth of the reactor (Figure 4-3). In addition, the length of the needle ensured sampling near the centre of the bulk volume. In addition, samples of reactor overflow were collected as a representative effluent sample. The multiple sampling points across the bulk volume facilitated the monitoring of physicochemical parameters, including aqueous species, throughout the reactor to evaluate system performance. They also provided insight into the mixing regime within the baffled system during operation where the presence of stratification or short-circuiting of the influent stream was easily detected. The samples were subjected to a range of solution chemistry and analytical methods, described in Section 3.2, to monitor reactor performance.

4.2.2 Hydrodynamic dye tracer study

The LFCR was constructed from clear poly(methyl methacrylate) (Perspex) to allow for easy visualisation of the hydrodynamic mixing patterns. After modifying the reactor with addition of baffles, a dye tracer experiment was conducted by filling the reactor to its standard operating volume with 2 mM sodium hydroxide to which ten drops of the pH indicator dye phenolphthalein were added to achieve a uniform pink colour. A solution of 42 mM hydrochloric acid was then pumped into the reactor at a pre-determined flow rate, representing the feed rate of interest. As acid feed flowed into the reactor a neutralisation reaction occurred which turned the pink reactor volume colourless. This allowed the fluid's path and mixing regime within the reactor to be visualised (Mooruth, 2013b). Over the course of the dye tracer experiments the mixing regime was photographed, and complete mixing time recorded. The study also evaluated the mixing regime in the presence of the polyurethane foam support matrix.

4.2.3 Effect of hydraulic residence time on process performance

For the demonstration of the proof of concept, the following experimental procedures were used. After successfully inoculating the hybrid baffled reactor with a mixture of active SRB and SOB cultures (Section 3.1, Section 4.2.1), the feed stream of modified Postgate B medium containing 1 g/L sulphate was supplied at a 6 day HRT (dilution rate: 0.0069/h) to the primary reactor using a speed controlled peristaltic pump. Lactate served as the electron donor and carbon source and was supplemented at 11 mM to achieve a COD:sulphate ratio of 0.7. The reactor was operated at room temperature. Samples (2 mL) were taken every second day from the sampling ports that were distributed across the length and depth of the bulk reactor volume, as well as from the effluent port. The performance was monitored until the system was sufficiently colonised and stable system performance was achieved.

FSB formation was visually observed and once a thick, stable biofilm had been formed, it was harvested after 3 HRTs. The FSB was harvested by physically disrupting the biofilm and allowing the fragments to settle on the harvesting screen. The sulphur product was recovered by removing the harvesting screen and collecting the accumulated biofilm. Thereafter, the system was allowed to operate for an additional 3 HRT after which a second biofilm harvest was performed. The feed rate was then incrementally adjusted in a step-wise approach from the initial operation at 6 days to 5, 4, 3, and 2 day HRT. At each HRT, the same operating procedure was followed with two biofilm harvesting occurring over a total of 6 HRTs.

A second experiment to investigate the effect of HRT using a higher feed sulphate concentration of 2 g/L was conducted. Similar to the 1 g/L feed sulphate concentration experiment, lactate was used as the electron donor and 2 mL samples were taken every second day. However, the 2 g/L study started from a 5 day HRT to a 4, 3 and 2 day HRT and the biofilm was harvested after 4 HRTs instead of three. Each residence time went through two runs with an FSB harvest occurring after each run. The same method used for biofilm harvesting at a 1 g/L feed sulphate concentration was used for the 2 g/L feed sulphate experiment.

4.3 Results and Discussion

4.3.1 Hydrodynamic in the hybrid baffled reactor

The hydrodynamics within the hybrid LFCR was previously evaluated as a function of the HRT. Mixing within the reactor was primarily governed by passive diffusion and was characterised as relatively well mixed system. In the current investigation the reactor was modified into a hybrid baffled reactor system; this was expected to change the fluid dynamics throughout the reactor substantially. The inclusion of baffles is expected to direct fluid flow pattern throughout the reactor, to create zoning based on depletion of reactants, to enhance active biomass contact with feed, and to enhance delivery of sulphide formed to the surface interface.

The fluid flow patterns were observed through the tracer studies and associated colour changes. These were recorded photographically.

As the acidic feed was pumped into the baffled reactor a concentration of acid (colourless) built up at the base of the reactor in the first compartment, indicated by the clear zone of dye neutralisation (Figure 4-4). As time progressed the zone of clearing began to move along toward the surface in a typical plug flow motion. As it reached the top of the first baffle, the clear acid font overflowed into the second and third compartment bypassing the region near the surface. The clear fluid proceeded to move along the base of the reactor until it reached the third baffle (Figure 4-4). As more acid was pumped into the reactor the clear zone began to rise to the surface and eventually complete neutralisation of the tracer dye was observed within the 1st, 2nd and 3rd compartments. Similarly, as the clear zone approached the top of the third baffle there was an overflow into the fourth compartment (Figure 4-4). A similar profile observed in the second compartment was repeated in the fourth compartment where the clear acid front overflowed to the base of the reactor followed by a gradually clearing toward the surface.

The reactor volume was gradually displaced from the inlet to the outlet port. There was clear indication of short-circuiting and dead zone formation where the liquid bypassed certain regions of the reactor and required a longer time to clear. This was particularly observed at the surface as the zone of clearing transitioned from the 1st to 2nd compartment and 3rd to 4th compartment. Under process operations this would not be ideal since the sulphide oxidation relies on effective transport of sulphide generated within the bulk volume to the air-liquid interface. Poor hydrodynamics within a reactor can have major implications on the overall process performance. Ideally there should not be any short-circuiting or dead zones within the reactor to ensure equal displacement and transport of the inlet feed throughout the reactor.

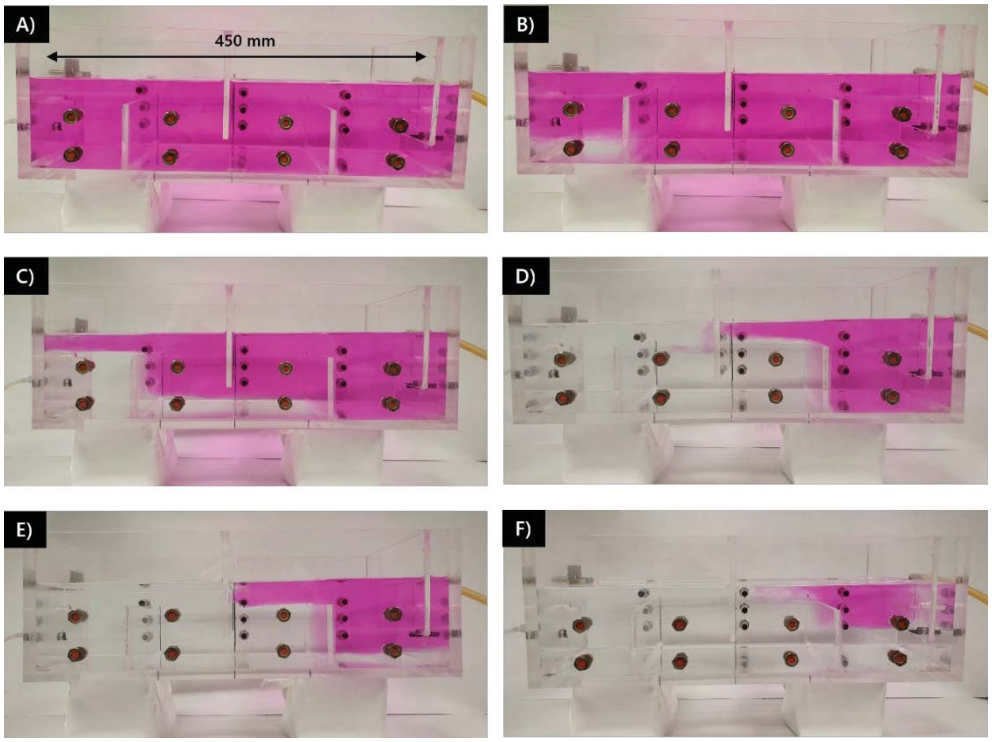


Figure 4-4: Dye tracer study showing photographic images on the progression of mixing over time in the baffled reactor operated at a flow rate equivalent to a 12 h HRT, photographs taken at A) 0 min B) 9 min C) 1 h 15 min D) 2 h 21 min E) 2 h 29 min F) 2 h 57 min.

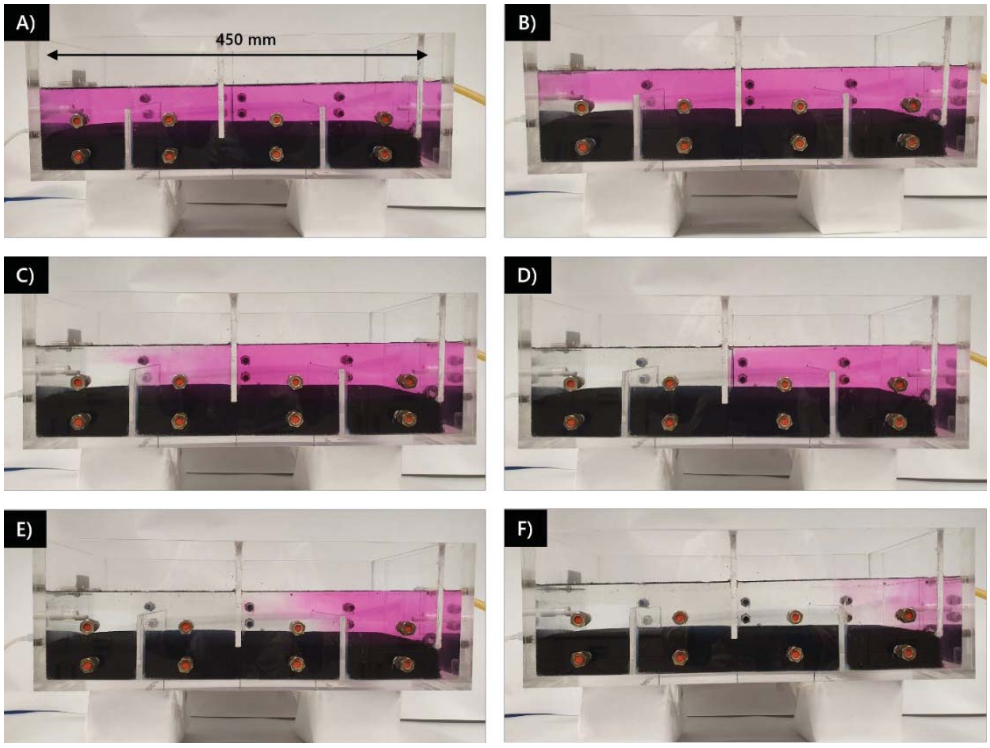


Figure 4-5: Dye tracer study showing photographic images on the progression of mixing over time in the baffled reactor with polyurethane foam operated at a flow rate equivalent to a 12 h HRT, photographs taken at A) 0 min B) 44 min C) 1 h 24 min D) 2 h 7 min E) 2 h 26 min F) 2 h 51 min.

The dye tracer experiment was repeated in the presence of the polyurethane foam matrix (Figure 4-5). The mixing progression along the reactor was distinctly different. The addition of polyurethane foam slowed the short-circuiting flow of the acidic solution in the 2nd and 4th compartments (likely caused by density difference) creating plug flow as the solution diffused through the matrix. The nature of this flow precluded short-circuiting of the interface zone as the incoming fluid travelled across the surface zone before diffusing into the foam matrix. Notably the concentrated acid front seen in the previous experiment that overflowed into the 2nd compartment along the baffle was not present. There was a clear progression in dye neutralisation from the 1st to 4th compartment (Figure 4-5). There was plug flow fluid displacement throughout the reactor with no indication of short-circuiting or dead zones within the reactor.

4.3.2 Effect of hydraulic residence time on the performance of the hybrid baffled reactor

On studying the effect of hydraulic residence time on reactor performance, the reactor system was set up to include a primary LFCR with four baffles and a polyurethane foam matrix for biomass retention as well as baskets for recovery of the FSB, a secondary LFCR unit of lower volume which lacked both baffles and the retention matrix ensure an increased sulphide removal efficiency, and a clarifier downstream of the second LFCR to enhance elemental sulphur recovery. This is detailed in Figure 4-6.

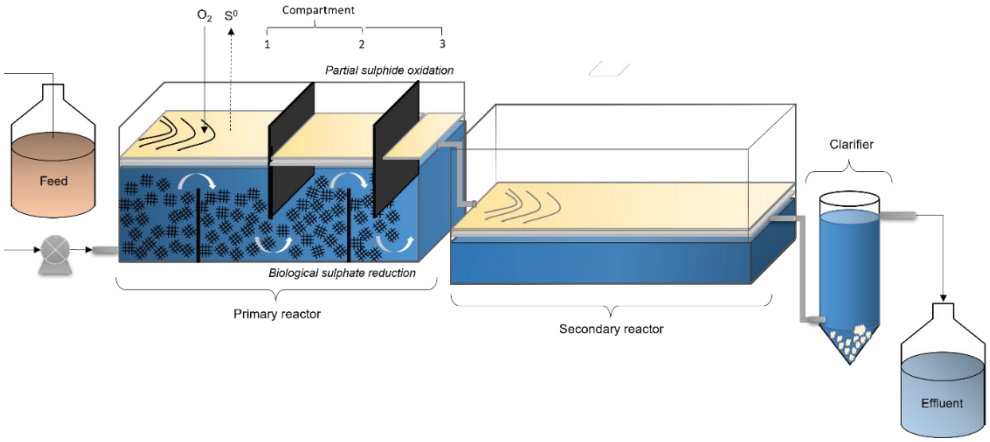


Figure 4-6: Hybrid baffled reactor configuration with the addition of a secondary sulphide oxidation unit and clarifieer downstream for enhance sulphur recovery.

At a feed concentration of 1 g/L (~10 mM)

The residual sulphate and dissolved sulphide concentrations measured over the duration of the HRT experiments are shown in Figure 4-7. During initial evaluation of the performance of the primary LFCR at a 6-day HRT, low residual sulphate concentrations were maintained (Figure 4-7A). These were maintained at HRTs of 5 and 4 days (~ 1 mM). The residual sulphate concentration gradually increased as HRT was incrementally decreased to 3 and 2 days. This was accompanied by a high generation of dissolved sulphide concentrations which ranged between 6 and 7 mM at 4 to 6 day HRT. As expected, the increase in sulphate concentration (to 3-4 mM) resulted in a decrease in the production of sulphide (to ~ 4 mM). As depicted in Figure 4-7A, the sulphur balance based on the measured sulphate and sulphide concentration revealed an unaccounted for fraction. In the context of this study, the unaccounted-for fraction represented the partially oxidised sulphide in the form of elemental sulphur. While other sulphur species may have formed within the reactor, such as thiosulphate, polysulphides and release of sulphide gas, for the purpose of this study it is assumed that elemental sulphur was the main product. Previous investigations, such as that of Mooruth (2013), have performed extensive studies on sulphur speciation within the integrated LFCR process. Under similar operating conditions, the formation of thiosulphate and polysulphide were not favourable and were shown to be negligible when quantified.

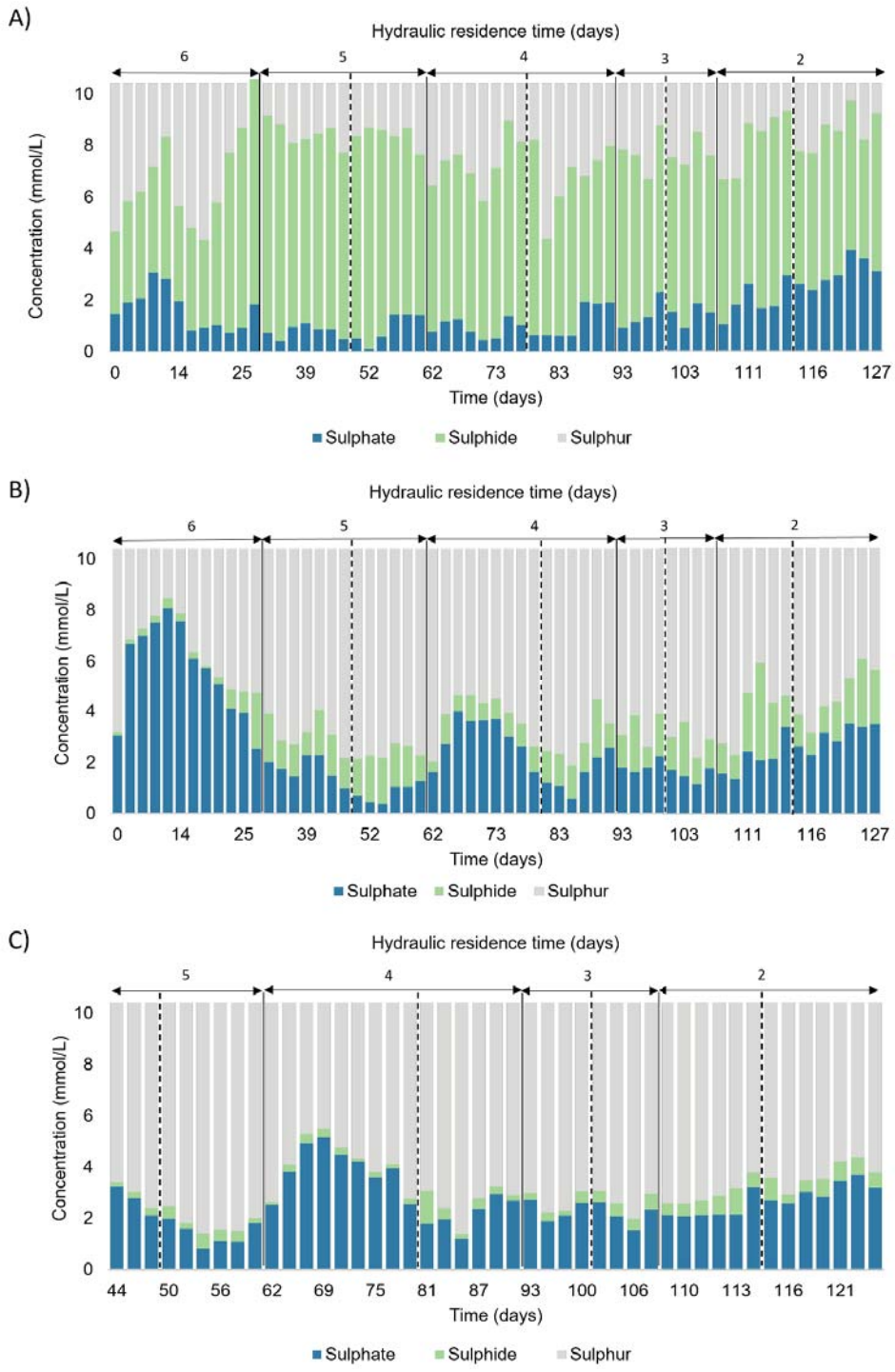


Figure 4-7: Effect of HRT on process performance showing measured resideual sulphate and dissoved sulphide concentration over time in the A) primary reactor B) secondary reactor C) clarifier. Elemental sulphur is assumed to be the main product of sulphide oxidation and was determined based on the sulphur mass balance and unacounted fraction. Solid and vertical lines define the FSB harvesting regime.

During initial start-up of the reactor at a 6 day HRT, high sulphate conversion was achieved at some 90% conversion within the first two compartments. A well-established FSB formed at each of the three exposed surfaces. After initial evaluation of process performance of the primary LFCR at a 6-day HRT, sulphide conversion accounted for 30% with significant untreated dissolved sulphide released into the effluent. The rate limiting step was sulphide oxidation as the FSB would gradually become oxygen limiting leading to a considerable reduction in the rate of sulphide oxidation. One strategy to increase sulphide oxidation and recovery of elemental sulphur is to increase the frequency of harvesting the biofilm. Alternatively, the addition of a second LFCR unit downstream would facilitate sulphide oxidation and elemental sulphur recovery. The latter was adapted in this study such that the addition of a secondary LFCR unit and clarifier downstream of the primary reactor, as shown in Figure 4-6, would ensure an increased sulphide removal efficiency and elemental sulphur recovery.

As expected, there was a considerable decrease in sulphide concentration within the secondary reactor (Figure 4-7B). The secondary reactor facilitated an average sulphide removal efficiency of 76±7% over the course of the study, with 85% and 68% removal efficiency at a 6- and 2-day HRT respectively. During operation at a 6-day HRT, there was a notable increase in sulphate concentration which indicated that complete sulphide oxidation to sulphate had occurred within the secondary reactor. This was due to both the time required for effective colonisation of the surface of the newly set up secondary LFCR and to the operation at a low dilution rate where the replenishment of dissolved sulphide into the secondary reactor was not sufficient to maintain the sulphide to oxygen ratio required to favour partial sulphide oxidation to elemental sulphur. As the HRT was decreased to 5, 4, 3 and 2 days, with concomitant increase of the dilution rate, the residual sulphate concentration between the primary and secondary reactor became less volatile with only slight increase in concentration. Further bot sulphate and sulphide concentrations were low (1 to 4 mM and 1 to 2 mM respectively), indicating that partial sulphide oxidation to elemental sulphur was favoured (Figure 4-7B). This was also evident by the well-established FSB that covered the surface of the reactor.

The addition of the clarifier was to ensure maximum recovery of suspended colloidal sulphur and fragments of FSB that would otherwise be released through the effluent and re-oxidised under environmental conditions. The loss of elemental sulphur to the effluent stream was a key observation during the initial development and operation of the hybrid LFCR (Marais *et al.,* 2020b). In the clarifier unit there was additional removal of residual sulphide with minimal change in sulphate concentration (Figure 4-7C).

Throughout the experimental operation pH and redox conditions were maintained for optimal sulphate reduction performance. Redox potential remained stable throughout the study with an average reading of -347±12 mV. The effluent pH was consistently higher than the inlet feed. At a 6-day HRT, a high pH reading of 8.0 was measured within the primary reactor. As HRT was incrementally decreased, there was a gradual decrease in the effluent pH reaching around 7.2 at a 2-day HRT. The increase in pH is strongly associated with the production of bicarbonate alkalinity as a result of sulphate reduction activity. These conditions were ideal for the operation of the baffled reactor system. In the secondary reactor there was a considerable increase in pH reaching a value of 8.5 during operation at a 6-day HRT. Similarly, as the HRT was incrementally decreased, there was a decline in the measured pH reaching a value of 8.0 at a 2-day HRT. The observed increase in pH between the two reactors can be attributed to the partial oxidation of sulphide to elemental sulphur which resulted in the release of hydroxyl ions.

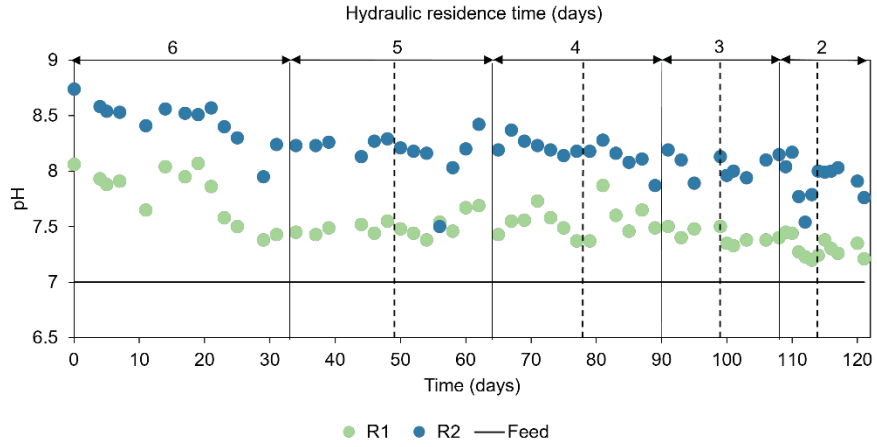


Figure 4-8: Effect of HRT on pH profile within the effluent samples taken from the primary and secondary reactors. Solid and verstical lines define the FSB harvesting regime.

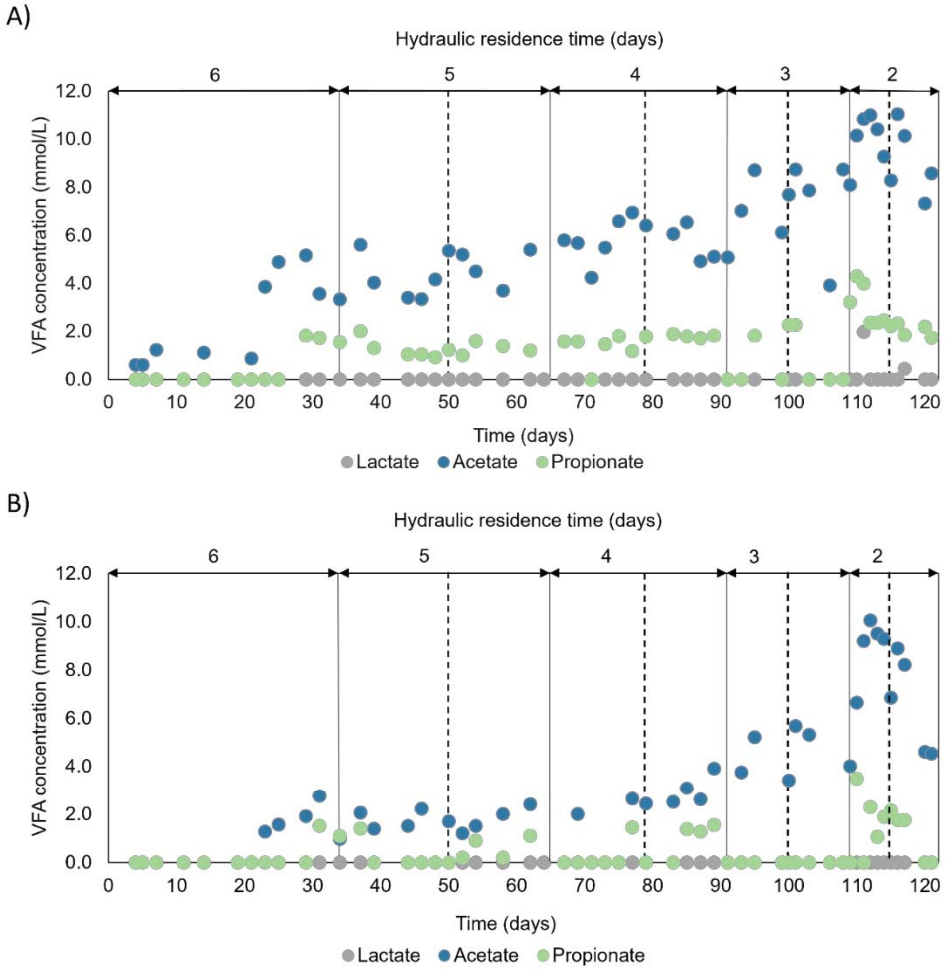


Figure 4-9: Effect of HRT on volatile fatty acid concentration profile measured over time showing A) primary reactor and B) secondary reactor. Solid and dotted vertical lines define FSB harvesting regime.

A decrease in HRT from 6 to 2 days led to an increase in volumetric sulphate reduction rate from 0.066 mmol/L.h to 0.169 mmol/L.h and decrease in sulphate conversion from 91 to 78% (Figure 4-10). Residual lactate concentrations were below the minimal detection limit indicating 100% lactate utilisation within the reactor

over the course of the experiment. The metabolism of lactate was accompanied by the production of acetate and propionate (Figure 4-9; Figure 4-10). Competition between SRB and fermentative microorganisms for lactate metabolism in mixed cultures has been extensively studied in CSTRs (Oyekola *et al.,* 2009; Bertolino *et al.,* 2012). During lactate fermentation, acetate and propionate are generated as a by-product. Additionally, SRB are capable of complete or incomplete lactate oxidation, with the latter generating acetate as a byproduct. Based on the high sulphate reduction and acetate generated, incomplete lactate oxidation was the major metabolic pathway occurring within the reactor. Low levels of propionate were generated which indicated that a small portion of lactate was utilised for fermentation (Figure 4-9). As the HRT was decreased incrementally, there was an increase in propionate production rate. These results are consistent with previous investigations that evaluated the effect of HRT in biosulphidogenic reactors including the well-mixed stirred tank reactor (Oyekola et al.*et al.,* 2012) and the hybrid LFCR (Marais et al.*et al.,* 2020b; Oyekola et al.*et al.,* 2012) . Under substrate limiting conditions resulting during low dilution rates SRB activity is favoured while at high dilution rates and concomitant increased residual VFA concentrations the proliferation of fermentative microorganisms is favoured. These finding are associated with the growth rate and affinity for the substrate. SRB are highly proficient at scavenging substrates at low concentrations. The effect of HRT has major implications not only on the overall process performance but also on the microbial community dynamics. This often results in a structural shift in microbial community composition in favour of either SRB or fermentative microbial populations. It is therefore crucial that optimal operating conditions are selected to ensure SRB activity is promoted over competing microbial populations, particularly to minimise demand for carbon source and electron donor which impacts process economics as well as outlet water quality. While lactate is an effective carbon substrate and electron donor to evaluate sulphate reduction performance during initial evaluation of novel bioreactor systems, it is not an effective carbon source for large scale application. Besides the economic challenges, a major drawback associated with the use of lactate is the generation and accumulation of acetate which contributes to downstream COD pollution.

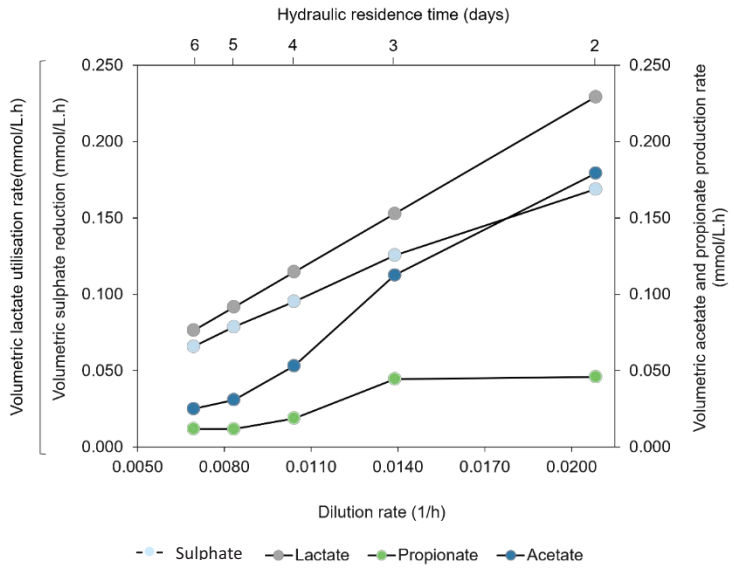


Figure 4-10: Effect of HRT on reaction kinetics showing the volumentric sulphate reduction rate, lactate utilisation rates and acetate/propionate production rates.

Progressive chemical analysis of samples taken from the ports distributed along the fluid pathway from the inlet to the outlet port provided insight into process dynamics (Figure 4-11). The addition of the baffles facilitated the zoning of the reactor creating a chemical gradient from the inlet to the outlet port. This was not observed in the un-baffled reactor. Tracing the sulphate concentration along the fluid pathway through the reactor revealed a clear trend in sulphate removal. The observed sulphate reduction activity across the reactor was consistent with the increasing dissolved sulphide concentrations. These results show that SRB activity occurred predominantly

within the first and second compartment (sampling ports 1-4), achieving approximately 90% sulphate removal approximately 90% sulphate removal. At a long residence time the concentration profiles were consistent while at a short HRT of 2 days the profiles were more variable between sampling times. The increase in sulphate concentration at sampling port 7 is due to its location (for reference Figure 4-3) in the 4th compartment near the air-liquid interface where sulphide oxidation takes place. This was consistent with the dramatic decrease in dissolved sulphide concentration which coincided with sampling port 7. Under these dynamic conditions, where the FSB is harvested intermittently and oxygen mass transfer into the bulk reactor volume can fluctuate significantly, complete sulphide oxidation conditions are favourable. The re-appearance of sulphate in the latter compartments is due to re-oxidation and suggests that improved design with respect to the positioning of the effluent port should be considered. Interestingly, as dynamic as the concentration profiles were along the reactor volume, the final effluent samples were relatively stable during operation at each HRT considered in this study. The ability to retain a high amount of biomass within the first two compartments enabled high sulphate conversion to be achieved. The high biomass retention allows the system to be operated at high dilution rates. Although not evaluated in this study, the operation at higher dilution rates beyond a 2-day HRT could be beneficial to evaluate its potential to treat higher volumes.

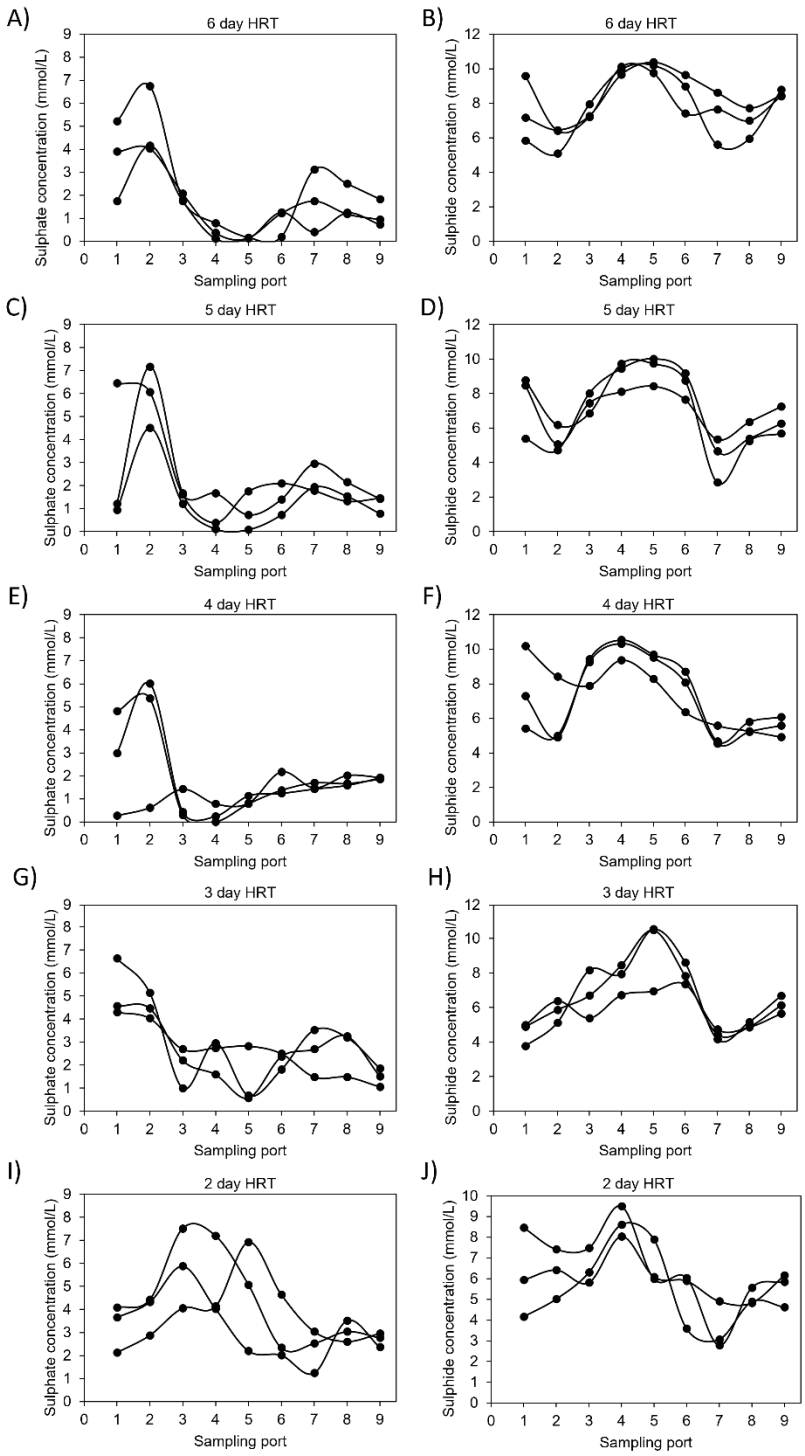


Figure 4-11: Residual sulphate (A, C, E, G, I) and dissolved sulphide (B, D, F, H, J) concentration profile across the baffled reactor sampling ports (1-9). Each data series represents a time point (day) of sampling.

The effect of HRT on FSB harvesting, and elemental composition is shown in Figure 4-12. The average amount of FSB harvested decreased from 11.9 to 4.6 g within the primary reactor as HRT decreased incrementally from 6 to 2 days (Figure 4-12). This was largely driven by the difference in the total time of operation between residence times evaluated. In the secondary reactor the amount of FSB harvested was lower compared to the primary with the exception during operation at a 3- and 2-day HRT. This was due to the additional harvesting regime adopted within the secondary reactor which facilitated the increase in sulphide oxidation and higher recovery of the FSB and elemental sulphur. The low FSB harvested during the initial run at a 4-day HRT within the secondary reactor was consistent with the elevated sulphate concentration which indicated complete sulphide oxidation was favoured over elemental sulphur production. Subsequently, the biofilm was not well developed with little sulphur deposition. These findings highlighted the importance of developing a structurally sound biofilm. When the integrity of the biofilm was compromised the unimpeded oxygen mass transfer into the bulk reactor volume created conditions that favoured complete sulphide oxidation to sulphate. Furthermore, by regulating the frequency of FSB harvesting within the second reactor high sulphur recovery could be achieved. Improved FSB structure at the 3- and 2-day HRTs led to improved elemental sulphur recovery.

Elemental composition of the FSB was predominantly made up of nitrogen, carbon, hydrogen, sulphur and an inorganic fraction (Figure 4-12B). The composition was relatively stable between samples with minimal difference observed when comparing samples derived from the same reactor. A key observation was the difference in elemental sulphur composition between the primary and secondary reactors which accounted for 25±2.2% and 15±5.7% respectively over the range of HRT tested.

A major component of the FSB was characterised by an inorganic crystalline mineral precipitate that accounted for 59±1.3 and 68±4.5% of the FSB in the primary and secondary reactors, respectively (Figure 4-12B). The crystals embedded within the sulphur biofilm were later identified through SEM-EDS as struvite. Struvite is comprised of equimolar concentrations of magnesium (Mg) ammonium (NH_4) and phosphorus (P). The high content of the struvite within the FSB was attributed to the supplementation of Mg, NH_4 and P within the modified Postgate feed used in the study. During operation, the FSB may serve as a nucleation site for struvite precipitation. The mechanism involved in the formation of struvite within the FSB still requires further investigation. However, it has been suggested that the biomineralisation of struvite through the action of microorganisms may be involved (Soares et al., 2014)(Soares et al., 2014)(Soares et al., 2014)(Soares et al., 2014). The formation of struvite presents a potential approach for recovery of key nutrients, especially phosphorus, where these present in wastewaters and environmentally contaminated waters.

Sulphide oxidation kinetics were largely dependent on the availability of sulphide generated through sulphate reduction and the ratio of sulphide to oxygen. Sulphur recovery via the harvesting of the FSB accounted for 24±9% on average across the HRT range tested (Table 4-1). The amount of sulphur recovered varied based on the sulphate conversion. The unaccounted-for sulphur fraction not recovered through the FSB was postulated to be in the form of colloidal sulphur and biofilm particles that were difficult to capture. Despite the addition of a secondary reactor and clarifier, a considerable fraction of elemental sulphur deposits and biofilm fragments settled at the base of the reactor and piping. The mechanism of recovering the elemental sulphur through harvesting the FSB is a key feature of the integrated semi-passive process and is highly attractive for process operation. Traditional sulphide oxidation processes are typically operated under active treatment conditions in a liquid suspension with mechanical mixing and controlled supply of oxygen. These processes require downstream separation to recover the sulphur though centrifugation and sedimentation techniques.

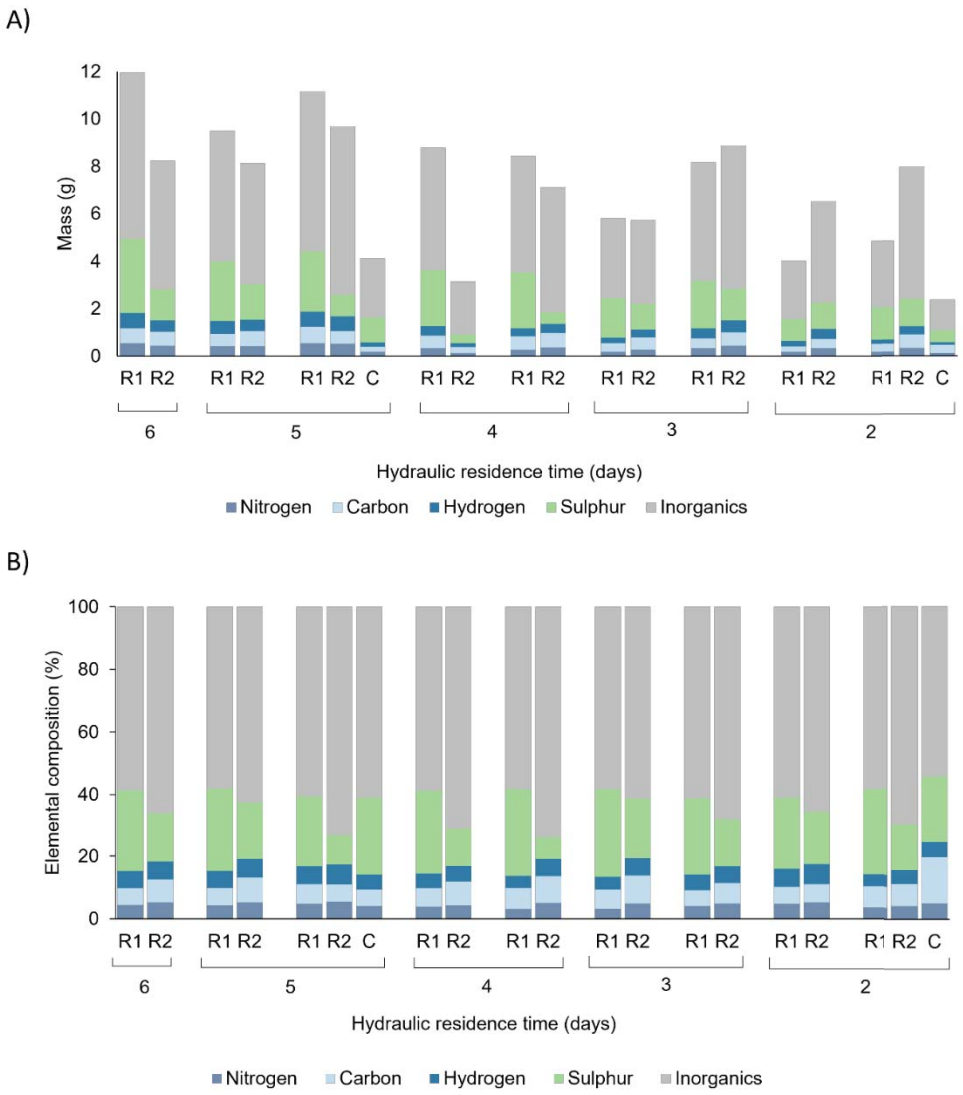


Figure 4-12: Effect of HRT on biofilm harvest and elemental sulphur recovery showing A) total harvested FSB B) elemental compostion (%) in the primary and secondary reactors as well as the clarifier.

Table 4-1: Effect of hydraulic residence time on overall process performance of the hybrid baffled reactor comparing primary reactor and addition of a secondary reactor.

	Primary reactor (R1)				Secondary reactor (R2)	
HRT (days)	VSRR (mmol/L.h)	Sulphate conversion (%)	Sulphide conversion [a] (%)	Sulphur Recovery [b] (%)	Sulphide conversion [a] (%)	Sulphur Recovery [b] (%)
6	0.066	91	36	19	87	30
5	0.079	91	24	37	81	55
4	0.095	88	35	27	86	37
3	0.126	87	31	23	78	44
2	0.169	78	23	13	68	36

[a] Cumulative sulphide conversion based on the expected theoretical sulphide concentration and final effluent sulphide concentration over the duration of each experimental run

[b] Elemental sulphur recovery from harvested FSB calculated based on sulphate conversion

Comparative assessment of the baffled reactor system against the performance of the original hybrid LFCR reactor configuration showed a 23 and 17% increase in sulphate conversion efficiency when operated at a 4 and 2 day HRT respectively (Marais *et al.,* 2021). This had positive impact on overall sulphide oxidation performance and sulphur recovery. The addition of the secondary reactor resulted in a 51 and 45% increase in sulphide conversion during operation at a 6- and 2-day HRT respectively (Table 4-1). The best performance in terms of sulphate conversion (91%) and sulphide removal (81%) with the recovery of elemental sulphur (55%) was achieved at a 4-day HRT.

At a feed concentration of 2 g/L

The effect of hydraulic residence time was also investigated at a higher sulphate concentration of 2 g/L (20.8 mM sulphate); multiple runs were conducted with each run lasting 4 HRTs, with an FSB harvest occurring after each run.

The residual sulphate and dissolved sulphide concentrations measured over the duration of the HRT experiments are shown in Figure 4-13. On evaluating the performance of the primary LFCR at a 5-day HRT, residual sulphate concentrations of 4-6 mM were maintained, representing 70 to 80% conversion (Figure 4-13A). These were maintained at HRTs of a 4-day HRT. The residual sulphate concentration gradually increased as HRT was decreased to 3 (5-6 mM) and 2 days (6-8 mM). This was accompanied by a high generation of dissolved sulphide concentrations which ranged between 12 and 16 mM. In Figure 4-13A, the unaccounted-for fraction in the sulphur balance based on the measured sulphate and sulphide concentration was assumed to represent elemental sulphur, with low recoveries shown.

Comparing the results for the 2 g/L sulphate feed with the 1 g/L sulphate feed to the primary LFCR, unlike the 1 g/L study which had the highest sulphate conversion occurring at the longest HRT, the highest sulphate conversion of 68.4% was achieved at a 3-day HRT across both the primary and secondary reactors for the 2 g/L sulphate feed. This was lower than the highest sulphate conversion of 90% across both reactors with a 1 g/L sulphate feed. This could be due to a limitation on reaction kinetics within the reactor or to sulphide toxicity as the higher sulphate feed concentration led to sulphide concentrations in the range 12-16 mM which may inhibit the SRB to a degree (Marais, 2020; Moosa & Harrison, 2006). Higher sulphate loading in the feed also results in a greater demand for carbon source and electron donor to maintain a COD:sulphate ratio above 0.67. High lactate concentrations may shift the balance between fermentation and oxidation. Further, accumulation of acetate which has also known to be toxic to SRB at high concentrations, above 45 mmol/L at a pH above 5, may inhibit the process (Koschorreck, 2008; Marais, 2020).

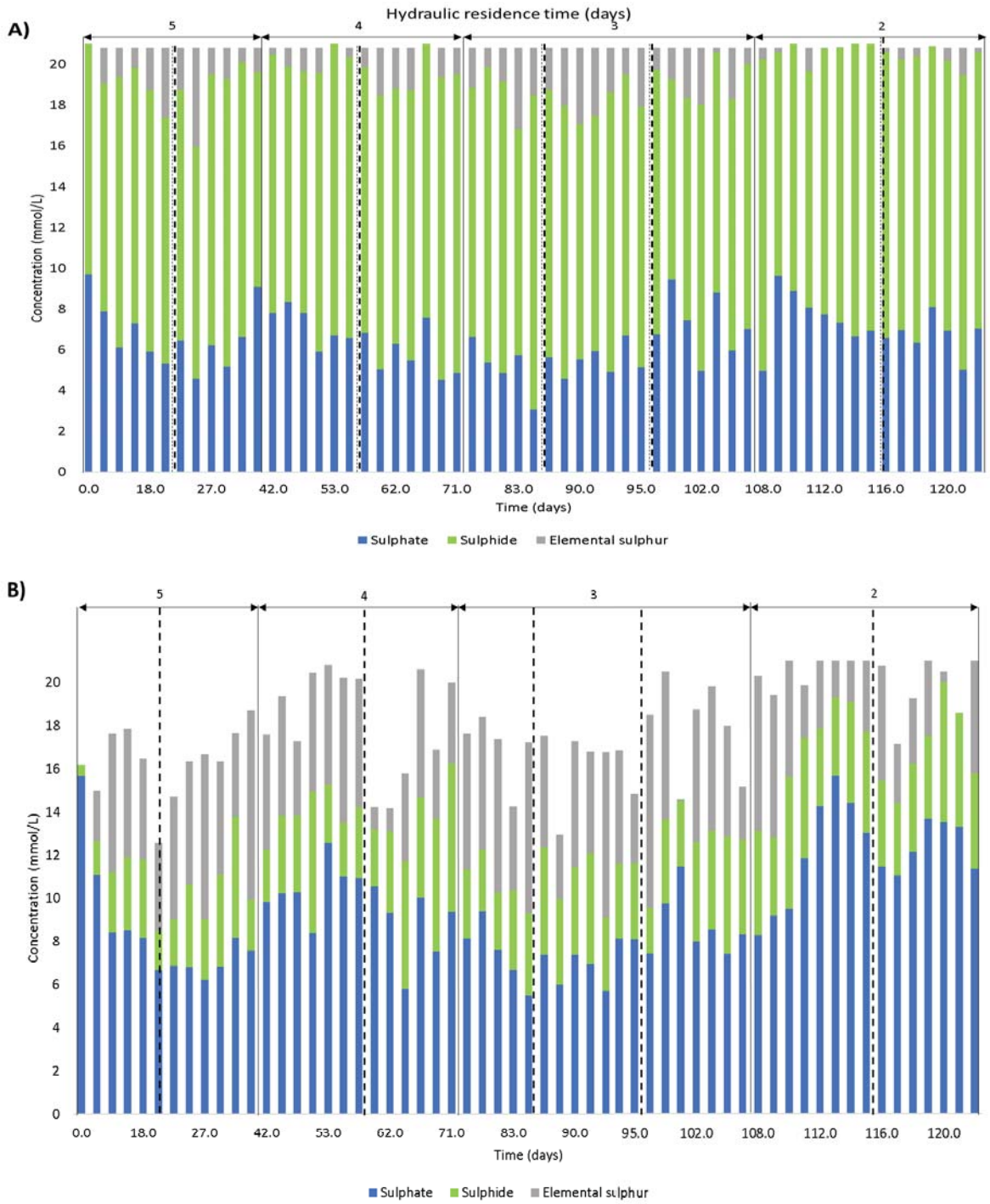


Figure 4-13: Effect of HRT on process performance showing measured residual sulphate and dissolved sulphide concentration over time in the A) primary reactor B) secondary reactor. Elemental sulphur is assumed to be the main product of sulphide oxidation and was determined based on the sulphur mass balance and unaccounted fraction. Solid vertical lines define the FSB harvesting regime.

Similar to the 1 g/L sulphate concentration system, there was sulphide in the effluent of both the primary and secondary reactor. The primary reactor had higher sulphide concentrations in its effluent than in the secondary reactor (Figure 4-13); as formation of the floating sulphur biofilm impedes oxygen from reaching the liquid surface, thus limiting the amount of sulphide partially oxidised to elemental sulphur.,

Increasing the frequency of harvests may increase sulphide conversion and elemental sulphur recovery; harvesting frequency increase would allow more oxygen to reach the liquid surface. However, increasing the frequency of harvesting would also result in more oxygen entering the liquid phase which must remain anoxic

for optimum functioning of facultatively anaerobic SRB. Excessive oxygen availability is responsible for reducing sulphate to sulphide. Thus, unlike the 1 g/L sulphate concentration system which harvested the FSB after 3 HRTs, the 2 g/L sulphate concentration system harvested the FSB after 4 HRTs to minimise re-oxidation.

Sulphide conversion in the secondary reactor was higher than that in the primary reactor for all HRTs at 2 g/L sulphate. Figure 4-13 also shows an increase in sulphate concentration in the secondary reactor showing that some of the sulphide was re-oxidised to sulphate. Sulphide conversions achieved are summarised in Table 4-2. The highest sulphide conversion in the primary reactor was 14.5±8% at a 3-day HRT while for the secondary reactor, the highest sulphide conversion was achieved at a 5-day HRT at 59.8±11%. Overall, across both reactors, the highest sulphide conversions of 35.9% and 35.4% were achieved at a 5-day and 4-day HRT respectively, while the lowest sulphide conversion of 19.4%. was found at a 2-day HRT.

At a 3-day HRT, the lowest sulphate concentrations were seen in the secondary reactor showing that the least amount of re-oxidation occurred at the 3-day HRT while the highest sulphate concentrations in the secondary reactor of the 2 g/L sulphate feed study were seen at the 2-day HRT (Figure 4-13B). It was also observed that the FSB formed at the 2-day HRT was not rigid; this could indicate less partial oxidation of sulphide to elemental sulphur compared to the complete oxidation of sulphide back to sulphate. This re-oxidation at a 2-day HRT could be due to the low sulphide concentration resulting in a low sulphide to oxygen ratio in the secondary reactor which in turn would cause complete oxidation to be favoured over partial oxidation. While high dilution rates at a 2-day HRT resulted in the highest volumetric sulphate reduction rate (0.286 mmol/L.h) (Table 4-2), the reduced fractional sulphate conversion in the primary reactor resulted in the observed low sulphide concentrations in the primary reactor. The higher dilution rate may also have depleted the microbial community necessary for good FSB development through wash out from the primary reactor, which would allow for more oxygen to enter the system favouring complete oxidation.

After harvesting, oxygen can enter the liquid phase causing complete oxidation which in turn decreases the sulphide concentration. From Figure 4-13, it can be observed that the sulphate concentrations in both the primary and secondary reactor increased after harvesting, then decreased as the FSB formed and impeded oxygen from entering the system. Formation of viscous sludge in and around the polyurethane foam used as a support structure in the primary reactor may have also decreased oxygen ingress into the primary reactor. This may explain why there is less re-oxidation seen in the primary reactor as compared to the secondary reactor (Campbell et al., 2019).

Throughout the HRT study, the average pH of the primary reactor was lower than the average pH of the secondary reactor (Figure 4-14); this is similar to the trend observed in Figure 4-8 detailing data for the 1 g/L sulphate feed case.

On the feed containing 2 g/L sulphate, the pH ranged between 7.0 and 8.5 for the primary reactor and between 8.0 and 9.0 for the secondary reactor. The higher pH in the secondary reactor is attributed to the higher sulphide conversion to elemental sulphur; a reaction that produces hydroxyl ions Further observation of the pH data showed that the average pH in both reactors generally stayed constant between the 5-day and the 3-day HRT but decreased at the 2-day HRT, owing to more re-oxidation occurring at a 2-day HRT and the oxidation of sulphide to sulphate producing protons which decrease pH.

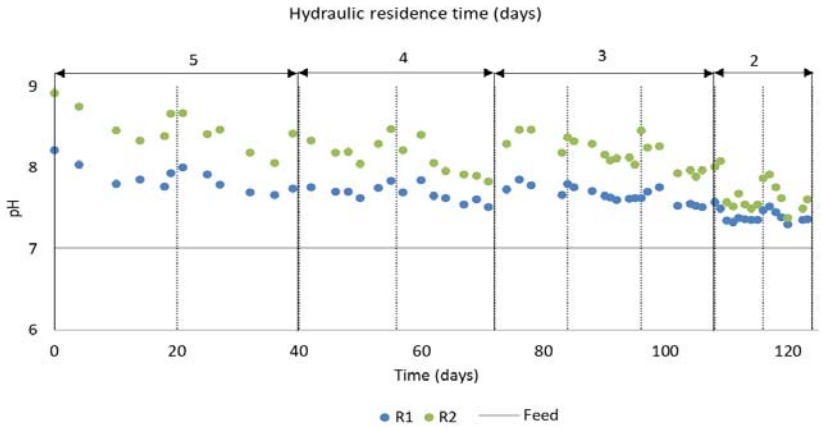


Figure 4-14: Effect of HRT on pH profile within the samples taken from the primary and secondary reactors. Solid and broken vertical lines define the FSB harvesting regime.

The redox remains negative in both the primary and secondary reactor, as shown in Figure 4-15, indicating a reducing and anoxic environment which is essential for the optimal performance of SRB (Postgate, 1984; White & Gadd, 1996; Oyekola *et al.*, 2010). Redox readings lower than -100 mV were reported by Postgate (1984) to be optimal for SRB function. On average the redox potential ranged between -400 and -250 mV for both reactors. The decrease of the HRT from 6 days to 2 days in the secondary reactor shows a slight increase in redox potential which may be due to the increase in sulphate concentration. The anoxic, reducing environment indicated by the negative redox potential and the pH range maintained in the reactors demonstrate that optimal conditions for SRB function were created.

At the liquid-air boundary a redox gradient is created which allows for the formation of elemental sulphur at the liquid surface. The conditions essential for elemental sulphur formation from sulphide are a pH range of 6 to 8 and a redox between -200 to -20 mV (Mooruth, 2013), however the redox in both reactors of the bulk fluid was lower than -200 mV, as shown in Figure 4-15.

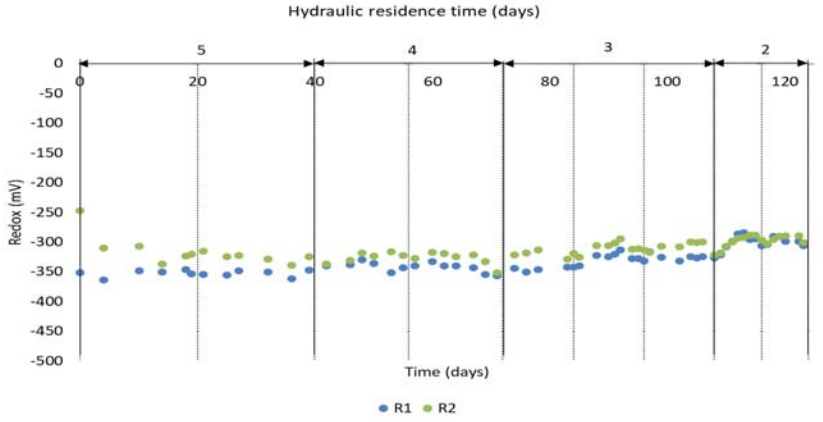


Figure 4-15: Effect of HRT on the redox potential profile within the effluent samples taken from the primary and secondary reactors. Solid and broken vertical lines define the FSB harvesting regime.

Similar to the 1 g/L feed sulphate concentration study, a progressive chemical analysis of samples taken from the ports distributed across the reactor longitudinally along the course of the fluid flow was undertaken to give an insight into the LFCR process dynamics. These results are presented in Figure 4-16.

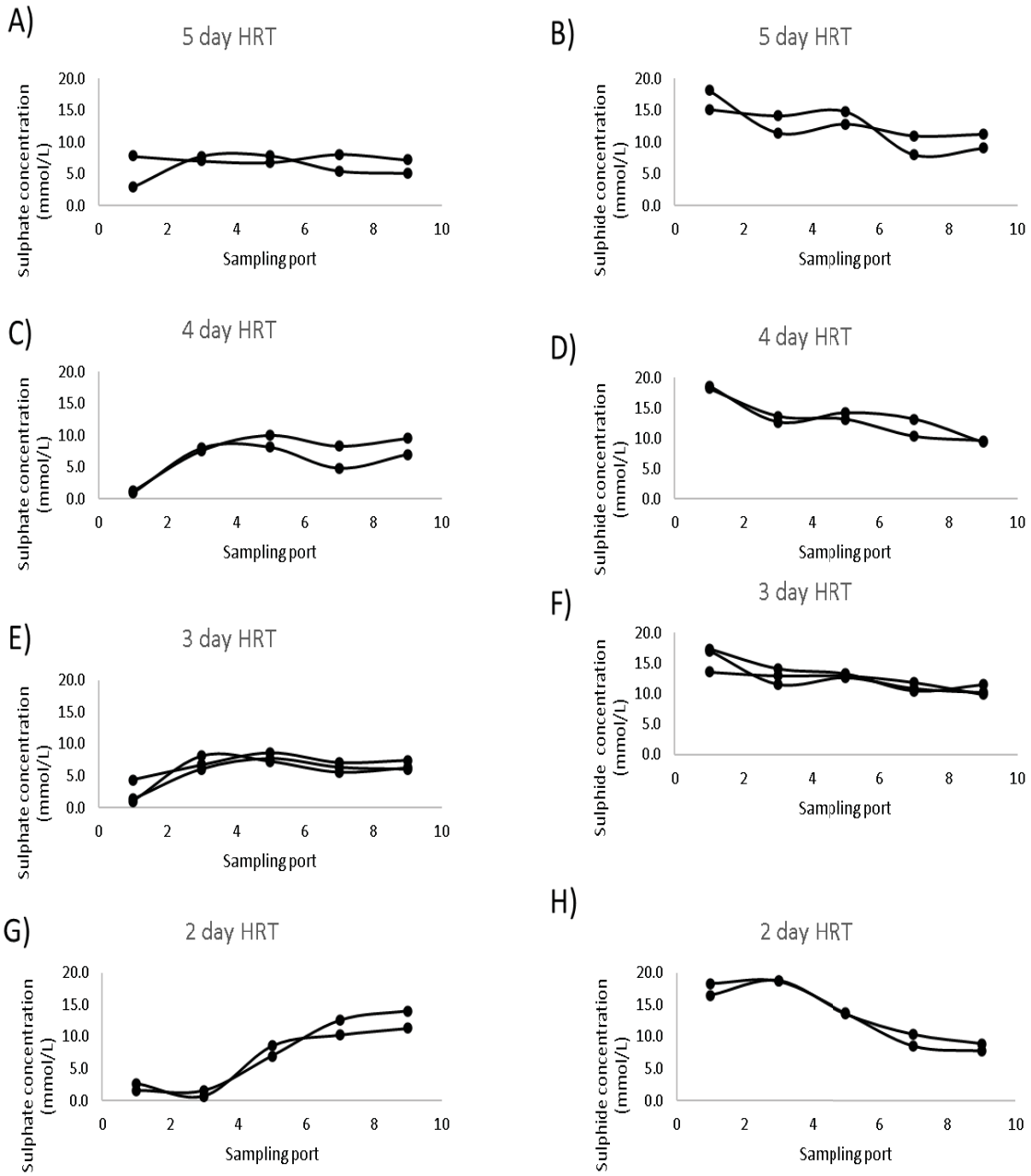


Figure 4-16: Residual sulphate (A, C, E, G) and dissolved sulphide (B, D, F, H) concentration profile across the baffled reactor sampling ports (1-9). Each data series represents a time point (day) of sampling.

Most of the sulphate reduction occurred in the 1st and 2nd compartment of the LFCR in both the 1 g/L and the 2 g/L concentration systems. This demonstrates that two compartments provided sufficient residence time for complete conversion and that the reactor should be able to handle higher sulphate loadings.

The sulphate concentration at the 1st sampling port of the primary reactor was on average at 2.68±3.09 mmol/L across the 5-day to 2-day HRT studies, equivalent to a sulphate conversion of 86.6%. The sulphate concentration then increased at the 3rd port whereafter the sulphate concentration remained reasonably constant for all HRTs.

The increase in sulphate at the surface of the 2nd compartment (3rd port) for HRTs of 3 to 5 days resulted in a decrease in overall conversion to an average of 69±7% in the primary reactor. This increase in sulphate concentration at the 3rd port is attributed to re-oxidation of the sulphide in the surface zone. Contrary to this, sampling port 7 at the surface of the 4th compartment did not show a significant increase in sulphate concentration.

The sulphide concentration trends corresponded to the sulphate trends where the increase in sulphate concentration at the 3rd sample point corresponded to a decrease in sulphide concentration at HRTs of 3 to 5 days. Significant re-oxidation was confirmed at the 2-day HRT in which a large decrease in sulphide from sample port 3 through to the effluent corresponded to an increase in sulphate concentration. While the effluent sulphide concentration decreased across sample points for the 5-day to the 3-day HRT, the sulphate concentrations remained fairly constant after the third sampling point, indicating that partial re-oxidation of sulphide occurred to produce elemental sulphur.

As with the 1 g/L sulphate feed study (Figure 4-12), the mass of FSB decreased with the incremental decrease of the HRT from a 5-day to a 2-day HRT when using the 2 g/L sulphate feed, as seen in Figure 4-17 which shows the mass of FSB harvested from the primary and secondary reactor and the total mass from both reactors. The total mass harvested at the 5-day and 2-day HRT were 24.6 g and 16.0 g respectively. For the 2 g/L feed sulphate concentration system, the mass of FSB harvested in the primary reactor was greater than the mass of FSB harvested from the secondary reactor at the 4-day to 2-day HRT. As the HRT was decreased the rigidity of the FSB in the primary and secondary reactor decreased; the complete oxidation of sulphide to sulphate increased resulting in less elemental sulphur being formed.

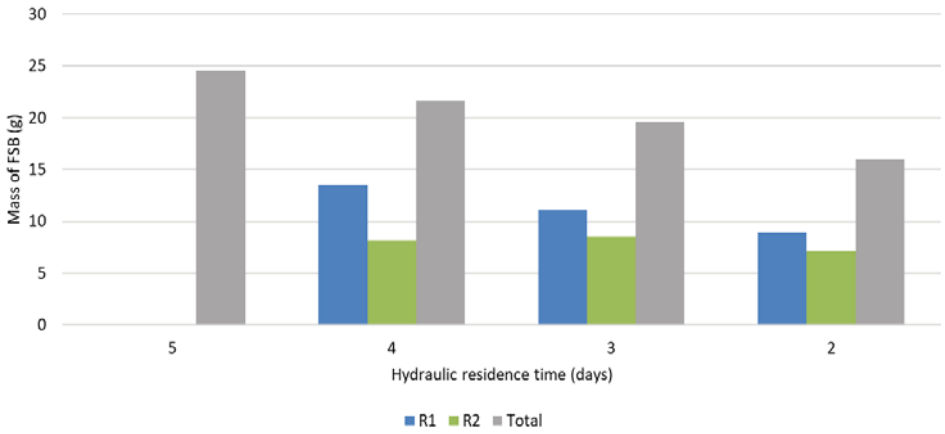


Figure 4-17: Effect of HRT on the biofilm harvest in the primary and secondary reactors.

Since the higher residence times allowed for longer runs, higher amounts of FSB were collected because the FSB had more time to form; more elemental sulphur was deposited into the biofilm. Elemental analysis of the FSB harvested from the 2 g/L feed sulphate concentration system is yet to be conducted; it will be used to determine the elemental sulphur within each harvested biofilm. Determination of its composition will give enough information for a sulphur balance to be carried out.

The performance of the 2 g/L sulphate feed study is summarised in Table 4-2. This shows an increase in volumetric sulphate reduction rate from 0.120 to 0.286 mmol/L.h with reduction in HRT from 5 to 2 days. The trend in sulphate conversion was not clear, with all conversions lying in the range 66 to 72%. Comparing the 1 g/L to the 2 g/L feed sulphate concentration studies (Table 4-1 and Table 4-2), the sulphate conversion in the

primary reactor at all the HRTs decreased by an average of 16.7% in the 2nd study; however the sulphate load doubled, indicated that a larger amount of sulphate was reduced. Sulphide conversion also decreased at all HRTs for the 2 g/L sulphate concentration study. VFA analysis are yet to be undertaken to determine if high acetate concentrations may be inhibiting the SRB (Marais, 2020). Importantly, the volumetric sulphate reduction rate in the 2nd study (0.120 mmol/L.h at a 5-day HRT and 0.286 mmol/L.h at a 2-day HRT) exceeded those with the 1 g/L sulphate feed (0.079 mmol/L.h at a 5-day HRT and 0.169 mmol/L.h at a 2-day HRT) by 52 and 69% respectively. The 2-day HRT at 1 /L sulphate feed and the 4-day HRT at 2 g/L sulphate feed represent the equivalent sulphate loading rate and gave comparable VSRRs of 0.169 mmol/L.h and 0.148 mmol/L.h respectively, with the small difference attributable to differing hydrodynamic environments.).

Table 4-2: Effect of hydraulic residence time on overall process performance of the hybrid baffled reactor comparing primary reactor and secondary reactor.

	Primary Reactor (R1)			Secondary Reactor (R2)	
HRT	VSRR	Sulphate Conversion	Sulphide Conversion [a]	Sulphate Conversion	Sulphide Conversion [a]
(days)	(mmol/L.h)	(%)	(%)	(%)	(%)
5	0.120	71.3%	12.0%	-39.2%	59.8%
4	0.148	68.4%	6.63%	-21.5%	46.9%
3	0.207	71.4%	14.5%	-31.4%	56.4%
2	0.286	66.0%	1.3%	-8.1%	37.5%

[a] Cumulative sulphide conversion based on the expected theoretical sulphide concentration and the final average sulphide concentration over the duration of each experimental run.

4.4 Conclusion

Owing to the potential shown to date of the hybrid LFCR for semi-passive treatment of sulphate-rich wastewaters and effluents, further development of its design and the impact of its critical operating variables on performance is being sought. Through expanding this understanding, applications for treatment of both end-of-pipe low grade effluents and long-term distributed ARD sources hold promise. The re-configuration of the LFCR into a baffled reactor system demonstrated improved overall conversion through sulphate reduction and sulphide oxidation performance with enhanced recovery of elemental sulphur via the FSB. Further, rates of volumetric sulphate reduction were increased by some 20% in accordance with the higher loading. The addition of a secondary reactor unit downstream facilitated near complete sulphide removal with higher elemental sulphur recovery. The study highlighted the importance of FSB management through regulating the frequency of biofilm harvesting to ensure maximum sulphide removal and sulphur recovery.

The hybrid, baffled reactor was able to sustain simultaneous sulphate reduction and partial sulphide oxidation at high levels of efficiency at hydraulic retention times as low as 2 days for the 1 g/L feed sulphate concentration system and 3 days for the 2 g/L feed sulphate concentration system. Maximum rates of biological sulphate reduction (VSRR) of 0.169 mmol/L.h and 0.286 mmol/L.h for the 1 g/L and 2 g/L feed sulphate concentration systems were reached, respectively. The zoning of the reactor and addition of polyurethane foam created niche environments and enhanced the overall contact of the inlet feed with the active biomass. The high biomass

retention, by attachment to and entrapment in the polyurethane foam and reconfiguration of the reactor with baffles prevented washout of the SRB. At 1 g/L sulphate and low HRTs there was evidence of a shift in lactate metabolism from partial oxidation by SRB toward fermentation by competing species.

The data suggests that the maximum VSRR may be further increased with a lower HRT, albeit at the cost of further reduction in conversion efficiency. Therefore, based on the compromise between rate and conversion, the choice of operating HRT should consider the desired water quality and treatment rate. It is envisaged that different microbial communities were spatially separated not only along the chemical gradient that formed across the bulk reactor volume but also preference for attached within the biofilm and planktonic phase. Further investigation into the microbial community dynamics and the effects of operating parameters will be important to further understanding process performance and determining optimal process conditions.

The key objective of this study was to ascertain the effect of polyurethane foam as a support-matrix for enhanced sulphate reduction kinetics and its ability to preferentially retain and select for an SRB microbial community within a reconfigured hybrid baffled reactor system. The study successfully demonstrated the potential of the system to achieve high volumetric sulphate reduction rates with simultaneous sulphide conversion and enhanced sulphur recovery. Further investigations will address the selection of a viable carbon substrate for large scale implementation and the impact of treating real AMD produced from coal discards and fines sourced from local mine sites.

5 Carbon substrate and mini-column bioreactor experiments

5.1 Introduction

Focusing on availability, cost effectiveness and suitability, an investigation into different carbon and electron donor sources for biological sulphate reduction was conducted to contribute toward the provision of a suitable carbon source for large scale implementation. The carbon sources investigated included both defined and complex sources selected based on cost and availability when compared to the more expensive defined alternatives such as ethanol and lactate. The carbon sources investigated included:

1) Acetate – a major constituent of organic mineralisation and a major component in the breakdown of complex organic material.
2) Molasses – often regarded as an inexpensive waste stream generated from the sugar industry and characterised by a high COD that can be used by SRB.
3) Honey – there is potential for rehabilitation of mine sites to designate the land as an apiculture zone which could stimulate local bee keeping, conservation of bees, pollination, and generation of honey. The honey, which is rich in simple sugars and may potentially contain toxins from the mine site, could also in part be supplemented as a viable carbon source for treating AMD instead of it being used for human consumption.
4) Algal lysate – algae products and biomass such as digestate in recent projects have shown potential as alternative carbon sources for biological sulphate reduction and can be produced on site. Algal lysate can be used as a carbon source and electron donor for this study as it contains VFAs and carbohydrates extracted through bead milling in the algal lysate production process. While algal digestate (following anaerobic digestion) has also been considered previously (Motleleng 2020), it is not considered here owing to limited availability.

The carbon sources and electron donors investigated were compared with the well-studied, but expensive, lactate substrate.

5.2 Materials and Methods

5.2.1 Substrate sourcing and characterisation

Undefined (complex) substrates chosen for consideration as carbon substrate were molasses, honey, and algae lysate. The type of molasses used was dark, blackstrap molasses. Raw unprocessed honey was used. For the algal lysate, Spirulina was chosen as the blue-green algae of choice as it has been shown to grow easily and contain both complex and simple sugars to act as a carbon source for anaerobic digestion and SRB mixed cultures (Inglesby, 2011).

Production of algae lysate

A culture of spirulina algae was grown in Zarrouk's media within bottles aerated with humidified air which was filtered through a 0.22 µm syringe filter. The bottles were illuminated by white, fluorescent lights. These algae were isolated from an abandoned wastewater treatment pond at a Western Tanning Company outside Wellington, South Africa and have been maintained at Centre of Bioprocessing Engineering Research (CeBER) in the Department of Chemical Engineering at the University of Cape Town.

The Zarrouk's media consists of 18 g/L $NaHCO_3$, 2.5 g/L $NaNO_3$, 0.5 g/L K_2HPO_4, 1 g/L K_2SO_4, 0.04 g/L $CaCl_2.2H_2O$, 1 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$ and 0.08 g/L EDTA, metal solution A5 (2.86 g/L H_3BO_3, 1.81 g/L

$MnCl_2.4H_2O$, 0.22 g/L, $ZnSO_4.7H_2O$, 0.08 g/L $CuSO_4.5H_2O$ and 0.0124 g/L Na_2MoO_4) at a concentration of 1 ml/L and metal solution B6 (56.6 mg/L K_2CrO_7, 47.8 mg/L $NiSO_4.7H_20$ and 4.2 mg/L $CoSO_4.7H_2O$) at a concentration of 1 ml/L.

The culture was first grown in a 200 ml bottle, then scaled up to a 1 L Schott bottle. Once densely populated (algae bottle had a dark green colour), the whole culture was harvested and transferred to 500 ml Beckman centrifuge bottles (Beckman Coulter Inc., California, USA) and centrifuged at 4000 rpm for 20 minutes in the Eppendorf MiniSpin plus mini-centrifuge with an angle 45° rotor. The algae pellet was resuspended in deionised water. The OD of the algae suspension was determined to calculate the concentration of the algae. The algae cells were then disrupted by bead milling for two hours using a Rushton turbine as a stirrer and glass beads A light microscope at a 100X setting was used to confirm that the spirulina cells had been lysed. The cell lysate was centrifuged for 20 mins at 4000 rpm to separate out the cell debris and obtain the algal lysate supernatant.

Characterisation

The COD concentrations of all the undefined, complex substrates were determined theoretically as well as experimentally using the method described in Section 3.2.7. The theoretical COD content of the undefined substrates was calculated using the different sugar compositions of honey and molasses obtained from literature. A maximum oxygen demand was calculated using from all major sugars in the undefined substrates obtained from literature (Palmonari *et al.,* 2020; De Beer *et al.,* 2021) .

Deviations of the experimentally determined COD from the theoretical COD of the undefined substrates were expected as the source and composition of these substrates varied. The theoretical COD was also assumed to likely be higher than the experimental as a maximum value was calculated and not all sugar within the carbon source is usable.

The experimental COD was used to determine concentrations required for a COD:sulphate ratio above 0.67 for optimal SRB function (Oyekola, 2008). The ratio of COD:sulphate was set to 1.

5.2.2 **Carbon substrate experiments**

Batch experiments for evaluation of carbon sources and electron donors
Evaluation of different carbon substrates was initially performed in batch culture. These batch tests were performed in 1 L Schott bottles at 30°C with an initial sulphate concentration of 2 g/L in Postgate media (Postgate', 1963) at the beginning of the 1st cycle. The batch reactors were agitated through manual shaking before sampling

Subculturing was performed when sulphate reduction tailed off, through a 50% draw and feed. Subculture feed contained Postgate media at a sulphate concentration of 2 g/L with the specific substrate at a COD:sulphate ratio of 1. Each substrate batch reactor went through a minimum of two cycles of successful sulphate reduction before the substrate was transferred to the mini-column reactor set-up.

This initial batch culture study into the potential use of alternative carbon sources (defined and undefined) provided an assessment on the effectiveness of a range of different carbon substrates for BSR. The results from this study were used to determine a suitable carbon substrate for the integrated passive process that can be used for large-scale and long-term implementation. Promising carbon sources from the batch reactor set up were also assessed in mini-column bioreactor experiments to give an indication of their performance in a continuous set-up.

Mini-column bioreactor set-up
After the initial assessment of different carbon sources and electron donors for BSR in batch cultures, a sub-set of these substrates were selected, based on effectiveness for sulphate reduction and suitability for large scale application, to be carried over to the mini-column bioreactor set-up. These carbon sources were evaluated under continuous operation within packed mini-column bioreactors using polyurethane foam as the support matrix (Figure 5-1). The columns had a working volume of 90 ml with inlet and outlet sampling ports.

A gradient profile across the length of the reactor was developed by feeding the reactor from the bottom of the column and having the effluent port at the top of the column.

The reactors were inoculated from the initial batch cultures that were fed on different carbon substrates, as well as from a range of lactate and acetate fed bioreactors that have been maintained at CeBER. The set-up is detailed in Figure 5-1.

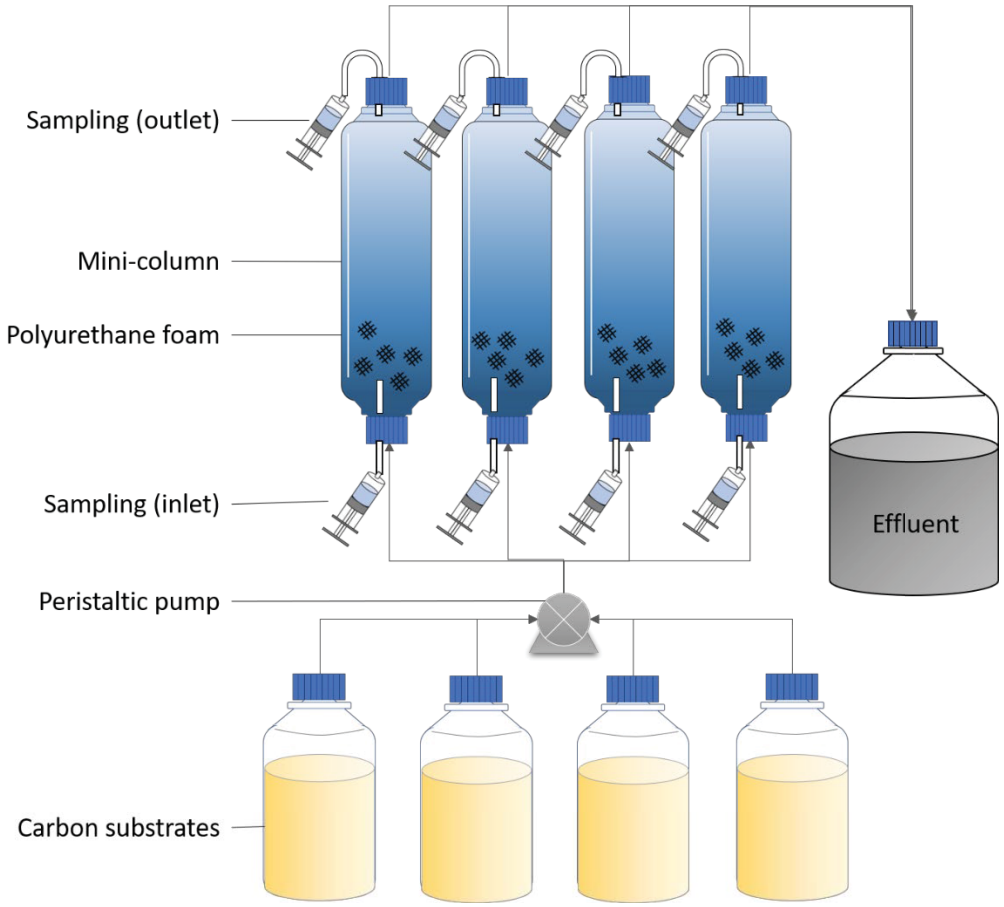


Figure 5-1: Mini-column bioreactor set-up used to evaluate selection of carbon sources (simple and complex).

5.3 Results and Discussion

5.3.1 Characterisation of natural organic materials

The theoretical COD content of the honey, molasses and algae lysate was determined and compared to the experimental COD content of the complex, organic substrates and the results are shown in Table 5-1. Experimentally determined COD content of the substrates differed from the theoretically calculated COD content, due to source and composition of the natural organic substrates being different for the literature obtained values. However, the difference between the honey, molasses and algae was relatively small, 0.049 g COD/g substrate, 0.083 g COD/g substrate and 0.0137 g COD/ml substrate respectively; two of which had a difference of 10% or less. The algae COD content may vary more due to the concentration of algae after harvesting, bead milling and resuspension being higher than that used in the values obtained from literature.

Table 5-1: Theoretical and experimental COD content of undefined, complex substrates.

Substrate	Theoretical COD	Experimental COD
Molasses (g COD/g substrate)	0.826 (De Beer *et al.,* 2021; Shugaba, 2012; Swallow & Low, 1990)	0.777
Honey (g COD/g substrate)	0.759 (Palmonari *et al.,* 2020)	0.842
Algal lysate (g COD/ml substrate)	0.0450 (Ingelsby , 2011)	0.0313[a]

[a]COD content of algal lysate varies as it is made in batches, but an average was taken.

The experimental COD content of the natural organic substrates was then used to ensure that the feed COD:sulphate ratio was around 1.

5.3.2 **Substrate batch reactor results**

Molasses

The molasses batch reactor concentration and pH trends are shown in Figure 5-2. A clear trend of sulphate reduction and sulphide formation is shown after each subculture which is represented by the vertical dotted lines on the graph. The sulphate concentration time trend shows a sudden increase in sulphate concentration following subculturing followed by a decrease in sulphate concentration till it reaches a constant after an average of 30 days. The sulphide concentration time trend complements the sulphate concentration time trend as it shows a sudden decrease in sulphide concentration following subculturing and thereafter, the sulphide concentration increases due to the reduction of sulphate to sulphide until it reaches a constant. An average sulphate reduction of 82.7% across the four cycles and a maximum sulphate conversion of 89.2% in the first cycle, as reached in the molasses batch reactor. The highest sulphide concentration after a cycle was calculated to be 13.0 mmol/L at the end of the second cycle while the average maximum sulphide concentration across all cycles was calculated to be 12.6 mmol/L.

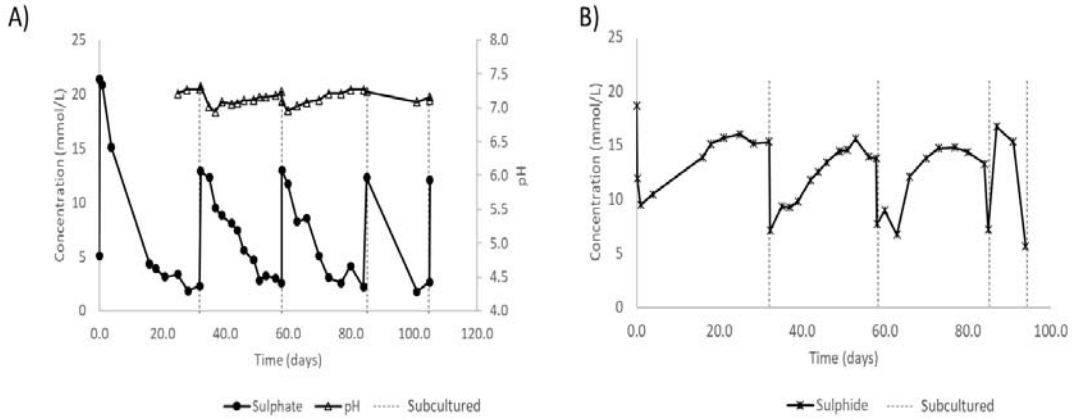


Figure 5-2: Molasses batch reactor A) sulphate concentration and pH time trends B) sulphide concentration time trend.

Unlike the lactate, ethanol, and algae lysate batch reactor (discussed later) that were inoculated at the beginning of the batch reactor study, the molasses batch reactor had been set up previously before the start of the batch reactor studies. The inoculum for all the substrate batch reactors was taken from a BSR reactor (8 L LFCR) which was fed on Postgate B media and used lactate as a substrate. Thus, the bacteria within the molasses batch reactor are expected to have already adjusted to the molasses substrate from the lactate substrate, upon the start of the study. This would explain the high sulphate reduction and short cycle time of 30 days experienced from the first cycle of the molasses batch reactor. The cycle time was shorter than most of the other first cycle times of the other substrate batch reactors discussed later.

The pH ranged between 7.0 and 7.5 throughout all 3 runs of the molasses batch reactor which is an optimal range for SRB performance as discussed by (Mooruth, 2013a). After subculturing, a slight decrease in pH was noticed before a minor steady increase occurred as time progressed in the cycle before the next subculture. The decrease in pH could be due to the addition of the molasses substrate which is originally acidic in nature (Mordenti *et al.,* 2021). Further fermentation of the sugars in the molasses to VFAs produces acidity (Singh *et al.,* 2011). The subsequent rise in pH is caused by SRB metabolism which produces sulphide and bicarbonate when they reduce sulphate and oxidise fermented sugars and VFAs in molasses causing the pH to rise (Marais, 2020).

Since the molasses reactor was performing well, a continuous mini-column reactor using molasses as a substrate was set-up.

Acetate

Like the molasses batch reactor, the acetate batch reactor also shows a clear trend of sulphate reduction and sulphide formation after subculturing in Figure 5-3. The acetate batch reactor presented a lower sulphate reduction and sulphide formation. The average sulphate reduction across the five cycles was calculated as 41.4% with a maximum sulphate reduction of 53.7% in the first cycle. The maximum sulphide concentration of 8.12 mmol/L was observed at the end of the fourth cycle with an average maximum sulphide concentration of 7.26 mmol/L across the second to last cycle. The sulphide concentration in the first cycle were not considered because even though there was a clear reduction of sulphate after subculturing on day zero, the sulphide concentration decreases instead of increasing (sulphate is reduced to sulphide in BSR systems). The decrease could be that the bottle was not airtight which caused the sulphide to be partially oxidised to elemental sulphur. However, this decrease was more likely an error in the sulphide analysis during the first days of the first cycle.

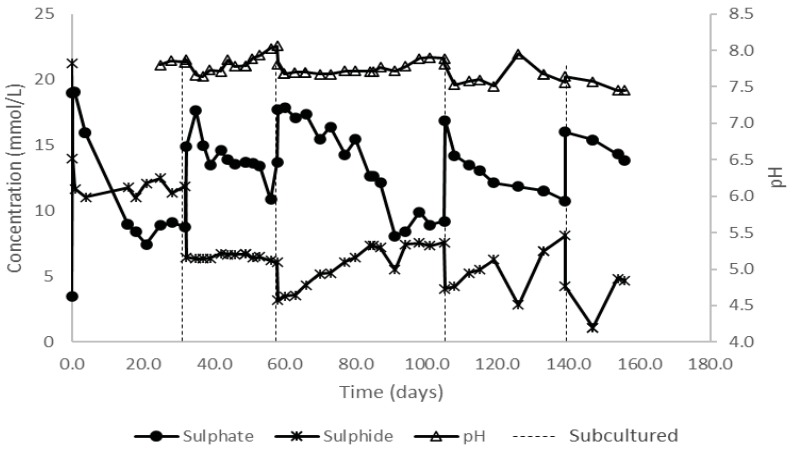


Figure 5-3: Acetate batch reactor sulphate concentration, sulphide concentration and pH time trends.

The acetate batch reactor time trends also showed a slight decrease in pH after subculturing; this could have been due to the stock feed pH being lower than the reactor pH. The reactor pH was higher than the feed pH because of the reduction of sulphate by SRB in the reactor bulk fluid which produced alkalinity. The acetate batch also displayed a wide pH range throughout its runs, between 7 and 8. It reached the highest pH of 8.06 amongst all the substrate batch reactors. This could be due to the oxidation of acetate which produces more alkalinity when compared to substrates such as lactate when they are incompletely oxidised (Table 2-3). An analysis of the samples obtained from the acetate batch reactor will be conducted through HPLC. The samples were taken at the beginning (just after subculturing) and the end (when sulphate and sulphide concentration remained constant) of each cycle. The acetate batch reactor ran for about 160 days with a total of four complete cycles and was carried on to the continuous mini-column set up.

Honey

The honey batch reactor performed poorly as shown in Figure 5-4. It had the lowest overall sulphate conversion of 7.36% and lowest maximum sulphate conversion of 11.1% among all the batch reactors fed with a natural organic substrate. Honey is known to have anti-microbial properties which may have contributed to the low sulphate reduction observed in the honey batch reactor (Mandal & Mandal, 2011).

It had a maximum sulphide concentration and overall average, maximum sulphide concentration of 4.26 mmol/L in cycle five and 2.10 mmol/L across all cycles, respectively. The concentrations of sulphide formed are quite high considering the low sulphate reduction occurring in the honey batch reactor. A possible explanation here could be linked to the sulphur which can be contained in honey being converted to sulphide in a BSR system (Samarghandian *et al.,* 2017).

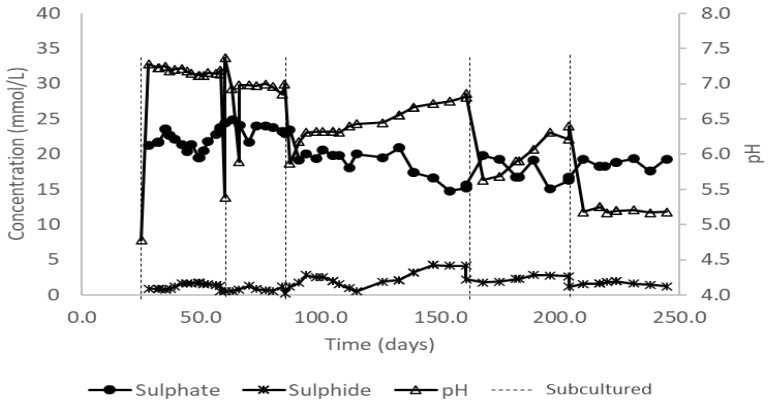


Figure 5-4: Honey batch reactor sulphate concentration, sulphide concentration and pH time trends.

Another observation was that the pH decreased sharply after the first and second subculture, to between pH 4.5 and 5.5, i.e. unfavourable for SRB function. Honey, like molasses, is known to be acidic, ranging between a pH of 3.2 and 4.5 (Mandal & Mandal, 2011). SRB thrive in conditions with a pH range between 7.5-8 (Brahmachariyum *et al.,* 2019). To provide a conducive operating window, the pH was increased using sodium hydroxide in the first and second run, but this did not improve sulphate reduction during these runs. After the second run, the pH was not adjusted after subculturing and the reactor was monitored. Even though sodium hydroxide was not added to increase the pH in the reactor, the pH rose on its own to a pH between 6 and 7 but this did not improve sulphate reduction. The pH increase was thought to be due to the slight decrease in sulphate concentration; sulphate reduction produces bicarbonate alkalinity which could have increased the pH in the reactor. The final feeding cycle of the honey batch reactor was, however, an exception as the pH did not increase on its own and remained below 5.5 throughout the entire cycle.

Honey was thus classified as a poor carbon source and electron donor for biological sulphate reducing systems, owing to the low conversions of sulphate. Tests on honey as a substrate were discontinued after the substrate batch experiments. The honey batch reactor ran for about 250 days and was not carried over to the continuous mini-column setup. However, an analysis of sugars and VFAs in the honey batch samples obtained at the beginning and end of each cycle will be conducted through HPLC to determine substrate utilisation and potential for inhibitions linked to sugars and VFAs in the system.

Algae lysate

The sulphate concentration trend in the algae lysate batch reactor differs from those in the batch reactors fed with molasses and acetate, as shown in Figure 5-5. The sulphate concentration decreased gradually over the first 100 days and then remained fairly constant till the 276th day regardless of the subculturing. The algal lysate batch reactor was intermittently fed (subcultured) and overall, an average conversion of 14.1% and a maximum conversion of 34.1% was achieved. The sulphide concentration also increased over the first 120 days reaching a maximum concentration of 6.51 mmol/L and generally remained constant after these first 120 days, with the exception of the deviations at the point of subculture.

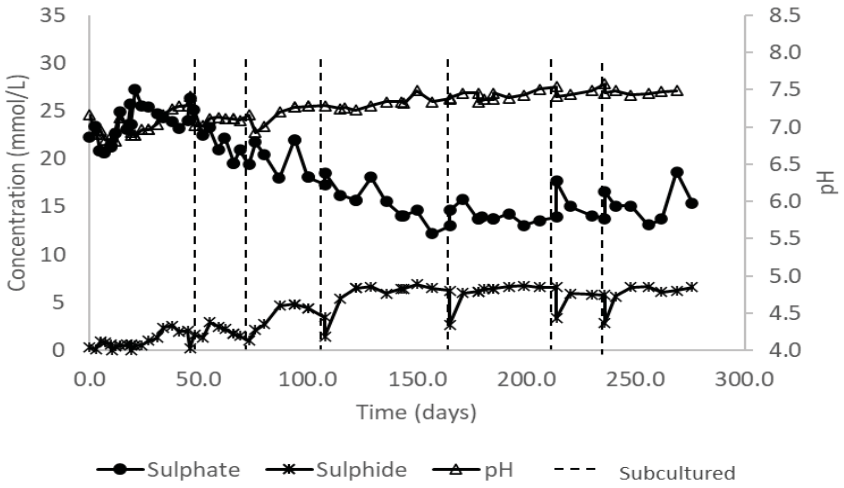


Figure 5-5: Algal lysate reactor sulphate concentration, sulphide concentration and pH time trends.

After subculturing, there was a slight increase in the sulphate concentration; however, it was marginal as the sulphate concentrations tended to fluctuate during the runs with small incremental decreases. This small increase in sulphate concentration can be attributed to the low average sulphate conversion across the individual algal lysate cycles. At these low conversions, subculturing does not affect the sulphate concentration significantly as the fresh feed and harvested culture suspension have similar sulphate concentrations. There was a sharp increase in the sulphide concentration at the very beginning of each cycle and then the concentration proceeded to remain constant for the rest of the cycle until the next subculture, which results in a decrease of the sulphide concentration, was done.

The reactor may have performed poorly during the first three runs due to the COD:sulphate ratio being below 0.67 due to an incorrect determination of COD concentration in the first batch of algal lysate produced. On correcting the COD:sulphate ratio to 1 using a new experimentally determined COD content of the algal lysate, after the 3rd cycle, the performance of the reactor improved from an average sulphate conversion of 8.29% to a conversion of 21.2%. After adjusting the COD:sulphate ratio to 1, the sharp increase in sulphide before it remains constant during each cycle, was observed. This may indicate that the algal lysate may contain a small concentration of VFAs and simple sugars that are metabolised by the SRB to reduce the sulphate which causes an increase in sulphide; these VFAs and simple sugars are probably being quickly and completely oxidised at the beginning of the cycles. However, Table 5-1 does show that algal lysate had a high COD content but it is possible that not all of this COD was usable and may have been in the form of complex carbohydrates and proteins which were not fermented well by the fermenters in the mixed culture. Thus, a much lower usable COD was available to SRB (Gouda *et al.,* 2022). A proposed solution is to anaerobically digest the algal lysate before feeding it to BSR systems so that the complex carbohydrates and proteins are broken down to simple sugars and VFAs that can be utilised in BSR systems with mixed cultures (Motleleng, 2020).

The pH within the reactor ranged between 6.5 and 7.6 for all the cycles. Average sulphate conversion improved due to the adjustment of the COD:sulphate ratio and an ideal pH range for SRB function. However, unlike the molasses, acetate and honey batch reactor which had been running previously before the batch reactor study started, the algal lysate batch reactor was started on day zero of the substrate batch study. The algal lysate batch reactor experiment lasted for almost 300 days which would have given it enough time to adapt to the new substrate from molasses. Thus, the performance observed in Figure 5-5 shows the optimum performance of the SRB culture using algal lysate as substrate with a COD:sulphate ratio of 1.

The algal lysate batch reactor's final sulphate conversion was low compared to that observed in the molasses and acetate batch even though the pH range and COD:sulphate ratio were conducive for SRB growth. Increasing the COD:sulphate ratio may be advantageous for this system using algal lysate as a substrate as this may in turn increase the usable COD content in the reactor resulting in a higher sulphate reduction. However, a large volume of algal lysate would have to be produced and added to the system which could increase the operating costs

and the final treated effluent stream would contain a high COD content. This would make BSR systems using algal lysate as a substrate costly and impractical considering the high COD concentration in the effluent stream. Thus, algal lysate was seen to not be a suitable substrate for large-scale and long-term treatment in the hybrid LFCR. Analysis of the sugars and the VFAs at the beginning and end of the algal lysate batch reactor cycles will be performed using HPLC to determine the substrate utilisation.

Lactate

A lactate batch reactor was set-up to be used as a base case comparison for the other organic carbon sources and electron donors being tested since lactate has been shown to be one of the most effective carbon sources and electron donors in biological sulphate reducing systems (Kijjanapanich et al., 2012). However, lactate is not cost-effective at a large scale and over a long term, driving the need for other organic compounds and streams to be used as substrates that have the potential to be less expensive and more practical alternatives as for the large scale and long-term treatment of AMD in the hybrid LFCR system.

The lactate batch reactor, unlike the molasses, acetate and honey batch reactors which were started before the batch reactor study and had cultures which were already adapted to the substrate and sulphate loading, was started at the beginning of the batch study. Hence the 1st cycle of the lactate batch had a lower conversion compared to the 2nd and 3rd cycle as seen in Figure 5-6. A clear trend of sulphate reduction and sulphide formation after subculturing was observed in Figure 5-6.

The average sulphate reduction over the three cycles was calculated to be 78.1% with a maximum sulphate conversion within a single cycle of 95.1%; this is the highest maximum conversion amongst all the substrate batch reactors. Similarly, sulphide formation was high, yielding a maximum sulphide concentration and an average maximum sulphide concentration that were equal across the last two cycles with a value of 19.2 mmol/L. Good sulphate reduction within the lactate batch reactor was expected as lactate is known to be an efficient carbon source for biological sulphate treatment.

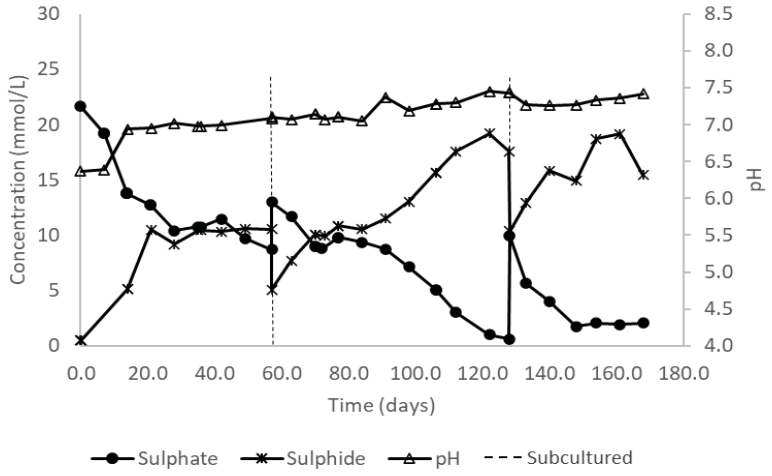


Figure 5-6: Lactate batch reactor sulphate concentration, sulphide concentration and pH time trends.

The pH within the reactor remained between pH 6.5 and 7.5 which is conducive for SRB bacteria function and as expected, during each cycle, the pH slightly increased due to the reduction of sulphate by SRB which produces alkalinity. Since lactate was being used as the base case substrate and performed well in the substrate batch reactor stud, a continuous mini-column with an immobilised microbial community was also set up on lactate as the carbon source and electron donor to be compared to the other mini-columns running on the other substrates.

Ethanol

The ethanol batch reactor was set up at the beginning of the batch reactor studies like the lactate and algal lysate batch. Surprisingly, as seen in Figure 5-7, it performed best in the first cycle and last cycle. The ethanol batch reactor had an average sulphate reduction of 22.2% and a maximum sulphate reduction of 32.7% across

all its cycles, both of which are much lower than those observed in the molasses and lactate batch reactors. There was a decrease in sulphide concentration after subculturing which increased as the cycle proceeded, and a maximum sulphide concentration of 8.87 mmol/L was reached in the last cycle.

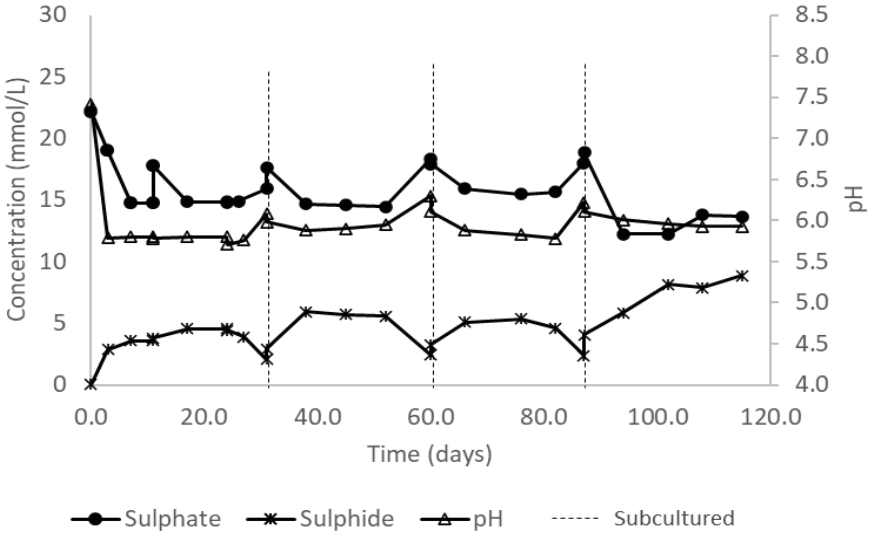


Figure 5-7: Ethanol batch reactor sulphate concentration, sulphide concentration and pH time trends.

The pH in the first cycle started out at around 7.0 but decreased to a final pH of about 6.1. In the second run, the pH remains between 6.0 and 6.5. A higher pH in the first run may be the reason for why there was better sulphate conversion in the first cycle, suggesting that conversion of sulphate to sulphide may be improved by pH adjustment to be between 7 and 7.5. However, in the final cycle, the pH ranged between 6.0 and 6.5 but the highest sulphate conversion reached in this cycle was 32.1% which is almost as high as the sulphate conversion in the first cycle at was at 32.7%. Thus, the pH did not seem to be the reason for ethanol having a lower overall, average sulphate reduction when compared to average sulphate reductions observed in the molasses and lactate batch reactor experiments. The slight decrease in pH as each cycle proceeds may indicate that ethanol is being incompletely oxidised to acetate which produces acidity (Bomberg *et al.,* 2017).

Though the sulphate conversion achieved in the ethanol batch reactor was higher than that obtained in the honey and algal lysate batch reactor experiments, it is an expensive substrate and would not be suitable for long-term and large-scale treatment. It also had lower sulphate conversions when compared to the molasses and lactate batch reactor experiments. After about 120 days of batch operation, subculturing after an average of 30 days when the sulphate concentration reached a constant the ethanol batch reactor was discontinued. Substrate utilisation for the ethanol batch reactor was not determined.

5.3.3 **Mini-column reactors**

Mini-columns of 90 ml volume were packed with SRB communities immobilised in polyurethane foam. They were run in the batch mode to achieve >50% sulphate conversion before they were switched to continuous.

The molasses column was the first to be set up. After switching the column from batch to continuous operation, there was immediate re-oxidation of sulphide to sulphate in the first 10 days; the sulphate concentration increased to 23.7 mmol/L from a concentration of 14.5 mmol/L (Figure 5-8). The molasses column was converted back to batch mode where it achieved a sulphate conversion of 76.4% and the sulphide concentration increased from 1.09 mmol/L to 7.96 mmol/L. Once again, when switched to continuous operation, the molasses column experienced re-oxidation of sulphide to sulphate. Sulphate concentration fluctuated during the molasses column's continuous operation but overall, after 56 days of continuous operation, no significant sulphate reduction had been observed. The pH in the molasses mini-column remained fairly constant between pH 7 and 8 but decreased to a pH just above 6 on day 44 after switching to continuous operation for the second time. It also decreased between day 70 and 77 and between day 98 and 113. The decrease pH corresponded to days

when the sulphate concentration was at its highest (>30 mmol/L). Thus, the decrease in pH could be linked to the release of protons when sulphide is oxidised to sulphate.

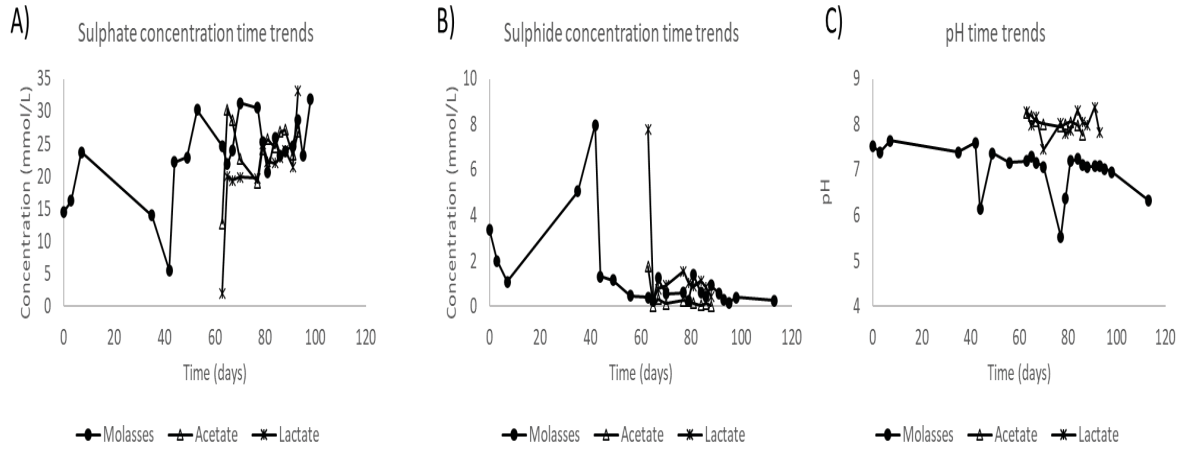


Figure 5-8: Molasses, acetate, and lactate mini-column time trends for A) sulphate concentration B) sulphide concentration C) pH.

The lactate and acetate columns were set-up after the molasses column. These columns were also operated in batch mode till the sulphate conversion was above 50% and like the molasses column, these columns experienced re-oxidation when switched from batch mode to continuous operation (Figure 5-8). The sulphate concentrations increased from 1.97 to 19.4 mmol/L and from 12.8 to 30.3 mmol/L for the lactate and acetate columns respectively after 4 days (day 63 to day 67). The sulphide concentration also drastically decreased between day 63 and 67 showing that re-oxidation had occurred. The sulphate concentration for the lactate and acetate column then fluctuated between 20 and 35 mmol for the rest of the days they were operated continuously, showing that, just like the molasses column reactor, they did not recover and overall had no significant sulphate reduction occurring. The pH for both the lactate and acetate column was within the pH range of 7.5 and 8.5.

The most likely reason for the reactor to fail is due to oxygen ingress into the columns or feed bottles or both through the tubing, the ports, or the reactor openings; the effect of this would be aggravated by the fact that the reactors are very small in size compared to reactors such as the 1 L batch reactors and the 8 L LFCR. Serrano *et al.* (2020) stated that the sample size of a reactor should not exceed 10% of the reactor volume as this may have a negative impact on reactor performance. In this study, 2 ml samples were taken from the bottom, top and occasionally, the middle of the mini-continuous columns using a hypodermic needle. With a working volume of 90 ml, the total sample volume in such a sampling time represents 6.6% of the reactor volume and exceeds the quoted 10% following multiple samplings. This high sampling volume also interferes with the low dilution rates at a 5-day HRT. Operating at a high HRT to replace the liquid lost to sampling could provide further negative impact through removal of active SRB biomass.

Owing to the small size of the reactors, the potential of oxygen ingress when operated in continuous flow was investigated. Tests were conducted using the oxygen-sensitive dye, resazurin, to identify areas where oxygen ingress occurred in the continuous mini-column system. Figure 5-9 and Figure 5-10 show the results from these tests.

When oxygen is introduced to a system containing resazurin it turns from colourless to pink. Figure 5-9 shows that the feed, before autoclaving, contains oxygen, the resazurin changed from a violet-purple to a fluorescent pink (an irreversible change) (Knapp et al., 2018) After autoclaving, the feed turned colourless showing that all oxygen from the feed was removed. The feed after being left to stand for 48 hours did not turn pink showing that there was no oxygen ingress into the feed bottle and feed liquid when it was standing (before feeding into the continuous system).

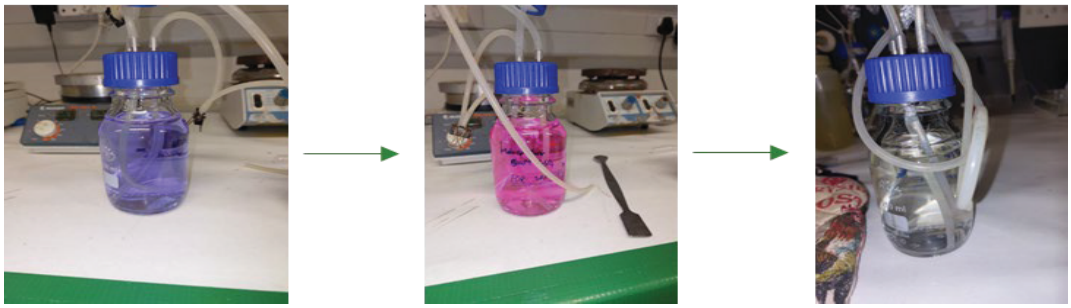


Figure 5-9: Feed (set-up and batch mode) dissolved oxygen test using resazurin.

Figure 5-10: Mini-column (set-up and batch mode) dissolved oxygen test using resazurin.

In Figure 5-10, the mini-column contains water without polyurethane foam packing. Resazurin was added to the water; the absence of polyurethane foam was to prevent obscuring the colour changes that would occur in the reactor since polyurethane foam is a dark grey-black colour. Figure 5-10 first shows the water in the reactor turning from violet-purple to a fluorescent pink indicating that during the set-up of the reactor there is oxygen in the column. The column was then placed in an anaerobic chamber containing a Thermo Scientific AnaeroGen™ bag which creates an anaerobic environment. The chamber was tightly sealed, and a vacuum was created within the chamber. The mini-column was left for 48 hours in the sealed chamber to represent the column operating on batch mode. After 48 hours the chamber was opened, and the column was retrieved. The liquid in the column had turned clear showing that no oxygen was present in bulk liquid of the column. This shows that, when operated in batch mode, the columns can maintain anaerobic conditions and reduce sulphate efficiently as there is no oxygen ingress into the reactor occurring during batch operation.

Figure 5-11: Mini-column dissolved oxygen tests using resazurin while on batch mode at room temperature.

Figure 5-11 shows the column after it had been placed in the cupboard and left to stand for 2 hours. The liquid at the ports in the clamped tubing turned pink showing that oxygen ingress was occurring at the ports of the

mini-columns. However, the bulk fluid in the reactor remained colourless which showed that the amount of oxygen entering through the ports was probably insignificant in the short-term considering the volume of the reactor when it is set up in batch mode at room temperature, in the cupboard.

The feed line was then connected to the column and all tubing, including the effluent tube, were filled with liquid from the feed bottle. At this point, once all tubing was filled with liquid, continuous operation was started. Figure 5-12 shows the feed and feed tubing during continuous operation.

Figure 5-12: Feed and feed tubing dissolved oxygen tests using resazurin during continuous operation.

One can see that the liquid in the feed bottle and tubing remained colourless indicating that in continuous operation, there was no oxygen ingress into the feed via the feed bottle ports, feed tubing feeding into the mini-column reactor and tubing connections.

At the initial startup, while filling all the tubes with liquid (including the effluent tubing), the bulk liquid in the mini-column and tubing connected to the column ports turned a fluorescent pink indicating oxygen was present in the system. This can be seen in Figure 5-13.

Figure 5-13: Mini-column dissolved oxygen test using resazurin during the startup of continuous operation.

After a short while, the pink colour did disappear, and the fluid became clear. This was after the effluent tubing had filled with fluid and some liquid had drained into the waste bottle. However, the water in the effluent tubing was a fluorescent pink showing that oxygen ingress into the effluent tubing was occurring. It was believed that the bulk fluid turned pink at the initial startup of continuous operation because of air that may have been in the tubing. This air was pushed out to the effluent which could have caused the colour change. After the tubing was

filled with fluid no more oxygen was entering the reactor. The air was replaced with oxygen free water from the feed bottle causing the pink colour of the bulk fluid to disappear.

A 2 ml sample of water was drawn out from the column while in continuous operation to mimic sampling and Figure 5-14 is an image of the bulk fluid just after the sample draw, from the bottom of the reactor, was made. A dark pink, fluorescent coloured liquid from the effluent entered from the top of the reactor and changed the bulk fluid entirely to a pink colour. This showed that sampling was the main cause of oxygen entering the reactor. It was thought that this was caused by the effluent being sucked back down into the reactor. Even after clamping the effluent tube during sampling, it did not stop the oxygen from entering the bulk fluid because as soon as the effluent tubing was unclamped, the low pressure created within the reactor due to sampling caused effluent liquid to be sucked back into the column. Due to the small size of the column, the amount of oxygen entering the reactor from the effluent was too high to be mopped up by the low sulphide concentrations, this in turn resulted in the reactor failing as seen in Figure 5-8.

Figure 5-14: Mini-column dissolved oxygen test using resazurin during continuous operation (while mimicking sampling).

5.4 Conclusion

Second to lactate, the molasses batch reactor performed the best among the substrates tested. Therefore, molasses, as carbon source and electron donor, having the highest average sulphate conversion observed, is a suitable and effective substrate for BSR. The metabolism of the SRB was slower for the acetate and honey batch reactor cultures compared to those in the molasses and lactate batch reactors. For the acetate batch reactor, the acetate consuming SRB have slower reaction kinetics, as discussed by Marais (2020). The honey batch reactor was acidic which inhibited the function of the SRB. After increasing the pH, sulphate conversion remained low suggesting inhibition through honey toxins. For the algal lysate batch, the complexity of the substrate may have resulted in a lower usable COD content for the SRB. According to Gouda et al. (2022) and Inglesby (2011), spirulina cells are composed primarily of carbohydrates, proteins, and lipids. These need prior hydrolysis and fermentation to be converted to VFAs. Hence, while the algal lysate may have a high COD content, this may not be directly available to the SRB. Spirulina lysate does contain VFAs from intracellular components and residual media, however this may be very low following lysate preparation. All these factors may have led to the low sulphate reduction in the algal lysate batch reactor. A recommendation would be to anaerobically digest the algal biomass to breakdown the complex carbohydrates, proteins, and lipids to form a VFA dense stream which would provide a readily available substrate to the SRB and achieve a higher sulphate conversion as reported by Motleleng (2020). The ethanol batch reactor was discontinued because it had a lower average

sulphate conversion when compared to the average sulphate conversion in the molasses and lactate batch reactors and it also is generally quite a costly substrate and thus would not be practical for large-scale, long-term BSR systems.

On operating the mini-columns, the reactor size greatly affected the performance of the reactors and oxygen entering the reactor through the sampling port connections and through sampling itself re-oxidation occurred and in turn the mini-column reactors continuously failed. Therefore, a different reactor configuration and scale was considered which involved switching the 1 L Schott bottle, batch reactors to a continuous operation. This was done to gather continuous data testing the different substrates under investigation.

The batch reactor results were used to select the suitable substrate for operation of the 8-litre hybrid LFCR system. Molasses performed best after lactate and is available at scale, and was selected for scale up.

6 Hybrid baffled reactor treatment of real acid mine (AMD) drainage

6.1 Introduction

The composition of true AMD is complex, potentially containing inhibitors as compared to the synthetic AMD feed that has been applied to date in the assessment of the hybrid LFCR. In this study, we consider the performance of the LFCR using real AMD. We consider the chemical composition of the AMD and address potential problems that may present during treatment such as metal precipitation and pH fluctuation. The study investigated the potential of the re-designed baffled packed bed horizontal reactor system to treat an actual AMD stream produced in a pilot study from coal waste and fines sourced from a mine site in Mpumalanga, South Africa.

6.2 Methods

6.2.1 **AMD source and pre-treatment**

The AMD stream was generated at the Centre for Bioprocess Engineering (CeBER) greenhouse at the University of Cape Town in which AMD generation from coal discards is studied at barrel scale in a pilot study to assess the prevention of AMD through packing regime (Kotsiopoulos *et al.,* 2023) .The actual AMD stream was characterised in terms of its chemical composition of major and trace metals through ICP-MS while the iron concentration, COD, and sulphate concentration were determined by wet chemistry methods. Additional measurements done on the actual AMD included pH, redox and electrical conductivity (EC). The actual AMD was tested in batch cultures which were fed with AMD that was untreated, pretreated with lime, pretreated with both lime and sodium sulphide and pretreated with lime sterilised before feeding to the batch reactors.

The AMD treated with lime used hydrated lime which had a composition of 40-42% calcium hydroxide, 29-30% magnesium oxide and a 25-27% water content (National Lime Association, 2023). Lime was chosen as the desired pretreatment process due to its efficiency in metal removal, neutralisation and the low cost of lime, the active ingredient, compared to other alternatives (Aubé, 2003). The AMD was treated in batches of a 10 L volume in a twenty litre PVC bucket using a Rushton blade stirrer for agitation. About 24 ml of a lime slurry made by mixing 100g of powdered lime with 200 ml of tap water was added to the 10 L of untreated AMD. Addition of the lime slurry increased the pH to be between 8.5 and 9. At a pH between 8.5 and 9 most metals are precipitated out (Aubé, 2003; Balladares *et al.,* 2018). After lime addition and stirring until the desired pH was reached, the suspension was left to stand allowing the metal precipitates to separate by gravitational settling. The clear treated AMD was then decanted from the treatment bucket and transferred to a storage bottle for later use as feed to BSR systems.

For the AMD batch fed with AMD treated with lime and sulphide, the AMD was first treated with lime and the metal precipitates were allowed to settle. The clear solution was then decanted and was then further treated with sodium sulphide to precipitate out the residual metals left after lime treatment. The metal sulphide precipitates that formed from the sulphide addition were removed using filtration and the filtrate of treated AMD was used as the feed for the third AMD batch reactor.

The sterilized AMD was treated with lime in1 L batches and autoclaved at 120°C for twenty minutes.

6.2.2 **Batch experiments on real AMD**

Initial batch experiments were performed on real AMD before introduction into the baffled reactor system. These batch tests were used to assess the SRB culture ability to perform sulphate reduction on the real AMD

stream which had been pretreated in different ways. Furthermore, the test was expected to adapt the SRB culture to the AMD and to stimulate the native SRB population within the AMD stream. The batch cultures were conducted in 1 L Schott bottles at 30°C, fed with either pre-treated or untreated AMD generated. Besides the AMD from the CeBER greenhouse, only the organic substrate (carbon source and electron donor) was substituted into the feed and no other nutrients or components from the modified Postgate B media were added. The substrate of choice within the batch reactors was lactate and the reactors were only agitated by shaking just before sampling. The cultures were monitored regularly over time to assess BSR performance.

Sub-culturing was performed to promote the selection of a highly active SRB culture adapted to the AMD. Sulphate reduction was monitored, and a 50% draw-and-feed was undertaken when rates were close to zero. Each batch experiment was taken through a minimum of two cycles of successful sulphate reduction.

The initial batch culture study on the treatment of the real AMD provided a comprehensive assessment on the effectiveness of SRB culture in treating real AMD and its need for pretreatment. The AMD pretreatment methods were assessed in the batch reactors before the actual AMD was introduced into the LFCR system.

6.2.3 Operating the hybrid reactor on real AMD

Following the selection of the pretreatment required for the AMD, the hybrid reactor described in Section 4.2.1 was operated on AMD. The first set of experiments used lactate as the carbon source and electron donor. Following assessment of performance, the carbon source was substituted with molasses, as selected from the batch studies presented in Section 5.3.2.

6.3 Preliminary Results and Discussion

6.3.1 Source and characterisation of real AMD

The AMD was sourced from the CeBER greenhouse, where a mixture of coal discards (CD) and fine waste (FW) tailings from the Mpumalanga region were mixed in CD:FW ratio of 3:2 and packed in PVC barrels with an aspect ratio of 1.8. Total mass of the mine waste in the barrels was 200 kg. The barrels were irrigated with either water or acidified water (Barrel 1 is non-agglomerated irrigated with water, barrel 2 and 3 are irrigated with acidified water, barrel 4 is agglomerated and irrigated with water, barrel 5 and 6 are agglomerated and irrigated with acidified water). This was done to mimic the leaching process that generates AMD (Kotsiopoulos *et al.,* 2022). This AMD was collected and the sulphate concentration, iron concentration, pH and conductivity of the AMD was determined. An elemental analysis was also conducted through inductively coupled plasma mass spectrometry (ICP-MS) to identify both the major and trace elements in the AMD. The AMD was then pretreated in different ways to remove metals through precipitation and to increase the pH. Pretreatment was assessed due to AMD having a high heavy metal content and acidic pH that is expected to be inhibitory to SRB (Koschorreck, 2008). The LFCR system was also designed for circum-neutral AMD and therefore a pretreatment of highly acidic AMD would be necessary for successful sulphate removal in the LFCR. Current treatment methods for AMD typically focus on the acid and heavy metal component of AMD while failing to remove sulphates to the required standards resulting, in the need for expensive additional polishing steps (Fernando *et al.,* 2018; Torres *et al.,* 2018). Thus, the LFCR system can also be seen as a more cost-effective treatment step that is able to handle significant sulphate loads while also polishing to required water quality.

Table 6-1 shows the compositions of the untreated AMD from the greenhouse and AMD from mines in Mpumalanga for comparison sourced from literature (Alegbe *et al.,* 2019; Sheridan *et al.,* 2021).

Table 6-1: Comparison of untreated AMD composition from Mpumalanga and the CeBER greenhouse

Description	Units	Mpumalanga Region AMD	Untreated Greenhouse AMD	Greenhouse AMD treated with lime
Sulphate	mg/L	4868	1701	1696
pH		2.48	2.43	8.42
Conductivity	mS/m	726.7	2.98	1.92
Al	mg/L	109.6	8.44	0.0616
As	mg/L	3.52	0.0029	0.0012
B	mg/L	0	0.0909	0.104
Ba	mg/L	0.034	0.0209	0.024
Be	mg/L	0.05	ND	ND
Ca	mg/L	747.5	512	683
Cd	mg/L	0.0034	0.00024	0
Co	mg/L	1.87	0.0071	0.0008
Cr	mg/L	0.222	0.0119	0.0006
Cu	mg/L	0.290	0.139	0
Fe	mg/L	282	52.3	0.0037
Hg	mg/L	0	0	0
K	mg/L	8.53	0	12.8
Mg	mg/L	308	8.57	87.6
Mn	mg/L	59.9	0.928	0.199
Mo	mg/L	0	0.0002	0.0002
Na	mg/L	74.7	9.61	0.0102
Ni	mg/L	1.85	0.0198	0.0062
Pb	mg/L	0.0831	0	0
Se	mg/L	0	0.0071	0.0029
Si	mg/L	11.7	25.1	13.7
Sn	mg/L	0.004	0	0
Sr	mg/L	4.38	2.52	2.58
Th	mg/L	0.46	ND	ND
Ti	mg/L	0.17	ND	ND
V	mg/L	0.053	0.00058	0.00008
Y	mg/L	0.33	ND	ND
Zn	mg/L	4.96	0.190	0.0091

One can see that both the AMD from the Mpumalanga region and from the CeBER greenhouse have an acidic pH between 2 and 3. SRB are highly sensitive to pH as toxins and inhibitors such as organic acids and hydrogen sulphide are more potent at low pH ranges (Koschorreck, 2008). Koschorreck (2008) and Watson-Craik & Senior (1996) report that at a pH of 4, both methanogens and SRB are inhibited when present as a mixed culture. The ideal pH range for SRB has been reported to be between 6 and 8 (Koschorreck, 2008). At low pH bacterial proteins denature while macromolecules can become destabilised (Fink *et al.,* 1994; Koschorreck, 2008). SRB generally have a low metabolic energy yield and so cannot benefit from the mechanism of acidophiles which can survive in acidic environments by maintaining a higher internal pH within their cells through proton transfer which is an energy taxing process. Another mechanism which could help SRB to cope in acidic environments is the formation of micro-niches with the BSR systems. However, in extremely acidic environments the proton gradient is far too steep and though SRB produce alkalinity when they reduce sulphate, it is not enough to combat the fast diffusion of protons (Koschorreck, 2008).

Both streams contain heavy metals such as Fe, Cr, Hg, Mn, Ni, Pb, Cd, Cu, Al and Zn which are known to be toxic to microorganisms as they can react with functional groups of enzymes resulting in their deactivation and also compete with essential cations (Utgikar *et al.,* 2002, 2003b). Some authors have reported that at low concentrations, heavy metals can improve the activity and growth of SRB (Akinpelu *et al.,* 2021; Del Busso Zampieri *et al.,* 2021.; Utgikar *et al.,* 2002, 2003b). Table 6-2 shows a summary of the concentration levels that are reported as toxic to SRB.

Table 6-2: Toxic heavy metal concentrations levels to SRB

Metal	Toxic concentrations (mg/L)	References
Al	13	(Akinpelu *et al.,* 2021; Martins *et al.,* 2012)
Cd	>4-20	(Utgikar *et al.,* 2003b)
Cr	60	(Utgikar *et al.,* 2003b)
Cu	4-20	(Utgikar *et al.,* 2003b)
Fe	475	(Gonzalez-Silva *et al.,* 2009)
Ni	10-20	(Utgikar *et al.,* 2003b)
Pb	75-80	(Utgikar *et al.,* 2003b)
Zn	20	(Utgikar *et al.,* 2003b)

All metals in the untreated AMD found in Table 6-1 were lower than the toxic concentrations quoted in Table 6-2, suggesting that the metal concentrations in the untreated greenhouse AMD should not be toxic to SRB. The calcium concentrations are higher than the WHO potable water limits of 100-300 mg/L; however, this concentration may not affect the efficiency of the mixed community's performance, but should be considered in terms of outlet water quality. Other metals that are in high concentrations, above 4 mg/L, in the untreated AMD from the greenhouse are Mg, K and Si. Cao *et al.* (2009) showed that an increase in Mg had a positive effect on SRB, increasing their sulphate conversion at concentrations as high as 100 g/L. The potassium concentration of 12 mg/L is lower than that found in Postgate media B which has been found to be an ideal growth media for SRB (Postgate', 1963).

6.3.2 AMD batch reactor results

At the beginning of the real AMD study, two AMD batch reactors were set-up, one fed with pre-treated AMD and the other with untreated AMD from the UCT CeBER greenhouse. Both used lactate as the carbon source to test the microbial community's ability to treat real AMD. No other Postgate B media components were supplemented and the COD:sulphate ratio was set to be around 1.0 which is optimal for SRB function in a mixed culture.

Untreated AMD batch reactor

Figure 6-1 shows the batch reactor fed with untreated AMD from the CeBER greenhouse. The reactor was given a period of 110 days to adapt to the introduction of real, untreated AMD before the batch study was started. The SRB was not able to function in the harsh conditions created by the introduction of untreated AMD and thus there was very low sulphate reduction within the batch reactor.

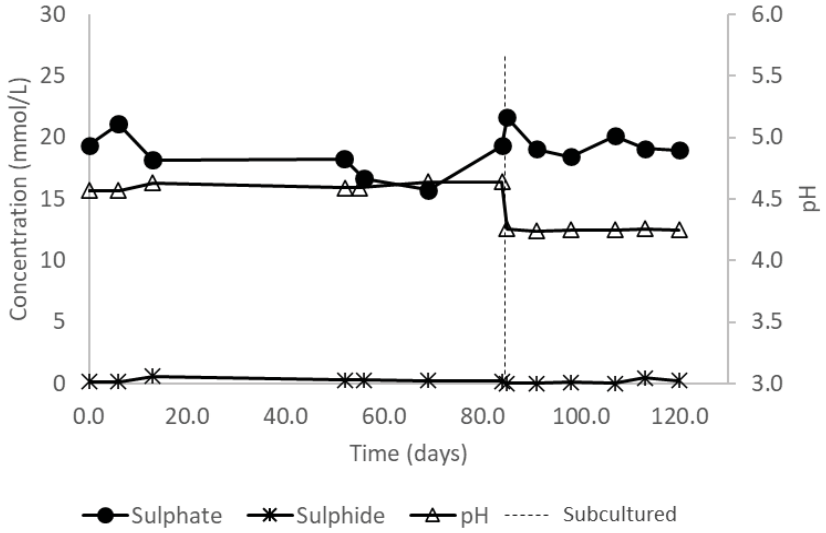


Figure 6-1: Untreated AMD batch reactor sulphate concentration, sulphide concentration and pH time trends

The untreated batch reactor had a maximum sulphate reduction of 19% and an average sulphate reduction of 9% across its two cycles. It had the lowest sulphate reduction among the four batch reactors receiving true AMD treated in various ways. Since Table 6-2 showed that the metal concentrations within the untreated greenhouse AMD were lower than the levels toxic to SRB, the poor performance of the batch reactor was expected to be caused by the low pH of the untreated AMD. From observations reported by Elliott *et al.* (1998b), it would only take a few days for SRB to adapt to acidic conditions (less than 50 days). However, the untreated AMD batch reactor culture did not adapt sufficiently over 100 days. Subculturing once after the sulphate concentration decreased and reached a constant failed to effectively reduce sulphate after a considerably long adaptation period. The mixed culture likely only contained SRB that thrive in sulphate rich waste streams that are circum-neutral and did not contain acidophilic SRBs. However, Elliott *et al.* (1998b) tested pH ranges between 4.5 and 3. At higher pH values 4.5, 4.0 and 3.5 the adaptation period was shorter (2-5 days) but at pH values lower than 3.5 the adaptation period increased to more than 20 days and at a pH of 3 there was some significant sulphate reduction observed but it was not maintained, and the effluent pH and sulphide concentration decreased (Elliott *et al.,* 1998b). This is what was observed in the untreated AMD batch reactor, where a low sulphate conversion is observed which increased the sulphide concentration (less than 1 mmol/L of sulphide formed) and pH slightly but after a short while the sulphide concentration drops as seen in Figure 6-1. Koschorreck (2008) also mentions how in acidic conditions, the type of substrate used is important. In acidic conditions non-ionic substrates such as methanol and glycerol were seen to improve sulphate reduction over ionic substrates such as lactate (Koschorreck, 2008). The low pH of the feed made of untreated AMD and lactate as a carbon source resulting in a bulk liquid pH ranging between 4.0 and 5.0 was detrimental to the untreated batch reactor resulting in its failure. Thus, it is necessary to treat AMD to increase its pH while simultaneously precipitating out metals in the system which will allow for efficient removal of sulphate by SRB.

AMD pretreated with lime batch reactor

A composition between the untreated and lime-treated AMD is given in Table 6-3.

Table 6-3: Comparison between untreated and lime-pretreated AMD compositions and characteristics

Description	Units	Untreated AMD	AMD treated with lime
Sulphate	mg/L	1701	1696
pH		2.43	8.42
Conductivity	mS/m	2.98	1.92
Al	mg/L	8.44	0.0616
As	mg/L	0.0029	0.0012
B	mg/L	0.0909	0.104
Ba	mg/L	0.0209	0.024
Ca	mg/L	512	683
Cd	mg/L	0.00024	0
Co	mg/L	0.0071	0.0008
Cr	mg/L	0.0119	0.0006
Cu	mg/L	0.139	0
Fe	mg/L	52.3	0.0037
Hg	mg/L	0	0
K	mg/L	0	12.8
Mg	mg/L	8.57	87.6
Mn	mg/L	0.928	0.199
Mo	mg/L	0.0002	0.0002
Na	mg/L	9.61	0.0102
Ni	mg/L	0.0198	0.0062
Pb	mg/L	0	0
Se	mg/L	0.0071	0.0029
Si	mg/L	25.1	13.7
Sn	mg/L	0	0
Sr	mg/L	2.52	2.58
V	mg/L	0.00058	0.00008
Zn	mg/L	0.190	0.0091

As can be seen from Table 6-3, the average sulphate concentration remained similar after lime treatment thus showing the need for an additional step to remove the sulphate. The conductivity decreased from 2.43 mS/m to 1.92 mS/m. The slight decrease in conductivity could have been caused by the addition of salts from the pretreatment step with hydrated lime which contains calcium, magnesium and potassium. However, the

conductivity level is still well below the upper limit of 2.5 mS/m for human consumption (Loock *et al.,* 2015). The pH increased from 2.43 to 8.42 allowing for several metals to precipitate out; their removal efficiencies are shown in Table 6-4. The removal efficiencies were only calculated for metals that had a concentration higher than 0.001 mg/L. All metal removal efficiencies were high including Fe which had the largest concentration of 52.3 mg/L. The removal of the metal ions through precipitation due to the increase of the pH created conditions that were suitable for sulphate removal by SRB.

Table 6-4: Removal efficiency of metals in AMD treated with lime

Metal	Removal efficiency after lime treatment
Al	99.3%
Co	88.7%
Cr	95.0%
Cu	100.0%
Fe	99.9%
Mn	78.6%
Ni	68.7%
Zn	95.2%

The lime-treated AMD was then fed to a 1 L Schott bottle batch reactor and the time trends for the sulphate concentration, sulphide concentration and pH are shown in Figure 6-2. The pre-treated AMD batch reactor was run for a total of 180 days excluding the first 110 days the reactor had to adapt to the partially treated AMD from synthetic AMD. In the batch experiments of the real AMD study, the AMD with pre-treated AMD went through two cycles where there was a decrease in the sulphate concentration within the first 20 days; however, there was re-oxidation of the sulphide to sulphate in the first cycle. The sulphate concentration began to decrease again on day 60 of the first cycle and a significant sulphate reduction was observed. At day 113, the batch reactor had reached its maximum sulphate reduction for the first cycle with a reduction of 89.8%. The decrease in sulphate concentration caused an increase in sulphide concentration reaching a maximum sulphide concentration of 14.6 mmol/L in the first cycle. The first cycle lasted about 140 days. The second cycle was significantly shorter when compared to the first cycle. Unlike the first cycle which showed little to no sulphate reduction in the first 60 days, the second cycle showed significant sulphate reduction occurring after 20 days.

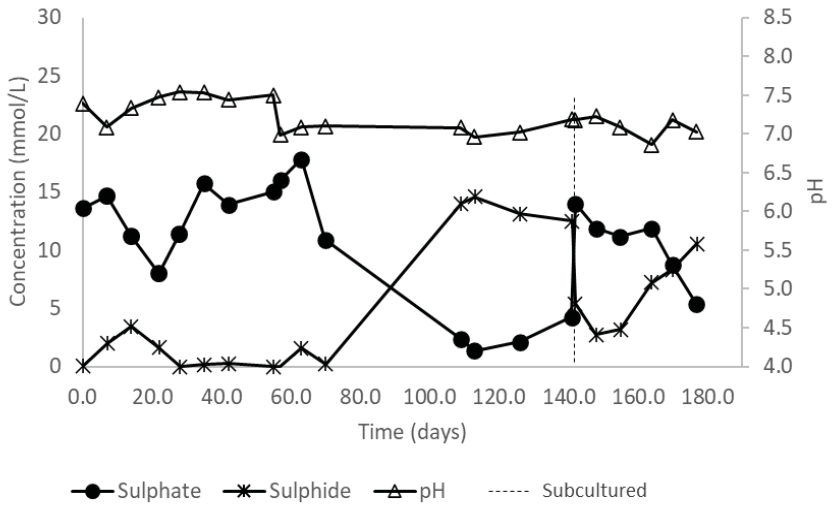


Figure 6-2: Pretreated with lime AMD batch reactor sulphate concentration, sulphide concentration and pH time trends

Overall, using only lactate and partially treated AMD had an average sulphate reduction of 75.8%. The delay in sulphate reduction at the beginning of the first cycle may have been caused by several factors such as inhibitions by metal sulphides that may have formed from the residual metals left after lime treatment, an alkaline pH of the feed which may have not been optimal for SRB activity, or competition between indigenous microorganisms present in the tailings and discards or used for the generation of the AMD. However, even though the feed had a pH above 8 the bulk fluid pH in the reactor was determined to be at 7.5 which is within the ideal pH range for SRB function. Thus, two more batch reactors were set up to test whether the residual metal sulphides formed from reaction of pretreated AMD and the sulphide inhibited the function of the SRB or if indigenous microorganisms competed with the SRB.

Batch reactor using AMD pretreated with lime and sulphide

The batch reactor used to test whether metal sulphides (formed from the reaction of residual metals left after lime treatment and the sulphides from sulphate reduction) reduced the metabolism of the SRB was fed with AMD that was treated with both lime and sodium sulphide. Figure 6-3 provides the concentration profiles for this batch reactor.

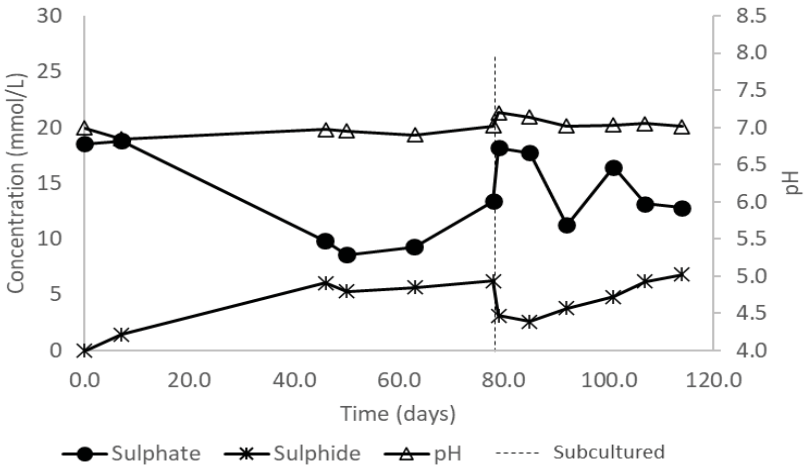


Figure 6-3: Pretreated with lime and sodium sulphide batch reactor sulphate concentration, sulphide concentration and pH time trends

The batch reactor was inoculated with a culture extracted from the AMD batch reactor fed with AMD treated with just lime. Thus, the culture had already been adapted to the partially lime treated AMD and was expected to perform better since less metal sulphides would form. The batch reactor achieved a maximum sulphate conversion of 54% in the first cycle around the 50th day of operation and the overall average sulphate reduction across its two cycles of operation of 29%. Decrease in the sulphate concentration resulted in an increase in sulphide concentration achieving a maximum sulphide concentration of 6.81 mmol/L in the second cycle. The pH remained between 7.0 and 7.5 which is optimum for SRB activity (Koschorreck, 2008).

Addition of sodium sulphide resulted in decreased sulphate reduction as the maximum and average sulphate reduction were lower than those observed in the batch reactor fed with just lime treated AMD. The decrease in sulphate reduction may have been due to an increase in sodium concentration as Vinícius & Vallero (2003) reported that high sodium concentrations can decrease sulphate reduction in a BSR system.

Limed-treated, sterilised AMD batch reactor

The batch reactor used to investigate competition between SRB and indigenous microorganisms treated used a feed of greenhouse AMD partially treated with lime and autoclaved at 120°C for twenty minutes to ensure all microorganisms in the partially treated AMD were killed. The AMD was autoclaved in 1 L batches. Time trends for the partially treated, sterilised AMD are shown in Figure 6-4.

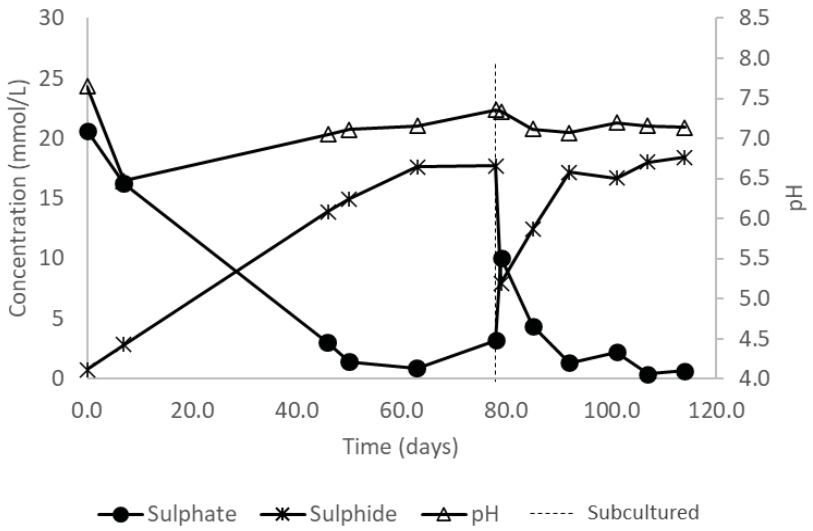


Figure 6-4: Pretreated with lime and sterilised AMD batch reactor sulphate concentration, sulphide concentration and pH time trends

The sterilised, partially treated AMD batch was inoculated with the mixed culture from the 8 L LFCR running on synthetic AMD with an initial sulphate concentration of 2 g/L and lactate. This was done to avoid carry over of the indigenous microorganisms from the other three AMD batch reactors which were fed with non-sterilised AMD treated in different ways. A maximum conversion of 96% in the first cycle and an average conversion of 89% across both two cycles was achieved in the batch reactor. This was the highest conversion achieved amongst the four AMD batch reactors and it achieved a maximum sulphide concentration of 18.4 mmol/L in the second cycle.

The first cycle had a longer run time with maximum sulphide conversion only reached after 40 days whereas the second cycle reached maximum conversion at day 92, about 12 days after the second cycle began. SRB in the batch reactor quickly adapted to the sterilised, partially treated AMD. The highest pH of 7.65 was observed at the start whereafter the pH remained between 7.0 and 7.5.

The increase in sulphate reduction and sulphide formation was probably due to the removal of indigenous microorganisms through sterilisation of the partially treated AMD. Leachate from coal discards and fines was used as the AMD, and iron and sulphur oxidisers are typically found in these heaps (Li *et al.,* 2023; Tambwe *et al.,* 2020). These oxidise sulphide converting it back to sulphate which in turn decreases the efficiency of a BSR system in reducing sulphate (Li *et al.,* 2023; Suzuki *et al.,* 1990; Tambwe *et al.,* 2020). However, these Fe- and S-oxidising microbes are mainly acidophiles, so unlikely competitors. Other competitors were not characterised. While sterilising the partially treated AMD eliminated any microbial competitors allowing for SRB activity to thrive resulting in a high sulphate conversion, this does not represent a practical approach, only one to shed understanding on our system. The sulphate reduction in the sterilised, pretreated AMD is higher than that observed in the lactate batch reactor from Figure 5-6 which was fed with synthetic AMD. This was unexpected considering the presence of residual heavy metals in the pretreated AMD compared to the synthetic AMD that contained additional nutrients from the Postgate B media. Utgikar *et al.* (2002) indicate that low concentrations of metals have been shown to improve the activity of the SRB. Thus, the minute concentrations of metals left in the sterilised, pretreated AMD may have improved overall sulphate reduction by the SRB.

Both sterilisation and pretreatment the AMD with lime before feeding it to the 8 L LFCR was used. Pre-treating with lime allowed for an increase in pH to reach the circumneutral conditions required by the SRB and precipitate out heavy metals. The sterilisation by autoclaving ensured the death of indigenous microorganisms expected to be at a higher level in the pilot study from which the AMD came than in environmental AMD. Further, it prevented the feed to the LFCR from fermenting after the addition of a carbon source. In other words, the sterilisation was a requirement of our set-up rather than a step planned at scale.

6.3.3 Linear flow channel reactor studies on treatment of AMD

Two LFCR studies were conducted in the 8 L LFCR connected to a secondary reactor as described in Figure 4-2. The secondary reactor was set up to improve elemental sulphur recovery. One study focused on the ability of the LFCR system to treat true AMD that had been pretreated with lime to increase the pH and remove heavy metals using lactate as the substrate. This study helped assess the capability of the LFCR system to treat actual, partially treated AMD on a simple carbon source that has been studied extensively and proven to be efficient in BSR. In the second study, the treatment of AMD pretreated with lime was carried out with molasses, the chosen alternative substrate from the substrate batch reactor tests discussed in Section 5.3.2. Molasses represents a cost effective and readily available substrate as a by-product of the sugar industry. The pretreated AMD fed in both studies was sterilised by autoclaving at 120°C for 20 mins.

LFCR studies with partially treated AMD and lactate as the feed

The mixed culture in the 8 L LFCR system was adapted to the new feed of real AMD by increasing the concentration of AMD in the feed in 20% increments from 20% AMD to 100% AMD. Although the feed had been pretreated to increase the pH and remove heavy metals, removal was not complete and the Postgate media components were removed such that supplementation was with a carbon source only. The pretreated AMD also had a higher concentration of calcium from lime pretreatment which could have an adverse effect on the SRB. Hence stagewise adaptation was employed.

The AMD concentration in the feed was increased after the FSB was harvested. Five different ratios of AMD:synthetic AMD (Postgate B media) were fed into the 8 L LFCR system of 20:80, 40:60, 60:40, 80:20 and 100% AMD. The sampling layout of the LFCR system with the primary reactor (8 L baffled LFCR) and the secondary reactor is shown in Figure 6-5.

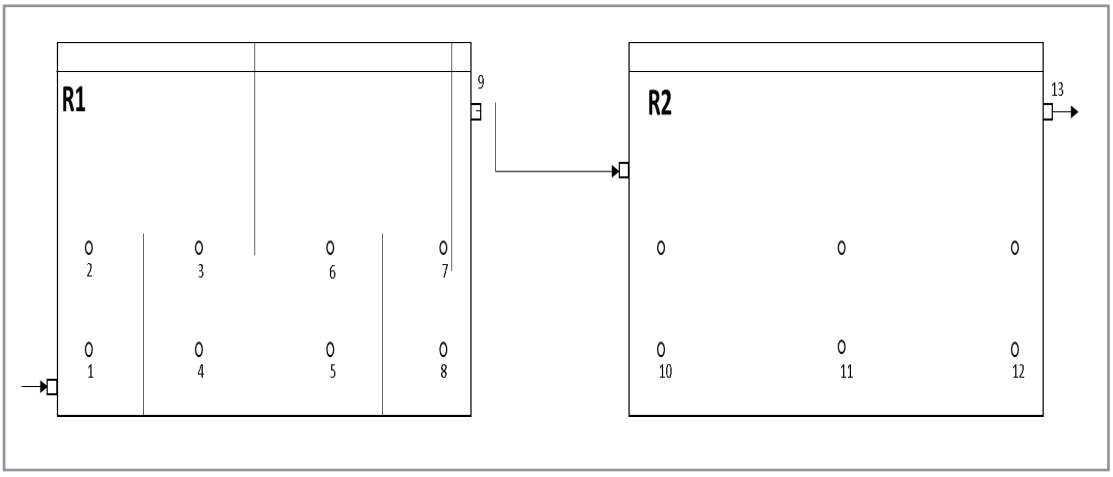


Figure 6-5: Sampling layout of the primary and secondary LFCRs (R1: primary reactor and R2: secondary reactor)

The sulphate concentration profiles of the LFCR sequence system fed with real, pretreated AMD and lactate as a substrate are shown in Figure 6-6.

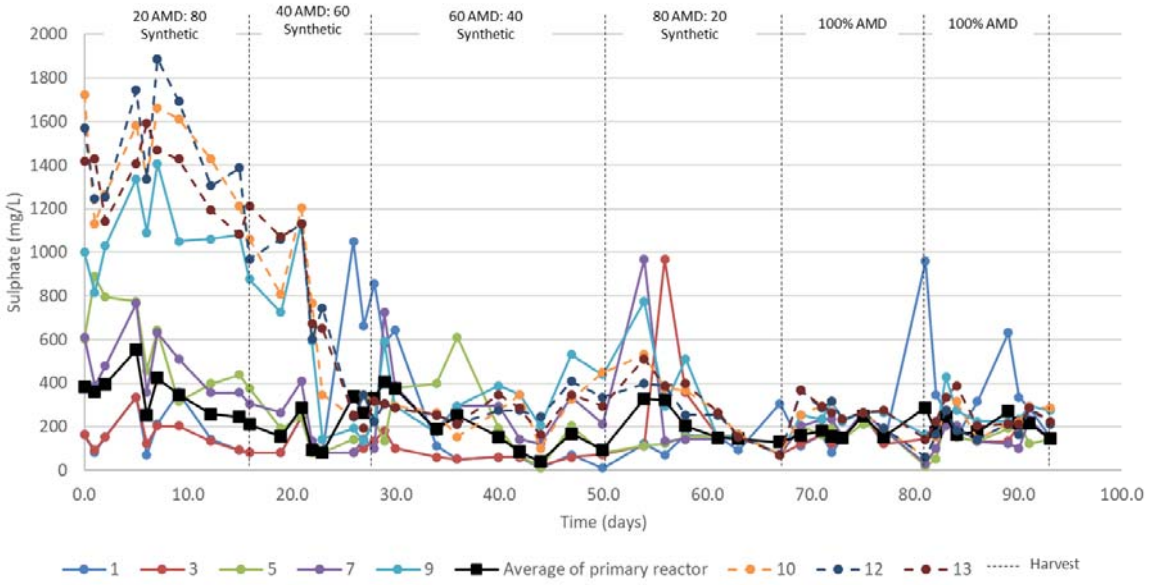


Figure 6-6: Sulphate time trends in the 8 L LFCR system with stepwise increases in real AMD, and lactate as electron donor.

The initial feed concentration to the 8 L LFCR had an average concentration of 1800 mg/L across all the AMD: synthetic ratios and all runs were conducted at a 3-day hydraulic residence time, the optimum HRT in Section 4.3.2. In the runs from 20% AMD to 80% AMD, the run time depended on the time to reach pseudo steady state before harvesting the FSB; thereafter the concentration of pretreated AMD was increased. The 100% AMD runs extended for 4 HRTs, a total of 12 days each, and as such are comparable to the 3-day HRT study using synthetic AMD with a 2 g/L sulphate loading (Section 4.3.2).

The sulphate reduction in the primary reactor increased with an increase in the real AMD fraction in the feed with the highest average sulphate reduction of 92% achieved at a ratio 80 AMD: 20 Synthetic run in the primary reactor. The lowest sulphate reduction of 82.8% was observed in the in the first run with a 20:80 AMD:Synthetic ratio. In all other runs, the sulphate reduction in the primary reactor stayed close to an average of 88%. Looking closer at the individual sample ports, most of the sulphate reduction occurred in the 1st compartment (sample point 1 and 3); this low sulphate concentration was maintained at points 5 and 7. However, re-oxidation occurring in the first run, 20 AMD: 80 Synthetic, at sample point 9 which is the primary reactor effluent port with concomitant increase in sulphate concentration. The high sulphate concentration was maintained through to the secondary reactor as seen in sample ports 10, 12 and 13.

As the concentration of the AMD in the feed increased, there was less re-oxidation at the primary reactor effluent port and in the secondary reactor. A few anomalies were seen; between day 50 and 60 the sulphate concentration spiked for sample point 7 and 3 and between day 75 and a 100 the sulphate concentration spiked at sample point 1. Overall, sulphate reduction in the LFCR sequence system was high despite the removal of the nutrients in Postgate B media. The average sulphate reduction achieved in the 100% AMD runs of the primary reactor was calculated to be 87.4% which was higher than the 71.4% conversion achieved in the primary LFCR fed with synthetic AMD at a sulphate concentration of 2 g/L at a 3-day HRT (Section 4.3.2). Unlike the synthetic AMD experiment reported in Section 4.3.2, harvesting of the biofilm had little effect on sulphate concentration. In the synthetic AMD experiment, the sulphate concentration would tend to increase after harvesting due to the influx of oxygen caused by the disruption of the biofilm.

The sulphide concentration time trends for the pretreated AMD with lactate as a substrate are shown in Figure 6-7. The sulphide concentration in the primary reactor was high throughout all the AMD runs with an average sulphide concentration of 546 mg/L (16.5 mM) in the primary reactor. The decrease in the concentration of sulphide in the secondary reactor for the 20 AMD: 80 Synthetic and 40 AMD: 60 Synthetic runs shows either the partial oxidation of sulphide to elemental sulphur or the complete oxidation of sulphide back to sulphate. Figure 6-6 showing the sulphate time trends, showed an increase in sulphate concentration in the secondary reactor

for the 20 AMD: 80 Synthetic and 40 AMD: 60 Synthetic runs. Since sulphate concentrations remained low for all the runs after the 40 AMD: 60 Synthetic run (Figure 6-6), the decrease in sulphide concentration in Figure 6-7 indicated the formation of elemental sulphur. However, the 80 AMD: 20 Synthetic run showed very little decrease in sulphide concentration compared to the other runs but like the other runs after the 40 AMD: 60 Synthetic run, maintained low sulphate concentrations which could indicate that little to no elemental sulphur is forming. This was an interesting observation as the formation of the FSB is necessary for oxygen to be impeded from entering the bulk liquid and in turn avoiding sulphide re-oxidation.

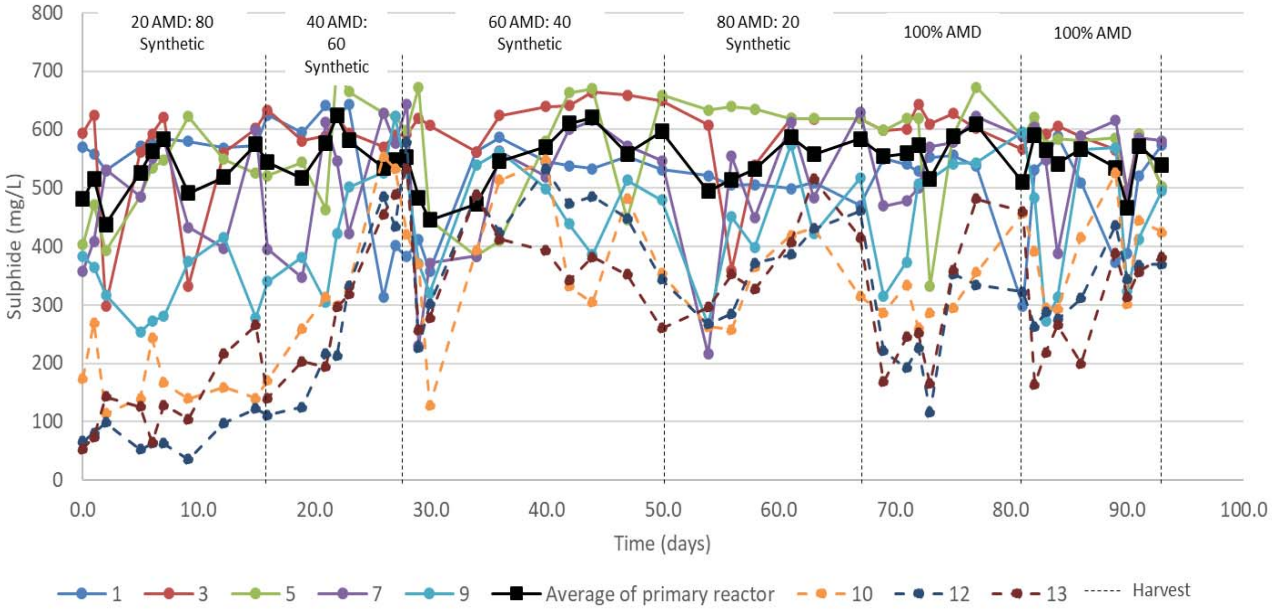


Figure 6-7: Sulphide time trends in the 8 L LFCR system with stepwise increases in real AMD, and lactate as electron donor.

The redox potential in both the primary and secondary reactor remained between -300 and -350 mV (Figure 6-8), well below -100 mV and therefore ideal for SRB activity (Hessler, 2020; Marais, 2020). This also shows that during the ratios which indicated poor sulphide oxidation to elemental sulphur, the environment remained anoxic and reducing despite a presumably thinner FSB. The redox readings also show that for runs after the 40 AMD: 60 Synthetic run, the redox for the secondary reactor is mostly equal to the redox in the primary reactor. Figure 4-15 which shows the redox for the experiment using synthetic AMD with a sulphate loading of 2 g/L using lactate as a carbon source, shows that the redox in the secondary reactor is higher than that in the primary reactor at a 3-day HRT. This may indicate that the study using synthetic AMD with a sulphate concentration of 2 g/L may have been experiencing more re-oxidation in the secondary reactor compared to the study using real, pretreated AMD and lactate as feed.

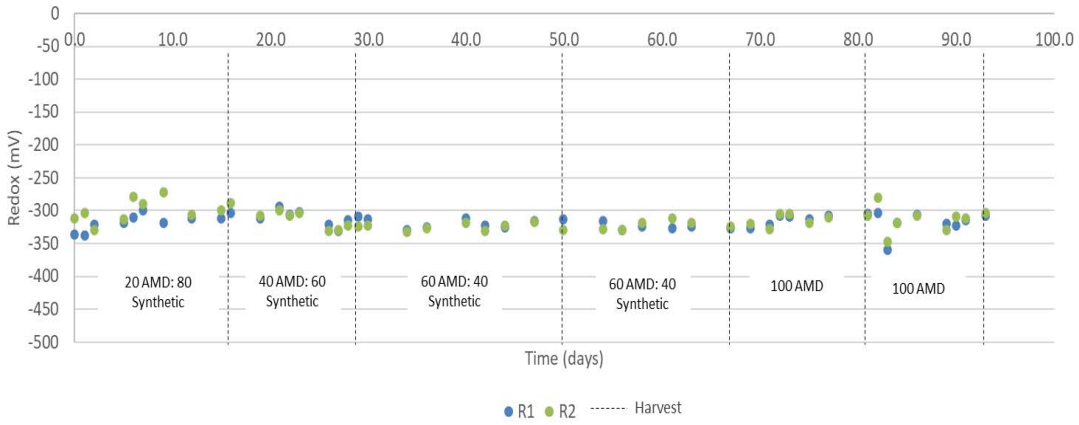


Figure 6-8: Redox time trends in the 8 L LFCR system with stepwise increases in real AMD, and lactate as electron donor.

The average pH values of the primary and secondary reactor were recorded over time (Figure 6-9) and showed a similar trend when compared to the pH trends of the synthetic AMD study with a 2 g/L sulphate feed concentration (Figure 4-14). As seen in Figure 6-9, the pH in the primary reactor was consistently lower than the pH in the secondary reactor and ranged between 7.0 and 8.5 for both reactors. Across all ratio runs, the pH generally increased from the first sampling point of the primary reactor till the last sampling point of the secondary reactor.

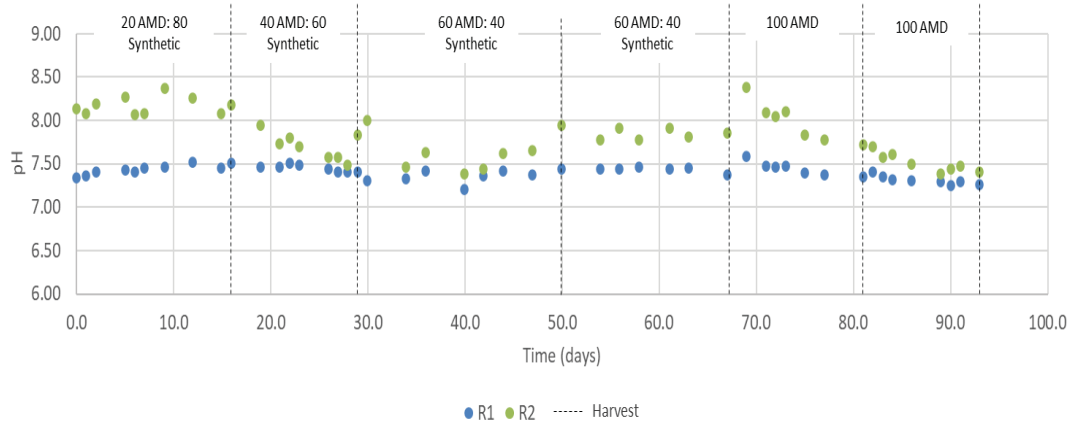


Figure 6-9: pH time trends in 8 L LFCR system with stepwise increases in real AMD, and lactate as electron donor.

Table 6-5 is a summary of the overall performance of the LFCR sequence system running on AMD and lactate. The highest sulphate conversion was at the 80 AMD:20 Synthetic run. However, this run had the second lowest sulphide conversion of 15.9% in the secondary reactor. The negative sulphide conversions seen in Table 6-5 for the 80 AMD: 20 Synthetic and 100% AMD run in the primary reactor may indicate that the initial feed sulphate concentration at these runs was underestimated thus resulting in the expected sulphide being lower than the measured sulphide. Sulphate concentration in these last two ratios was not expected to be far off from the calculated and so it was also concluded that the sulphide conversion to sulphate was negligible at these runs in the primary reactor.

Table 6-5: Effect of the stepwise AMD addition on the overall process performance of the hybrid baffled reactor at a 3-day HRT

	Primary Reactor (R1)			Secondary Reactor (R2)	
Ratio (AMD:Synthetic)	VSRR (mmol/L.h)	Sulphate Conversion (%)	Sulphide Conversion (%)	Sulphate Conversion (%)	Sulphide Conversion (%)
20:80	0.239	82.8%	7.95%	-39.2%	37.5%
40:60	0.257	88.9%	8.53%	-21.5%	17.3%
60:40	0.234	88.6%	3.87%	-31.4%	14.0%
80:20	0.235	92.0%	-1.76%	-26.34%	15.9%
100	0.216	87.4%	-5.82%	18.1%	29.9%

The highest sulphide conversion in the secondary reactor was achieved at the 20 AMD: 80 Synthetic run with a conversion of 37.5%. However, as the concentration of AMD increased in the feed, sulphide conversion in the secondary reactor generally decreased, and then increased again from run 80 AMD: 20 Synthetic to the 100% AMD run achieving a conversion of 29.9%. The sulphide conversion in the secondary reactor with a 100% AMD and lactate feed is lower than that observed for the synthetic study (section 4.3.2) at a 3-day HRT which had a conversion of 56.4% (Table 4-2). Thus, even though the sulphate reduction is higher in the primary reactor when real, pretreated AMD is present in the feed the sulphide conversion in both reactors has decreased when compared to the synthetic experiment. Re-oxidation of sulphate in the secondary reactor is similar to that observed in the synthetic AMD and lactate experiment (Section 4.3.2). However, the 100% AMD run is an exception and showed no re-oxidation occurring in the secondary reactor but rather an 18.1% sulphate reduction. Thus, the experiment with real, pretreated AMD as the feed when compared to the experiment fed with synthetic AMD, both using lactate as a carbon source, had better sulphate reduction but had less sulphide oxidation. This is interesting as even though less sulphide was being oxidised to elemental sulphur, the liquid phase remained anoxic and higher sulphate conversions were achieved and maintained in the real, pretreated AMD experiment.

Figure 6-10 shows top view pictures taken from different experiments running in the LFCR sequence system to visually evaluate formation of the FSB in the three different cases.

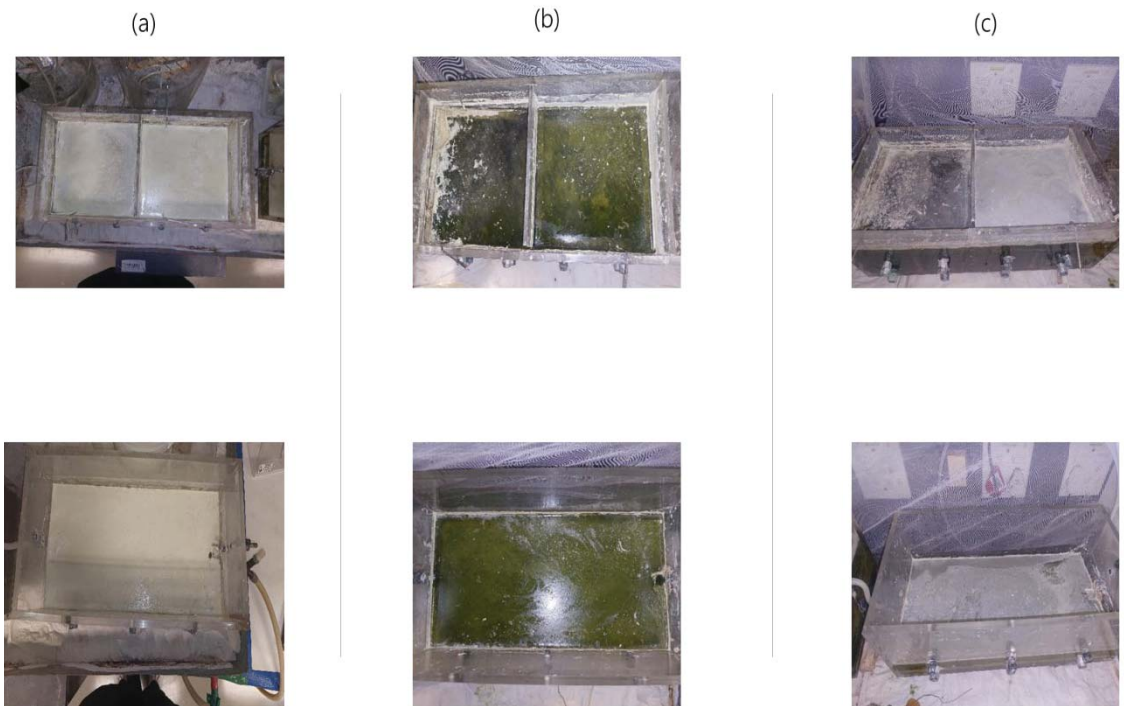


Figure 6-10: Top view of the primary (top) and secondary (bottom) reactor showing (a) FSB formed during a 3-day HRT experiment with a 100% synthetic AMD feed, (b) thin film formed during a 3-day HRT experiment with a 80% AMD and 20% synthetic AMD feed and (c) FSB and thin film mixture formed during a 3-day HRT experiment with a 100% AMD feed

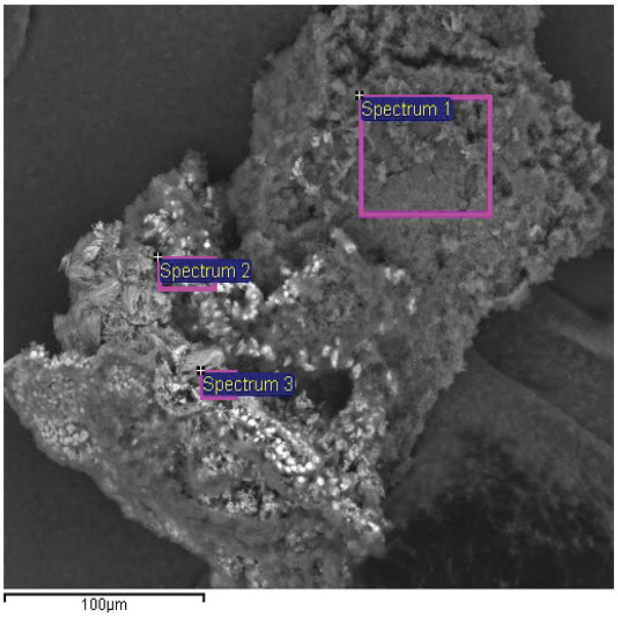
Figure 6-10(a) shows the top view of the primary and secondary reactor just before harvesting when it was fed synthetic AMD and lactate at a 3-day HRT. As can be seen from (a) an opaque, rigid FSB formed in both the primary and secondary reactor. This indicated partial oxidation of sulphide to elemental sulphur which was incorporated into the floating biofilm forming a mature FSB. However, in Figure 6-10(b) there was little to no sulphur biofilm visible in both the primary and secondary reactor after more than four 3-day HRTs had passed. This observation tallies with the decrease in sulphide oxidation during the 80 AMD: 20 Synthetic run. A closer look at (b) shows that there was a lustrous, transparent coat on top of the liquid phase in this run. This barrier seemed sufficient to prevent oxygen ingress into the liquid phase, thus, promoting efficient sulphate reduction and preventing complete sulphide re-oxidation to sulphate. It is a possibility that the film layer impeded oxygen ingress to the extent of preventing the microaerophilic zone necessary for partial oxidation of sulphide to elemental sulphur at the surface of the bulk volume.

If interrogating only the 80 AMD: 20 Synthetic run, an alternate explanation for the poor formation of the FSB would be the introduction of the pretreated AMD. It could be suggested that the AMD contains SOB inhibitors preventing elemental sulphur being produced, or the SOB may be more fastidious with respect to nutrient requirements, no longer supplied by Postgate media. However, at the 100% AMD run with lactate, the top view of the LFCR system shown in Figure 6-10(c) showed an FSB that had formed. The FSB had not yet reached maturity at the time of harvest as it was not as brittle as that in Figure 6-10(a) and was still somewhat “sticky” when harvested.

Thus, it was theorised that there were compounds in the synthetic AMD interacting with a substance in the pretreated AMD which were forming the thin transparent film that was in some way impeding partial sulphide oxidation. This would explain why an FSB formed at the 100% AMD run but had not yet reached maturity. As can be seen from Figure 6-10(c), the FSB in the first compartment of the primary reactor is more translucent than in the second compartment. As this image was taken just before the harvest time of the first 100% AMD run, some of the substances from the synthetic AMD that may be interacting with the real AMD may have been left over from the 80 AMD: 20 Synthetic run and carried over to the first 100% AMD run. These substances from the synthetic AMD may then have been mostly used up in the first compartment to form the transparent film

but some formation of the FSB was seen here, and a fuller FSB from the second compartment onwards was allowed to form.

A sample from the primary reactor with both the transparent film and the pale-yellow opaque film (FSB) was taken for observation using scanning electron microscopy (SEM) and SEM pictures were taken together with elemental analysis. Figure 6-11 shows the SEM image taken of this sample and the table next to the image shows the elemental analysis done on different spectrums of the image and the figures are an elemental percentage.



Spectrum	C	N	O	Mg	S	Ca
Spectrum 1	58.36		33.28		0.7	4.26
Spectrum 2	50.68	14.8	7.93		26.24	0.36
Spectrum 3	40.99		31.56	0.31	11.68	15.45

Figure 6-11: SEM image of a biofilm sample from the primary reactor of the 100% AMD run containing both the thin, transparent film and the opaque FSB together with a table showing the elemental analysis of the sample. Figures in the table are elemental percentages.

The highest amount of sulphur was found in spectrum 2 which, as can be seen from the image, has a high concentration of bright spheres or circles. Spectrum 2 also has a high concentration of carbon which could be due to the microorganisms also contained within the FSB and which mainly consist of carbon, hydrogen, nitrogen, and oxygen. All spectrums contained some calcium, with spectrum 3 having the highest calcium concentration. From previous studies which involved the use of synthetic AMD, there was no calcium found when elemental analysis was conducted on the FSB (Fernandes, 2020). The presence of calcium in the real AMD biofilm can be attributed to both the calcium found in the AMD (Table 6-1) and calcium from the lime treatment process. Thus, it was concluded that the transparent, thin biofilm may be a calcium complex containing a compound found in the synthetic AMD, Postgate media B from the observations made in Figure 6-10. The presence of a 0.31% magnesium could also indicate that magnesium may have played a part in the formation of the calcium complex.

Spectrum 1 consists of very little sulphur and no nitrogen but is mostly dominated by carbon and oxygen with a bit of sulphur. The presence of an 11.68 element% of sulphur in spectrum 3 is surprising as an assumption was made that the thin film did not contain any sulphur as it was transparent; sulphur is expected for form an opaque biofilm with a pale-yellow colour (Fernandes, 2020; Marais, 2020).

Figure 6-12 shows a magnified view of spectrum 3 in Figure 6-11 which seems to contain crystals. A more magnified view of spectrum 3 shows a combination of crystals that have a petal like shape shown as (a) and bright sphere-like clusters shown as (b) on Figure 6-12. The structure seen at the sections labelled (b) closely resembles the bright spheres in Figure 6-11 (spectrum 2) which were assumed to be sulphur. Thus, the petal shaped crystals could be the calcium complex. This would explain why spectrum 3 in Figure 6-11 has significant concentrations of both sulphur and calcium, 11.68 element% and 15.45 element% respectively as it contains a mixture of both the calcium complex and the sulphur spheres.

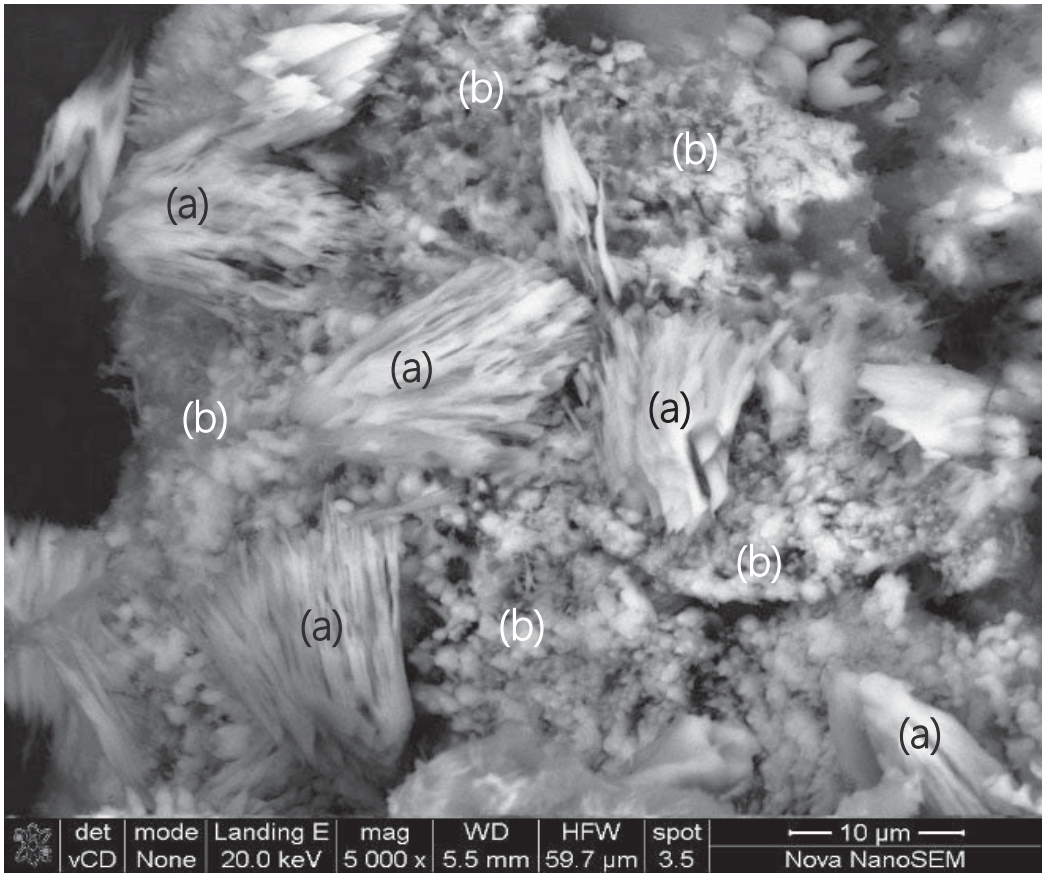
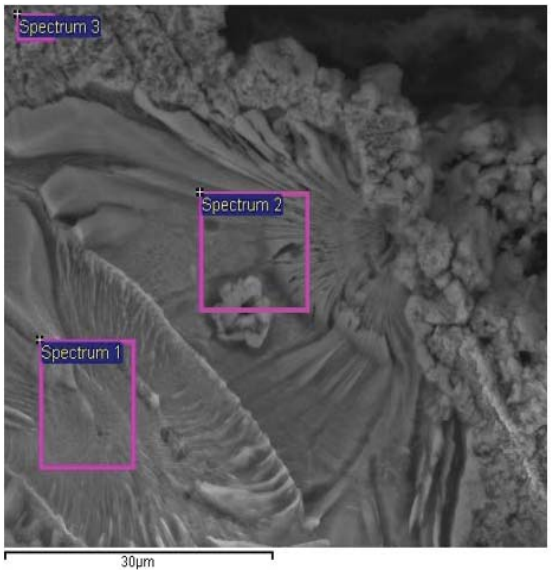


Figure 6-12: Magnified SEM image of spectrum 2 from Figure 6-11 with a composition of (a) crystals and (b) bright spherical clusters

The presence of both sulphur and calcium could also indicate that the two types of film, the FSB known to be pale yellow and the transparent, thin film, could have mixed together which would explain why the crystal petals are spaced out and not aggregated together. Figure 6-13 shows what was assumed to be an intact transparent, thin film and the elemental analysis of it is shown in a table.



Spectrum	C	O	Mg	S	Ca
Spectrum 1	24.21	59.17	0.95	0.13	15.55
Spectrum 2	20.92	51.47	0.47	0.24	26.9
Spectrum 3	19.92	49.86	0.26	0.36	29.59

Figure 6-13: SEM image of intact section of thin, transparent film

There are no bright spheres seen in Figure 6-13 and as can be seen from the table with the elemental analysis results, the sulphur present was the lowest element% (<0.4 element%) for all spectrums. Magnesium was present at all spectrums which further supported the assumption that the calcium complex also contained small concentrations of magnesium and again the high concentrations of carbon and oxygen could have been due to the presence of microorganisms in the film. Spectrum 3 in Figure 6-13 has the highest element% of calcium at 29.6 element%.

Thus, it was concluded that the transparent, thin film was a calcium complex which contained magnesium and substances from trace Postgate B media that was in the form of crystals. It was assumed that the presence of crystals would explain why the thin film had a lustre (Figure 6-10(b)) and the FSB which has more sulphur is opaque and dull due to it having clustered sulphur-containing spheres. The lustrous, thin film could have also been much better at impeding oxygen than the FSB because the crystal structure from Figure 6-13 seemed to be uniform and flat with little to no breaks or pores compared to the clustered spheres and thus, the thin, transparent film may have impeded more oxygen from entering the liquid phase. The SEM image in Figure 6-11, showing a piece which was partly FSB, shows the structure of the biofilm being irregular and textured which may allow for more breaks or pores to be present which could result in more oxygen ingress into the liquid phase.

The presence of calcium may have allowed for more stable biofilms to form as cations can from crosslinks and bridges with the EPS as reported by Körstgens *et al.* (2001) and could explain the higher sulphate conversion in the real AMD run. Fernandes (2020) reports that magnesium affects the conversion of sulphide to elemental sulphur, reporting that lower concentrations of magnesium increased the conversion of sulphide to elemental sulphur. Since the pretreatment method used for the AMD used hydrated lime containing magnesium, the increase in concentration of magnesium could have decreased the conversion of sulphide to sulphur.

SRB have also been known to take part in microbially-induced calcium carbonate precipitation (MICP) and so the crystals containing calcium could be calcium carbonate crystals (Wang *et al.,* 2023). MICP crystals have also been described to form flower, petal-like structures which could explain the petal-like crystals containing calcium from Dikshit *et al.* (2020). The petal shaped crystals in Figure 6-12 also look like they could be a cluster of thin crystal needles forming a petal shape, suggesting that these could be calcium phosphate crystals known to form thin needle shaped crystals (Lin *et al.,* 2014). Since there is phosphate in one of the residual Postgate B media compounds, K_2HPO4, this could be interacting with the calcium in the pretreated AMD resulting in the formation of some calcium phosphate crystals.

In conclusion, the introduction of real, pretreated AMD with lactate as a carbon source into the LFCR sequence system resulted in higher sulphate conversion to sulphide but there was a decrease in sulphide conversion to elemental sulphur by SOB. This gave the benefit of less re-oxidation occurring in the secondary reactor which

was an issue observed in the synthetic AMD and lactate experiment (Section 4.3.2). The decrease in the conversion of sulphide was assumed to be caused by one or a combination of factors such as the increased magnesium concentration from the hydrated lime in the pretreatment step, MICP and/or calcium phosphate crystal formation. The emergence of the new transparent film has been assumed to be linked to the high calcium concentration in the pretreated AMD forming a calcium complex crystal. Formation of this crystal is postulated to depend on a substance found in Postgate B media.

LFCR studies with partially treated AMD and molasses as the feed

The above study on the treatment of actual AMD in the LFCR system used lactate as a substrate. This is not a cost-effective substrate for the large scale and long-term treatment of AMD in the LFCR system. Thus, a second experiment on the LFCR system used pretreated AMD and molasses, the substrate chosen from the substrate batch reactor tests and a byproduct of the sugar, as the carbon source which could be a cheaper, effective alternative.

The feed to the LFCR system was switched to contain molasses as the substrate instead of lactate and the same pretreated AMD from the CeBER greenhouse was used as the sulphate source. Unlike lactate which only contains carbon, hydrogen and oxygen and does not degrade or react at high temperatures (autoclave holding temperature of 121°C), molasses contains carbon and nitrogen compounds which when exposed to high temperatures can cause the carbon and nitrogen compounds to react together in the Maillard Reaction with usable sugars being lost (Van Boekel, 2001). To avoid losing the usable sugars in molasses, a concentrated 500g/L solution of molasses was pasteurised at 106°C for 30 mins separately before it was aseptically added to sterilised AMD to meet the desired COD:SO_4 ratio of 1. The mixed feed was then fed to the LFCR system.

The reactor was first given 10 days (3.3 residence times) for adaption to the new carbon source and to allow for the whole reactor volume to be replaced with the new feed containing molasses. The sulphate concentration profiles for the LFCR system with a molasses and real, pretreated AMD feed is shown in Figure 6-14.

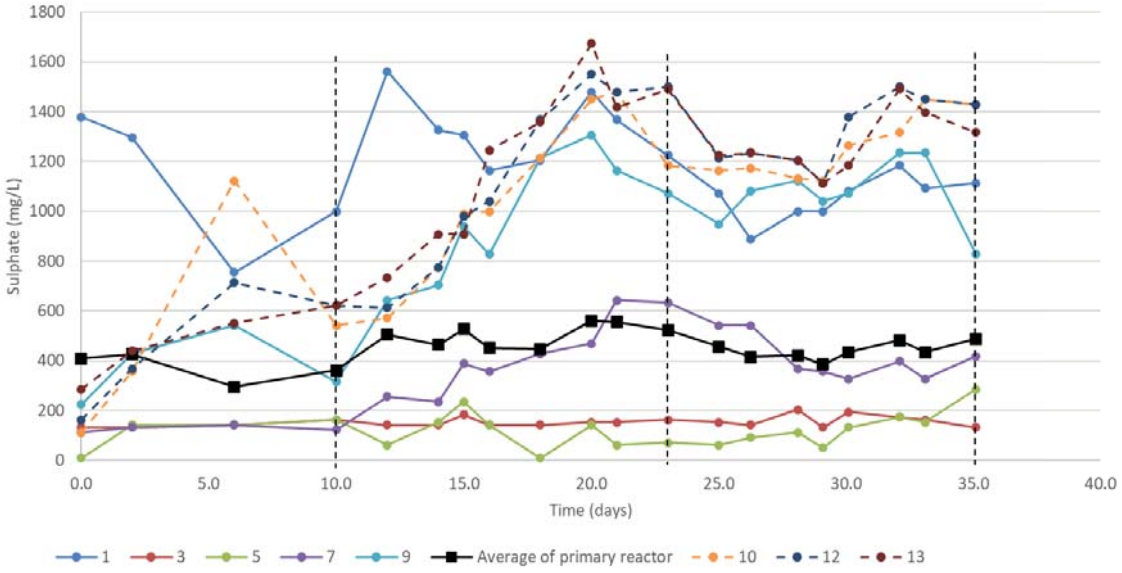


Figure 6-14: Sulphate time trends for 8 L LFCR system fed with pretreated AMD and molasses.

The average sulphate concentration in the feed was calculated to be 1720 mg/L and all runs for the alternative substrate study were conducted at a 3-day HRT with the biofilm harvested after 4 residence times, similar to the real, pretreated AMD and lactate study. Unlike the study where the LFCR system was fed with real, pretreated AMD and lactate which had high sulphate reduction at the first sample point of the primary reactor, the molasses fed LFCR run had a high sulphate concentration at sample point 1 of the primary reactor. Thus, there was low sulphate reduction occurring at the feed entry side of the primary reactor of the pretreated AMD study with molasses as a substrate. This could have been due to molasses being a more complex substrate when compared to lactate; molasses contains a mixture of sugars which are mostly sucrose, glucose and fructose (Palmonari *et*

al., 2020). Thus, the delay in sulphate reduction could have been from the need for breakdown of the complex substrate by fermenters and acidogens in the mixed culture, to give VFAs as an electron donor for the SRB.

Sample points 3, 5 and 7 had low sulphate concentrations, all below 800 mg/L. However, there was re-oxidation of sulphate occurring at the effluent port, sample point 9, of the primary reactor with some sulphate concentrations going above 1200 mg/L in the first cycle after the 10 days of adaptation. The high sulphate concentration from the primary reactor effluent port was carried over to the secondary reactor for both the first and second cycle after adaptation, where more sulphide was re-oxidised to sulphate resulting in high sulphate concentrations in the secondary reactor for both the first and second cycle. This was very different to the real, pretreated AMD and lactate study at a 100% AMD feed which maintained low sulphate concentrations in both the primary and secondary reactor (Figure 6-6). The lower sulphide re-oxidation in the secondary reactor during adaptation was thought to be due to the presence of residual lactate from the previous. Another reason for an increase in sulphide re-oxidation could have been the introduction of an inhibitory compound from the molasses substrate which is complex and contains other non-carbon substances (Palmonari *et al.,* 2020).

The average sulphate reduction in the primary and secondary reactor across the two runs after the adaptation was calculated to be 71.6% and -25.1% respectively. A negative sulphate reduction means that there was re-oxidation occurring in the secondary reactor. Even though the sulphate reduction across the two runs for the LFCR system study with real, pretreated AMD and molasses is lower than the average sulphate reduction of 87.4% for the real, pretreated AMD and lactate, it was comparable to the sulphate reduction of 71.4% achieved when the LFCR system was fed with synthetic AMD and lactate (Table 4-2). Thus, molasses, though complex, is suitable for the treatment of sulphate rich wastewaters but resulted in poor elemental sulphur recovery.

Figure 6-15 shows the sulphide concentration profiles of the LFCR system running on real, pretreated AMD and molasses as a carbon source. The sulphide time trends complement the sulphate time trends with a low sulphide concentration at sample point 1 confirming that there was low sulphate reduction at the feed entry side of the primary reactor. Low sulphide concentrations at the primary reactor effluent, sample point 9, and the secondary reactor sample points were due to the re-oxidation of sulphide. Sample points 3, 5 and 7 had a high sulphide concentration showing that there was significant sulphate reduction in the primary reactor. Both the sulphate and sulphide time trends showed a pseudo-steady state was reached when looking at the average of the primary reactor sulphide and sulphate concentration time trends. The highest sulphide concentration achieved in the two runs using molasses as a carbon substrate was 346 mg/L (10.5 mM) and the average sulphide concentration across the two runs, when they had reached pseudo steady state was calculated to be 253 mg/L (7.7 mM).

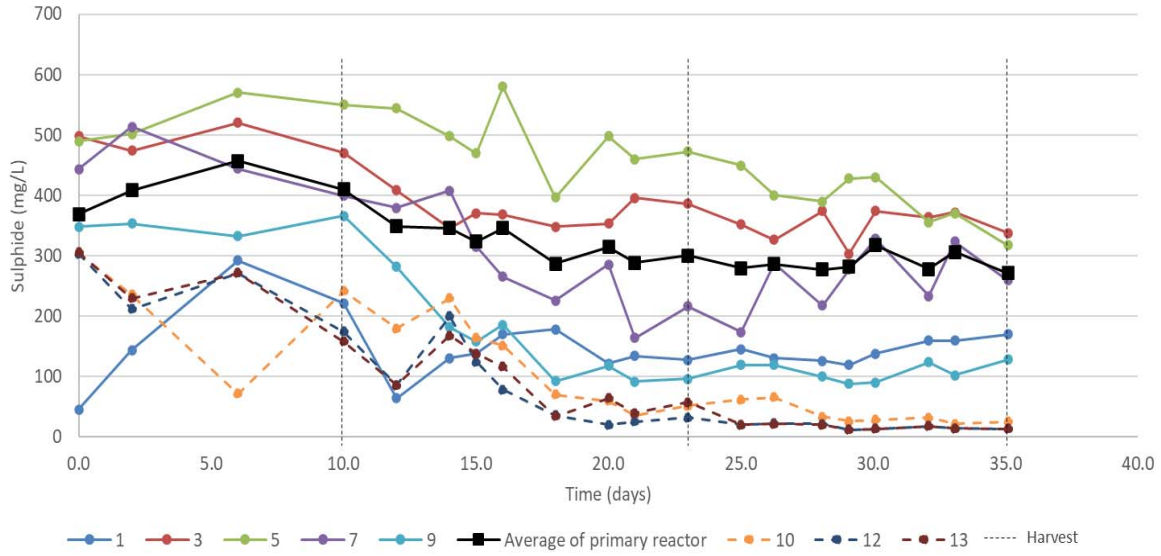


Figure 6-15: Sulphide time trends for 8 L LFCR system fed with pretreated AMD and molasses.

As time progressed in the first run of the LFCR system running on molasses after 10 days of adaptation in the secondary reactor, the sulphide concentration decreased to be below 50 mg/L and continued to decrease in the second run to lower than 15 mg/L. Decrease in the sulphide concentration in the secondary reactor over time showed that the liquid phase of the secondary reactor may have become oxidising and the pH may be out of the ideal range for optimum function of SRB, thus, resulting in almost complete re-oxidation of all the sulphide that entered the secondary reactor from the primary reactor.

Figure 6-16 shows the redox time trends in the primary and secondary reactor. As can be seen in Figure 6-16, the redox potential in the primary reactor, R1, was between -300 mV and -200 mV. However, the redox potential of the secondary reactor was increasing with time which was making the environment less reducing which could be the cause of the re-oxidation in the secondary reactor.

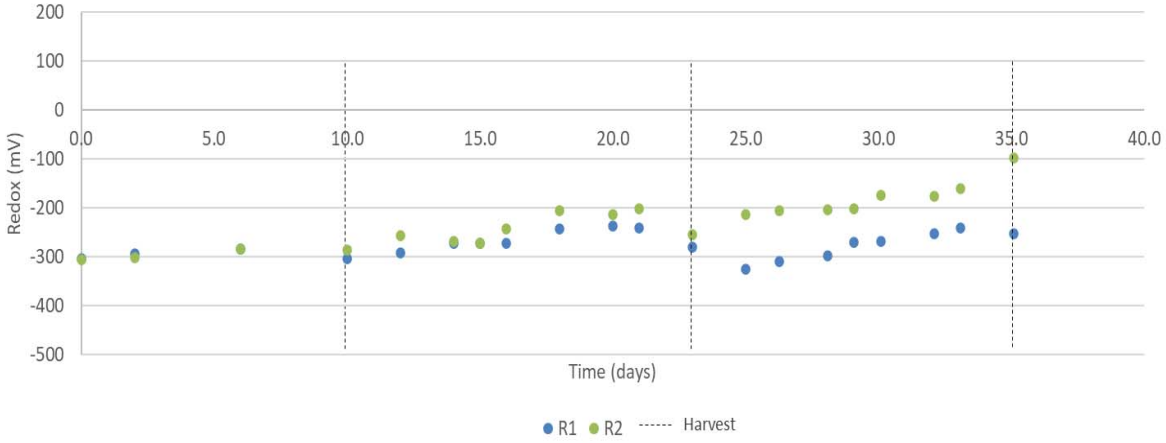


Figure 6-16: LFCR system fed with pretreated AMD with a molasses substrate - redox time trends

The average pH in the primary reactor ranged between 6.50 and 7.50 while the secondary reactor had a pH range between 7.00 and 8.00 (Figure 6-17). Both reactors have pH ranges that are ideal for SRB activity. However, as seen from the sulphate and sulphide time trends, there is high sulphide re-oxidation in the secondary reactor to sulphate.

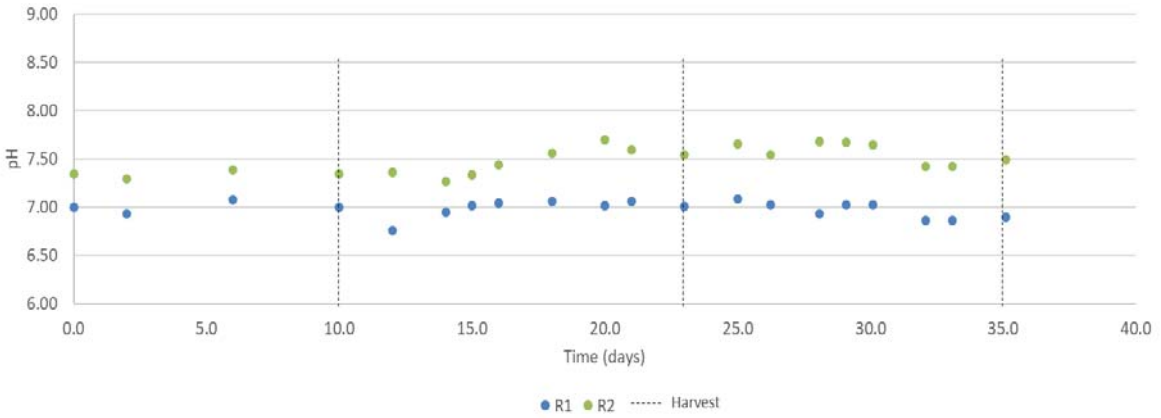


Figure 6-17: LFCR system fed with pretreated AMD with a molasses substrate - pH time trends

The FSB forming in the secondary reactor may not have impeded oxygen well enough to prevent sulphide re-oxidation. Figure 6-18 shows the FSB formed in (a), the first run and, (b) the second run in both the primary and secondary reactors. It can be seen that an FSB was forming in both runs when the LFCR was fed with real, pretreated AMD and molasses as a substrate. However, in the secondary reactor the FSB formation is not consistent across top of the reactor, and some areas on the liquid surface were not covered by the FSB.

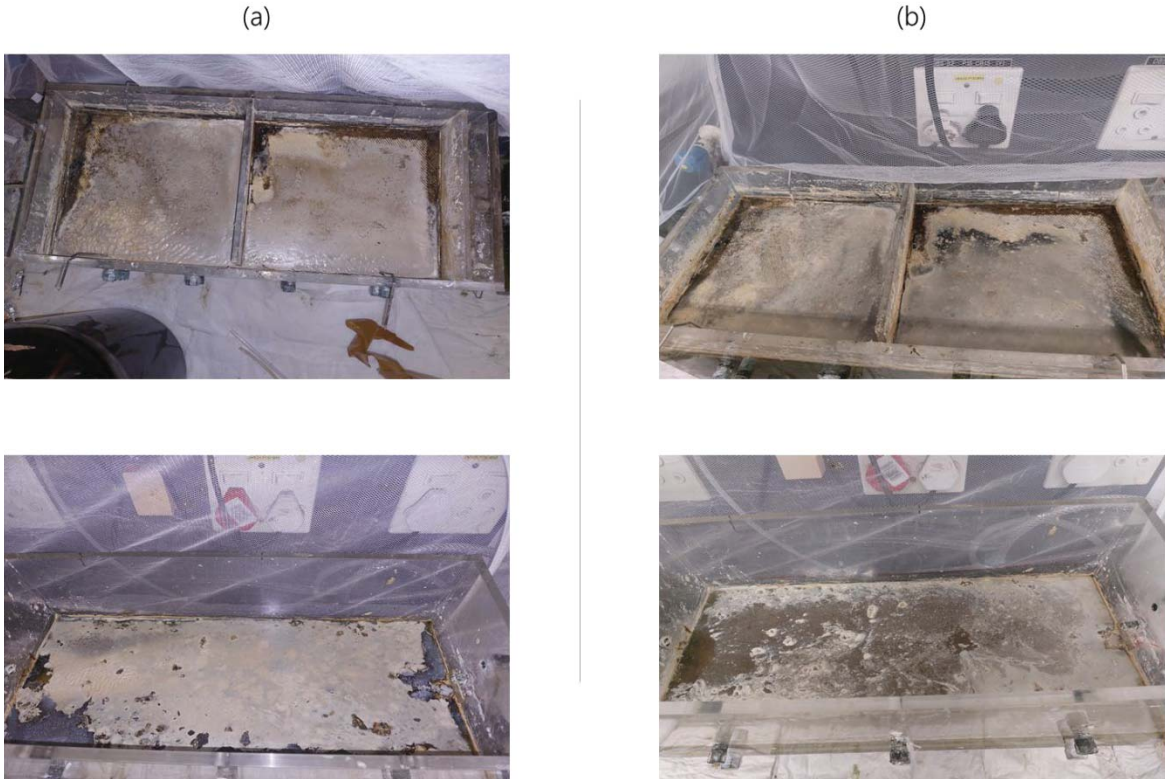


Figure 6-18: Top view of the primary (top) and secondary (bottom) reactor running on pretreated AMD and molasses, showing (a) the first run FSB formation (b) the second run FSB formation.

It was assumed that the amount of usable COD in molasses after pasteurisation may have decreased and so the COD:SO4 ratio of 1 was not met. This would have resulted in a lower concentration of usable COD for the SRB and SOB which may have mostly been used up in the primary reactor and less would have been available to the secondary reactor causing poor FSB formation in the secondary reactor. Heterotrophic bacteria are necessary for initial stages of FSB formation (Molwantwa, 2007) and in conditions where the carbon source is limited there

is poor biofilm formation. This is what could be occurring in the secondary reactor; the usable COD in molasses is low and may be causing poor biofilm formation in the secondary reactor. Without the initial sticky phase occurring, SOB cannot be incorporated which would result in less elemental sulphur being deposited into the FSB.

To test whether there was a carbon limitation in the system, two approximately 50 ml volumes of bulk liquid from the secondary reactor in areas where there was no biofilm formation, was taken and placed in glass sample bottles. Molasses was added to one of the bottles to supply extra carbon source. After a week, with the bottles partially closed and left at ambient conditions, the bottles were observed for any changes. Figure 6-19 shows an image of the sample bottles after a week.

Figure 6-19: Testing for FSB formation when additional carbon source is added to a seemingly carbon depleted SRB system which cannot form an FSB: (a) full view and (b) close-up view

One can see from the image that an FSB was beginning to form in the sample bottle that had molasses added to it while the surface of the sample botte without any carbon source added to it did not show any signs of a FSB forming. This observation could suggest that the poor formation of the FSB in the secondary reactor for the study running on real, pretreated AMD and molasses could be due to carbon source limitation which is adversely affecting the heterotrophic SOB in the LFCR system.

6.4 Conclusion

Introduction of the pretreated AMD to the LFCR system while using lactate as a substrate resulted in a high sulphate reduction in the primary reactor of 87.4% which is higher than the sulphate reduction of 71.4% obtained when the LFCR system was fed with synthetic AMD made of Postgate media B and lactate as a carbon source. Pretreatment of the AMD was necessary as the low pH of the AMD was not conducive for SRB growth and function, as observed in the AMD batch reactor studies where different treatment methods were explored. It was also necessary for the AMD to be sterilised due to the presence of native bacteria which competed with the SRB, and in turn reduced sulphate reduction as shown in the AMD batch reactor studies. Thus, pretreated AMD did not compromise the performance of the reactor even when lacking the additional nutrients provided by the Postgate media B as seen in the 100% AMD run of the LFCR system on pretreated AMD and lactate. In fact, a higher sulphate conversion was achieved when a 100% pretreated AMD and lactate feed was used. The higher conversion was attributed to the presence of low concentrations of metals which have been reported to positively influence the activity of SRB. However, the sulphur recovery in the pretreated AMD with lactate as a carbon source was lower when compared to the synthetic AMD with lactate as a carbon source at a 3-day HRT. This is an interesting observation as low sulphate concentrations were maintained in both the primary and secondary reactor of the pretreated AMD and lactate study. Further investigations revealed the formation of a calcium crystal complex that formed a thin film on the surface of the primary and secondary reactor of the pretreated AMD and lactate study. It was suggested that this thin transparent film impeded oxygen from entering the liquid phase better than the FSB formed during the synthetic AMD and lactate study. This would explain how the low sulphate concentrations were maintained. It was theorised that the formation of the calcium crystal complex was dependent on a substance from the Postgate B media, introduced in previous runs before the 100% AMD run, and the high calcium concentration from the AMD due to the lime treatment step.

Substitution of lactate with a more cost-effective substrate, molasses, a by-product of the sugar industry, in the hybrid LFCR system resulted in a lower sulphate reduction when compared to the pretreated AMD and lactate study. This could be due to the complex nature of molasses potentially requiring some breakdown for SRB use. An average sulphate reduction of 71.6% was achieved for the primary reactor. This reduction though lower than the pretreated AMD and lactate study, is comparable to the average sulphate reduction in the primary reactor for the synthetic AMD and lactate 3-day runs at 71.4%. However, FSB formation was poor when molasses was used as the substrate for the treatment of AMD in the LFCR system and this could have been due to a limited carbon source which affected the function of the heterotrophic microbes in the FSB.

In summary, the introduction of partially treated AMD into the LFCR system did not compromise its efficiency, even when lacking the additional nutrients present in the SRB-specific feed. Furthermore, utilising a complex waste stream as a substrate maintained a high sulphate reduction in the primary reactor. These findings demonstrate the LFCR system's effectiveness in treating circum-neutral, sulphate-laden mining impacted waters cost effectively, with however, some compromise in elemental sulphur recovery.

7 Conclusions

Biological sulphate reduction followed by partial sulphide oxidation has potential to yield upgraded and fit-for-purpose water as well as an elemental sulphur product with potential application as a fertiliser from acid rock drainage or acid mine drainage streams. The technology has potential value to treat low volume AMD streams emanating from sulphidic mine waste and disturbed lands. While active technologies available are not cost effective for these applications, passive technologies have tended to lack predictability. In this study, we explore further semi-passive technology through use of the linear flow channel reactor (LFCR) for interlinked BSR and partial sulphide oxidation.

Three factors limiting its application are the identification of a suitable carbon source and electron donor that is cheap and readily available, optimising reaction kinetics and introducing appropriate sulphide handling.

To address the first challenge of carbon source, we compared BSR in batch reactors, using the preferred lactate carbon source as reference and comparing it to the defined carbon sources acetate and ethanol, and the complex carbon sources algal lysate, honey and molasses. Molasses was selected as the carbon source with best potential.

On addressing reaction kinetics of BSR, the LFCR was modified to introduce baffles with the aim of introducing a plug flow and zoning of the reactor into regions to allow stratified microbial adaptation and maximise performance of each stage. The reactor was additionally packed with polyurethane foam for cell retention, allowing the whole reactor to be used. Hydrodynamic studies showed channelling in the baffled reactor in the absence of packing with surface zones bypassed. However, on introducing the packing, plug flow was observed, as desired.

Operation of the packed, baffled reactor with lactate as carbon source and electron donor and synthetic AMD demonstrated improvements over the conventional LFCR with carbon microfibrils. A 20% average improvement in conversion efficiency of sulphate was observed. By adding a second LFCR without baffles or packing, sulphide conversion was optimised in the secondary reactor. At 1 g/L sulphate feed, best performance was attained at a 4-day HRT, yielding 91% sulphate conversion, 81% sulphide removal and 55% sulphur recovery. While lower sulphate conversions were found when feeding 2 g/L sulphate (66 to 72%), this represented a larger amount of sulphate reacted: 12.7 to 14.9 mmol/L on feeding 2 g/L sulphate and 8.2 to 9.2 mmol/L on feeding 1 g/L sulphate. At a 1 g/L feed, VSRR ranged from 0.079 mmol/L.h at a 5-day HRT to 0.169 mmol/L.h at a 2-day HRT. At a 2 g/L sulphate feed, these increased to 0.120 mmol/L.h at a 5-day HRT and 0.286 mmol/L.h at a 2-day HRT. On comparing performance at the same loading (1 g/L and 2-day HRT vs 2 g/L and 4-day HRT) similar VSRRs of 0.169 and 0.148 mmol/L.h were obtained. These performances surpass those of the unbaffled LFCR with microfibrils.

The packed, baffled reactor was then operated at a 3-day HRT with lactate as carbon source and electron donor and AMD generated in a pilot study of coal discards. This highly acidic AMD was pre-treated with lime to enable BSR. Good BSR was obtained with a sulphate conversion efficiency of 87.4% and VSRRs in the range 0.235 to 0.257 mmol/L.h were seen as the real AMD was phased in and the Postgate nutrients removed, and of 0.216 mmol/L.h when treating pre-treated AMD in the presence of only lactate (no nutrients). Under these conditions, the FSB formation was limited. A calcium-based glassy biofilm replaced this. It is expected that the calcium surface film precluded oxygen transfer, preventing both partial sulphide oxidation and complete sulphide oxidation. Further characterisation of the surface film is required to control its formation. Additionally, further analysis of the compromise of formation of the FSB is required through understanding factors curtailing partial sulphide biooxidation.

On replacing the lactate substrate with molasses for treatment of pre-treated AMD in the packed baffled LFCR, satisfactory BSR was obtained, with 71.6% sulphate conversion achieved. Under similar conditions and processing synthetic AMD with lactate as substrate, an equivalent 71.4% sulphate conversion was achieved at the 3-day HRT. However, again partial oxidation of sulphide to form the FSB was compromised. In this case, the calcium film was not dominant, yet the robust FSB did not occur to control oxygen availability and limit oxidation to partial oxidation. Possible factors that may influence the SOB include the potential presence of inhibitory compounds with respect to the SOB in either the AMD stream or the molasses stream, fastidious nutrient

requirements that are not met on removal of Postgate media components, insufficient supply of carbon sources used by the SOB or inadvertent washout of the SOB. These factors require further investigation.

In conclusion, this study has shown the promise of a baffled flow channel reactor, packed with polyurethane foam or similar porous biomass entrapment matrix, to provide a well-functioning and robust system for treatment of real AMD by biological sulphate reduction using molasses as a carbon source and electron donor. The need for prior neutralisation of the AMD was shown. While done with lime here, potential exists to use bicarbonate generating biosystems to achieve this. While the sulphide conversion to sulphur and its recovery as elemental sulphur has been demonstrated with synthetic AMD, further analysis of constraints on SOBs found on treating real AMD and removing nutrient addition require further analysis to achieve a robust FSB production system. Further it is expected that the packed baffled flow channel reactor can handle a higher sulphate loading than demonstrated here, especially if the design of the reactor outlet is re-thought to avoid sulphide re-oxidation observed. It is envisaged that the introduction of baffles and packing of the full bulk volume of the reactor will facilitate further scale up, proposed as a next step.

8 Dissemination of knowledge

The current work is expected to generate new insight into the development of the integrated process and its potential application in the remediation of AMD. The results from the project will be disseminated across several platforms that includes presentations at conferences and publications within peer-reviewed journals. The ideal audience are stakeholders within the mining industry and the scientific community interested in the application of semi-passive BSR technologies,

The research will lead to several publications targeting high-impact journals. These are still to be prepared with one planned on the packed baffled flow channel reactor, a second on carbon source and a final on performance of the packed baffled flow channel reactor on processing real AMD using molasses.

The current work will be presented at local and international conference proceedings. A variety of conferences covering topics from water remediation to microbial processes can be targeted. Local conferences include the South African Society of Microbiology (SASM), the SAIMM Minerals Research Showcase, and Water Institute of Southern Africa (WISA) conferences while ideal international conferences could be the International Mine Water Association (IMWA) Congress, International Biohydrometallurgy Symposium (IBS) as well as Biomining and Sustainable Minerals international conferences.

In addition, the research will also be showcased on the CeBER website and shared across social media networks. It is proposed that an article for a trade journal be developed.

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