Investigation of Alternative Carbon Sources for the Biological Treatment of Synthetic Sulphate-laden Water and Mine Impacted Water in a Linear Flow Channel Reactor

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Abstract

South Africa grapples with the generation of acid mine drainage (AMD), which adversely affects surface and ground water quality. Existing treatment methods typically treat the acid and heavy metal components of AMD but often fail to meet sulphate removal standards, necessitating additional polishing steps that add to expense. They also carry drawbacks such as high operational costs and metal sludge generation for active treatment and reduced process control and the need for large land areas for installation for passive treatment. These treatment methods are not cost-efficient when treating low-volume, circum-neutral wastewater. Biological sulphate reduction (BSR) offers a sustainable alternative for sulphate removal and is applicable to partially treated AMD as well as circum-neutral, mine-impacted water. Coupled with partial sulphide oxidation, it also has the potential to convert sulphate to elemental sulphur, touching on waste valorisation as sulphur is a value-added product. However, BSR systems require supplementation with organic carbon and are characterised as slow, while partial oxidation of sulphide is difficult to control in many reactor systems; these are key drawbacks in the economic feasibility of these processes.

A semi-passive linear flow channel reactor (LFCR) which simultaneously reduces sulphate to sulphide using sulphate reducing bacteria (SRB) and partially oxidises the sulphide formed to elemental sulphur using sulphur oxidising bacteria (SOB) within a floating sulphur biofilm, was developed at the Centre for Bioprocess Engineering Research at the University of Cape Town, South Africa. It may be operated as a one- or two-stage reactor system, with the second stage providing additional surface area for partial sulphide oxidation. During its development, carbon fibres were added to enable biomass retention and thereby enhance reaction rates. For this study, the primary reactor was further modified to include baffles for improved directional flow and enhanced contacting and the carbon microfibre biomass support was replaced with polyurethane foam (PUF) previously shown to effect efficient biomass retention. A secondary reactor was included to provide 30% of operating volumes with no baffles; it was connected in series to increase surface area for sulphur recovery. The baffled hybrid linear flow channel reactor (BaH-LFCR) was supplied with an organic substrate, lactate, and was tested for its treatment of a synthetic sulphate laden feed. Lactate has been shown to be highly effective carbon source and electron donor for sulphate reduction but is expensive and not available at sufficient scale or low enough cost to be a cost-effective option at an industrial scale. The synthetic feed, unlike AMD from the field, was nutrient-rich with a stable, neutral pH that promoted SRB function.

This study investigated the selection and use of an alternative, cheap and readily available carbon source and electron donor for BSR as well as the treatment of AMD from the field in the BaH-LFCR. Alternative carbon sources investigated included molasses, acetate, honey and algal biomass; each have a high chemical oxygen demand making them potentially suitable organic carbon sources for BSR. Honey and algal biomass can be produced on-site enhancing availability and negating transportation costs. As a byproduct of the sugar industry, the equivalent COD as molasses costs less than 0.1% of that of lactate. Acetate is a byproduct of most fermentation processes making it readily available.

Field AMD presents several challenges for BSR due to its acidic nature, lack of nutrients, and potential toxins. To address these challenges, the AMD was characterised and pre-treated to increase the pH before introduction into the BSR system. Use of an alternative substrate and real AMD from the field demonstrates the ability of the LFCR to achieve real-world application and implementation.

Three small-scale reactor configurations were tested with lactate and sulphate-laden feed: a 1 L fed-batch Schott bottle, a 93 mL continuous mini column, and a 1 L continuous Schott bottle. Continuous reactors performed poorly due to oxygen ingress, achieving only 43.4% sulphate conversion in the Schott bottle and no conversion after 23 days in the mini column. The fed-batch reactor demonstrated better stability with 78.1% conversion. Oxygen ingress impact was found to be inversely proportional to reactor size. Based on superior stability and conversion efficiency, the fed-batch reactor was selected for carbon source testing.

The four alternative substrates were tested against lactate, as base case, in the fed-batch reactor. Molasses showed the highest performance among the alternative carbon sources, achieving 82.7%

sulphate conversion and producing the highest sulphide concentration. In contrast, honey and algal lysate performed poorly, with average sulphate conversions of 7.4% and 14.1% respectively. The poor performance of honey was attributed to its antimicrobial properties and acidic nature. Poor performance of algal lysate likely resulted from its composition of predominantly unusable COD which would require fermentation to produce more accessible compounds. Acetate had an average conversion of 41.4% which was approximately half the conversion achieved using molasses as a carbon source. VFA analysis revealed that molasses was the only substrate where all measurable sugars and VFAs were consumed by the end of each cycle, as indicated by HPLC analysis. Additionally, molasses contained fermenting microorganisms that converted sugars into small concentrations of lactate, enhancing sulphate reduction. These fermenters were introduced into the BSR system along with the molasses substrate.

Before introducing AMD from the field to the BaH-LFCR, various AMD pretreatment methods were evaluated using lactate-fed batch reactors. Four AMD conditions were tested: untreated AMD, lime-treated AMD, lime and sulphate-treated AMD, and lime-treated sterilised AMD. The untreated AMD batch showed the lowest sulphate conversion (9%) due to its acidic pH, which inhibited SRB activity. The highest conversion of 89% was achieved with lime-treated, sterilised AMD. Sterilisation eliminated competition for the carbon source between native microorganisms present in AMD and the SRB, resulting in enhanced sulphate conversion.

Three experiments were conducted in the BaH-LFCR system to evaluate its performance using different combinations of carbon sources (lactate vs. molasses) and feed solutions (synthetic Postgate media vs. pre-treated AMD from the field). The first experiment established a base case using lactate and sulphate-rich synthetic feed to determine the optimal hydraulic residence time (HRT). At the optimal 3-day HRT, this base case achieved 64.8% sulphate conversion and the highest volumetric sulphate reduction rate (VSRR) of 0.187 mmol/L.h in the primary reactor, nearly two-fold that achieved previously in the LFCR. The second experiment, using lactate with partially treated AMD, achieved the highest sulphate conversion of 87.4% and the second-highest VSRR (0.216 mmol/L.h) in the primary reactor.

It had 41.6% of the sulphur entering the system through the feed converted to sulphur. Sulphur formation was observed to decline, likely due to the development of a thin, impervious surface film hypothesised to consist of calcium crystal complexes. This film may have impaired oxygen diffusion at the air-liquid interface more severely than the typical floating sulphur biofilm (FSB), thereby reducing the efficiency of sulphide oxidation to elemental sulphur. The third experiment, combining molasses with partially treated AMD, achieved second highest sulphate conversion of 85.6% in the primary reactor. Overall sulphate conversion however dropped to 27.2% due to extensive re-oxidation in the secondary reactor. This re-oxidation linked to poor FSB formation and limited carbon availability in the secondary reactor. However, in the primary reactor the molasses configuration achieved the highest proportion of expected sulphide converted to sulphur of 30.7%, with a comparable expected sulphide amount of 518 mmol. The synthetic feed + lactate experiment achieved approximately only 9.4% sulphide conversion in the primary reactor, with an expected sulphide amount of 428 mmol while the AMD + lactate experiment had the highest expected sulphide amount of 543 mmol.

In summary, the introduction of partially treated AMD into the LFCR system on a lactate carbon source not only maintained but enhanced system performance, achieving the highest sulphate conversion despite lacking the additional nutrients present in the SRB-specific feed. While using molasses as a complex waste stream carbon source achieved high sulphate conversion in the primary reactor, sulphur recovery was compromised due to re-oxidation at the primary reactor effluent port and because of limited carbon availability there was poor sulphur formation and high sulphate concentrations. The effective treatment of circum-neutral, sulphate-laden mine-impacted water using a readily available, cost-effective substrate demonstrates the system's suitability for industrial-scale deployment.

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Acronyms and Abbreviations

ALD	Anoxic lime drains
AMD	Acid mine drainage
BaH-LFCR	Baffled hybrid linear flow channel reactor
BSR	Biological sulphate reduction
CD	Coal discards
CeBER	Centre for Bioprocess Engineering Research
CHNS	Carbon-Hydrogen-Nitrogen-Sulphur
COD	Chemical oxygen demand
D	Dilution rate
RS	Residual sulphide (amount)
EPS	Extracellular polymeric substance
ES	Expected sulphide (amount)
FSB	Floating sulphur biofilm
FW	Fine waste
HPLC	High pressure liquid chromatography
HRT	Hydraulic residence time
ICP-MS	Inductively coupled plasma mass spectrometry
LFCR	Linear flow channel reactor
MICP	Microbially-induced calcium carbonate precipitation
OLC	Open limestone channels
PUF	Polyurethane foam
PUF-BSPs	Polyurethane foam biomass support particles
SEM	Scanning electron microscopy
SOB	Sulphur oxidising bacteria
SRB	Sulphate reducing bacteria
UAPBR	Up-flow anaerobic packed bed reactor
VFA	Volatile fatty acid
VSFR	Volumetric sulphur formation rate
VSLR	Volumetric sulphur loading rate
VSRR	Volumetric sulphate reduction rate
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
X	Conversion

1 Introduction

1.1 Background

Acid mine drainage (AMD) continues to be a major pollutant in South Africa due to the mining of sulphidic rock to recover a variety of metals and other natural processes exposing mineral sulphides to oxidation. The associated mining industries are either still running or have closed down with many abandoned legacy sites contributing to AMD. AMD is acidic and toxic to the environment (Akcil and Koldas, 2006). Many studies and technologies have been investigated and implemented and can fall under active treatment or passive treatment (Moosa, Nemati and Harrison, 2002; Johnson and Hallberg, 2005; Thisani, von Kallon and Byrne, 2021). These technologies face multiple drawbacks such as high operational costs and metal sludge generation in active treatment and poor process control and need for large areas to install treatment technology for passive treatments (Thisani *et al.*, 2021). Thus, there is a need for further innovation to develop AMD treatment technologies that combat the drawbacks faced by the existing technologies. Biological treatment of sulphides provides a cost effective and sustainable process to reduce sulphate concentrations in biological sulphate reduction (BSR) systems (Molwantwa, 2007; Van Hille *et al.*, 2011; Marais, 2020).

BSR systems are treatment technologies that have come to light due to their sustainability, lower sludge generation rates and lower amounts of chemical input into the system when compared to most of the physicochemical treatment processes (Ayangbenro *et al.*, 2018; Marais, 2020; Thisani *et al.*, 2021). However, biological treatment also has disadvantages that present challenges for large scale implementation of the process. The BSR system is governed by several factors such as temperature, pH, feed composition and availability of carbon source and presence of inhibitors or toxins (Marais, 2020). The three factors providing greatest challenge to implementation at scale are the conversion rates, the cost of carbon source and electron donor and the management of the sulphide formed. The BSR system generates toxic sulphides through sulphate reduction, which must be removed from the system. This can be accomplished through contained methods such as metal sulphide precipitation or through conversion to elemental sulphur products (Moosa and Harrison, 2006; Tang *et al.*, 2009; Van Hille *et al.*, 2011).

Many technologies have been investigated for the treatment of sulphides, but they have mostly been physicochemical and have disadvantages of high operational costs and need of specialty chemicals (Tang *et al.*, 2009)

An integrated biological treatment system has been proposed that simultaneously reduces sulphates and partially oxidises the resultant sulphide to elemental sulphur (Van Hille *et al.*, 2011; Marais, 2020). Sulphur is a useful product, and thus, the remediation process also incorporates waste valorisation and potential for the cyclic use of sulphur released through the mining process. This integrated, hybrid technology was developed at the Centre for Bioprocess Engineering Research (CeBER), University of Cape Town, building off the knowledge base of previous technologies and studies which included ThiopaqTM, Biosulphide®, Rhodes BioSURE® and Pulles Howard and de Lange integrated passive treatment process (IMPI) (Van Hille *et al.*, 2011; Marais, 2020). The novel technology is a hybrid linear flow channel reactor (LFCR) which reduces sulphates anaerobically in the bulk fluid using sulphate reducing bacteria (SRB) and then partially oxidises the sulphide formed aerobically at the air-liquid boundary using sulphur oxidising bacteria (SOB) to form a floating sulphur biofilm at the surface of the open reactor (Marais *et al.*, 2020). The floating sulphur biofilm limits oxygen transfer rates, thereby ensuring partial oxidation to sulphur dominates with minimal re-oxidation to sulphate.

Proof of concept of the LFCR was provided and the factors that affected the BSR system in the LFCR were optimised leading to enhanced sulphate reduction and sulphur recovery (Marais *et al.*, 2024). The system uses gravity for flow and so saves on energy costs and it contains a support matrix, initially using carbon fibres and thereafter polyurethane foam (PUF), which allows for the decoupling of hydraulic residence time (HRT) and biomass retention time (Marais *et al.*, 2024). The LFCR was

modified to include baffles that improve the flow pattern within the reactor and allow for highly efficient contact of the feed with the biomass on the support matrix (Marais *et al.*, 2024). Thus, it became the baffled hybrid linear flow channel reactor (BaH-LFCR). The system used lactate as its main carbon source of choice for the SRB together with Postgate media B and a synthetic AMD, as the feed (Postgate, 1963; Marais, 2020; Marais *et al.*, 2024).

Although the BaH-LFCR has been optimised and modified to achieve good conversion and recovery, there is still need for improvement as the system is set to operate semi-passively at an industrial scale on real-world AMD (Marais, 2020). To date the system has only been run on synthetic AMD. Further, it uses a lactate as a carbon source and electron donor, which is highly effective for sulphate reduction but is economically unviable for long-term industrial application.

Thus, there is need for the BaH-LFCR to be tested on real AMD and run on an alternative substrate that is effective, readily available and is cost efficient. Several substrates need to be investigated and compared to the already well established, lactate substrate. The substrates need to have a high sugar content and should be readily available to function effectively in reducing sulphates and recovering sulphur. Complex organic substrates: molasses, honey, and algal biomass, are potential substrates that could be highly effective in the BSR system and of lower cost compared to lactate when considering treatment at an industrial scale.

Thus, the main aims of the study are to:

- Test complex organic substances with a high sugar content for their efficiency as carbon source and electron donor in a BaH-LFCR system for sulphate reduction and partial sulphur oxidation
- Characterise and test real AMD in a BaH-LFCR system

This study will bring the BaH-LFCR process closer to its application in industry, in the real world.

1.2 Scope and constraints

The study focused on investigating the efficiency of complex organic substrates: molasses, honey, and algal biomass, with high sugar content, in the reduction of sulphate in fed-batch and continuous BSR systems. The complex organic substrates were also characterised and compared to lactate and to each other. The most effective substrate was then introduced into the BaH-LFCR for testing. Optimal conditions established in prior studies of the LFCR were maintained such as temperature, pH, sulphate loading and COD:Sulphate ratio (Marais *et al.*, 2024). The BaH-LFCR was operated across a range of decreasing HRT from a 5-day HRT to a 2-day HRT in a unit stepwise manner.

The study also focused on the characterisation and selection of a suitable pre-treatment method for the introduction of AMD into BSR systems. The AMD was first tested in fed-batch reactors before it was introduced into the BaH-LFCR.

1.3 Structure of dissertation

The dissertation begins with the introduction (Chapter 1) and the literature review (Chapter 2) which gives an insight into the formation of acid mine drainage (AMD), its effects on the environment and the different AMD treatment methods that have been established. It then dives deeper into biological treatment and its advantages over traditional non-biological passive and active treatments such as reduction of sludge production. Biological treatment of AMD is based on the sulphur cycle and looks at the reduction of sulphate to sulphide which can be partially oxidised to elemental sulphur or re-oxidised back to sulphur. During sulphate reduction, a carbon source is required to act as both a carbon source and an electron donor to sulphate which is reduced to sulphide. Conditions optimal for SRB and the factors that influence sulphate reduction by SRB such as chemical oxygen demand, hydraulic residence time, pH, temperature and the type of carbon source are reviewed. The literature review then goes into

explaining how active treatment and passive treatment do not fully treat AMD and are usually suitable for high volume wastewater generation; a polishing step or a cost-effective treatment for low volume, circum-neutral wastewater is needed. It details a semi passive biological technology that linearises the sulphur cycle, the baffled hybrid linear flow channel reactor (BaH-LFCR), which allows for sulphate reduction in the anaerobic liquid phase and sulphur formation at the air-liquid boundary. The BaH-LFCR system has traditionally used a synthetic feed and a lactate substrate and thus the chapter concludes with a motivation for the research project to test the system on AMD and a cheaper alternative substrate. Problem statements, objectives, hypotheses and key questions are provided.

Chapter 3 focuses on the materials and methods used for the study and Chapter 4 on the use of alternative substrates in BSR systems. Fed-batch reactors are used to test five different substrates: acetate, honey, molasses, algal lysate and lactate (as the base case). Each substrate fed-batch reactor goes through a full cycle before it is subcultured and the substrate that achieves the best sulphate reduction and is comparable to lactate is selected to be tested in the BaH-LFCR system. Chapter 5 looks at the treatment of AMD in the BaH-LFCR using the alternative substrate. A base case experiment where the BaH-LFCR is fed with synthetic feed and a lactate carbon source is conducted. Before AMD is introduced to the system, pre-treatment methods for the AMD are investigated in fed-batch reactors. The pre-treatment method that allows for the highest sulphate conversion is chosen for the AMD fed into the BaH-LFCR. Pre-treated AMD is fed into the BaH-LFCR together with a lactate carbon source as the second experiment to monitor the effect in the change of the feed to AMD. A third experiment was then conducted which used an AMD feed and the alternative carbon source. Results for the 3 different BaH-LFCR experiments were compared and discussed. Chapter 6 is the final chapter that draws conclusions and recommendations from the study.

2 Literature Review

2.1 Acid Mine Drainage

South Africa's mining industry grapples with the problem of acid mine drainage (AMD) generation in regions where sulphidic minerals abound, producing 7.0-20.0 Ml/day of AMD from a single abandoned mine (Baloyi *et al.*, 2023). AMD forms when sulphidic rock is exposed to oxygen and water in the presence of iron- and sulphur-oxidising micro-organisms which play an important role of catalysing the reaction process (Moosa *et al.*, 2005; Akcil and Koldas, 2006). Examples of micro-organisms responsible for catalysing AMD formation are Acidit*hiobacillus ferrooxidans*, *Leptospirillum ferriphilum* and *Ferroplasma* sp.; these which provide a mechanism that lowers the activation energy for the oxidation of iron and sulphur (Pozo-Antonio *et al.*, 2014).

AMD generation occurs naturally at a very slow rate, but mining accelerates generation by increasing the exposed surface area of sulphidic rocks (Johnson and Hallberg, 2005; Moosa *et al.*, 2005; Akcil and Koldas, 2006). Post closure of mines, AMD continues to form from tailings and discards left behind at the sites for decades to centuries; rain falls on stockpiles, tailings or open pits. The runoff formed is acidic, high in specific conductivity, sulphate concentrations and heavy metals (Maillacheruvu and Parkin, 1996; Kaksonen *et al.*, 2004; Akcil and Koldas, 2006; Kijjanapanich *et al.*, 2012; Celis *et al.*, 2013; Hessler *et al.*, 2018).

Other industries that produce acidic, sulphate rich wastewater are the power industry through the scrubbing of flue gas, pulp and paper industry and industries that use the galvanic process (Johnson and Hallberg, 2005). Gas from the power industry contains SO_3 which, when contacted with water, forms sulphuric acid (Johnson and Hallberg, 2005). The pulp and paper industry produces sulphites while the petrochemical industry produces an oily wastewater which contains sulphates, sulphites and chloride resulting in the production of wastewater which is toxic and acidic in nature (Johnson and Hallberg, 2003; Akcil and Koldas, 2006). Galvanic processes contain reactive metal sulphides which after processing also generate acidic wastewater containing sulphates and heavy metals (Johnson and Hallberg, 2003; Petrov *et al.*, 2020).

The continuous production of AMD and acidic, sulphate rich wastewaters create an ever-growing need to establish effective techniques to treat these waters over long periods of time at active, closed and abandoned mine sites, as well as in industry (Thisani *et al.*, 2021).

Due to its acidity and toxicity, AMD pollutes the environment, and its actual extent of pollution is difficult to determine (Johnson and Hallberg, 2005). AMD can be uninhabitable, leaches and contaminates rocks, soils, surface water and groundwater (Akcil and Koldas, 2006; Kijjanapanich *et al.*, 2012). AMD that has leaked into water sources has been known to decrease biodiversity by interrupting food chains and killing water life (Feris and Kotze, 2015). The effects of AMD on the environment can range from moderate to highly severe depending on the toxicity and polluting levels of the AMD. If AMD pH ranges between 2 and 4 and the sulphate concentration is below 1000 mg/L, the impact it will have on the environment will be minimised (Akcil and Koldas, 2006). However, highly acidic AMD with high concentrations of sulphate (<1000 mg/L) will result in severe negative impacts (Akcil and Koldas, 2006).

Soluble heavy metals in AMD pose a risk of bioaccumulation and biomagnification if not treated which is detrimental to both flora and fauna (Kijjanapanich *et al.*, 2012). Some metals in AMD found in high concentrations (in the range of hundreds of ppm) are Fe^{2+}, Cu^{2+}, Al^{3+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} (Dinu *et al.*, 2014; Alegbe *et al.*, 2019; Sheridan *et al.*, 2021). Other metals in AMD that are toxic to human, aquatic life and microorganisms in minute concentrations (ppb) are As^{3-}, Cd^{2+}, Pb^{2+}, Hg^{2+} and Sb^{2+} (Dinu *et al.*, 2014).

Some of the negative effects caused by heavy metals on humans and plants are listed in Table 2-1 (Cabot *et al.*, 2019; Li *et al.*, 2019; Rambabu *et al.*, 2020).

Table 2-1: Negative effects of heavy metals found in AMD on humans and plants

Heavy Metals	Effects on Humans	Effects on Plants	References
Arsenic	Cancer of the skin and bladder, kidney failure, bronchitis	Inhibition of growth which decreases fruit production and low yields	(Rambabu *et al.*, 2020)
Cadmium	Cancer of the lung, lung disease, renal dysfunction	Decrease of seed germination and lipid content	(Rambabu *et al.*, 2020)
Chromium	Necrosis of the liver/kidney, skin ulcers	Membrane damage which results in a decrease in plant growth, root damage and chlorosis	(Rambabu *et al.*, 2020)
Copper	Damage of the liver and kidney, anaemia	Inhibition of the reproductive process and photosynthesis	(Rambabu *et al.*, 2020)
Lead	Developmental delay, children experience mental retardation	Plant growth and the production of chlorophyll is decreased	(Rambabu *et al.*, 2020)
Manganese	Nervous system can become damaged	Plant growth is inhibited by chlorosis of young leaves, necrotic dark spots on mature leaves and crinkled leaves	(Li *et al.*, 2019; Rambabu *et al.*, 2020)
Mercury	Memory is decreased; neurodevelopment is impaired	Photosynthetic activity and water uptake is decreased	(Rambabu *et al.*, 2020)
Nickel	Contact causes dermatitis, cancer of the lung and nasal passage, chronic bronchitis	Enzyme, protein and seed germination is decreased	(Rambabu *et al.*, 2020)
Zinc	Nervous membrane damage	Yield is reduced and growth is stunted	(Cabot *et al.*, 2019; Rambabu *et al.*, 2020)

2.2 AMD formation chemistry

When looking at sulphide containing compounds found in the rocks at mining sites, iron sulphides are the most abundant and these are usually in the form pyrite, FeS_2 (Johnson and Hallberg, 2005; Akcil and Koldas, 2006).

Equation 2-1, shows the chemical reaction of AMD production through the oxidation of pyrite (Johnson and Hallberg, 2005; Akcil and Koldas, 2006):

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

Equation 2-2, shows the formation of ferric iron which also oxidises additional pyrite (Akcil and Koldas, 2006):

$$8FeS_2 + 15O_2 + 52Fe^{3+} + 34H_2O \rightarrow 60Fe^{2+} + 16SO_4^{2+} + 68H^+ \quad \text{Equation 2-2}$$

The process, in more detail, begins with pyrite oxidation in the presence of moisture. This results in the dissolvement of iron into ferric, decrease in pH as sulphuric acid is formed and dissociates in water leading to increase in the total dissolved solids (TDS) (Akcil and Koldas, 2006). The ferrous iron is further oxidised to ferric iron which precipitates as iron hydroxide ($Fe(OH)_3$) at pH 2.3 – 3.5 as shown in Equation 2-1 (Akcil and Koldas, 2006). Unprecipitated ferric iron oxidises additional pyrite (Equation 2-2) and serves as the primary pyrite oxidising agent of pyrite (Johnson and Hallberg, 2005). The oxidation process is pH dependant. At pH levels above 4, both abiotic and biotic oxidation of ferrous to ferric iron occurs. Below pH 4, acidophilic bacteria are responsible for oxidation (Johnson and Hallberg, 2005).

While pyrite is the most studied mineral in AMD formation, other minerals also generate AMD and research on these non-pyrite compounds is less extensive (Akcil and Koldas, 2006). These include chalcopyrite ($CuFeS_2$), galena (PbS), cinabar (HgS), spalerite (ZnS) and millerite (NiS) (Dinu *et al.*, 2014; Zipper *et al.*, 2018).

2.3 Treatment of AMD

Akcil and Koldas, (2006) outline three basic levels for minimising the effect and impact of AMD. Primary control focuses on preventing generation of acidic wastewater. Secondary control prevents the migration of the AMD to different locations and tertiary control involves the collection of AMD for treatment. These strategies can be further categorised into two main approaches: prevention (primary and secondary control) and treatment (tertiary control). Figure 2-1 illustrates the different ways AMD can be minimised.

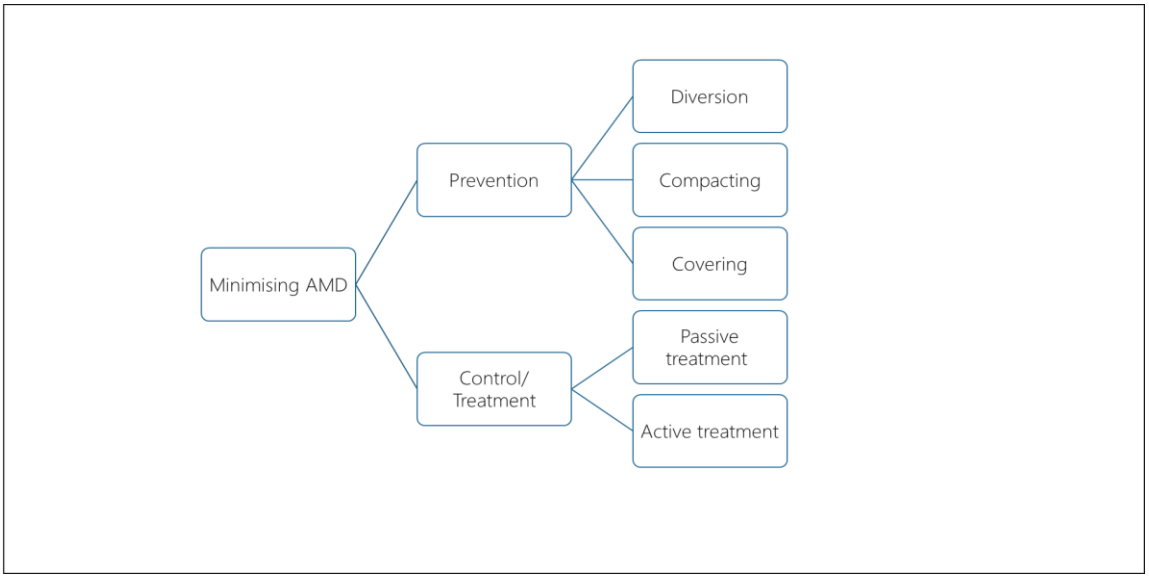


Figure 2-1: Methods for minimising AMD. Adapted from (Akcil and Koldas, 2006)

AMD prevention methods aim to remove oxygen, water or the bacteria which are all necessary for the accelerated AMD formation (Pozo-Antonio *et al.*, 2014). All three conditions must be present for AMD to form. Prevention strategies include various approaches. Migration focuses on the diversion of water using piping, channelling, and slope alteration around the polluting area, though this method can fail without detailed hydrological and hydrogeological studies (Akcil and Koldas, 2006; Pozo-Antonio *et al.*, 2014). Flooding and sealing underground mines is another preventative method which limits amount of dissolved oxygen available and reduces bacterial activity (Johnson and Hallberg, 2005).

Compacting decreases acidic soil permeability limiting air and water flow. Saturation of the compacted soil potentially decreases oxygen diffusion by 3-4 times (Pozo-Antonio *et al.*, 2014). The dry cover method decreases oxygen exposure to the pollutant, reduces run off, prevents erosion and improves appearance (Johnson and Hallberg, 2005; Pozo-Antonio *et al.*, 2014). Desulphurisation produces desulphurised coal concentrate with a high sulphide concentrate and benign tailings which are safe for disposal (Fagan-Endres *et al.*, 2018).

These preventative methods reduce contamination of surface water, groundwater and reduce water seepage into acid generating material. However, preventative measures are not permanent and can wear and tear over time. Thus, corrective techniques are essential as the formation of AMD is inevitable.

Corrective treatment of AMD typically involves passive and active methods as well as semi-passive approaches that combine elements of both.

2.3.1 Active treatment

Active treatments require significant mechanical and human input, chemicals, control, and energy compared to passive treatments which are usually self-sustaining (Thisani *et al.*, 2021). While associated with high operational costs and high sludge production from metal precipitation, active treatments offer advantages such as easy process control, high volume treatment capacity, and the ability to treat highly acidic wastewater (Johnson and Hallberg, 2005; Thisani *et al*., 2021).

Most active treatment processes are abiotic, using physiochemical methods (Kijjanapanich *et al.*, 2012). These include aeration, lime addition, ion exchange and reverse osmosis (Johnsaboon and Hallberg, 2005; Pozo-Antonio *et al*., 2014; Thisani *et al*., 2021).

Aeration oxidises metals, causing them to precipitate out and create sludge (Johnson and Hallberg, 2005). Lime addition technologies include conventional neutralisation, the high-density sludge (HDS) process and chemical desalination (Thisani *et al.*, 2021). Conventional treatment uses stirred reactors with alkaline chemicals like limestone, dolomite or sodium hydroxide to neutralise and precipitate metal; low density sludge forms at the bottom of the stirred reactor while the liquid on top has a lower metal content and a neutral pH (Dinu *et al*., 2014; Thisani *et al*., 2021). The HDS process improves on the conventional treatment method by forming flocs that create sludge with a higher solid content and use neutralising agents more efficiently (Thisani *et al.*, 2021).

Ion exchange uses synthetic resin regenerated using alkali solutions (Pozo-Antonio *et al.*, 2014). Reverse osmosis, a more expensive technology, uses semi-permeable membranes that allow water to pass through while retaining solid particles and dissolved substances (Pozo-Antonio *et al*., 2014). These membranes use positive hydrostatic pressure and may require multiple stages of to improve water quality (Thisani *et al.*, 2021).

Biotic active treatments also exist, such as active anaerobic digestion which produces less sludge compared conventional treatments and the HDS process (Thisani *et al.*, 2021). These processes involve sulphate reducing bacteria which convert sulphate to sulphide. However, these processes do require constant control of parameters such as pH, temperature, sulphate concentration and flowrate and like other active treatments, has disadvantages such as high operational costs (Thisani *et al.*, 2021).

2.3.2 Passive and semi-passive treatment

Passive treatment occurs near AMD generation sources, requiring minimal inputs and process control compared to active treatment, making it more cost effective (Johnson and Hallberg, 2005; Thisani *et al.*, 2021). It often utilises naturally occurring physiochemical and biological processes (Zipper *et al.*, 2018).

Advantages of passive treatments include low operational cost due to the use of low-cost substrates, suitability for long-term treatment, reduced precipitate formation and gravity-driven operations (Johnson and Hallberg, 2005; Zipper *et al.*, 2018; Thisani *et al.*, 2021). However, disadvantages include large

land requirements, unpredictable processes that are difficult to control, limited treatment of high acidity loads, process efficiency decreases with time due to the decrease in reactive substances, and challenges in long-term sludge disposal (Johnson and Hallberg, 2005; Thisani *et al.*, 2021).

Passive treatments can be abiotic or biotic. Abiotic methods include anoxic limestone drains (ALD) and open limestone channels (OLC). ALDs are trenches that are filled with limestone to neutralise AMD and then sealed (Pozo-Antonio *et al.*, 2014). There is an increase in carbon dioxide partial pressure which helps accelerate limestone dissolution rate (Pozo-Antonio *et al.*, 2014). Dissolved iron in the AMD can decrease the neutralising power of the alkali by coating it. (Johnson and Hallberg, 2005). OLCs are similar to ALDs but utilise the alkalis such as limestone which continue to dissolve after armouring which facilitates further neutralisation(Ziemkiewicz and Skousen, 1994).

Biotic methods include wetlands, compost wetlands, permeable reactive barriers and sulphate reducing bioreactors (Johnson and Hallberg, 2005; Thisani *et al.*, 2021). Wetlands use aerobic or anaerobic bacteria to produce alkalinity and immobilise metals present in AMD (Johnson and Hallberg, 2005; Zipper *et al.*, 2018). Native vegetation in the wetlands can be used as a substrate for the bacteria. Compost bioreactors employ sulphate reducing bacteria (SRB) and fermentative bacteria (Johnson and Hallberg, 2005). Fermentative bacteria breakdown the compost to a useable form for the SRB such as in aerobic wetlands but anaerobic conditions such as those in compost bioreactors do not support vegetation and thus need for substrate substitution. Anaerobic systems cannot support vegetation and so need substrate substitution.

Recent improvements in biological passive treatments involve supplying of a carbon rich substrate to act as both a carbon source and an electron donor which allows for more efficient sulphate reduction and alkalinity production which helps further neutralise acidic wastewater. These modified treatments combine the low costs of passive treatment with better process control (Marais, 2020). They also still retain the advantages of traditional biological treatments such as sustainability and minimal waste production (Kleinmann *et al.*, 2023). Examples include the IMPI process developed by Pulles Howard and de Lange and the hybrid linear flow channel reactor (Coetser *et al.*, 2004; Mooruth, 2013). The hyrid linear flow channel reactor is referred to as a semi-passive process as it can be run with gravity but requires inputs such as substrate substitution and floating sulphur biofilm harvesting.

2.3.3 Biological treatment

Many AMD treatment processes incompletely address the problems associated with AMD. They only treat the acidity and heavy metals but leave high sulphate concentrations(Akcil and Koldas, 2006; Arnold *et al.*, 2016). Ongoing development of more efficient and cost-effective processes is crucial in mitigating AMD pollution worldwide. Biological treatments of sulphate rich wastewaters can serve as a polishing step or as the main treatment for circum-neutral, low volume, high sulphate wastewater.

Sulphur Cycle

Microorganisms play a crucial role in transforming various sulphur compounds and oxidation states (Tang *et al.*, 2009). They catalyse both the formation of AMD by oxidising sulphide to sulphate and the reduction of sulphate back to sulphide. Understanding the sulphur cycle is essential in comprehending biological sulphur systems. The sulphur cycle consists of different reduction and oxidation reactions as seen in Figure 2-2 below (Tang *et al.*, 2009).

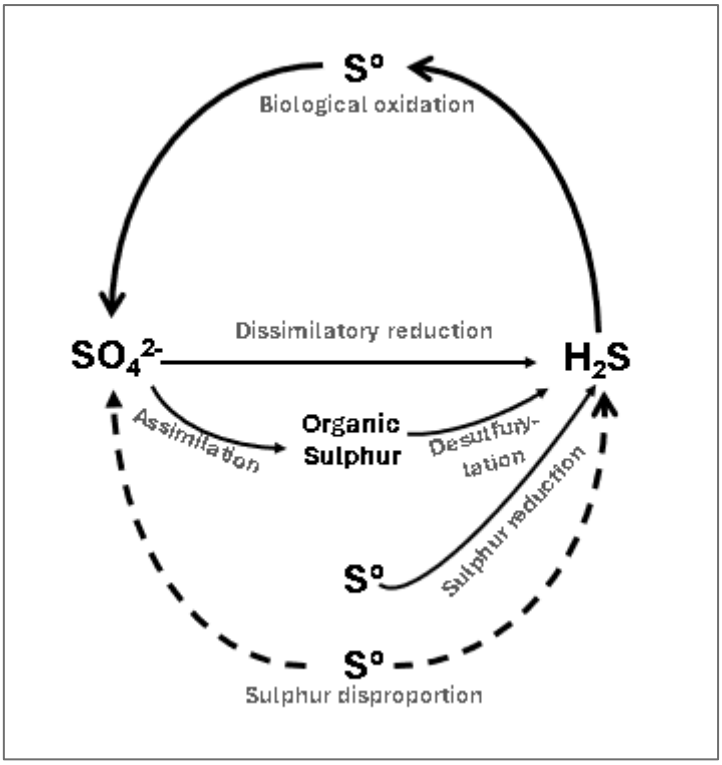


Figure 2-2: Sulphur cycle. Adapted from Tang *et al.* (2009)

The main reactions of the sulphur cycle are listed below.

- The conversion of organic sulphur into inorganic forms which include hydrogen sulphide, sulphide minerals and elemental sulphur.
- The partial oxidation of hydrogen sulphide to elemental sulphur which can further be completely oxidised to sulphate by sulphur oxidising bacteria (SOB). Oxidation of sulphide can be harmful as seen in the oxidation of sulphides to high concentration of sulphates found in AMD.
- The reduction of sulphate to sulphide by sulphate reducing bacteria (SRB).
- The incorporation of sulphide and organic compounds into metal compounds which results in the formation of precipitates as seen in the biological treatment of sulphate rich wastewater which contains significant amounts of metals.
- The process of sulphur disproportionation where sulphur, thiosulphate and sulphite act as electron donors and electron acceptors and SRB or highly specialised bacteria transform the ions to hydrogen sulphide and sulphate (Finster, 2008; Tang *et al.*, 2009). In this process both sulphate and sulphide are produced.

Biological sulphate reduction (BSR)

Sulphate reduction was first identified in ponds that released hydrogen sulphide (Boshoff *et al.*, 2004b). Previously, sulphate reduction was undesirable due to the release of toxic H_2S but was eventually shown to be an efficient process in removing sulphate from wastewater and thus, became more widely accepted (Liamleam and Annachhatre, 2007). Biological sulphate reduction (BSR) systems not only reduce sulphate but also oxidise organic matter removing organic chemical oxygen demand (COD). They also remove nitrogen and heavy metals. Heavy metals are removed as precipitates through sulphide precipitation. This compares to physiochemical treatment where metals are removed as hydroxides which are often not as stable as metal sulphides (Kaksonen *et al.*, 2004; Liamleam and Annachhatre, 2007). Biological treatment of AMD also produces much less sludge waste compared to chemical treatment (Kaksonen *et al.*, 2004; Liamleam and Annachhatre, 2007). There is potential for

metal recovery from metals precipitated out in BSR systems which can be used as raw materials. Optimising sulphide production ensures maximum heavy metal removal and decreases concentrations of toxic sulphide. Sulphide not removed via metal precipitation can be partially oxidised to elemental sulphur (Kaksonen *et al.*, 2004; Liamleam and Annachhatre, 2007). BSR systems also offer long-term treatment, sustainability and low costs. Use of a semi-passive process is advantageous as it offers better process control and kinetics and operates at a lower cost when compared to active treatment (Hessler *et al.*, 2018).

In the biological treatment of AMD in anaerobic conditions, the terminal electron acceptor is sulphate (Moosa *et al.*, 2002). The sulphate is reduced while the substrate is oxidised. This process produces energy which is used for growth by microorganisms (Moosa *et al.*, 2002). Since sulphate is an electron acceptor there is need for an electron donor and organic compounds can act as both electron donors and carbon sources (Maillacheruvu and Parkin, 1996; Moosa *et al.*, 2002). Examples of these organic compounds include organic acids, propionate, acetate, lactate, glucose, fructose, and alcohols (Maillacheruvu and Parkin, 1996; Ayangbenro *et al.*, 2018). Non-organic compounds only act as electron donors and so need to be coupled with carbon sources (Maillacheruvu and Parkin, 1996). Hydrogen is an example of a non-organic electron donor that needs to be paired with a carbon source such as carbon dioxide or carbon monoxide (Moosa *et al.*, 2002). Other nutrients are also required by the SRB communities such as nitrogen and trace metals which are incorporated into proteins and cofactors respectively (Moosa *et al.*, 2002).

Sulphate reduction can either be assimilatory or dissimilatory and Table 2-2 shows the different processes that assimilatory and dissimilatory bacteria go through (Tang *et al.*, 2009).

Table 2-2: Assimilatory and dissimilatory processes of SRB (Tang *et al.*, 2009)

	Assimilatory	Dissimilatory
1	Attach to ATP to form adenosine phosphosulphate (APS)	Attach to ATP to form adenosine phosphosulphate (APS)
2	Attachment of phosphorus atom forming phosphoadenosine phophosulphate (PAPS)	Sulphate portion directly reduced to sulphite (enzyme: APS reductase)
3	PAPS reduced to sulphite	Sulphite converted to sulphide (enzyme: sulphite reductase)
4	Sulphite converted to sulphide (enzyme: sulphite reductase) and incorporated into organic compounds	Sulphide excreted

Both pathways start with activation, attaching a sulphate ion to adenosine triphosphate (ATP) to form adenosine phosphosulphate (APS). The assimilatory SRB then attach a phosphorus atom forming phosphoadenosine phosphosulphate (PAPS). The dissimilatory SRB instead directly reduces sulphate to sulphite in the presence of the APS reductase enzyme, following which sulphite is converted to sulphide and externally excreted (Tang *et al.*, 2009). PAPS formed by the assimilatory SRB is reduced to sulphite and then the sulphite is converted to sulphide and incorporated into organic compounds (Tang *et al.*, 2009). In BSR systems, most of the sulphate reduction is dissimilatory and the sulphide released can either dissolve, form H_2S which is liberated as a gas or can form precipitates with metals. The physical state of the sulphide released is dependent on pH. At lower pH values, sulphide is mostly a gas and as the pH increases to neutral and above it is in its aqueous state or dissociates in water to form its respective ions HS^- and S^{2-} (Yongsiri *et al.*, 2004).

Most SRB are obligate anaerobes, but some SRB can function in small concentrations of oxygen (Johnson *et al.*, 1997). As mentioned before, sulphate is used as a source for oxygen and the terminal electron acceptor for SRB as they metabolise substrates. The oxygen obtained from the sulphate is used to oxidise the substrates (Moosa *et al.*, 2002).

Reduction of sulphate can be carried out by both mesophilic (25-35ºC) and thermophilic (35-70ºC) bacteria (Liamleam and Annachhatre, 2007). Mesophilic bacteria are useful for wastewater treated at ambient conditions and come from sulphide producing ponds. Thermophilic bacteria can treat warm

wastewaters such as those produced in the paper and pulp industry. SRB that operate at different temperatures provide the benefit of not having to cool or heat of the wastewater. Liamleam and Annachhatre, (2007) state that the conversion rate of thermophilic bacteria is much higher than that of mesophilic bacteria, however, operating at higher temperatures is energy intensive and incurs more operational costs when compared to a system that uses mesophilic bacteria. Thermophilic SRB are also mostly found in hard to reach, deep offshore reservoirs while mesophilic SRB are found in shallow reservoirs such as ponds which are more accessible (Tang *et al.*, 2009).

SRB are prokaryotes, including both bacterial and archaeal species (Tang *et al.*, 2009). Earlier studies of BSR systems did not identify which types of SRB were in the mixed communities but since the early 2010s there have been studies conducted identifying the microorganisms involved (Tang *et al.*, 2009). Knowledge of the microorganisms within the mixed cultures that drive BSR systems allow for insight and knowledge into the organisms' roles, functionality, and metabolic pathway (Sheoran *et al.*, 2010; Marais, 2020). This will in turn help improve the system performance and allow for efficient removal of sulphate (Sheoran *et al.*, 2010).

Below, Table 2-3 shows the taxonomic groups that are involved in the biological reduction of sulphate and their characteristics (Johnson and Hallberg, 2003; Tang *et al.*, 2009).

Table 2-3: SRB taxonomic groups' characteristics (Johnson and Hallberg, 2003; Tang *et al.*, 2009)

Taxonomic Group	Examples	Operating Temperature	Characteristics
δ-subclass of proteobacteria	*Desulphovibrio, Desulphomicrobium, Desulphobulbus, Desulphobacter, Desulphobacterium, Desulphococcus, Desulphosarcina, Desulphomonile, Desulphonema, Desulphobotulus, Desulphoarculus*	20 to 40ºC (psychrophilic, mesophilic, and thermophilic)	Diverse and shape varies
Gram positive bacteria	*Desulphotomaculum*	Most: 20 to 40ºC Some: >40ºC (mesophilic and thermophilic)	Form heat resistant endospores
Bacterial thermophilic	*Thermodesulphobacterium, Thermodesulphovibrio*	65 to 70ºC (thermophilic)	Inhabit high temperature environments
Archaea	*Archaeoglobus, Thermocladium, Caldivigara*	>80ºC (thermophilic)	Found in marine regions

In dissimilatory sulphate reduction, metabolism may vary depending on the organisms present and the substrate oxidised.

Equation 2-3 is a general equation for the oxidation of substrate by dissimilatory sulphate reducers.

$$2CH_2O + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \qquad \text{Equation 2-3}$$

Sulphates oxidise the substrate, producing alkalinity in the form of hydrogen carbonate as well as hydrogen sulphide. An example of the oxidation process on a commonly used substrate, lactate, in BSR systems is shown below in Equation 2-4 and Equation 2-5.

$$2\ lactate + SO_4^{2-} + H^+ \rightarrow 2\ acetate + 2HCO_3^- + 2H_2O + HS^- \quad \text{Equation 2-4}$$

$$acetate + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad \text{Equation 2-5}$$

Equation 2-4 shows the incomplete oxidation of lactate while Equation 2-5 shows the oxidation of the acetate produced. Putting together Equation 2-4 and Equation 2-5 shows the complete oxidation of lactate. Incomplete oxidation yields less alkalinity compared to the complete oxidation of lactate. The alkalinity is produced by the formation of hydrogen carbonate from the oxidation of substrates and the more alkalinity produced the better the performance of the BSR system as this will more efficiently neutralise the sulphate rich wastewater which is mostly acidic in nature (Kaksonen *et al.*, 2004). Incomplete oxidation also results in a higher COD content in the effluent as it still contains acetate. This is harmful if released to the environment and thus complete oxidation is more favourable (Celis *et al.*, 2013).

Incomplete oxidisers tend to have a faster growth rate compared to complete oxidisers and so when both groups are present in a system, the incomplete oxidisers will multiply faster and push the system towards incomplete oxidation which will result in an effluent with a higher COD content (Celis *et al.*, 2013). Figure 2-3 shows examples of incomplete, complete and hybrid (both complete and incomplete oxidisers) (Tang *et al.*, 2009).

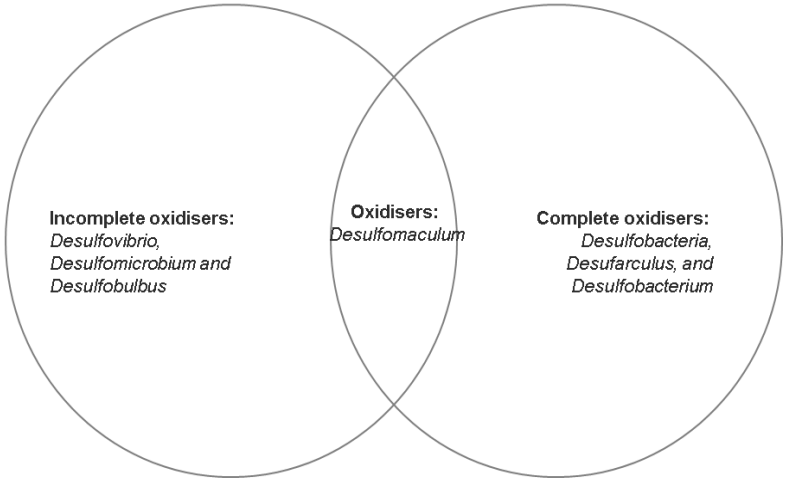


Figure 2-3: Venn diagram of incomplete, complete and hybrid substrate oxidisers (Tang *et al.*, 2009)

Sulphide Oxidation

In the sulphur cycle, sulphide can be partially oxidised to elemental sulphur, form polysulphide or completely oxidised to sulphate. The two equations, Equation 2-6 and Equation 2-7, show partial and complete oxidation of sulphide by SOB respectively (Guerrero *et al.*, 2015).

$$H_2S + \frac{1}{2}O_2 \rightarrow S^0 + H_2O \quad \text{Equation 2-6}$$

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \quad \text{Equation 2-7}$$

The redox potential and pH range in which partial oxidation of sulphide occurs, and elemental sulphur is formed is small and pH and redox values outside these ranges will result in the formation of the undesired product, sulphate (Molwantwa, 2007; van Hille *et al*., 2011; Marais, 2020). Thus, it is important to maintain the environment at the desirable conditions to allow for elemental sulphur formation, oxygen must be limiting, and the concentration of sulphides must be high for elemental sulphur to form and avoid complete oxidation (Visser *et al.*, 1997).

In the biological treatment of AMD, partial oxidation of sulphide to elemental sulphur is beneficial as it reduces the concentration of toxic sulphide which allows for better performance of both SRB and SOB (Molwantwa, 2007). Production of elemental sulphur also allows for sulphur containing ions and molecules to be extracted from wastewaters in a separate phase during treatment which is not achieved in most active and passive treatment processes (Arnold *et al.*, 2016). The most abundant ion, sulphate, in solution is stable under all pH values and so is hard to remove from wastewater (Montes-Atenas, 2022).

2.4 Factors influencing BSR

2.4.1 BSR system communities

Mixed communities are important in BSR systems as microbial diversity with complementary ecological niches improves stability of the bioreactor performance under changing environmental conditions (Kaksonen *et al.*, 2004). Presence of fermenters and methane producing bacteria in mixed cultures is advantageous in BSR systems that use complex substrates as they can breakdown the substrates to compounds that are more easily metabolised by SRB. However, these fermenters and methane producing bacteria can compete with SRB for the substrate, and thus, establishing an optimal chemical oxygen demand to sulphate ratio (COD:Sulphate) that favours the growth and function of SRB is essential (Maillacheruvu and Parkin, 1996). Hussain *et al*. (2014) conducted experiments using pure cultures and noted poor system performance using a complex substrate, molasses. The author observed that the pure culture systems had long lag phases which were due to the presence of complex carbohydrates (molasses substrate) which are difficult to metabolise and also only achieved high sulphate reduction at the beginning of the experiments because of the concentration of simple carbohydrate molecules is at its highest at the beginning of the experiment (Hussain *et al.*, 2014). Thus, mixed culture systems tend to have higher process efficiency compared to single culture systems especially if complex substrates are used.

2.4.2 Chemical oxygen demand to sulphate ratio

Chemical oxygen demand (COD) is the amount of oxygen that is required to oxidise an organic substrate. In BSR systems, to achieve an optimal removal of sulphate, a minimum COD/SO_4 ratio of 0.67 is required (Liamleam and Annachhatre, 2007). However, AMD lacks electron donors and carbon sources and thus, needs to be supplemented with these so as to achieve the optimal COD:Sulphate. In mixed culture systems the COD:Sulphate ratio is very important as it may determine the success or failure of a system; thus, the ratio of COD to sulphate needs to promote sulphate reduction by SRB over the action of fermenters and methanogens (Maillacheruvu and Parkin, 1996). As seen from Oyekola *et al*., (2009, 2012), a ratio which is ≤1 promotes sulphate reduction; SRB favour a higher sulphate concentration to COD concentration and tend to have a higher affinity for carbon sources and so can tolerate the lower substrate concentrations (Oyekola *et al.*, 2009). Thus, in BSR systems that contain mixed communities it is important to establish a COD:Sulphate that promotes sulphate reduction instead of fermentation or methane production.

2.4.3 Hydraulic residence time

The hydraulic residence time (HRT) determines the amount of time the feed stream is in contact with the bacteria in the reactor system. In reactor systems that are dependent on planktonic microorganisms, HRT has a great influence on the system performance. If the HRT is too short (<2-day HRT) there is

risk of cell washout and there may not be enough contact time between the SRB and the wastewater stream which results in poor sulphate conversion (Zaiat *et al.*, 1996). If a system requires a short HRT, cell washout can be avoided by the placement of a support matrix which allows for biomass retention through the formation of biofilm (Van Hille *et al.*, 2015). Another problem associated with a short HRTs reported by Mukwevho *et al*. (2020) was poor system performance due to substrate washout when substrate was fed periodically (fed-batch – once a week) and a short HRT was used. In this study, the sulphate reduction rate increased when the substrate was replenished (Mukwevho *et al.*, 2020). Long HRTs result in slower rates and if the HRT is too long there is a risk of depletion of essential reactants that promote the reduction of sulphates such as a decrease in substrate concentration and nitrogen sources (Oyekola *et al.*, 2009). Another problem that may arise from a long HRT is the accumulation of toxic substances in the BSR system such as sulphide which may heavily deplete the functionality of the bacteria in the BSR system (Maillacheruvu and Parkin, 1996).

2.4.4 Temperature

As discussed before, SRB range from psychrophilics to thermophiles and thus their optimum operating temperature conditions vary (Tang *et al.*, 2009). Psychrophilic bacteria thrive in cold temperatures below 20ºC, while mesophilic bacteria favour temperatures that range between 20 and 40ºC and thermophiles prefer temperature that are above 40ºC, usually in the range 40 to 70ºC (Tang *et al.*, 2009). Sheoran *et al*. (2010) go on to mention that mesophilic SRB are most common. Moosa *et al*. (2005) investigated the effect of temperature on a BSR system between 20 and 35ºC (mesophilic SRB) and concluded that an increase in temperature resulted in enhanced sulphate reduction rates. Too high a temperature in BSR systems can denature proteins which can result in cell death, decompose substrates and affect the solubility of certain substances such as hydrogen sulphide in BSR systems (Sheoran *et al.*, 2010). At higher temperatures, solubility of H2S decreases; dissolved H_2S is less harmful compared to liberated, gaseous H_2S which when inhaled is toxic at levels higher than >700 ppm (AFROX Hydrogen Sulphide MSDS, 2011). Low temperatures usually result in very slow rates of sulphate reduction due to slowed activity of SRB resulting in a less efficient BSR system. Mukwevho *et al*. (2020) reports on how decreases in temperature below 10ºC can result in a decrease of sulphate reduction by more than half the expected reduction at temperatures above 20ºC.

2.4.5 pH

Most SRB are neutrophilic and operate at pH values between 6 and 9 and will be inhibited outside this range (Marais, 2020). However, there have been SRB that have been identified to be acidophilic and act by creating a small neutral environment by using hydrogen as their electron donor thus, in turn increasing the pH around them (Elliott *et al.*, 1998). Though there has been acidophilic SRB identified their conversion rates are much lower than those obtained at neutral pH values by the neutrophilic SRB and since AMD is acidic there is need for pre-treatment to increase the pH to the neutrophilic SRBs' range so as to achieve efficient sulphate reduction (Elliott *et al.*, 1998; Moosa *et al.*, 2005; Marais, 2020).

2.4.6 Support matrix

Table 2-4 below describes the characteristics of systems without a support matrix and those with a chemically inert support matrix (Marais *et al.*, 2024). Matrices such as PUF and carbon microfibres are inert and so do not offer any nutritional value to microorganisms but rather are only to promote biomass retention through entrapment or attachment (Mattos de Oliveira Cruz *et al.*, 2024). From Table 2-4, one can see that BSR systems have favourable reaction kinetics when a support matrix is present.

Table 2-4: Characteristics of BSR systems with and without a support matrix

No Support	Support Matrix Present
• Free cells present (prokaryotic cells)	• Both free cells and attached cells present (biofilm)

No Support	Support Matrix Present
• Prone to washout thus short HRTs should be avoided • Less contact between the wastewater and the bacteria compared to systems with a support matrix present • Lower sulphate conversions at high HRT	• Decouples the HRT and the biomass retention time • Promotes biofilm formation • Higher sulphate conversions compared to systems without a support matrix at higher HRT • Decreases the working volume • Support matrix has to be inert

2.4.7 Nutrient Requirements

Nutrients such as nitrogen, phosphorus and potassium are essential in the proliferation of SRB as they have been reported to promote enzymatic activity and growth (Thisani *et al.*, 2021). However, elevated concentrations of nitrates have been shown to be inhibitory to SRB as they cause osmotic and nitrite stress (He *et al.*, 2010). SRB-specific feed such as Postgate media B (Postgate, 1963; Marais, 2020) contains various nutrients such as nitrogen from yeast extract and ammonium chloride, phosphorus and potassium from di-potassium hydrogen phosphate, citrate from sodium citrate, magnesium from magnesium sulphate heptahydrate and chloride from ammonium chloride. A combination of these nutrients promotes SRB activity. Though chlorine is known to be fatal to bacteria, it has been shown to increase SRB growth in moderate concentrations but at high concentrations it is toxic to SRB (Xie *et al.*, 2019). Citrate acts as an additional carbon source and has also been shown to have toxin masking abilities towards SRB when heavy metals are present (Gu *et al.*, 2021).

Currently, literature on nutrients beneficial for SRB is sparse, but there is evidence indicating that presence of these additional nutrients in moderate concentrations in BSR systems has been shown to be beneficial for SRB growth and activity. However, unlike the SRB-specific, synthetic feed which contains additional nutrients, AMD lacks these nutrients which may decrease sulphate reduction. Other nutrients may be in too high a concentration in AMD and in turn be inhibitory to the SRB.

2.4.8 Potential inhibitors from AMD

Due to AMD being acidic in nature and containing significant concentrations of heavy metals, it can be toxic and inhibitory to SRB (Koschorreck, 2008). Biological treatment systems are more geared towards low acidic wastewaters and so highly acidic AMD may be detrimental to BSR processes and thus, a pre-treatment step would be essential to increase the pH of acidic AMD. As discussed in Section 2.4.5, most SRB bacteria are neutrophilic and SRB which are acidophilic and can tolerate low pH environments have slower conversion rates (Elliott *et al.*, 1998).

Heavy metals such as Fe, Cr, Hg, Mn, Ni, Pb, Cd, Al and Zn are present in AMD and are known to be toxic to microorganisms as they can react with functional groups of enzymes resulting in their deactivation and also the metals can compete with essential cations (Utgikar *et al.*, 2002, 2003). However, some authors have reported that very low concentrations of heavy metals can improve the activity and growth of SRB (Del Busso Zampieri *et al.*, no date; Utgikar *et al.*, 2002, 2003; Akinpelu *et al.*, 2021). A summary of the concentration levels that are reported as toxic to SRB are shown in Table 2-5.

Table 2-5: Toxic heavy metal concentrations for SRB

Heavy Metals	Toxic Concentrations (mg/L)	Source
Al	13	(Martins *et al.*, 2012; Akinpelu *et al.*, 2021)
Cd	>4-20	(Utgikar *et al.*, 2003)
Cr	25, 60	(Marsh *et al.*, 2000; Utgikar *et al.*, 2003)
Cu	4-20	(Utgikar *et al.*, 2003; Karri *et al.*, 2006)
Fe	475	(Gonzalez-Silva *et al.*, 2009)
Ni	10-20	(Utgikar *et al.*, 2003)
Pb	75-80	(Sani *et al.*, 2003; Utgikar *et al.*, 2003)
Zn	20	(Utgikar *et al.*, 2003)

2.4.9 Type of carbon source

Kijjanapanich *et al*. (2012) reports that AMD has low concentrations of carbon and electron donors and requires substrate supplementation for optimal sulphate removal. Substrates can be organic (carbon source and electron donors) or inorganic (only electron donors) (Maillacheruvu and Parkin, 1996). Maillacheruvu and Parkin (1996) mentions that the microbial kinetics in the reduction of sulphates may differ depending on the substrate used. Various organic compounds can act as electron donors such as sugars (fructose, glucose, maltose), amino acids, alcohols (methanol, ethanol), dicarboxylic acids (fumarate, malate, succinate), VFA (lactic acid, acetic acid, propionic acid) and aromatic compounds (benzoate, phenol: Hussain *et al*., 2016).

Substrate selection depends on sulphate reduction efficiency which is dependent on COD and nitrogen content (Boshoff *et al.*, 2004a). Simple organics are more easily metabolised by SRB than complex organics (Hussain *et al.*, 2014). Complex substrates are often waste streams that contain mixtures of carbohydrates and other non-digestible substances. They offer the advantage of remediating two waste streams but are usually more efficient in BSR systems that have mixed cultures (Boshoff *et al.*, 2004a; Marais, 2020).

This study focuses on lactate (for comparison as the base case), molasses, acetate, algal lysate, and honey. The latter two have potential for on-site production, reducing transportation costs while molasses and acetate are by-products of common processes that would make them readily available and cheaper alternatives.

Lactate

Lactate has been regarded as a highly effective electron donor in terms of biomass yield and energy provision (Kaksonen *et al*., 2004;Hessler *et al*., 2018;Celis *et al*., 2013). The study by Celis *et al*., (2013) focussed on reducing the start-up time of BSR systems by the addition of lactate at the start of the BSR process. Lactate accelerated reactor start-up, and this was also supported by Kaksonen *et al*., (2004). This acceleration of start-up time could be due to the fact that lactate produces a diverse culture and is readily available to SRB (Celis *et al*., 2013). However, it was seen that most simple organic compounds supported a mixed community (Kaksonen *et al*., 2004; Celis *et al*., 2013; Hessler *et al*., 2018). Celis *et al*, (2013) also concluded that lactate consuming microorganisms initiate sulphate reduction and biofilm formation which makes lactate an important substrate in the start-up phase of BSR systems. Lactate as a substrate has been broadly studied in different reactor types and has often been used as the substrate of choice when conducting kinetic studies on new reactor types and configurations (Oyekola *et al*., 2012; Marais, 2020). As reported by Maillacheruvu and Parkin, (1996), lactate-fed SRBs are less sensitive to sulphide toxicity compared to acetate-fed SRBs and the COD:sulphate in lactate fed systems alter the system behaviour.

Due to incomplete substrate oxidisers having a faster growth rate than complete oxidisers, incomplete oxidation of lactate is usually observed in BSR systems. Incomplete oxidation produces less alkalinity and an effluent stream with a high COD content. However, recent developments such as the up-flow anaerobic packed bed reactor (UAPBR), support complete lactate oxidation through microbial.

While lactate remains efficient for BSR systems, its high cost necessitates research into alternative, more cost-effective substrates for large-scale applications (Marais, 2020).

Acetate

Acetate can be produced within a BSR system from the incomplete oxidation of subtrates such as lactate, ethanol and propionate, or can be fed directly into the system. Acetate is readily available and a cost-effective alternative to lactate as it is produced from hydrolysis and fermentation of organic compounds. However, many studies have corroborated that acetate is a less effective electron donor compared to lactate (Maillacheruvu and Parkin, 1996; Kaksonen *et al.*, 2004; Celis *et al.*, 2013; Hessler *et al.*, 2018). Acetate removal usually occurs at slower rates which results in low alkaline production; sulphate-reducing activity obtained with acetate was lower than the activity obtained with lactate for all experiments conducted by Celis *et al.*, (2013). Maillacheruvu and Parkin, (1996) mention that acetate oxidising SRB are more sensitive to sulphide toxicity than lactate oxidising SRB. It was also observed that acetate oxidation is favoured in conditions with limited lactate concentrations (Maillacheruvu and Parkin, 1996; Celis *et al.*, 2013; Hessler *et al.*, 2018). However, methanogens tend to be more competitive in mixed cultures fed with acetate (Celis *et al.*, 2013). Celis *et al.*, (2013) go on to show that SRBs which oxidise acetate do not multiply as fast as those that are fed with lactate. However, oxidation of acetate produces less COD in the treatment effluent than the incomplete oxidation of lactate (Maillacheruvu and Parkin, 1996; Kaksonen *et al.*, 2004). Acetate utilisation can be improved when introduced as the sole carbon source in systems and the activity of competitive methanogens is decreased by creating a substrate limiting environment which is favoured by SRB as they are known to be better substrate scavengers than fermenters and methanogens in mixed communities and methanogens are also more sensitive to sulphide compared to SRB (Oyekola *et al.*, 2009, 2012).

Molasses

Molasses is a waste product produced from paper and sugar industries. It contains 50% organic compounds and chlorides, sulphates and potassium (Waligórska *et al.*, 2000). Some compounds found in molasses are challenging to breakdown and thus, when used for biological sulphate reduction, use of a mixed community may be beneficial in the breakdown of complex compounds in molasses making them more readily available to SRB.

Molasses has been mostly used to produce yeast, (bio)ethanol, citric acid, protein substances and in the industries of agriculture, baking, and building. However, even though molasses has multiple uses in different industries there is still large concentrations of molasses that remain unused and enter water bodies causing eutrophication (Waligórska *et al.*, 2000; Mordenti *et al.*, 2021).

Molasses is cost effective and easily available when compared to other substrates such as lactateupport. It can be utilised by mixed communities that contain both SRB and fermenters, where fermenters aid in the breakdown of the complex molecules. Waligórska *et al*. (2000) also go on to mention that preliminary studies of molasses have shown that the BSR process is significantly influenced by the usable COD content in molasses. Their results showed that a change in molasses concentration from 2% to 5% vol. caused change in the degree of sulphate reduction from 60% to 92% and the largest decrease of COD occurred at 5. Molasses concentrations between 0.2% and 0.4% vol. yielded low conversions of sulphates attributed to the lack/low concentration of usable substrate. pH, at a 5% vol. concentration of molasses, decreased from 7.0 to 4.51 and this decrease was attributed to the formation of acidic products of fermentation which caused a decrease in the conversion of sulphates. Changes in pH were shown to be completely dependent on the concentration of molasses and molasses was shown to be a suitable carbon source and electron donor for the reduction of sulphates by SRB communities (Waligórska *et al.*, 2000;Hussain *et al.*, 2014).

In studies done by Annachhatre and Suktrakoolvait, (2001) in mixed cultures, there was competition between the SRB and methane producing bacteria (MPB) when molasses was used as the substrate of choice. The COD:sulphate ratio determined the dominant species. Hussain *et al*., (2014) used pure SRB cultures in their experiments and concluded that the use of these pure cultures may have caused poor sulphate conversions of around 20%. This was also supported by other studies done such as that by Kaksonen *et al*., (2004) where it was said that diversity in communities improved their stability. It was observed that there was increased sulphate reduction at the beginning of experiments which was attributed to the presence of simpler molecules at the start of the anaerobic process it was followed by a lag phase which may have been dure to the complexity of the substrate which would need to be further broke down (Hussain *et al.*, 2014).

Poor conversion in the molasses fed reactors by Hussain *et al*., (2014) was attributed to the presence of non-biodegradable substances such as caramelised products which may have slowed the action of the SRB. When the molasses conversion was compared to conversion obtained using lactate, lactate achieved maximum conversions of 100% and this supported the claims of the author that molasses is a complex molecule and thus its complexity may hinder fast action by the SRB isolates (Hussain *et al.*, 2014). Lactate is a simple molecule that is easily oxidised. Hussain *et al*., (2014) also commented that better conversion could be achieved through bio-activation by simple molecule substrates such as lactate at the beginning of incubation and then replacing the simple substrate with the chosen complex molecule substrate. Celis *et al*., (2013) used a similar approach where lactate was used as the activation molecule in experiments fed with acetate as the substrate for SRB, a substrate that is not usually easily oxidised.

Honey

Honey sourced from honeybees, contains various sugars like fructose, glucose, sucrose and maltose, some water, trace elements, vitamins, proteins and organic acids(Swallow and Low, 1990; Shugaba, 2012; De Beer *et al.*, 2021). Its composition varies depending on harvest time, geographical and botanical origin. Additional molecules found in the honey such as proteins, vitamins and organic acids support SRB growth, with proteins serving as a nitrogen source which could improve BSR efficiency.

However, honey faces challenges as a BSR organic substrate. It's in high demand with limited availability in South Africa (de Beer *et al*., 2021). Its acidic nature and anti-microbial properties may also pose issues, though these could be mitigated by using low concentrations of honey or pH control could be employed, albeit at an added cost (Mandal and Mandal, 2011)

There is potential for on-site honey production by converting rehabilitated mines into apiaries. Seitz *et al*., (2019) mention the potential of successful beekeeping in anthropologically altered sites such as sand mines, limestone quarries and power line strips and similar habitats. This includes rehabilitation areas in mines or close to mines. Seitz *et al*., (2019) does however, mention that the landscapes to protect the bees are still not well understood and that further study in these anthropologically altered sites should be conducted to ascertain successful beekeeping and honey production.

A report from JONATI Environmental Services, (2019) detailed the advantages of having an apiary in areas that are undergoing rehabilitation. Advantages of beekeeping in these areas included biodiversity, pollination of wild plants, production of food for consumption by humans and animals and genetic diversity of plants which contributes to plant resilience (JONATI Environmental Services, 2019). The report emphasised the need for forage for the bees and thus sites with grasslands that have been growing for years could be more suitable for beekeeping. However, there was concern raised towards a venture into beekeeping on rehabilitated mine sites stating that there were too many variables to consider that would affect the progress and quality of these apiaries, thus, a conclusion was made that the benefit would be more from honey yield rather than improvement in biodiversity and ground cover JONATI Environmental Services, (2019). Additionally, concerns about the quality of the honey were raised. Since the bees are being kept in mine rehabilitation areas, there may be issues of toxins in the honey such as heavy metals. Thus, honey produced at the rehabilitated mine sites may not be safe for consumption by humans and animals. However, it would be suitable for use as substrates in BSR

systems in close proximity to the AMD polluted site; any toxins in the honey would likely be similar to those found in AMD wastewater.

Algal biomass

Algal biomass contains a high COD content from studies conducted by Inglesby, (2011) and Motleleng, (2020). This makes algal biomass a potential substrate for BSR systems. It can be introduced to the system in many different forms such as a bead milled algal biomass mixture containing both solids and liquids, an algal liquid substrate with no solids or an anaerobically digested algal substrate which could potentially have more SRB available COD when compared to the other two algal substrate forms mentioned (Boshoff *et al.*, 2004b; Inglesby, 2011; Motleleng, 2020).

A study by Boshoff *et al.*, (2004b) which investigated the use of algal biomass as a substrate, was conducted from an observation made in algae ponds. Settling of algae in ponds led to active fermentation with the release of hydrogen sulphide in the water, thus, indicating the natural generation of sulphide from the degradation of algal biomass by bacteria. This demonstrated the potential of algal biomass as a substrate in BSR systems. Boshoff *et al.*, (2004b) concluded that the load ratio was an important factor in an algae fed BSR system; lower organic loading rates result in higher sulphate removal. The lower COD:Sulphate ratios decreased the fermenting population as SRB are known to thrive in substrate limiting environments. It was also shown that only 31% of the digestible compounds was used for sulphate reduction (Boshoff *et al.*, 2004b). This suggests that even if the COD content of a complex organic substrate is known, it does not directly show the amount of carbon that could be oxidised in BSR systems. Thus, determining the composition of the algal biomass and the breakdown products may help in determining the actual available COD to the SRB.

Another study used algal biomass in two different forms in BSR systems. Molwantwa *et al.*, (2000) carried out the experiments in a stirred batch reactor system and used extracellular polysaccharide in both a solid form and soluble liquid form from algal cultures as a substrate, with lactate as a control substrate.There was dependence of SRB on the presence of hydrolytic bacteria such as fermenters to breakdown complex molecules in complex organic algal substrates (Molwantwa *et al.*, 2000; Waligórska *et al.*, 2000). The study by (Molwantwa *et al.*, 2000) showed that algal extracellular polysaccharides could be used to treat AMD and may be a low-cost carbon source for BSR systems. For both the solid and liquid extracellular polysaccharides, the COD removal increased with time, but the extracellular polysaccharide supernatant had higher hydrolytic activity compared to the extracellular polysaccharide precipitate (Molwantwa *et al.*, 2000). This could be due to the sugars being more accessible in the liquid extracellular polysaccharide compared to the solid extracellular polysaccharide and the presence of more simple sugars in the liquid extracellular polysaccharide than solid extracellular polysaccharide. Sulphate reduction for both extracellular polysaccharides increased over time with higher sulphate reduction in the mixed culture, thus, showing the supernatant to be a more readily available carbon source. A summary of the results from (Molwantwa *et al.*, 2000)are shown in Table 2-6.

Table 2-6: Sulphate removal and COD removal of pure and mixed sulphate reducing cultures fed on algal extracellular polysaccharide supernatant or precipitate substrate

	Extracellular Polysaccharide Supernatant		Extracellular Polysaccharide Precipitate	
	Sulphate Removal	COD Removal	Sulphate Removal	COD Removal
Pure	40%	33%	6.0%	4.7%
Mixed	57%	52%	22%	21%

Low sulphate removal in extracellular polysaccharide precipitate fed reactors could be from the substrate being a colloidal suspension, with carbohydrates that are not easily accessible to SRB. The mixed culture still showed higher conversions, likely due to the presence of hydrolysing bacteria (Molwantwa *et al.*, 2000). Thus, algal biomass, specifically the liquid extracellular polysaccharide supernatant, has been demonstrated as a suitable substrate for BSR systems.

Another algal biomass option is to digest the algae and produce algal digestate which will contain more usable sugars for the SRB in the BSR systems. Figure 2-4 below from Milledge *et al.*, (2019) shows

some of the simple sugars that can be obtained from the anaerobic digestion of algal biomass which hydrolyses its complex sugars such as cellulose and starch to simple sugars such as glucose and fructose.

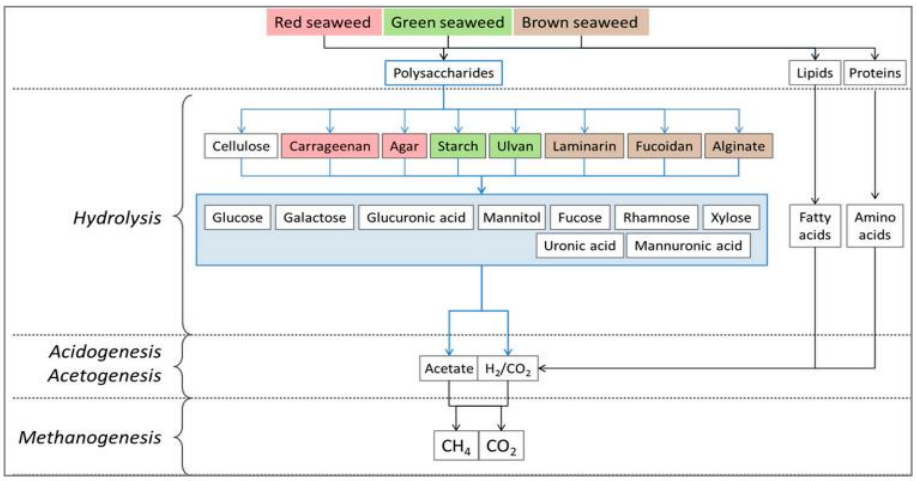


Figure 2-4: Potential simple molecules that can be obtained from the hydrolysis, acidogenesis, acetogenesis and methanogenesis of algal biomass taken from (Milledge *et al*., 2019)

Studies by Motleleng (2020) have shown successful sulphate reduction by SRB using algal digestate with sulphate reductions as high as 95% at a dilution rate of 0.0083 h^{-1} with a feed sulphate concentration of 5 g/l. There were also high conversions of volatile fatty acids (VFAs) and the nitrogen content in the algal digestate could have also improved microbial growth and activity (Motleleng, 2020).

Advantages and disadvantages of inorganic and organic substrates for BSR systems

Table 2-7 shows a summary of the advantages and disadvantages of established substrates and potential alternatives for BSR systems (Kaksonen and Puhakka, 2007).

Table 2-7: Advantages and disadvantages of inorganic and organic substrates for BSR systems

Substrate	Advantages	Disadvantages
H_2 and CO_2	• Supports multiple kinds of SRB • Thermodynamically more favourable to grow SRB on H_2 as opposed other simple carbon sources • SRB outcompete fermenters and methanogens • Economically favourable when high sulphate loads are involved	• Investment costs are high from natural gas reformer • H_2 has a low solubility in aqueous solutions and thus H_2 mass transfer may be a rate limiting step • Safety hazard as H_2 is explosive • Availability may be limited
Lactate	• Is an effective substrate for multiple kinds of SRB • Supports a diverse culture • Has a high biomass yield • Complete oxidation produces high concentrations of alkalinity	• Costly substrate • Low alkalinity concentrations during incomplete oxidation • High COD content in effluent when incompletely oxidised
Acetate	• Acetate consuming bacteria allow for high sulphate removal rates in systems that incompletely oxidise a higher chain substrate	• Metabolised by few SRB • Acetate consuming SRB have a slower growth rate compared to lactate • Methanogens are more competitive in acetate fed systems
Molasses	• Molasses is a waste stream with a high COD content • It is a low-cost option and is readily available • Contains nitrogen which is beneficial to SRB	• Contain complex carbohydrates that may require degradation to simpler, readily available compounds • Competition in system may result in lower sulphate conversions • May contain non-digestible compounds that may accumulate • High COD content in effluent
Algal digestate	• Low-cost production with high VFA and COD content • Simple production process • May contain nitrogen	• High COD in effluent • Extra costs from processing
Honey	• Contains readily available sugars that can be metabolised by SRB	• Not practical to source for BSR systems

Substrate	Advantages	Disadvantages
	• Potential to be generated from the construction of apiaries at rehabilitated mine sites	• At present, costly for BSR systems

Complex, substrates such as honey, molasses and algal substrates have great potential as efficient carbon sources and electron donors in BSR systems. They are generally high in COD content and offer advantages such as being a more low-cost option when compared to lactate and also contain additional nutrients such as nitrogen which are beneficial to SRB. However, most require mixed communities to allow for the breakdown of the substrates so that their COD is more accessible to the SRB and the carbon sources may contain small amounts of toxins which when they accumulate in the bioreactors may be harmful to the SRB; continual removal of these toxins may be necessary to ensure optimum performance in BSR systems.

2.4.10 Sulphide inhibition

In BSR systems sulphate is reduced to sulphide and since most of the SRB in AMD treatment are dissimilatory, sulphide is released externally as dissolved or free hydrogen sulphide and can combine with metals to form sulphide containing metal compounds. Equation 2-8 shows the formation of hydrogen sulphide (Johnson and Hallberg, 2005).

$$substrate + SO_4^{2-} \rightarrow HCO_3^- + HS^- \qquad \text{Equation 2-8}$$

Equation 2-9 below shows the precipitation of metals by hydrogen sulphide ions(Johnson and Hallberg, 2005).

$$Me^{2+} + HS^- \rightarrow MeS_s + H^+ \qquad \text{Equation 2-9}$$

Sulphide is toxic and a pollutant and if not reacted with metals to form solid metal sulphides, it can be released as hydrogen sulphide (Marais, 2020). Hydrogen sulphide tends to have a rotten egg smell which is very distinguishable but upon prolonged exposure gaseous hydrogen sulphide, one can lose the ability to smell the gas which makes it highly dangerous. High exposure will inhibit cell respiration leading to pulmonary paralysis which in turn can result in death (AFROX Hydrogen Sulphide MSDS, 2011). Hydrogen sulphide is fatal at concentrations higher than 700 ppm (AFROX Hydrogen Sulphide MSDS, 2011). In small concentrations it can cause irritation of the mucous membranes, headaches, dizziness, and nausea. It is also toxic to aquatic life. At pH levels between 6 and 8, hydrogen sulphide remains dissolved and so its release as a gas is minimal in a neutral system (Brahmacharimayum *et al.*, 2019). Dissolved sulphide is toxic to aquatic life and may damage submerged metal structures.

Sulphide is an undesirable product in the BSR, therefore, it is necessary to treat hydrogen sulphide to avoid its release into the environment. Table 2-8 below shows the different ways in which sulphide is treated and the advantages and disadvantages associated with each treatment (Tang *et al.*, 2009).

Table 2-8: Physiochemical and biological sulphide treatment methods, advantages and disadvantages

Physiochemical	Biological
Uses chemicals, metal compounds, oxygen (catalysts, solvents, alkalis)	Uses microorganisms (partial and complete oxidation)
Advantages • Highly effective • Simple and efficient process control Disadvantages • Metal sludge formation • Costly and energy demanding	Advantages • Sustainable • Comparably more cost effective compared to physiochemical methods • Waste valorisation (production of elemental sulphur)

Physiochemical	Biological
• Specialty chemicals required	Disadvantages • Process control is challenging and not as predictable compared to physiochemical processes • May result in complete oxidation which produces sulphate; another pollutant

Biological sulphide treatment has shown promising results (Molwantwa, 2007; Tang *et al.*, 2009; Marais, 2020) and there have been many technologies developed for the oxidation of sulphide. The one that is of interest for this research is the sulphur floating biofilm, which was incorporated into a system that reduces sulphate, thus, forming a hybrid system, the hybrid linear flow channel reactor. It is a semi-passive process which seeks to eliminate the problem of sulphide pollutant generation in BSR systems (Molwantwa, 2007; Marais, 2020).

2.5 Hybrid linear flow channel reactor (Hybrid LFCR)

Even though active treatment is efficient in the treatment of high volume and highly acidic AMD, it tends to produce high volumes of sludge waste and is not cost effective in the treatment of low volume AMD which is not highly acidic. Semi-passive biological treatments are an alternative that offer a low-cost solution in the treatment of low volume, circum-neutral AMD and also offer easier process control when compared to completely passive treatment methods. Since the semi-passive biological treatments are also biological, they are more sustainable as they do not pollutant chemicals as seen in active treatments. Hybrid, semi-passive systems are a modification which offer another advantage of linearising the sulphur cycle by partially oxidising the sulphide formed from sulphate reduction to elemental sulphur. Partial oxidation reduces concentrations of sulphide which is known to be toxic and inhibitory to SRB.

A hybrid, semi-passive technology that has been developed is the hybrid linear flow channel reactor (LFCR). It was developed from previous studies and processes such as the Biosulphide® and Pulles Howard and de Lange integrated passive treatment process (Coetser *et al.*, 2004).

The technology simultaneously reduces sulphate to sulphide in the bulk liquid through the metabolism of SRB and oxidises sulphide to sulphur at the liquid-air boundary through the metabolism of SOB. Elemental sulphur produced from the partial oxidation of sulphide is deposited into a floating sulphur biofilm (FSB) which forms at the air-liquid boundary of the linear flow channel reactor (Marais, 2020). Thus, two regions are formed in one reactor which are the anaerobic zone in the liquid phase which remains anaerobic due to the formation of the FSB which impedes oxygen ingress into the liquid phase and the aerobic zone at the air-liquid boundary which is an oxygen limited environment and supports partial oxidation of sulphide (Marais, 2020). The reactor mostly works with gravity, only needing the feed to be pumped into the system. Thus, the BaH-LFCR requires little energy input which decreases operational costs (Marais, 2020).

Sulphide oxidation is facilitated by SOB which can form a floating sulphur biofilm which is structurally sound. The biofilm, as reported by (Molwantwa, 2007) goes through three stages to reach maturation. The first is the thin stage which takes a few hours to form and is transparent, the second is the sticky stage which is opaque and slimy, and the third is the brittle stage which forms a clean break when disturbed (Molwantwa, 2007). Harvesting screens have been used to improve sulphur recovery while reducing the disturbance to the system (Marais, 2020). However, disruption using these screens has been shown to still have adverse effects on hybrid systems as they introduced oxygen into the bulk liquid which has to be maintained at anaerobic conditions (Marais, 2020). Thus, an optimal FSB disrupting and harvesting schedule has to be established to achieve optimal sulphate reduction and sulphur recovery. Some major groups of SOB identified in hybrid semi-passive systems were *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria, Chlorobi*, *Bacteroidetes, Synergistetes* and *Deltaproteobacteria* (Marais, 2020).

In the LFCR, the SRB have a support matrix where they can attach and form a biofilm, thus, the system is able to retain biomass. There are also planktonic cells and associated cells in the reactor. The hybrid LFCR first had carbon microfibres as its support structure to help in biomass retention through attachment (Marais, 2020). However, it was observed that the carbon microfibers would tend to select non-SRB communities over SRB communities (Marais *et al.*, 2024). Drawbacks associated with micro-carbon fibres resulted in PUF being used instead. The foam was used over the carbon microfibers as it works by entrapping cells which form a biofilm and thus, retain more SRB as opposed to the carbon microfibers which require active attachment (Marais *et al.*, 2024). Use of this foam enhanced biomass retention which in turn improved the performance of the system (Marais *et al.*, 2024). Addition of baffles helped with direction of flow within the system and thus increased contact of the wastewater being treated with the active biomass (Marais *et al.*, 2024). The BaH-LFCR configuration also promoted more plug flow and so a sulphate concentration gradient was expected (Hessler, 2020). The baffled reactor overall had higher sulphate reduction, sulphide oxidation and more efficient sulphur harvesting from the floating sulphur biofilm than before the baffles were added (Marais *et al.*, 2024). Zoning and the addition of PUF created different environments for optimum sulphate reduction, increased contact with active microorganisms and also allowed for better substrate utilisation (complete substrate oxidation is more likely) (Marais *et al.*, 2024). The major groups of SRB identified in the LFCR were the *Desulfovibrio* and *Desulfomicrobium* in the lactate fed reactors, the *Desulfobacter* in the acetate fed reactor and small quantities of *Desulfocurvus, Desulfarculus* and *Ruminoccoccacae* (Marais, 2020).

The LFCR system was mostly fed on lactate which at first was mostly incompletely oxidised but the introduction of zoning and stratification in the LFCR allowed for complete oxidation to occur in the LFCR. Before the modification of the LFCR with foam and baffles, it was concluded that the system performed best at low dilution rates with an optimal COD:Sulphate ratio of ≤1 and HRT of 2 days (Marais, 2020). This favoured the proliferation, maturation, and activity of both SRB and SOB. The BaH-LFCR showed high levels of efficiency in both sulphate reduction and partial oxidation of sulphide in HRTs as low as 2 days and had maximum VSRR of 0.169 mmol/L.h and a maximum sulphate conversion of 71% when fed on lactate in a 8 L LFCR (Marais *et al.*, 2024).

2.6 Conclusions on the BaH-LFCR

Though there has been proof of concept shown for the BaH-LFCR, there is still need for improvement in the system as it is set to operate semi-passively at an industrial scale with little to no temperature, pH and sulphate concentration control (Marais *et al.*, 2024). The BaH-LFCR hosts complex, mixed SRB and SOB microbial communities which contain fermenters, and the cells are planktonic, associated or attached creating a robust system that is not as sensitive to change when grown and matured (Marais, 2020). Table 2-9 shows some of the benefits and drawbacks that the lactate fed BaH-LFCR system has.

Table 2-9: Benefits and drawbacks of lactate fed LFCRs

Benefits	Drawbacks
• Low operational costs due to low maintenance requirements, chemical usage and no energy required for heating or cooling • Low waste sludge production • Conversion of toxic sulphide to elemental sulphur • Seeks to increase the recovery of elemental sulphur • Promotes complete utilisation of substrate.	• It uses a lactate as a substrate which is costly over a long period and at a large scale • The BaH-LFCR has only been tested using synthetic, sulphate-rich feed made of Postgate media and has not received real AMD

The technology currently, has mostly used lactate as its substrate of choice and though it is efficient for sulphate reduction, it is not cost effective at a large scale and long term. The BaH-LFCR system requires inexpensive, locally available substrates to run at a large scale over long periods of time. Thus, it is necessary to investigate different substrates to feed into the BaH-LFCR system that also facilitate high sulphate reductions and sulphur recoveries. The BaH-LFCR system has also used a synthetic, sulphate rich feed as opposed to treating true AMD. The synthetic feed is Postgate media which is known to contain additional nutrients for SRB (see Section 3.2). Thus, improvement of the BaH-LFCR in respect to the substrate fed and the treatment of true AMD is imperative to allow for the technology to be applied at a large-scale and over a long-term and determine if the BaH-LFCR is effective in treating sulphate laden, mine impacted water.

2.7 Defining the Research Project

2.7.1 Motivation for research project

The baffled, hybrid linear flow channel reactor (BaH-LFCR) has helped address several of the major disadvantages faced by previous treatment technologies that treat acidic, sulphate rich wastewater. These are the costs associated with active treatment and the lack of process control in passive treatments. The process also facilitates both the reduction of sulphate and the oxidation of sulphide at the air-liquid boundary, where elemental sulphur can be used as a raw material for products such as fertiliser and antifungals. Biomass retention is increased by the presence of a PUF which acts as a support matrix to allow biomass formation. Optimal conditions which include sulphate loading, hydraulic residence time (HRT), temperature, chemical oxygen demand to sulphate (COD:Sulphate) ratio and pH have been established which allow for the best sulphate reduction and sulphide oxidation. Baffles were also added to the system which allowed for control over the flow direction and promoted contact with active biomass and formation of a gradient profile which enhanced the kinetics of the system.

The system has been run on Postgate B media which is a synthetic feed with a set sulphate concentration, contains additional nutrients and is at an optimal pH that supports the development of SRB (Postgate, 1963). However, true AMD does not contain the full suite of macro- and micro- nutrients, and its composition tends to vary depending on the areas it has been collected from. AMD can also contain heavy metals. This, along with fluctuations in pH can result in perturbations to a biological sulphate reduction (BSR) system. Thus, the system needs to be run on real AMD which has been pre-treated to neutralise the stream and simultaneously allow for the removal of heavy metals.

The system has also mostly used the substrate lactate which is not as readily available and is costly on a large-scale, over a long period. Thus, there is need to identify a cost-effective substrate with a high chemical oxygen demand (COD) content that is readily available to feed into the BaH-LFCR system and effectively reduce sulphate.

This research will be a continuation of previous work completed at the (CeBER) on the BaH-LFCR (Marais *et al*., 2024). The project proposed aims to:

- Characterise AMD and test the efficiency of the BaH-LFCR to treat AMD
- Investigate alternative organic substrates with a high sugar content for their efficiency as substrates for SRB in a BSR system

The research study will help achieve the following sustainability developmental goals (SDGs):

- SDG 5: Clean water and sanitation
- SDG 9: Industry, innovation, and infrastructure
- SDG 12: Responsible consumption and production

This is especially important in South Africa which continues to face shortages in water supply but requires large volumes of water for its mining industry. AMD produced from active and inactive mines

can be treated and reused at the mine sites or treated to reach portable water standards (lower limit of 250 mg/l sulphate concentration) (World Health Organization, 2004).

This study will first focus on the investigation of different carbon sources in BSR systems in small-scale reactors. Testing in small-scale reactors allows for multiple reactors to be set-up and tested concurrently allowing for different variables to be compared and also optimises time utilisation. The small-scale tests can be run in fed-batch mode or in small continuous reactors where performance indicators will show which substrate performs the best in the small-scale reactors. The substrate which performs the best will then be the selected alternative substrate which will be fed to the BaH-LFCR.

The AMD will also be pre-treated in various ways and tested in small scale fed-batch reactors concurrently. This will again allow for better comparison of the efficiency of the systems fed with AMD pre-treated in various ways and will optimise time utilisation. Using performance indicators, the best performing reactor's pre-treatment method will be used for the AMD fed to the hybrid LFCR system.

Testing in small scale reactors for both the substrates and AMD will also help assess the mixed culture's ability to utilise an alternative substrate and also its ability to efficiently reduce sulphate and treat AMD. Preliminary tests in small-scale reactors are essential for high throughput which allows for rapid and efficient analysis of many tests, samples, and data points. It also allows for different conditions to be tested concurrently.

2.7.2 Problem Statements and objectives

Problem statements

Lactate, though presented to be a highly effective substrate (electron donor and carbon source) in comparison to substrates such as acetate, ethanol, propionate, and complex organic substrates, cannot be used as a substrate in the large-scale and long-term treatment of acid mine drainage (AMD) as it is not cost effective. This presents a challenge for large scale implementation of the BaH-LFCR as the process may become economically unfeasible if lactate is used. Sourcing of an alternative, complex organic substrate for the AMD treatment process is recognised as a key requirement for implementation but may cause alteration in the microbial community, process dynamics and efficiency which will differ from the established lactate fed system.

True AMD, unlike a synthetic, sulphate-rich feed made up of Postgate B media, lacks some micro- and macro- nutrients and varies in composition depending on where it is sourced from. Thus, the use of real AMD instead of synthetic feed in the BaH-LFCR may negatively impact system performance due to the lack of these nutrients, presence of heavy metals and fluctuations pH.

Research Project Objectives

- Determine the COD content of the different substrates to help establish a COD:sulphate of one in all substrate reactors and compare sugar profiles of the organic substrates (molasses, honey and algal digestate)
- Set up small-scale reactors in different configurations (fed-batch 1 L Schott bottles, continuous 93 ml mini columns and continuous 1L Schott bottles) with PUF-BSPs as a support matrix and a lactate substrate to assess the different reactor configurations' performance in terms of sulphate reduction and susceptibility to oxygen ingress
- Select the best performing reactor configuration and reactor type to assess and compare the performance of mixed cultures on different carbon sources using performance indicators such as sulphate reduction, sulphide concentration, sugar and volatile fatty acid (VFA) utilisation and pH.
- Select a suitable carbon source for use as the alternative organic substrate for the LFCR system based on the small-scale reactor which performed the best and the suitability of the substrate for long-term and large-scale application.

- Determine the chemical composition, physical qualities (pH, redox and conductivity) and trace elements of the supplied AMD which will help inform the suitable pre-treatment methods for the AMD before it is fed to the mixed culture.
- Setup and assess different pre-treatment methods in small-scale fed-batch reactors (1 L Schott bottles) packed with PUF-BSPs as a support matrix using performance indicators such as sulphate reduction, sulphide concentration, sugar and VFA utilisation and pH.
- Select a suitable pre-treatment method for AMD fed to the LFCR system based on the AMD small-scale fed-batch reactor which performed the best.
- Evaluate the performance of the LFCR system on a synthetic feed based on Postgate media B (Postgate, 1963) with a lactate substrate to be used as the base case. Performance of the system is determined using performance indicators (sulphate reduction, sulphide concentration, sugar and VFA utilisation, volumetric sulphate reduction rate (VSRR), sulphide oxidised, pH, redox and sulphur recovered).
- Evaluate the performance of the LFCR system on pre-treated AMD with a lactate substrate and compare it to the LFCR base case. Performance of the system will be determined using performance indicators.
- Evaluate the performance of the LFCR system on pre-treated AMD and the alterative substrate and compare it to the LFCR fed with pre-treated AMD with a lactate substrate. Performance of the system will be determined using performance indicators.

2.7.3 Research hypothesis and key questions

Hypothesis 1

Locally available, organic substances such as molasses, honey or algal biomass with high sugar content can be used as an alternative carbon source to lactate in BSR systems. The high COD content of complex and simple sugars in these substrates will result in readily available and accessible carbon sources for efficient sulphate reduction in a mixed culture which contains SRB and fermenters.

Hypothesis 2

Characterisation and preliminary testing of different AMD pre-treatment methods in BSR systems will enable identification of the AMD pre-treatment method required to enable the use of established optimal conditions similar to those used on the synthetic feed in the BaH-LFCR which allow for effective sulphate reduction by SRB and sulphide oxidation by SOB.

Key Research Questions

- How readily available are the complex organic substrates?
- Do complex molecules (carbohydrates) in the organic substrates require conversion to simple sugars?
- Is the presence and activity of fermenters in the mixed culture sufficient to breakdown the complex molecules to simple sugars readily available to SRB?
- How much of the complex organic substrate is required to provide enough COD for sulphate reduction and sulphur recovery?
- Can the ideal hydraulic residence times and COD:Sulphate ratios obtained using lactate be maintained using the complex organic substrates?

- Are the sulphate conversions obtained in a system fed with the alternative organic substrates similar to those of the lactate fed system?
- Will the real AMD require pre-treatment before being introduced into the BSR system and if so, what pre-treatment steps will be required?
- Can the ideal HRT and COD:Sulphate ratios determined for a synthetic AMD feed containing sulphate and lactate be maintained using real AMD?
- Is there evidence of inhibitors present in the real AMD?
- Is there evidence of side reactions occurring in the real AMD?
- Does the switch to real AMD from synthetic feed and the switch from lactate to an alternative substrate affect the efficiency of the LFCR system?

3 Materials and Methods

This study, primarily quantitative in nature, was conducted at the Centre for Bioprocessing Research (CeBER) laboratories at the University of Cape Town (UCT). It built upon the work of Marais (2020), comparing system performance of the hybrid LFCR.

The research focused on collecting and analysing numerical, measurable data. Qualitative observations were also noted to supplement the quantitative data.

The experimental setup involved four reactor types: fed-batch 1 L Schott bottles, continuous 1 L Schott bottles, continuous 93.3 ml mini-columns, and an LFCR system comprising of a primary and secondary reactor. Small-scale reactors (Schott bottles and mini-columns) were employed to test different organic substrates as well as various AMD pre-treatments before scaling up to the BaH-LFCR system. The best-performing organic substrate and pre-treatment method from these tests was applied to the BaH-LFCR system. Results were compared with literature and lactate-fed systems serving as a base case.

3.1 Inoculum

Linear flow channel reactors and up-flow anaerobic packed bed reactors (UAPBRs) at UCT were originally inoculated with a mixed sulphate-reducing culture originally sourced from Professor Peter Rose (Department of Microbiology, Biochemistry and Biotechnology – Rhodes University). This culture, extracted from an anaerobic compartment of a facultative pond located at the Grahamstown sewage treatment works in 2001 was maintained at UCT's CeBER labs on modified Postgate Media (Postgate, 1963; Marais, 2020). These reactors subsequently inoculated all fed-batch reactors in this study. Marais, (2020)'s previous work has revealed dominant SRB species to be *Desulfarculus*, *Desulfovibrio*, *Desulfocurvus* and *Desulfomicrobium* when using a lactate substrate (Marais, 2020). Other species belonged to *Bacteroidetes, Fimicutes*, *Synergistetes* and *Veilloella classes* (Marais, 2020). Dominant sulphur oxidising species included *Paracoccus*, *Hallothiobacillus* and *Arcobacter* while *Rhizobium* and *Pannonibacter dominated the FSB*.

Two reactor types, mini-columns and Schott bottles, were used for the small-scale continuous substrate testing. Both were inoculated with 20% culture inoculum from the fed-batch reactors which were adapted to the different substrates. Each fed-batch reactor, originally inoculated from LFCR and UAPBRs, contained PUF-BSPs to increase biomass retention. The foam was also transferred to the continuous reactors as it contained immobilised cells.

Four AMD fed-batch reactors were set up and fed with AMD that had been pre-treated in different ways. Two of the four fed-batch reactors, the one fed with untreated AMD and the other fed with lime-treated AMD were inoculated with 30% SRB culture from the LFCR and UAPBRs. The reactor fed with lime and sodium sulphate treated AMD was inoculated with 50% subculture from lime-treated AMD fed-batch reactor and the fourth fed-batch reactor which received sterilized and lime-treated AMD, was inoculated with 50% subculture from the lactate-fed-batch reactor.

3.2 Media Composition

Conventional Postgate B media (Postgate, 1963; Marais, 2020) - referred to as synthetic feed - was used for reactor maintenance and base case experiments. Synthetic feed was also used in the substrate small-scale reactors described in Section 3.3 and 3.4.

To investigate a real-world scenario, sulphate-rich AMD, generated from fine waste and discards sourced from coal mines in the Mpumalanga region, was used as a feed instead of synthetic feed. The AMD was fed to small-scale fed-batch reactors and the BaH-LFCR which are described in Section 3.5 and 3.6 respectively.

The synthetic feed was neutral with a sulphate concentration of 2 g/L and contained 0.42 g/L KH_2PO_4, 1 g/L NH_4Cl, 2 g/L $MgSO_4.7H_2O$, 1.8 g/L Na_2SO_4, 0.2 g/L yeast extract and 0.3 g/L sodium citrate (Postgate, 1963). The media was autoclaved (121°C, 20 minutes) and cooled before use. This feed was then supplemented with one of the following carbon sources at a COD:sulphate ratio of 1: lactate, acetate, honey, molasses or algal lysate.

The AMD used was produced at UCT's CeBER greenhouse in a pilot study that was assessing the prevention of AMD through packing regime at a barrel scale (Kotsiopoulos *et al.*, 2022). Coal discards (CD) and fine waste (FW) tailings were mixed in a CD:FW ratio of 3:2 and packed in PVC barrels (200 kg, aspect ratio 1.8) and irrigated with neutral or acidic water (Kotsiopoulos *et al.*, 2022). AMD from 6 differently packed and irrigated barrels was collected, mixed, and stored in a 100 L barrel. Composition and characteristics (sulphate concentration, iron concentration, heavy metal content, pH and conductivity) were determined as per Section 3.7.

Three treatment methods were used to treat the AMD and four AMD fed-batch reactors were started. The four different feeds were untreated AMD, lime-treated AMD, lime and sulphide-treated AMD and lime-treated AMD that was sterilised at 120°C for 20 minutes. Hydrated lime (40-42% Ca(OH)2, 29-30% MgO, 25-27% H2O) was used (National Lime Association, 2023) to make a 24 ml lime slurry (100g lime/200ml water) which was added to 10L of AMD. The pH was adjusted to be between 8.5-9 for metal precipitation (Aubé, 2003; Balladares *et al*., 2018). The precipitates settled at the bottom and the clarified water on top was collected. For the reactor that received both lime and sulphide treatment, sodium sulphide was added in excess to the collected clarified water to remove more dissolved metals by precipitation. The metal sulphide precipitates were then removed by filtration.

The LFCR reactor system experiments used three types of feed prepared in 10 L Schott bottles. The first feed was the synthetic feed made of Postgate B media with a 2 g/l (20.8 mmol/L) sulphate concentration and sodium lactate (3.2 ml/l of 60% w/w) was used as the organic carbon source. The second feed was AMD that was pre-treated based on the AMD fed-batch reactor results and substituted with lactate. The third experiment received the same sterilised, pre-treated AMD feed but was substituted with molasses as the carbon source. Molasses was pasteurized separately (106°C, 30 minutes) to avoid Maillard reactions and was then added aseptically to the autoclaved AMD.

All reactors, regardless of feed type, received a 1:1 COD:sulphate ratio to favour SRB over fermenters (Maillacheruvu and Parkin, 1996; Oyekola, 2008).

3.3 Substrate Fed-batch Reactor Experiments

3.3.1 Reactor Set-up, Operating Conditions, and Sampling

Five fed-batch reactors were set up with a different substrate: lactate, molasses, honey, acetate or algal lysate. The molasses, honey and acetate reactors were inoculated several months before the study and algal lysate and lactate fed-batch reactors were inoculated at the start of the experiments (30% culture inoculum from the UAPBRs and the LFCR (Hessler, 2020; Marais, 2020)).

The fed-batch reactors (1 L Schott bottles) with PUF-BSP packing were used to evaluate different carbon sources at 30ºC. Foam was used as it works by entrapping cells which form a biofilm and thus, retain more SRB as opposed to the carbon microfibers which require active attachment (Marais *et al*., 2024). Reactors were manually shaken before sampling to ensure homogeneity. Cycles ended when residual sulphate concentration stabilized, indicating minimal sulphate reduction. Subculturing was performed using a 50% draw and feed method. Each substrate reactor underwent a minimum of two cycles during the experiment.

3.3.2 Substrates and Substrate Preparation

Four substrates were tested: three were undefined/complex (honey, molasses, algal lysate) and one defined (acetate). Lactate served as a base case.

Dark, blackstrap molasses (stored at 4ºC) and raw, unprocessed honey (stored at room temperature) were used. Algal lysate was produced from Spirulina, a cyanobacterial species that is simple to grow and contains both complex and simple sugars (Inglesby, 2011).

Spirulina, isolated from a wastewater pond at a Western Tanning Company outside Wellington, South Africa, was cultivated in Zarrouk's media. 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 $NiSO_4.7H_2O$ and 4.2 mg/L $CoSO_4.7H_2O$) at a concentration of 1 ml/L. The culture was grown in a 200 ml glass bottle which was aerated with humidified air that had been filtered through a 0.22 µm syringe filter and illuminated by white, fluorescent lamps (approx. 120 µmol photons.m^{-2}.s^{-1}).

After cultivation in the 200 ml bottle, the culture was transferred to a 1 L Schott bottle aerated with filtered air and grown until densely populated (a dark, opaque green culture). Next, the whole culture was harvested and transferred to 500 ml Beckman centrifuge bottles (Beckman Coulter Inc., California, USA). The Beckman bottles were centrifuged at 4000 rpm for 20 minutes in the Beckman Coulter, Avanti J-E centrifuge with a JA-10 rotor and the supernatant was discarded. The algae pellet at the bottom of the bottle was re-suspended in deionised water and the concentration was determined spectrophotometrically. A Jenway 7305 spectrophotometer was used to determine the OD at a wavelength of 750 nm. The algae cells were then disrupted by bead milling for two hours using 1 mm glass beads and a Rushton turbine with a diameter of 20 mm stirring at a speed of approximately 900 rpm. To determine whether the Spirulina cells had been lysed, an Olympus CX40 light microscope at a 100X magnification setting was used to observe the bead milled algal suspension. The cell lysate was then centrifuged for 20 mins at 4000 rpm using the Beckman Coulter, Avanti J-E centrifuge with a JA-10 rotor to separate out the cell debris and obtain the supernatant which will be referred to as the algal lysate substrate.

3.3.3 Substrate Characterisation

Sugar and COD concentrations of the undefined substrates were determined theoretically and experimentally, using the Merck COD reagent set 1.14555 HR and a digestion block, and through high performance liquid chromatography (HPLC) (see Section 3.7.7 and 3.7.7). Theoretical sugar content and a maximum theoretical oxygen demand were calculated from literature data.

Experimental COD values were expected to deviate from theoretical maximums due to potential variations in substrate composition. The experimentally determined COD was used to calculate substrate amounts needed for a COD:sulphate ratio of 1, optimal for SRB function (Oyekola, 2008).

3.4 Substrate Continuous Experiments

3.4.1 Reactor Set-Up, Operating Conditions and Sampling

Mini-column reactors

Mini-column reactors (glass, 19 cm height, 2.5 cm diameter, 93.3 ml volume) were packed with colonised PUF-BSPs taken from the relevant 1L Schott bottle reactors. Rubber rings on lids were to minimize oxygen ingress. Two ports on the bottom and top of the reactor were for a feed inlet, effluent output, and sampling. Up-flow feeding was to promote some degree of stratification and improve substrate utilization (see Figure 3-1).

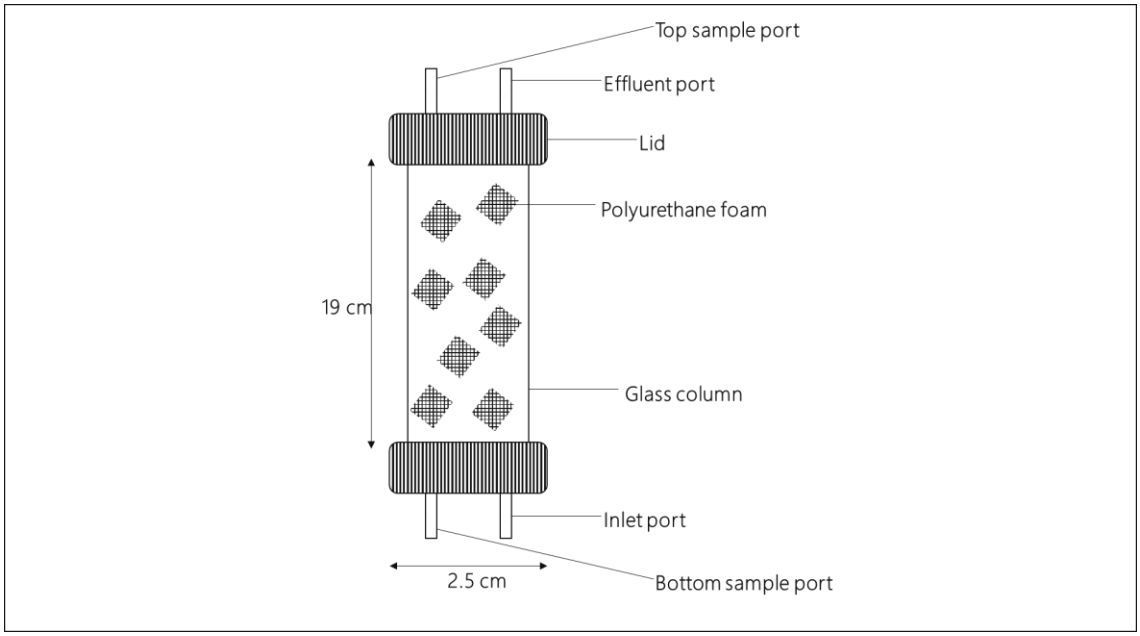


Figure 3-1: Continuous mini-column reactor schematic used in the continuous reactor substrate experiments

The start-up procedure started with pumping synthetic feed into the tubing until it was filled and then clamping the tubing before moving the reactor to a 30°C room. The reactor was then operated in batch mode until >50% sulphate conversion had occurred and then it was switched to continuous operation at a 5-day HRT. Reactors were maintained at room temperature during continuous operation.

Schott bottle reactors

Schott bottle reactors (1 L) were packed with PUF-BSPs for biomass retention. Feed was entered from the bottom to promote stratification, with effluent discharged from the top by gravity (see Figure 3-2).

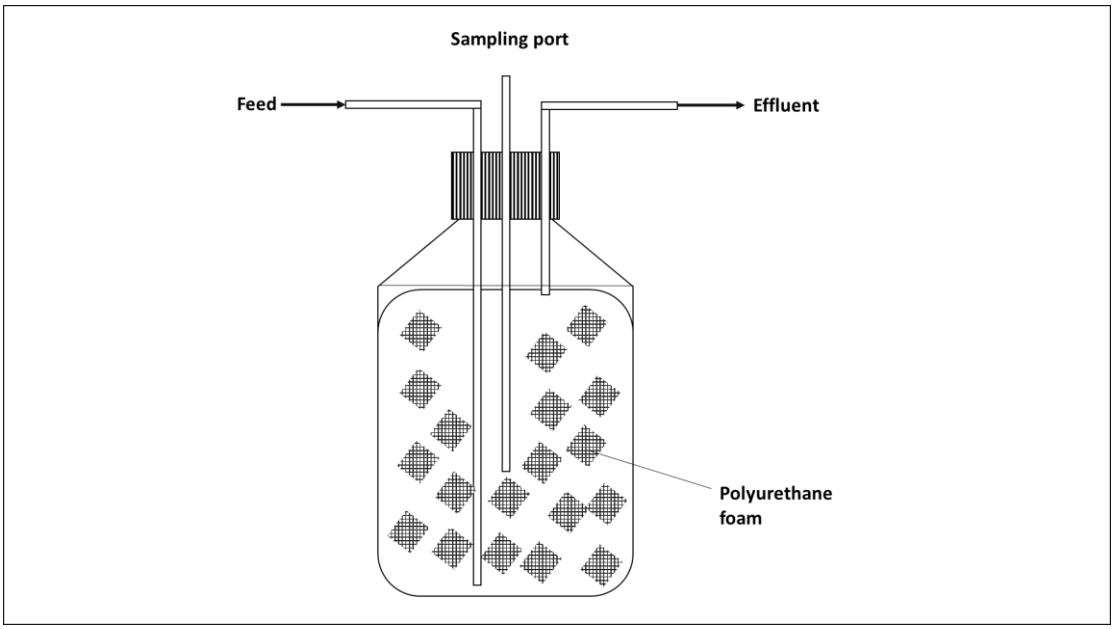


Figure 3-2: Continuous Schott bottle reactor schematic used in the continuous reactor substrate experiments

Two millilitre samples were taken from the middle and top for analysis. Start-up and operation were similar to the column reactors where the reactor was run in batch mode at 30°C until >50% sulphate conversion was achieved. It was then switched to continuous operation at room temperature where a 5-day HRT was maintained.

3.5 AMD Fed-batch Reactor Experiments

3.5.1 Reactor Set-Up, Operating Conditions and Sampling

AMD fed-batch tests assessed SRB culture performance on differently pre-treated AMD streams and their setup included 1 L Schott bottles kept at 30°C and packed with PUF-BSPs. They were agitated before sampling.

Cycles ran until the sulphate concentration stabilized and subculturing was the 50% draw and feed method, supplementing AMD and carbon source. Each fed-batch underwent a minimum of two cycles of successful sulphate reduction.

3.6 Linear Flow Channel Reactor Experiments

3.6.1 Reactor Set-Up, Operating Conditions and Sampling

The LFCR system consisted of two semi-passive, open Perspex channel reactors which had a wall thickness of 11 mm and are connected in series (Figure 3-3). The primary reactor (8 L volume, 450 mm (L) x 200 mm (W) x 150 mm (H)) had four baffles placed across the width for zoning and promotion of plug flow to improve sulphate reduction and substrate utilization by creating a gradient profile. PUF between the baffles acted as a support matrix to allow for better biomass retention. It had 9 sampling ports, with samples taken from ports 1, 3, 5, 7 and 9 (see Figure 3-3). Feed stored in 10 L bottles, was pumped into the reactor by a Cole-Parmer 7519-25 Master Flex L/s Cartridge pump and two screens placed just below the liquid surface were used to harvest the FSB.

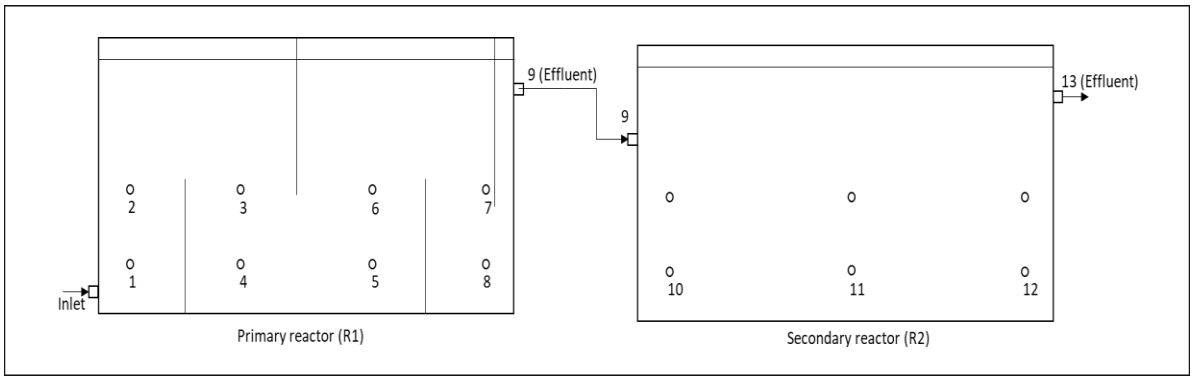


Figure 3-3: Schematic of the primary LFCR with baffles and secondary LFCR connected in series.

The secondary reactor, designed to increase sulphide removal efficiency and sulphur recovery, had a lower volume without baffles or foam. It had a single harvesting screen and four functional sampling ports. Samples were taken from port 10, 12 and 13. Effluent port 13 first went to a clarifier to increase sulphur recovery and then to a waste bottle. Both reactors were sampled every 2-3 days, with a minimum of 5 samples per cycle.

3.6.2 Reaction cycles, pseudo-steady state, and harvesting

Experiments were run in reaction cycles defined as the period between consecutive FSB harvests. Each run consisted of four residence time cycles to allow FSB maturation. Pseudo steady state was determined from the final 1.5 residence times of each cycle, during which sulphate conversion in the primary reactor had at most 10% variation across samples. At least 3 samples were obtained within this period, and their average was used to calculate average conversions and concentrations (sulphate, sulphide and VFAs), while data from the entire four residence time cycle informed sulphide-to-elemental sulphur conversion. Standard deviation of pseudo-steady state data includes only the sample points from Section 3.8.1 during the specified time period.

FSB harvesting involved lifting and scraping trays placed just below the liquid surface. Harvested biofilm was dried at 37°C for at least 8 days and ground to a powder before representative samples were taken and submitted for Carbon-Hydrogen-Nitrogen-Sulphur (CHNS) analysis (Section 3.7.4).

3.6.3 Synthetic feed with lactate substrate experimental operation

A hydraulic residence time study was conducted on the LFCR system experiment with a synthetic, sulphate rich feed and a lactate substrate to find the optimum HRT. HRTs of 5, 4, 3 and 2 days were tested, based on the primary reactor's 8 L working volume. Each HRT condition ran for at least two cycles, each of which consisted of 4 HRTs with the biofilm harvested after each cycle. This duration allowed for FSB maturation even at the shortest 2-day HRT.

3.6.4 AMD feed with lactate substrate experimental operation

For the AMD + lactate experiment, the mixed culture was gradually adapted to the pre-treated AMD feed by increasing the proportion of AMD in the feed from 20% to 100% in 20% intervals and decreasing the proportion of Postgate media by the same degree. A 1:1 COD:sulphate ratio was maintained. The adaptation process involved:

The use of an optimal HRT of 3 days which achieved sufficient sulphate reduction in the synthetic feed + lactate LFCR system experiment. For the adaptation period, which ran from 20% to 80% AMD feed concentration, the cycle ran for a varied amount of HRTs and was stopped when a significantly high sulphate conversion was achieved and the last three points of conversion were within less than 5% standard deviation. There were two 100% AMD feed runs which were also run at a 3-day HRT for 4 HRTs to allow for comparison and repeatability demonstration of the synthetic feed + lactate experiment and the AMD + molasses experiment run at a 3-day HRT.

3.7 Analytical Methods

3.7.1 Chemical Reagents

All chemicals and reagents used throughout all experiments were analytical grade and were supplied by Merck, Kimix, Sigma-Aldrich, Accsen Instrumental, Thermo Fisher Scientific and LabChem.

3.7.2 Sulphate Analysis

Residual sulphate concentrations were determined turbidometrically using the barium sulphate assay (American Public Health Association (APHA), 1975). Sulphate ions react with barium chloride to form barium sulphate, a white precipitate with a turbidity proportional to sulphate concentration.

$$SO_{4,aq}^{2-} + BaCl_{2,S} \xrightarrow{HCl} BaSO_{4,s} + 2Cl_{aq}^{-} \qquad \text{Equation 3-1}$$

The sulphate analysis procedure started with dissolved sulphide being removed by the addition 40 ul of 10% $ZnCl_2$ to a 2 ml sample. The sample was then centrifuged at 12,000 rpm for 10 minutes in an Eppendorf mini-Spin plus centrifuge (diameter: 8.5 cm) at room temperature to pellet ZnS. After, 50 µl supernatant was extracted and mixed with 4.95 ml deionized water and 250 µl conditioning solution (75 g NaCl, 30 ml 32% HCl, 50 ml glycerol, 100 ml ethanol, 300 ml deionized water). A micro-scoop of $BaCl_2$ powder was then added and the mixture was vortexed for >20 seconds. The absorbance was measured at 420 nm.

Sulphate concentration was calculated using a standard curve (0-50 mg/L) prepared with Na_2SO_4 solution (Figure A.1 1).

3.7.3 Sulphide Analysis

Dissolved sulphide was quantified using the colorimetric methylene blue technique (CLINE, 1969). Figure 3-4 taken from (Hassan *et al.*, 2002) shows the reaction that occurs for methylene blue to form.

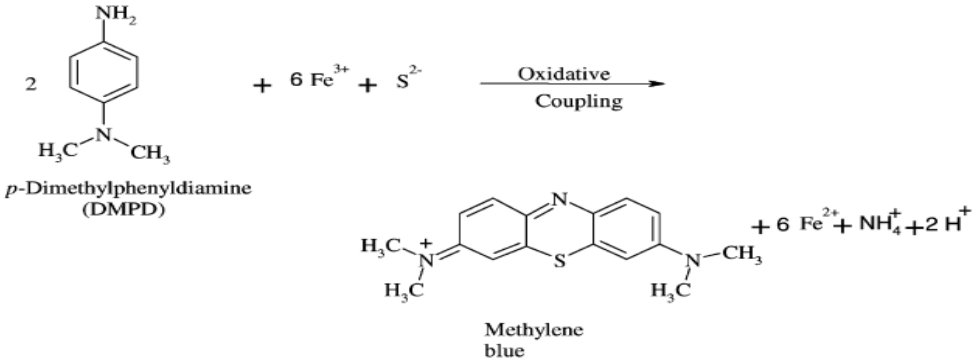


Figure 3-4: Reaction for the quantification of dissolved sulphide through the formation of methylene blue taken from (Hassan *et al.*, 2002)

Sulphide analysis was performed by mixing 200 µl of 1% zinc acetate with 5 µl of the reactor sample to stabilize dissolved sulphide. The volume was made up to 5 ml using deionized water before the addition of 500 µl each of 4 g/L N,N dimethyl-p-phenylenediamine dihydrochloride (DMPD) solution (4 g/L) and ferric chloride (16 g/L), both which were dissolved in 6 mM HCl. The mixture was then vortexed for 5 seconds, allowed to stand for 5 minutes for colour development and then absorbance was measured at 670 nm.

A standard curve (0-1 mg/L) was prepared using $Na_2S.9H_2O$ (Figure A.2 1, Section A.2). Methylene blue forms when sulphide reacts with DMPD in acidic conditions.

3.7.4 Floating Sulphur Biofilm Analysis

Representative samples of the FSB that had been dried as described in Section 3.6.2, were sent for Carbon-Hydrogen-Nitrogen-Sulphur (CHNS) analysis at the Central Analytical Facilities (CAF) at the University of Stellenbosch, South Africa. The elemental percentage of sulphur relative to carbon, hydrogen, nitrogen and inorganics, was determined through combustion using an Elementar EL Cube Elemental Analyzer. All species of sulphur were oxidised to sulphur dioxide gas which was measured using an RI detector.

3.7.5 pH, Redox and Conductivity Measurements

All samples from the fed-batch reactors, continuous reactors and the LFCR system were measured for pH using the Jenway 3510 pH meter fitted with a XS sensor 2-pore T DHS pH probe and the pH probe was calibrated using standard buffering solutions of pH 4.0 and 7.0 from Accsen Instrumetal. For the redox measurements, only samples taken from the effluent port of the primary and secondary reactor were measured. Redox was measured using a Metrohm Ion Analyser 827 pH lab meter fitted with a Ag-ring. Conductivity was measured using 86501 AZ Multi-parameter Benchtop Water Quality Meter – pH/ORP/Cond./TDS/Salinity. Samples of the AMD before and after pre-treatment were the only samples measured for conductivity.

3.7.6 COD Analysis

In the analysis of COD content, the COD measured is the amount of oxygen that originates from potassium dichromate that has reacted with oxidisable substances contained in a volume. Merck COD reagent set (1.14555 HR) for concentrations between 100 – 1500 mg/L COD was used in conjunction with a digestion block and a spectrophotometer to measure the COD content of the selected substrates and samples from the BSR reactors. The measurement was dependent on the oxidation of the sample

by a hot sulphuric acid solution which also contained potassium dichromate and a silver sulphate catalyst. Chloride was masked by mercury sulphate and the concentration of the unconsumed $Cr_2O_7^{2-}$ ions or of the Cr^{3+} ions was determined spectrophotometrically at a wavelength of 605 nm. Potassium hydrogen phthalate was used to prepare a standard curve (Figure A.3 1).

3.7.7 Sugars and Volatile Fatty Acids (VFA) Analysis

Samples were prepared for analysis by centrifugation for 10 minutes at 12,000 rpm in an Eppendorf MiniSpin® plus centrifuge (diameter: 8.5 cm). Samples were then diluted at a 1:1 ratio with mobile phase and well mixed before being filtered through a 0.22 µl syringe filter into a vial.

Sugars and VFAs were measured using high performance liquid chromatography (HPLC) and included sucrose, glucose, fructose, lactic acid, acetic acid, propionic acid, citric acid and ethanol. A Thermo Scientific System was used with the refractive index (RI) detector for sugars and ethanol and the ultra-violet (UV) detector for VFAs. A Bio-Rad organic acids column (Aminex HPX-87H, 30 cm x 7.8mm, 9 µm) was used for the analysis and acidified ultra-pure water (5 mM H_2SO_4) pumped at 0.5 ml/min was used as mobile phase. A column temperature of 40°C and a UV detection wavelength of 210 nm was maintained. Run time was 30 minutes and concentrations of the sugars, VFAs and alcohol were determined using standard curves with concentration ranges between (0-1.0 g/L) which were generated at the beginning of each run. The retention times are shown in Table A.4 2 in Section A.4.

3.7.8 Cation Analysis

Major, minor and trace elements were measured at Central Analytical Facilities, Stellenbosch University, South Africa. This was done through inductively coupled plasma mass spectrometry (ICP-MS) on a Thermo ICap 6300 ICP AES for major and minor elements and on the Agilent 7900 ICP-MS or Agilent 8800 QQQ ICP-MS for trace elements. Quantification of the data was done using calibration solutions prepared from the National Institute of Standards and Technology (NIST) traceable standards.

3.7.9 Total Iron Analysis

Total iron concentrations were determined using hydroxylamine and 1-10 Phenanthroline in a colorimetric orange red complex between Fe^{2+} and 1-10 phenanthroline technique. Equation 3-2 shows the conversion of Fe^{3+} ions to Fe^{2+} using hydroxylamine which allows for the total iron to be measured.

$$2NH_2OH + 4Fe^{3+} \rightarrow N_2O + 4Fe^{2+} + 4H^+ + H_2O \qquad \text{Equation 3-2}$$

The procedure involves mixing 2 ml acetate buffer (700 ml glacial acetic acid + 250 g ammonium acetate in 150 ml deionized water) and 2 ml 1-10 phenanthroline indicator solution (2.13 g 1-10 phenanthroline, $C_{12}H_8N_2.H_2O$, in 1 L deionized water) together in a test-tube. One millilitre of diluted sample is then added to the test-tube together with a micro-scoop of hydroxylamine. The mixture is vortexed for 10-20 seconds. After 5 minutes of colour development, absorbance was measured at 510 nm.

A standard curve, shown in Figure A.5 1, Section A.5, for concentrations between 0-50 mg/L Fe^{2+} was used to determine the iron concentration. An alternative method using ICP-MS was also used (detailed in Section 3.7.8).

3.7.10 Scanning Electron Microscopy and Elemental Analysis

As has been reported previously in (Marais, (2020), samples of the FSB from the LFCR were prepared for scanning electron microscopy (SEM) analysis and elemental analysis. FSB samples extracted from the LFCR during harvesting were fixed in 2.5% (v/v) glutaraldehyde solution at 4°C overnight before being rinsed with 1x phosphate buffer solution. The sample was then dehydrated by soaking in increasing concentrations of ethanol (30%, 50%, 90%, 95% and finally 100% (v/v)) for approximately 10 minutes. After drying, the samples were stuck onto a 10 mm aluminium pin stub using carbon glue and treated with a few drops of hexamethyldisilazane (HDMS) to bring them to a critical drying point.

The stub was then coated with gold palladium alloy before being placed on the scanning electron microscope stand. A FEI NovaNano SEM was used to visualise the FSB sample while energy dispersive X-ray spectroscopy (EDS) was performed to determine elemental percentage of selected sections of the FSB sample.

3.8 Data Handling

3.8.1 Bulk Residual Concentrations

The bulk residual concentrations determined were sulphate, sulphide and VFAs.

Fed-batch reactors

Each fed-batch reactor was sampled at least once a week. For each sample assayed, duplicates or triplicates were taken and averaged to calculate the bulk residual concentrations. Conversions for each cycle were calculated using the initial sulphate concentration taken just after subculturing and the final concentration taken just before subculturing.

Continuous reactors

The continuous columns were sampled at the bottom and the top of the reactor while the continuous Schott bottle reactors were sampled at the middle and top of the reactor. The bulk residual concentrations were the average of the duplicates or triplicates of the bottom and top sample points and the middle and top sample points from the different assays of the continuous columns and Schott bottle reactors respectively.

LFCR system

For the primary LFCR, the bulk residual concentrations were the average of sample point 1,3,5, and 7. The bulk residual concentrations for the secondary reactor were the average of sample points 10,12, and 13.

3.8.2 Sulphate Conversion

The sulphate conversion, given as a percentage, was calculated as follows:

$$X_{sulphate}(\%) = \frac{[sulphate_0]\left(\frac{mmol}{L}\right) - [residual\ sulphate]\left(\frac{mmol}{L}\right)}{[sulphate_0]\left(\frac{mmol}{L}\right)} \times 100\% \qquad \text{Equation 3-3}$$

Where $[sulphate_0]$ is the initial concentration of sulphate

[residual sulphate] is the bulk average sulphate concentration as described in Section 3.8.1.

For the secondary reactor, the sulphate conversion was calculated as follows:

$$X_{sulphate}(\%) = \frac{[sulphate_9]\left(\frac{mmol}{L}\right) - [residual\ sulphate]\left(\frac{mmol}{L}\right)}{[sulphate_9]\left(\frac{mmol}{L}\right)} \times 100\% \qquad \text{Equation 3-4}$$

Where $[sulphate_9]$ is the concentration of sulphate in the effluent port of the primary LFCR feeding the secondary reactor

3.8.3 Expected Sulphide Concentration

The expected sulphide was a theoretical calculation based on the sulphate conversion. It was the sulphide expected if no partial oxidation of sulphide occurred and all sulphur in sulphate went to sulphide. The expected sulphide in the primary LFCR was calculated as follows:

$$[expected\ sulphide\ ]\left(\frac{mmol}{L}\right) = \frac{[sulphate_0]\left(\frac{mg}{L}\right) - [residual\ sulphate]\left(\frac{mg}{L}\right)}{M_{sulphate}} \quad \text{Equation 3-5}$$

Where m is a theoretical ratio of sulphate to sulphide

$M_{sulphate}$ is the molar mass of sulphate (96,06 mg/mmol)

For the secondary LFCR, the expected sulphide was calculated as follows:

$$[expected\ sulphide\ ]\left(\frac{mmol}{L}\right) = \left(\frac{[sulphide_9]\left(\frac{mg}{L}\right)}{M_{sulphide}}\right) + \left(\frac{[sulphate_9]\left(\frac{mg}{L}\right) - [residual\ sulphate]\left(\frac{mg}{L}\right)}{M_{sulphate}}\right) \quad \text{Equation 3-6}$$

Where $[sulphide_9]$ is the concentration of sulphide in the effluent port of the primary LFCR feeding the secondary reactor

$M_{sulphide}$ is the molar mass of the hydrosulphide ion (33.08 mg/mmol)

3.8.4 Sulphide Oxidised

The sulphide oxidised to elemental sulphur in the primary and secondary LFCRs was calculated as follows:

$$X_{sulphide}(\%) = \frac{[expected\ sulphide]\left(\frac{mmol}{L}\right) - [residual\ sulphide]\left(\frac{mmol}{L}\right)}{[residual\ sulphide]\left(\frac{mmol}{L}\right)} \times 100\% \quad \text{Equation 3-7}$$

Where the residual sulphide is the bulk average as described in Section 3.8.1.

3.8.5 Sulphide converted to elemental sulphur

The sulphide converted to sulphur in the primary reactor was calculated using the concentration of feed sulphate, residual sulphate and sulphide concentration in the reactor effluent stream as shown in the Equation 3-8.

$$sulphide\ converted\ to\ sulphur_{R1}\left(\frac{mmol}{L}\right) = [expected\ sulphide_{R1}]\left(\frac{mmol}{L}\right) - [residual\ sulphide_{R1}]\left(\frac{mmol}{L}\right) \quad \text{Equation 3-8}$$

For the secondary reactor, the effluent sulphate and sulphide concentration of the two LFCR reactors were used as shown in Equation 3-9.

$$sulphide\ converted\ to\ suphur_{R2}\left(\frac{mmol}{L}\right) = [expected\ sulphide_{R2}]\left(\frac{mmol}{L}\right) - [residual\ sulphide_{R2}]\left(\frac{mmol}{L}\right) \quad \text{Equation 3-9}$$

3.8.6 Total sulphur amount predicted (amount sulphide converted to sulphur)

The expected elemental sulphur produced in the LFCRs per run could not be calculated as a bulk average as seen with the sulphate, sulphide and VFA concentrations and the elemental sulphur produced was a function of the expected sulphide concentration and the effluent sulphide concentration. The FSB accumulated over time and so the sulphur produced between each data point had to be accounted and thus the expected sulphide and effluent sulphide concentrations were a function of time in days, the dilution rate and the volume of the reactor. The expected sulphide for calculating the expected sulphur was calculated as follows:

$$ES_n(mmol) = [expected\ sulphide]_{n,p}\left(\frac{mmol}{L}\right) \times (T_n - T_{n-1})(h) \times D\left(\frac{1}{h}\right) \times V(L) \quad \text{Equation 3-10}$$

Where ES is the expected sulphide amount for calculating the expected sulphur

n is the sample point

n-1 is the sample point before sample point n

p is either primary or secondary LFCR

T is time in days

V is the volume of the reactor

The effluent sulphide for calculating the expected sulphur was calculated as follows:

$$RS_n(mmol) = [\ residual\ sulphide]_{n,q}\left(\frac{mmol}{L}\right) \times (T_n - T_{n-1})(h) \times D\left(\frac{1}{h}\right) \times V(L) \quad \text{Equation 3-11}$$

Where EFS is the effluent sulphide amount for calculating the expected sulphur

q is the effluent port for the primary reactor (9) or the secondary reactor (13)

Thus, the expected sulphur for each run was calculated as follows:

$$Sulphide\ converted\ to\ sulphur\ (mmol) = \left(\sum_{i=1}^{n} ES\right)(mmol) - \left(\sum_{i=1}^{n} RS\right)(mmol) \quad \text{Equation 3-12}$$

It was assumed that all the sulphide that did not exit the reactor in the effluent was converted to elemental sulphur.

3.8.7 Sulphur Recovered

Sulphur recovery was calculated in terms of the feed sulphate as shown in Equation 3-13 for the primary reactor and Equation 3-14 for the secondary reactor.

$$Sulphur\ recovery(\%) = \frac{[sulphide\ converted\ to\ sulphur_{R1}]\left(\frac{mmol}{L}\right)}{[sulphate_0]\left(\frac{mmol}{L}\right)} \times 100\% \quad \text{Equation 3-13}$$

$$Sulphur\ recovery(\%) = \frac{[sulphide\ converted\ to\ sulphur_{R2}]\left(\frac{mmol}{L}\right)}{[sulphate_0]\left(\frac{mmol}{L}\right)} \times 100\% \quad \text{Equation 3-14}$$

3.8.8 Volumetric Sulphate Loading Rate (VSLR)

Amount of sulphate available for reaction per volume per time calculated as follows:

$$VSLR\left(\frac{mmol}{L \cdot h}\right) = [sulphate_0]\left(\frac{mmol}{L}\right) \times D\left(\frac{1}{h}\right) \quad \text{Equation 3-15}$$

Where D is the dilution rate

For the secondary LFCR, the VSLR was calculated as follows:

$$VSLR\left(\frac{mmol}{L \cdot h}\right) = [sulphate_9 \frac{mmol}{L}] \cdot D\left(\frac{1}{h}\right) \quad \text{Equation 3-16}$$

3.8.9 Volumetric Sulphate Reduction Rate (VSRR)

The VSRR was the rate at which sulphate was removed in the BSR system per time and volume and was calculated as follows:

$$VSRR\left(\frac{mmol}{L \cdot h}\right) = \left(\left[sulphate_0 \frac{mmol}{L}\right] - \left[residual\ sulphate \frac{mmol}{L}\right]\right) \cdot D\left(\frac{1}{h}\right) \quad \text{Equation 3-17}$$

For the secondary reactor, the VSRR was calculated as follows:

$$VSRR\left(\frac{mmol}{L \cdot h}\right) = \left(\left[sulphate_9 \frac{mmol}{L}\right] - \left[residual\ sulphate \frac{mmol}{L}\right]\right) \cdot D\left(\frac{1}{h}\right) \quad \text{Equation 3-18}$$

3.8.10 Volumetric sulphur formation rate (VSFR)

Rate at which sulphur is formed within the reactor is calculated as follows:

$$VSFR\left(\frac{mmol}{L \cdot h}\right) = [sulphide\ converted\ to\ sulphur]\left(\frac{mmol}{L}\right) \cdot D\left(\frac{1}{h}\right) \quad \text{Equation 3-19}$$

3.8.11 Substrate Utilisation

Substrate conversion was calculated as follows:

$$X_{substrate}(\%) = \frac{[substrate_0]\left(\frac{mmol}{L}\right) - [residual\ substrate]\left(\frac{mmol}{L}\right)}{[substrate_0]\left(\frac{mmol}{L}\right)} \times 100\% = \quad \text{Equation 3-20}$$

Where [substrate$_0$] is the initial concentration of substrate such as sugars, VFAs or ethanol

[residual substrate] is the bulk average sulphate concentration as described in Section 3.8.1

The volumetric substrate reduction rate (VSRR) was calculated as follows:

$$VSRR\left(\frac{mmol}{L\cdot h}\right)=\left([substrate_0]\left(\frac{mmol}{L}\right)-[residual\ substrate]\left(\frac{mmol}{L}\right)\right)\cdot D\left(\frac{1}{h}\right) \quad \text{Equation 3-21}$$

3.8.12 Metal Iron Removal

Using data gathered from the ICP-MS analysis detailed in Section 3.7.8 of the AMD before and after pre-treatment, the percentage of metals removed from the AMD during pre-treatment was calculated as follows:

$$Metal\ removal\ (\%)=\frac{[metal_0]\left(\frac{mmol}{L}\right)-[metal]\left(\frac{mmo}{L}\right)}{[metal_0]\left(\frac{mmol}{L}\right)}\times 100\% \quad \text{Equation 3-22}$$

Where [metal$_0$] is the initial concentration of a specific metal before pre-treatment in the AMD

[metal] is the final concentration of the metal after pre-treatment in the AMD

4 Investigation of Alternative Carbon Sources

4.1 Introduction

The choice of carbon source and electron donor has been highlighted as a key consideration to enable a techno-economically favourable BSR process (Gopal, 2004; Harrison, 2014). This chapter focuses on the further investigation of carbon and electron donor sources for BSR to find an alternative, suitable carbon source for large-scale and long-term implementation in a baffled hybrid linear flow channel reactor (BaH-LFCR) for treatment of sulphate-rich waters with the recovery of elemental sulphur. A defined substrate and several complex substrates were investigated, selected based on their cost and availability. These were compared to the well-established substrate, lactate, which is known to be effective in BSR systems and to contribute to process robustness. Lactate is expensive when considering treatment over a long-term and large-scale. Performance of each carbon source was determined in both fed-batch and continuous configuration in small-scale reactors.

Several small-scale reactor configurations were considered for this study. Selection of the best performing reactor configuration was based on sulphate reduction efficiency, substrate utilisation and susceptibility to oxygen ingress when using a lactate carbon source and a synthetic, sulphate laden feed. Two reactor types were tested to find the best configuration: the mini column reactor (93 ml) operated with a SRB community retained on PUF-BSPs and run in a continuous configuration; and the Schott bottle (1L), run in both fed-batch and continuous configuration. The selected configuration and reactor type was then used to test the four organic substrates (acetate, molasses, honey and algal lysate) and compare their performance to the base case organic source, lactate. The best performing substrate in terms of sulphate reduction efficiency and sulphide formation in comparison to the other carbon sources and the lactate base case while on a synthetic, sulphate-laden feed, was then used as the alternative carbon source in the LFCR system.

The specific objectives addressed in this chapter are:

- Determine the COD content of each substrate (carbon source and electron donor) to establish the desired COD:sulphate ratio of 1.0 in all substrate reactors.
- Compare sugar profiles of the organic substrates (molasses, honey and algal digestate)
- Set up small-scale reactors in each configuration (fed-batch 1 L Schott bottles, continuous 93 ml mini columns and continuous 1L Schott bottles) with PUF-BSPs and lactate as the carbon source and electron donor to assess the performance of the reactor configurations in terms of sulphate reduction and susceptibility to oxygen ingress.
- Select the most robust and best performing reactor configuration and reactor type for use in the assessment and compare the performance of the mixed cultures on different carbon sources using performance indicators such as sulphate reduction, sulphide concentration, sugar, and volatile fatty acid (VFA) utilisation and pH.
- Select a suitable carbon source and electron donor for use as the alternative organic substrate for the LFCR system based on the small-scale reactor performance and the suitability of the substrate for long-term and large-scale application.

4.2 Characterisation of natural complex organic materials

4.2.1 Introduction

The substrates chosen for consideration as alternative carbon source to lactate were molasses, honey, and algal lysate. Dark, blackstrap molasses and raw unprocessed honey were used. Molasses, being a by-product of the sugar industry, is a cheaper alternative than lactate which has been the substrate used extensively to develop the LFCR and deliver proof of concept. Lactate is very effective in sulphate reduction; however, it is impractical for large-scale and long-term treatment as it is not cost effective. Natural honey was used as the second complex substrate owing to the potential for its production at rehabilitated mine sites would make it a readily available substrate produced close to AMD generating sites to cut down transport costs, while the danger that environmental toxins from the mine site would preclude it from use as a food. The third complex substrate used was an algal lysate as algal biomass and has potential to be produced on-site, making it readily available.

Preliminary tests on the degradability and suitability of these complex substrates as well as acetate and lactate in small-scale BSR reactors were done. This assisted in determining which substrate was most effective in biological sulphate reduction. Since the complex organic substrates were undefined, they were characterised in terms of their COD content to enable maintenance of a COD:sulphate ratio above 1.0 which supports oxidation of the carbon source with concomitant biological sulphate reduction over its fermentation. Characterisation also enabled determination of the amount of substrate required (volume/mass) for sulphate treatment as it is a major contributor to raw material and transportation costs. Acetate was also tested as a potential substrate. As a by-product of many fermentation processes and often present in anaerobic digestate, it is readily available and a cheaper alternative than lactate.

4.2.2 Results and Discussion

A literature-based estimate of the COD content of the complex, organic substrates was determined by sourcing a representative composition of each substrate from literature and calculating the theoretical oxygen requirement for complete oxidation of each sugar or VFA in the substrate to yield a theoretical COD. A sample calculation of the theoretical COD can be found in Appendix A.6. To determine the experimental COD content, two methods were used: (1) the direct experimental COD analysis described in Section 3.7.6 and (2) the calculation of COD from the sugar and VFA analysis as described in Section 3.7.7. On analysis, VFAs and alcohols were found to be present in the complex substrates in negligible concentrations and so did not contribute to the final COD concentration. Thus, the final COD concentration using approach (2) was calculated from the sugar concentration measured by HPLC using the theoretical COD method described in Appendix A.6. Table 4-1 compares the three COD concentration estimates obtained for each substrate.

Table 4-1: Literature derived and experimentally determined COD content of complex, organic carbon sources. Sample calculation for the COD estimated from the sugar and VFA content can be found in Appendix A.6.

Substrate	COD Estimate derived from Literature	COD Estimate derived by COD Analysis	COD Estimate derived by Sugar & VFA Analysis and associated conversion to COD*
Molasses (g COD/g substrate)	0.558-1.79 (Palmonari *et al.*, 2020)	0.759	0.511
Honey (g COD/g substrate)	0.752-0.909 (Swallow and Low, 1990)	0.826	0.538
Algal lysate (g COD/ml substrate)	0.0412-0.0450 (Inglesby, 2011)	0.0313[a]	0.000198[b]

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

[b]COD content calculated directly from a sample of pure algal lysate 0.02 g biomass/L (resuspension at a 1:100 ratio and measured at an OD of 750 nm to give a 0.020±0.001 absorbance)

*COD values derived from HPLC analysis represent only the quantifiable organic components (e.g., sugars, VFAs) and do not capture total oxidisable organic content. Thus, the actual total COD may be higher than indicated, especially for complex substrates such as molasses and algal lysate

Some variation was found between COD values determined experimentally using COD analysis and the average of the COD contents derived from literature, likely due to source and composition of the natural organic substrates being different for the literature obtained values. The experimentally determined COD analysis for molasses and honey agreed with the range given in literature. As the biomass concentration of the algal culture used to provide the literature-derived COD quoted for algal lysate in Table 4-1 was only measured at 605 nm and not 750 nm, the algal lysate experimental COD data could not be compared directly.

The COD values for the molasses and honey determined by theoretical calculation based on sugar concentrations determined by HPLC, were the lowest compared to the literature derived values and those from COD analysis. This is likely due to only considering the sugars, VFAs and alcohols, as mentioned in Section 3.7.7 while other compounds such as aconitic acid, pyrocarbonic acid, and unhydrolyzed proteins were not considered (Swallow and Low, 1990; Palmonari *et al.*, 2020). Unlike honey and molasses which are composed mostly of sucrose, glucose and fructose, algal lysate contains both simple molecules and complex long chain molecules (Gouda *et al.*, 2022). These complex molecules such as long chain carbohydrates, proteins and lipids were not assayed. Thus, the COD concentration for algal lysate obtained from HPLC under-estimates that present greatly.

From the three methods used to calculate the COD concentration of the different complex substrates, the values from the experimental COD analysis were used to determine the amount of organic substrate required to achieve a COD:sulphate ratio of 1.0. Neculita and Zagury (2008) report HPLC analysis of sugars and VFAs to provide a more accurate estimation of readily degradable carbon sources, for fairly well-defined carbon sources and where hydrolysis of all complex molecules is undertaken. When a wider range of molecules is needed for a carbon source with long chain polysaccharides and polypeptides that have not been hydrolysed such as algal lysate, use of HPLC may not be suitable is they are not easily eluted or may not be detectable. Using a COD concentration measurement that fully accounts for available COD is important to achieve a COD:sulphate ratio which ensures that the carbon source remains limiting; this allows for sulphate reduction to dominate over fermentation making the BSR system more efficient (Maillacheruvu and Parkin, 1996; Oyekola *et al.*, 2010).

4.3 Selection of Small-scale Reactors using Lactate

4.3.1 Introduction

In BSR processes, many small-scale reactors have been assessed to monitor whether they are effective in sulphate reduction. For efficient biological sulphate reduction to be achieved, maintaining an anaerobic environment is important as many SRB are obligate anaerobes, with the performance of those that are facilitative also impacted by oxygen concentration. To maintain a high sulphate conversion rate in BSR systems, fill and draw systems and continuous systems are used to accommodate ongoing supply of sulphate at dilute concentrations. SRB are slow growing and volumetric sulphate reduction rates (VSRRs) are the product of the specific reduction rate and biomass concentration; thus, cell retention is important in continuous reactors. Owing to the development of BSR systems for use in the continuous flow LFCR, all reactors used in this study contained biomass support structures. Presence of a support structure within continuous reactors greatly improves biomass retention and biofilm formation allowing for higher sulphate reduction rates.

Small-scale benchtop bioreactors have been used for many years to assist in providing optimal growth conditions for microorganisms (Obom *et al.*, 2013). An advantage of using multiple small volume reactors for the carbon source experiments is the ability to test different carbon sources simultaneously at the same conditions: pH, temperature, and feed sulphate loading. They allow for easy control of reactor conditions such as temperature or pH while also allowing multiple conditions to be tested simultaneously. In the case where new conditions are introduced e.g. the concentration or nature of substrate, small-scale reactors allow optimisation in a smaller volume prior to the desirable change being introduced at a large-scale or pilot scale, thereby avoiding system failure where the change in condition has a negative effect.

Two reactor configurations were considered for BSR, fed-batch and continuous reactors. Batch reactors are less susceptible to contamination, and they are also less prone to washout of essential species and subsequent overgrowth of unwanted species which often occurs in continuous configurations (Brahmacharimayum *et al.*, 2019). Inclusion of support structures in the reactors allows for better cell retention both on subculturing the fed-batch reactor and in the continuous bioreactor. PUF-BSPs was used as a support structure within the Schott bottle fed-batch reactors allowing cells to be retained within the interstitial spaces in the foam. Fed-batch reactors have the disadvantage of build-up of unwanted substances that are toxic or inhibitory to SRB such as sulphide or incompletely oxidised substrate such as acetate (Marais, 2020). Further, the substrates and other nutrients become depleted with time and thereby limiting which makes the BSR process less efficient. A combination of the build-up of toxins and the limitation of nutrients and substrates results in lower cell growth.

Continuous reactors offer an advantage of continuous supply of reactants and removal of products. This allows steady state conditions to be established such that the system can be studied under defined conditions. Further, it allows better reaction kinetics to be obtained, allowing for higher sulphate reduction rates, owing to replenishment. SRB communities can be held in the exponential phase in continuous reactors and, where retention is effective, be held in the stationary phase as an alternative. Where stratification is enabled within the reactors (plug flow configurations), continuous reactors also allow for better substrate utilisation (Hessler *et al.*, 2018). Owing to their continuous configuration, these reactors may be more prone to contamination, build-up of unwanted microorganisms to compete with SRB and oxygen ingress when operated on a small scale, compared to fed-batch operation.

Similar to the fed-batch reactors, the continuous reactors also contained PUF-BSPs. These BSPs were pre-colonised with the mixed sulphate reducing community which had been adapted to the specific organic substrates used in each continuous reactor. Improved cell retention is important in continuous operation to prevent washout of bacteria where their growth rate is below their removal rate. In reactors with support structures, both planktonic and attached cells are present. In reactors where no support structures are present, the planktonic cell culture travels with the liquid phase, making planktonic cells more vulnerable to washout. The support matrix may also protect both planktonic and retained cells from toxins and inhibitors owing to diffusion limitations. In continuous systems with planktonic cells, the

HRT has strong influence on cell retention. A short HRT increases the chances of washout and decreases contact time between the SRB and the sulphate-rich feed, decreasing sulphate conversion. Too long an HRT may result in substrate and nutrient shortage and the build-up of toxins (Oyekola *et al.*, 2009). The presence of support structures in BSR reactors promotes biomass retention which improves the efficiency of sulphate reduction and decouples the HRT from the biomass retention (Hessler, 2020; Marais *et al.*, 2020).

Since both the fed-batch and continuous configurations have benefits and drawbacks or limitations, both reactor configurations and the three reactor set-ups were used to investigate the different organic substrates.

4.3.2 Material and methods

The following small-scale reactors were tested for the selection of a suitable small scale reactor system for the carbon source experiments:

- 1 L Schott bottles as fed-batch reactors
- 1 L Schott bottles in continuous configuration,
- 93 mL mini columns in continuous configuration.

Lactate, as a base case in subsequent sections, was used as the substate in these tests. All three reactor set-ups contained PUF-BSPs as a support. The fed-batch reactors were not continuously agitated but were mixed by swirling directly before sampling to extract a representative sample. The continuous reactors were also not mixed throughout the experiment. The feed was introduced at the bottom of the reactor with effluent tubing positioned at the top to create some degree of stratification within the reactor. This allowed a gradient profile to form within the reactor, promoting substrate utilisation and sulphate reduction in a manner supportive of the culture conditions (Hessler, 2020).

The 1 L Schott bottle fed-batch reactors were easy to set-up with a small head space and formed a tight seal when closed which allowed for an anaerobic environment. The fed-batch reactors were sampled once a week to minimise the number of times the reactor was open to the air.

The Schott bottle and mini column reactors operating continuously were tightly sealed with lids that had rubber seals to reduce oxygen ingress and were sampled from the top or middle and bottom. The continuous reactors were run in batch mode until ≥50% of the initial sulphate was reduced before they were switched to continuous operation. This combined with the filling of the feeding and effluent tubing was done to help curb reactor failure due to oxygen ingress. Achieving a ≥50% conversion of sulphate, increased the sulphide concentration in the small-scale reactors which helped eliminate oxygen introduced from the switch to continuous operation.

4.3.3 Results and Discussion

Fed-Batch Schott Bottle Reactor

The lactate fed-batch Schott bottle reactor (1 L) ran for 3 cycles, almost 180 days, subculturing between each cycle. From Figure 4-1, a clear trend of sulphate reduction and sulphide formation after subculturing was observed. The 1st cycle had the poorest sulphate conversion out of the three cycles. This could have been due to the culture adjusting to the new conditions of a higher sulphate loading and an increase in temperature to 30°C. Previously the culture had been maintained in a LFCR on a synthetic feed with a 1 g/L feed sulphate concentration at room temperature.

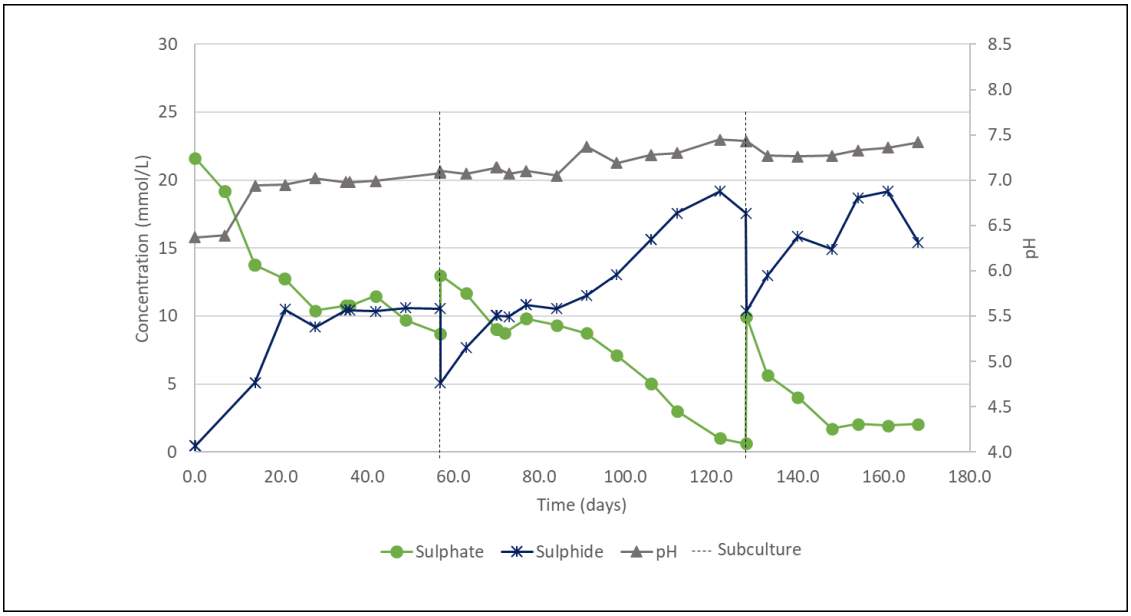


Figure 4-1: Sulphate and sulphide concentrations and pH time trends of the lactate Schott bottle fed-batch reactor

The average sulphate conversion over the three cycles was calculated to be 78.1% with a maximum sulphate conversion of 95.1% in the second cycle; this was the highest maximum conversion amongst all the other organic substrate fed-batch reactors. Similarly, sulphide formation was high, yielding a highest maximum sulphide concentration and an average maximum sulphide concentration that were equal across the last two cycles of 19.2 mmol/L. Good sulphate conversion within the lactate fed-batch reactor was expected as lactate is known to be an efficient carbon source for biological sulphate treatment. The pH within the reactor remained between pH 6.5 and 7.5 which is conducive for SRB function and as expected, during each cycle, the pH slightly increased due to the reduction of sulphate by SRB which produces alkalinity as shown in Equation 2-8. Overall, the fed-batch reactor running on a lactate substrate was effective in reducing the sulphate in the synthetic, sulphate-laden feed. However, an average cycle length of 30 days shows that the fed-batch Schott bottle reactor had poor reaction kinetics.

Continuous Mini Column Reactors

The mini column reactor (93 ml) was fed with a synthetic, sulphate-laden (20.8 mmol/L sulphate) feed which had a COD:sulphate ratio of 1 using a lactate substrate. The reactor ran for 50 days at a 5-day HRT in continuous operation at room temperature. Figure 4-2 A shows the sulphate trends of the mini column reactor running on a lactate substrate.

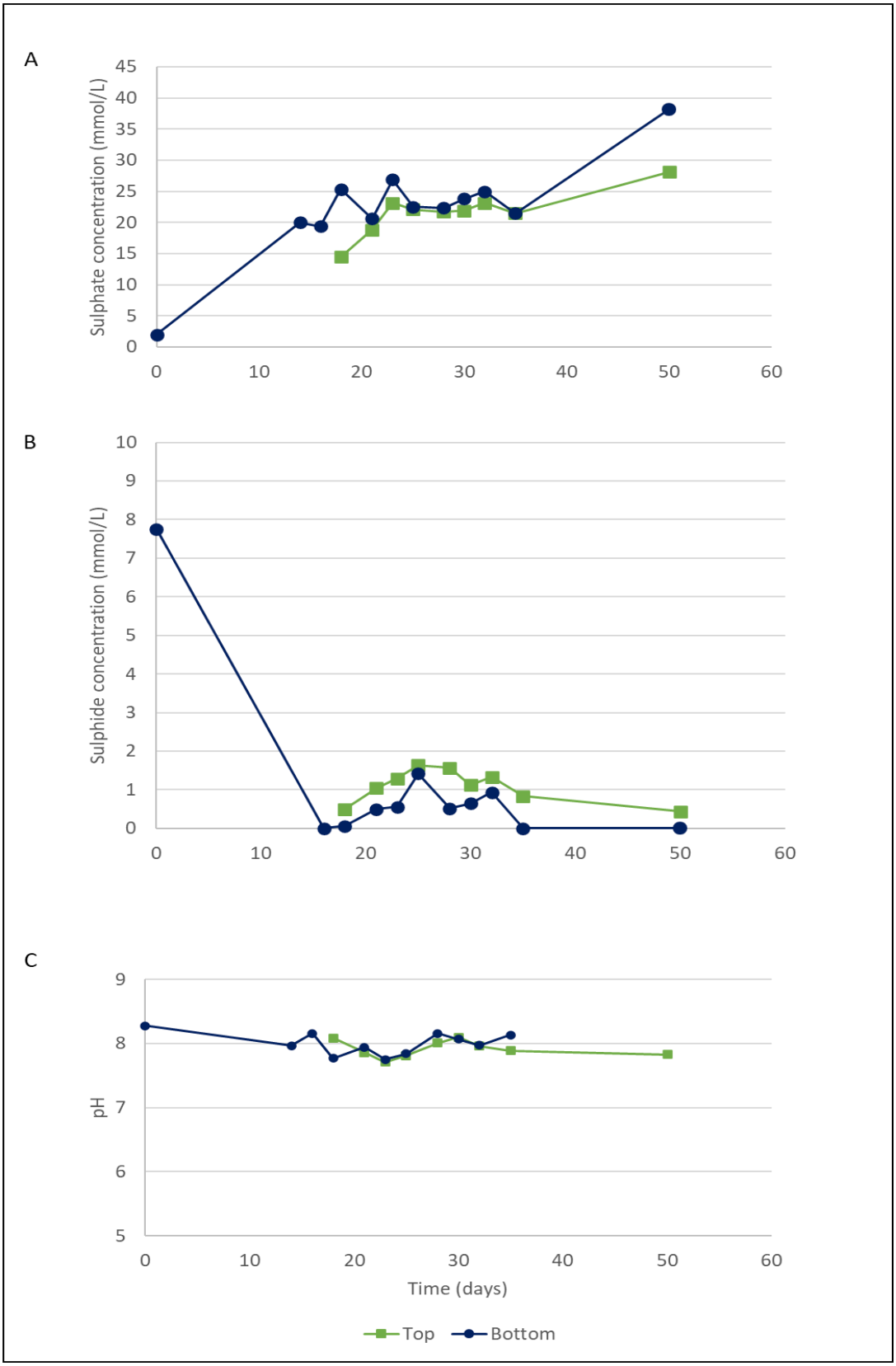


Figure 4-2: Sulphate and sulphide concentrations and pH time trends of the lactate-fed continuous, mini column reactor.

Analysing the graph, the sulphate concentration, before it was switched to continuous operation at day zero, was lower than 5 mmol/L at the bottom of the reactor where the feed entered. This showed that, in batch operation the mini column was able to reduce the sulphate feed by 90.5%. The sample from the top of the reactor was not taken at this time point as there was not yet enough volume to allow this

sample to be extracted. However, a similar sulphate concentration is assumed due to the presence of the foam across the length of the reactor with immobilised cells from the mixed, sulphate reducing community.

Once switched to continuous operation, the reactor was left to run for 14 days without sampling to minimise oxygen ingress from sampling. Samples taken on day 18 showed sulphate concentration had increased to 19.4 mmol/L and 14.5 mmol/L at the bottom and top respectively. A lower concentration at the top of the reactor showed that sulphate conversion was occurring along the reactor, but the sulphate conversion had decreased in continuous operation when compared to the conversion in batch operation. Regular sampling showed the sulphate concentration from the bottom and top of the reactor increase to above 20 mmol/L after day 18 and fluctuated between 20 mmol/L and 30 mmol/L from day 18 to around day 35. This showed that re-oxidation was occurring within the reactor as the sulphate concentrations at the bottom and top of the reactor were higher than the feed sulphate concentration of about 20 mmol/L. Sulphide that had previously been formed from sulphate reduction had been re-oxidised back to sulphate and combining with the sulphate entering the reactor, likely caused the sulphate concentration to increase above 20 mmol/L. Sampling was stopped for 15 days, to assess if sulphate conversion would recover with the elimination of oxygen ingress from sampling. A sample taken after the 15 days, on day 50, from the top of the reactor was above 25 mmol/L indicating the reactor was not reducing any sulphate.

Analysis of sulphide trends of the same reactor complemented the sulphate trends (Figure 4-2 B). Just after switching from batch to continuous operation, on day zero, the sulphide concentration was high at roughly 8 mmol/L. After the first 18 days the sulphide concentration had dropped to almost 0 mmol/L for both the bottom and top of the column. Sulphide concentration throughout the experiment, after the first 18 days, remained below 2 mmol/L showing that there was very poor sulphate reduction in the reactor.

The pH of the mini column reactor was also monitored, and the trend is shown in Figure 4-2 C. The pH remained largely within the optimal range, fairly neutral, between 7.7 and 8.0 but was slightly high at the beginning because of significant sulphate reduction that occurred in batch configuration before the switch to continuous.

Re-oxidation within the reactor may have been due to oxygen ingress into the columns or feed bottles through the tubing, the ports, or the reactor openings; the effect of this would be aggravated by the reactors small size in comparison to reactors such as the 1 L Schott bottles and the 8 L LFCR.

Owing to the small size of the reactors and the extensive re-oxidation, 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 4-3 and Figure 4-4 show the results from these tests.

Dissolving resazurin in water containing oxygen makes a violet-purple solution which irreversibly turns pink when oxygen is still present (Knapp *et al.*, 2018). When oxygen is removed the solution turns colourless and can turn back to pink when oxygen is re-introduced. Figure 4-3 shows that the feed, before autoclaving, contains oxygen, the resazurin changed from a violet-purple to a fluorescent pink. 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, remained colourless showing that there was no oxygen ingress into the feed bottle and feed liquid when it was left standing (before feeding into the continuous system).

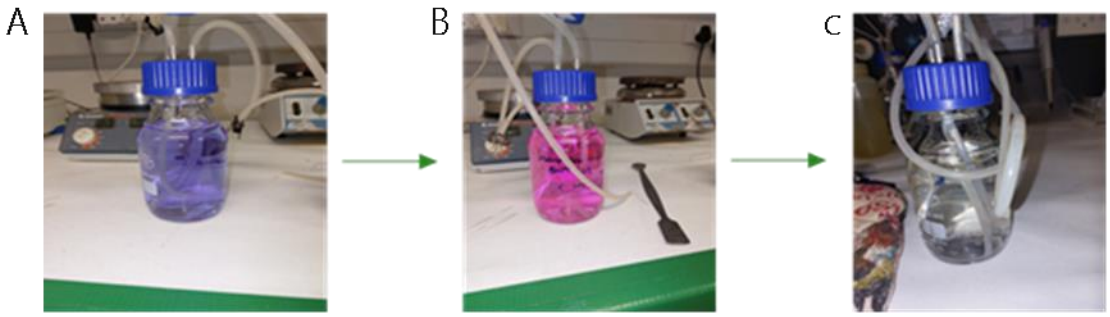


Figure 4-3: Oxygen ingress monitoring of feed bottle using resazurin dye: A) feed bottle after dissolving resazurin dye turned violet-purple in the presence of oxygen, B) feed bottle left to sit, irreversibly turns from violet-purple to pink in the presence of oxygen and C) feed bottle turns from pink to colourless post autoclaving showing all oxygen from the feed had been removed

Figure 4-4: Mini column (set-up and batch operation) dissolved oxygen test using resazurin dye: A) mini-column reactor after dissolving resazurin dye turned violet-purple in the presence of oxygen, B) transition change from violet-purple to pink in the presence of oxygen C) mini-column reactor left to sit, irreversibly turns from violet-purple to pink in the presence of oxygen and D) mini-column reactor left to sit in an anaerobic chamber to remove dissolved oxygen E) mini-column reactor turns from pink to colourless after sitting in the chamber for 48 hours showing all dissolved oxygen had been removed

Figure 4-4 shows resazurin tests in a mini column which contained water without PUF-BSPs to allow clearer dye visibility. It 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. 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.

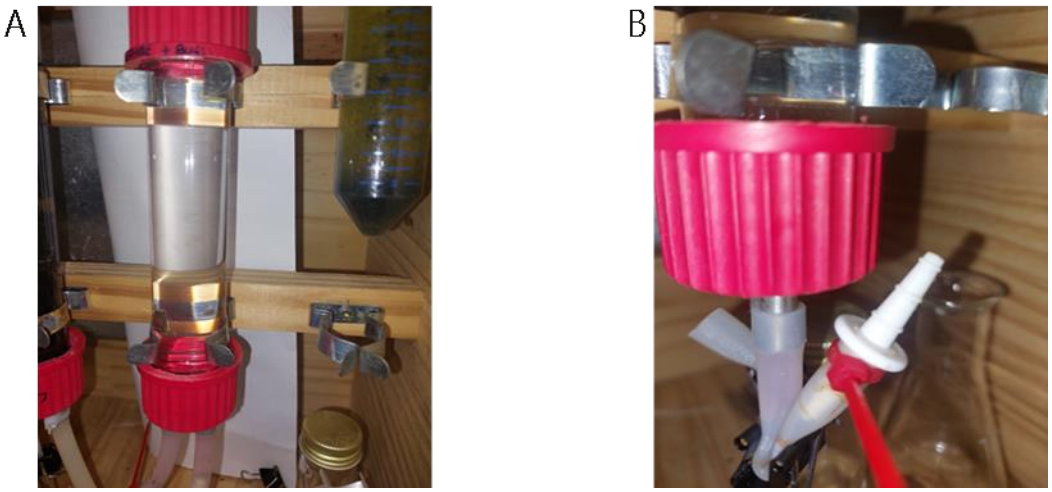


Figure 4-5: A) Mini-column reactor containing dissolved resazurin dye after being placed in an anaerobic chamber and removing dissolved oxygen in batch operation at room temperature and B) pink coloured liquid in mini-column tubing at feeding port showing oxygen had entered the tubing (dissolved oxygen test using resazurin dye)

Figure 4-5 shows the column after having been removed from the anaerobic chamber but still in batch operation. 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; the was no flow into the reactor.

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 4-6 shows the feed and feed tubing during continuous operation.

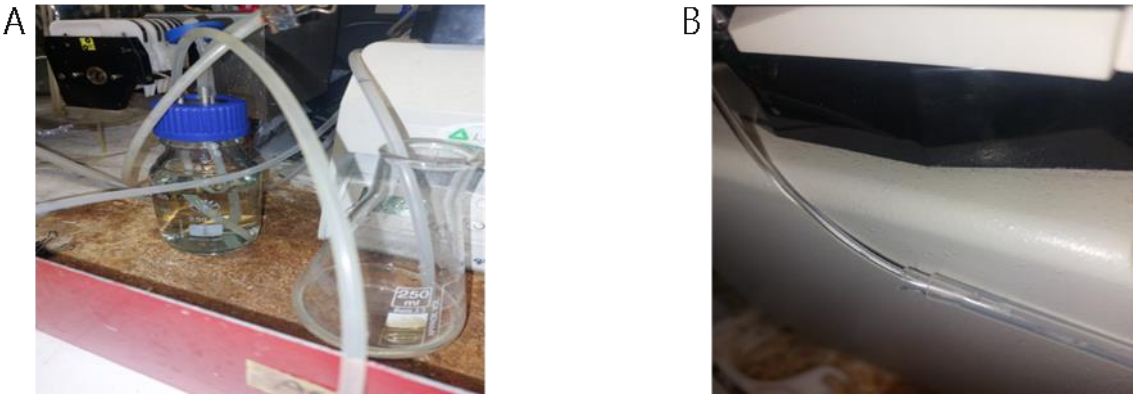


Figure 4-6: A) Feed and feed tubing and B) feed tubing in continuous operation with colourless liquid showing no oxygen is present (dissolved oxygen test using resazurin dye)

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 start-up of continuous operation, 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(Figure 4-7). Oxygen entering from the feed ports seen from Figure 4-6 was being pumped into the bulk liquid causing the colour to change to pink.

Figure 4-7: Mini column reactor at start-up of continuous operation with bulk liquid turned a faint pink from oxygen entering through the feed ports (dissolved oxygen test using resazurin dye)

After a short while, the pink colour disappeared, 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 trapped in the feed tubing (Figure 4-5 B) that was released into the reactor upon commencing continuous operation. After the tubing was filled with fluid from the feed bottle, 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 4-8 is an image of the bulk fluid just after the sample draw, from the bottom of the reactor, was made.

Figure 4-8: Sampling from the bottom of the mini-column reactor in continuous operation showing a colour change to pink from the top of the reactor (dissolved oxygen test using resazurin dye)

Dark pink, fluorescent coloured liquid from the effluent entered from the top of the reactor and changed the bulk fluid entirely to a deep pink colour. This showed that sampling was the main cause of oxygen

entering the reactor from liquid in the effluent pipe being sucked back down into the reactor. Intermittently clamping the effluent tubing while sampling did not stop oxygen from entering the bulk fluid as low pressure created within the reactor caused the same result upon unclamping. Due to the small size of the column, the amount of oxygen entering the reactor had a big impact on the sulphide reoxidation and on the performance of the SRB as little to no sulphate reduction was observed during continuous operation. Thus, the mini column reactors were not suitable for testing the different organic substrates due to uncontrollable re-oxidation.

Continuous Schott Bottle Reactors

The 1 L Schott bottle reactor on lactate ran for almost 80 days at a 5-day HRT in continuous operation at room temperature. With a larger volume, the Schott bottle continuous reactor was set-up to combat reactor failure due to oxygen ingress upon sampling a very small reactor volume as was observed in the mini column reactor. Similar to the mini column continuous reactor, the continuous Schott bottle reactor was also inoculated with sludge and PUF-BSPs from a fed-batch reactor adapted to the specific substrate being tested. The foam contained immobilised cells from the mixed, sulphate reducing community. The sulphate trend of the lactate fed continuous Schott bottle reactor is shown in Figure 4-9 A.

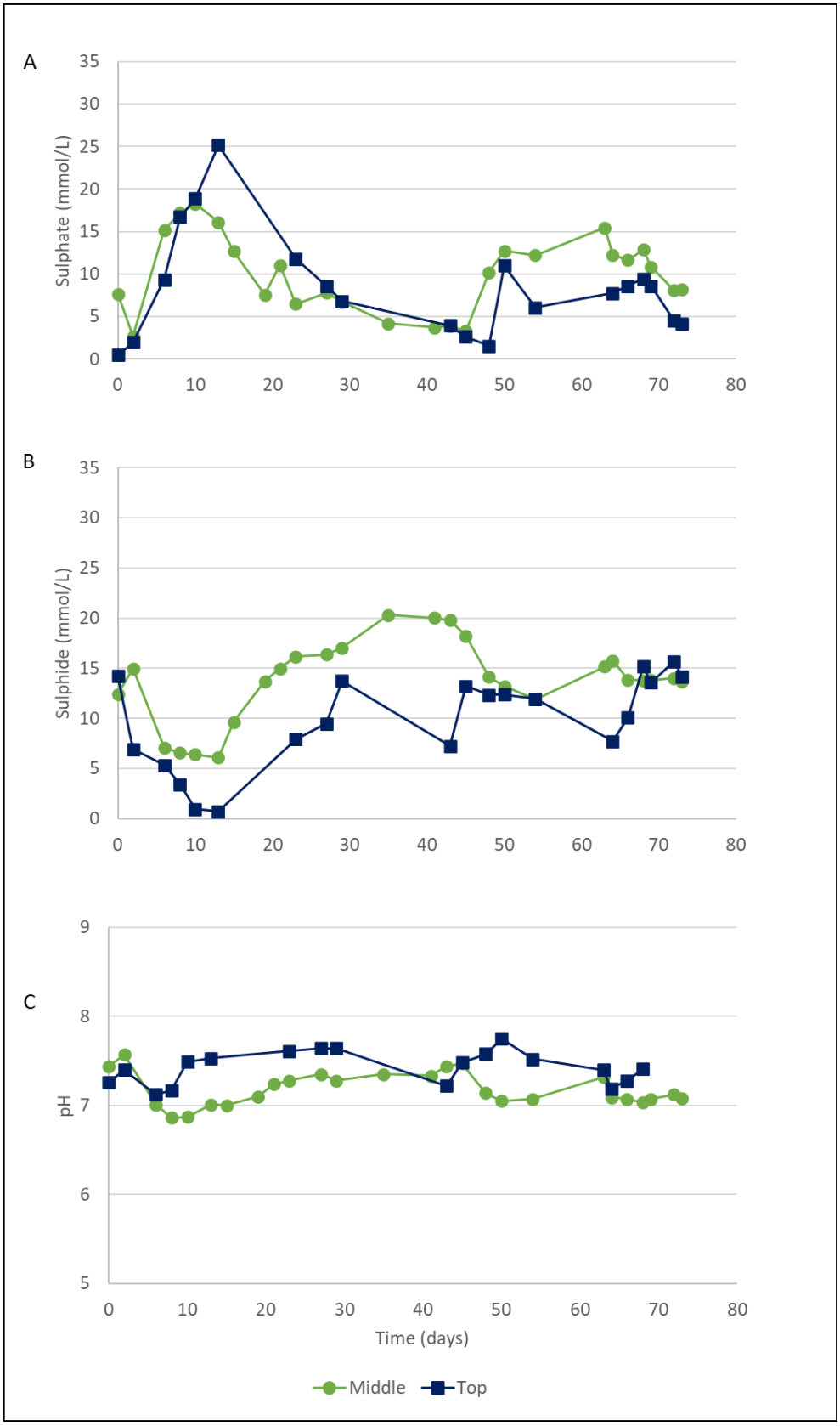


Figure 4-9: Sulphate and sulphide concentrations and pH time trends of the lactate-fed continuous, Schott bottle reactor.

After a sulphate conversion ≥50% was achieved in batch operation, a switch was made to continuous operation at day 0. In the first 13 days, both the middle and top samples sulphate concentrations increased. Increase in the concentrations may have been due to the reactor adapting to the change in

configuration and some oxygen being introduced to the system from the feed tubing when first pumping in the feed. Introduction of oxygen into the reactor re-oxidised sulphide to sulphate as can be seen by the top of the reactor having higher sulphate concentrations than the middle of the reactor in the first 13-20 days of continuous operation. A sulphate concentration of 25.1 mmol/L on day 13, higher than the feed sulphate concentration, also indicates re-oxidation had occurred. After the first 10 days, the sulphate concentrations began to decrease and reached its lowest value of 1.54 mmol/L on day 48. Measuring the concentration from the top sampling point, the highest sulphate conversion of 92.6% was achieved on day 48.

The continuous Schott bottle reactor had higher sulphate conversions compared to the mini column which confirms that in a reactor with a larger volume, oxygen ingress has a less negative impact on the performance of the BSR system. Due to oxygen ingress from sampling and continuous operation, when a reactor is too small there is not enough sulphide to eliminate the oxygen that enters the reactor without severely increasing and decreasing sulphate and sulphide concentrations respectively, resulting in reactor failure.

The sulphide trend graph of the continuous Schott bottle is shown in Figure 4-9 B. The sulphide concentration time trend graph compliments the continuous Schott bottle reactor sulphate trend graph with a drop in sulphide concentration in the first 13 days where an increase in sulphate concentration was observed. The lowest sulphide concentration was observed on day 13 at 0.72 mmol/L at the top of the reactor further indicating re-oxidation occurring just after the switch from batch to continuous configuration at day zero. The sulphide concentration was also fluctuating showing that there was re-oxidation in the reactor. The highest sulphide concentrations were observed at the middle of the reactor (closer to the feed point) rather than the top which indicates that throughout the entire run of the continuous Schott bottle reactor marginal re-oxidation was occurring at the top of the reactor.

The pH of the reactor was also recorded and graphed into Figure 4-9 C. The pH remained neutral with lowest pH recorded at 6.86 and the highest pH at 7.75. Decrease in pH in the first 10 days could have been due to the re-oxidation which causes the release of protons resulting in a lower pH. Though the pH fluctuated it remained fairly neutral and thus, did not have any negative impact on the SRB's performance. Performance data of the continuous reactors on other organic substrates are discussed in Appendix A.8.

4.3.4 Reactor and configuration selection conclusion

The fed-batch Schott bottle reactor had higher sulphate conversions due to the system having more time to reduce sulphate. The continuous Schott bottle reactor had the highest VSRR showing that it had the better reaction kinetics. Cultures in continuous systems are pushed to their limits to operate at maximum capacity achieving faster rates. The mini column reactors had little to no sulphate reduction, they were too small and thus, suffered from sampling which introduced too much oxygen into the system. Sulphide in the 93 ml reactor was limited and could not eliminate the oxygen entering the reactor through sampling from the effluent. The continuous Schott bottle reactors had a larger volume compared to the mini columns and so they had better sulphate conversion and sulphate reduction rates. However, they were still affected by oxygen ingress into the system which potentially caused sulphate concentrations to fluctuate and decreased efficiency of the system due to re-oxidation. Thus, small (90-1000 mL) continuous reactors are not suitable for BSR due to their susceptibility to oxygen ingress through sampling and minute openings at tube and port connections. The fed-batch reactor offered an advantage of better oxygen ingress control in a small volume than in the continuous reactor and would offer better consistency in the organic substrate testing. Consistency is an important factor in comparing different variables' effect on the performance of BSR systems. Therefore, the fed-batch Schott bottle studies were used to compare the alternative carbon sources and to select the best performing substrate to take forward into the LFCR system.

4.4 Alternative carbon source selection (Fed-batch Schott bottle reactor tests)

4.4.1 Results and Discussion

Lactate (Base Case)

As shown in Section 4.3 the lactate fed-batch reactor had a high average and maximum sulphate conversion of 78.1% and 95.1% respectively. Good performance of the lactate fed-batch reactor was expected as lactate is known to be an effective carbon source in BSR systems. The VFA content in the reactor at the start and end of three cycles was measured through HPLC and results are shown in Figure 4-10.

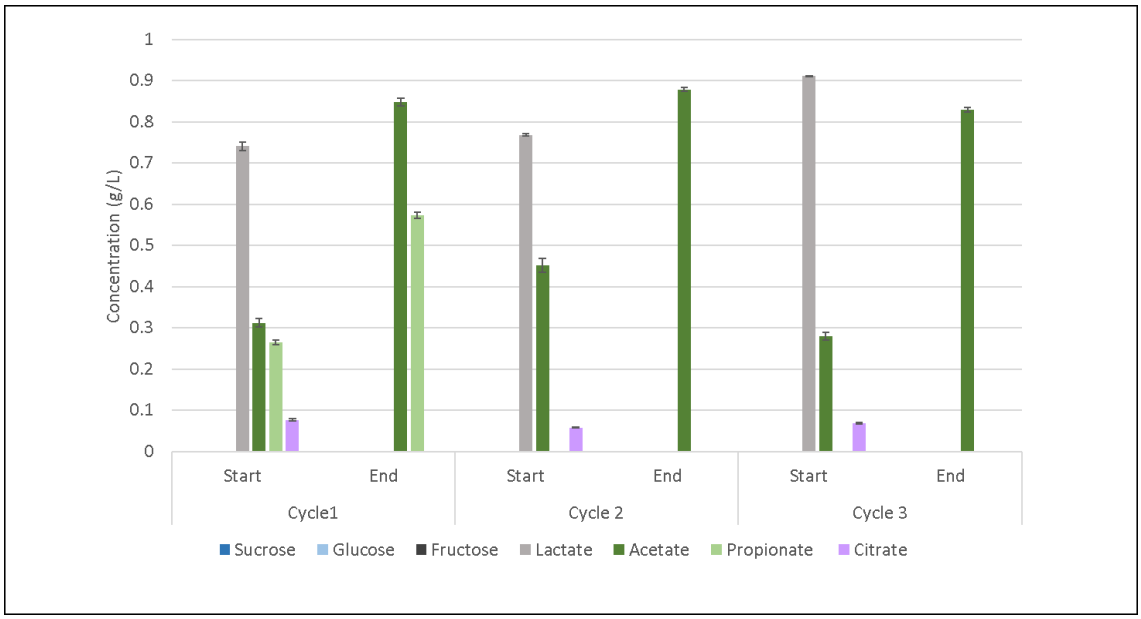


Figure 4-10: Sugar and VFA concentration time trends in a lactate-fed-batch, Schott bottle reactor at the start and end of three cycles.

All lactate was oxidised by the end of each cycle. The build-up of acetate indicates that the lactate was incompletely oxidised. An average of 0.85 g/L of acetate was found in the reactor before subculturing. There was also propionate measured after the first cycle at 0.572 mg/L which compliments the lower sulphate conversion of the first cycle observed in Figure 4-1 when compared to the other 2 cycles that succeeded it. Presence of propionate in the first cycle indicates that there was some fermentation of lactate occurring. Propionate is poorly utilised by SRB as a carbon source, but partial oxidation of propionate produces acetate and other compounds such as HS^-, H^+, H_2O and H_2 (Liamleam and Annachhatre, 2007; Muyzer and Stams, 2008; Fernandes, 2020). Citrate was also completely used up in each cycle.

Molasses

The sulphate and sulphide concentrations, and pH trends of the reactor are shown in Figure 4-11. The reactor ran for 4 cycles, with an average of about 30 days for each cycle. 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. An average sulphate conversion of 82.7% across the four cycles from the start of each cycle (just after subculturing) to the end (just before subculturing) and a maximum sulphate conversion of 89.2% in the first cycle was reached in the molasses fed-batch reactor. The average highest sulphide concentration across all cycles was approximately 13 mmol/L.

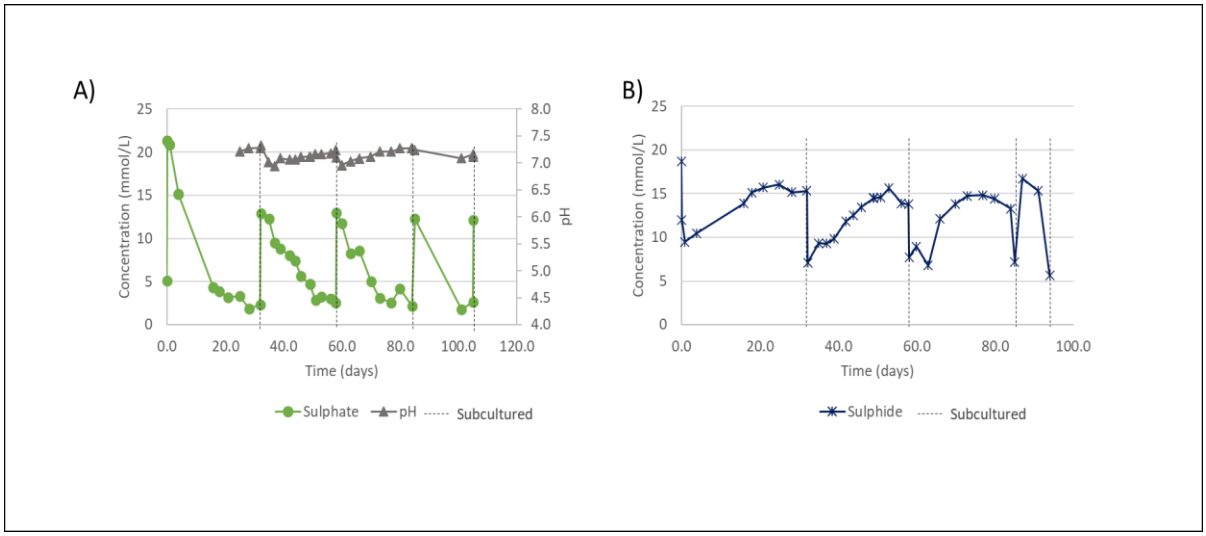


Figure 4-11: Sulphate and sulphide concentrations and pH time trends of the molasses fed-batch reactor.

The molasses fed-batch reactor had been in operation for some months before the start of the fed-batch reactor studies. Thus, the bacteria within the molasses fed-batch reactor had already adapted to the molasses substrate from a lactate substrate, upon the start of the study. This would explain the high sulphate conversion and short cycle time of 30 days experienced from the first cycle of the molasses fed-batch reactor. The cycle time was shorter than most of the other first cycle times of the other substrate fed-batch reactors discussed later.

The pH ranged between 7.0 and 7.5 throughout all 4 runs of the molasses fed-batch reactor which is an optimal range for SRB performance as discussed by (Mooruth, 2013). 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 have been 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 likely due to SRB metabolism which produces sulphide and bicarbonate when sulphate reduction and organic substrate oxidation occurs (Marais, 2020).

HPLC analysis of the samples taken from the molasses fed-batch reactor indicated that all sugars and VFAs in the system had been used up by the end of each cycle. Thus, all usable sugars and VFAs in the fed-batch reactor were utilised as can be seen in Appendix A.9.1 and Figure 4-12 below.

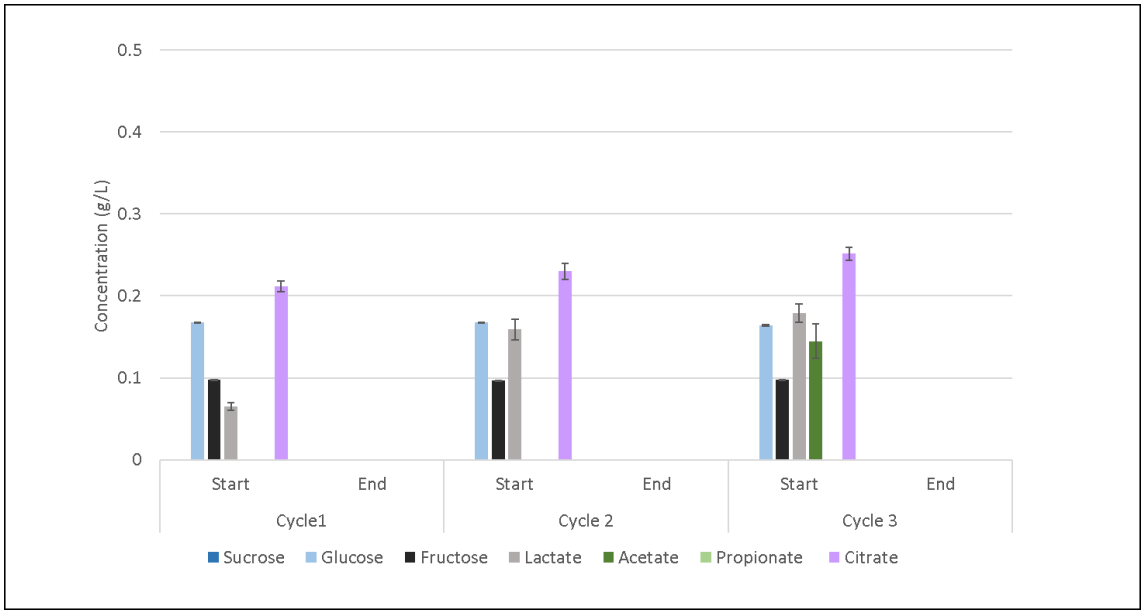


Figure 4-12: Sugar and VFA concentrations in the molasses fed-batch reactor at the start and end of three cycles.

Though molasses was contains sucrose (Table B.1.1 1), addition of the molasses to the fed-batch reactor may have caused a quick break down of sucrose to its respective monomers, glucose and fructose, which showed consistent starting concentrations of approximately 170 mg/L and 100 mg/L respectively. The samples were not run through the HPLC straight after extraction and so the delay may have contributed to the breakdown of sucrose. Lactate was also present in the samples taken at the beginning of the cycle with the highest lactate concentration observed in the final cycle at 0.179 mg/L.

According to Equation 4-1 (Liamleam and Annachhatre, 2007), molasses can be converted to 4 moles of lactate. Sucrose, fructose and glucose in molasses can be fermented to produce lactate (Abedi and Hashemi, 2020).

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4C_3H_6O_3 \qquad \text{Equation 4-1}$$

Although the exact concentration of lactate was not measured, Figure B.2 1 in appendix A.7shows a peak around 15 minutes in the UV chromatogram which from Table A.4 1 is known to be the retention time of lactate. This showed that the pure molasses carbon source, outside of the reactor systems, contained fermenters which converted some of the sugars to lactate before the carbon source was introduced into BSR system. The fermenters were then introduced into the BSR system which allowed for the fermentation of more sugars to lactate resulting in the high concentrations seen in Figure 4-12 at the start of each cycle. The increase in lactate measured between cycle 1 and cycle 2 may indicated the quick adaptation of the fermenters to the BSR system.

The presence of lactate may have aided in the high performance of the molasses fed-batch reactor when compared to the other organic substrates as lactate in small concentrations in combination with other carbon sources at a higher concentration within a reactor can help improve sulphate reduction as seen in (Maillacheruvu and Parkin, 1996; Celis *et al.*, 2013). Pre-conversion of sugars to lactate could have also increased conversion as lactate is known to be one of the most effective substrates in BSR systems.

Citrate was present as an additional nutrient in the synthetic feed added as sodium citrate, and that is why it was present after subculturing at the beginning of the cycle at an average of 0.231 mg/L. Citrate can also be formed from the fermentation of sugars such as glucose and thus, some citrate may have been formed from fermentation which may have contributed to its high concentration. The concentration of lactate and citrate increased after each cycle, and this may have been due to the time difference between the subculturing of the reactor and the extraction of the samples for HPLC. For the first cycle,

the HPLC sample was taken 4.8 hours after subculture, while for the third cycle it was taken after 24 hours. The third cycle sample was also the only sample that contained acetate at a concentration of 0.144 mg/L and this may have also been caused by the delay in extraction of the sample after subculturing. Acetate would have been formed from the oxidation of sugars and lactate which could have occurred over the 24 hours before sample extraction. The absence of acetate at the end of cycle 3 also indicates that acetate utilising SRB with a highly effective acetate oxidation rate may be present in the molasses fed-batch reactor.

Overall, the molasses fed-batch reactor had high sulphate conversions with an average sulphate conversion that was higher than the average sulphate conversion in the lactate fed-batch reactor. However, the maximum sulphate conversion of the molasses fed-batch reactor was lower than the maximum sulphate conversion of the lactate fed-batch reactor. A benefit arising from the use of molasses over lactate was the complete utilisation of the usable sugars and VFAs detected through HPLC from the molasses substrate and the fermentation of molasses sugars to lactate. In the lactate fed-batch reactor, there was incomplete utilisation of the lactate substrate added as seen from the presence of acetate and propionate at the end of cycles. Maximum utilisation of the usable sugars and VFAs in the molasses substrate is an advantage in AMD treatment as the COD content of the effluent stream will be lower and thus, causes less harm when discharged into the environment.

Acetate

The fed-batch reactor on acetate ran for 4 complete cycles, 140 days and like the molasses fed-batch reactor, the showed a clear trend of sulphate reduction and sulphide formation after subculturing in Figure 4-13. The acetate fed-batch reactor presented lower sulphate conversions and sulphide concentrations when compared to the lactate and molasses fed-batch reactors. The average sulphate conversion across the four cycles was calculated to be 41.4% with a maximum sulphate conversion of 53.7% in the first cycle. The maximum sulphide concentration of 11.8 mmol/L was observed across all four cycles and an average highest sulphide concentration of 7.26 mmol/L across the second to last cycle. The sulphide concentration in the first 10 days of the first cycle was 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. In other cycles succeeding the first, the sulphide concentration fluctuates which does not form a clear trend of sulphide formation as seen with the other cycles.

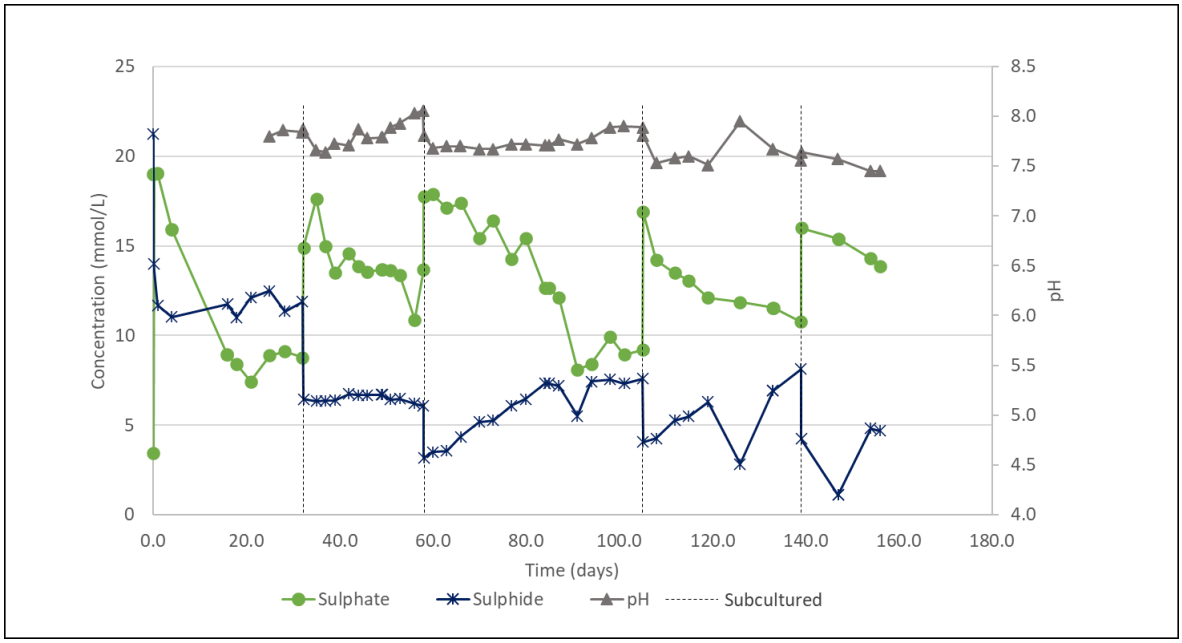


Figure 4-13: Sulphate and sulphide concentrations and pH time trends for the acetate fed-batch reactor.

The acetate fed-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 bulk fluid pH in the reactor was higher than the feed pH because of the reduction of sulphate by SRB which produced alkalinity. The acetate fed-batch also displayed a fairly neutral pH range throughout its runs, between 7 and 8. It had the highest measured pH amongst all the organic substrate fed-batch reactors at 8.06. This could be due to the exclusive oxidation of acetate which produces more alkalinity when compared to substrates such as lactate when they are incompletely oxidised.

Samples taken at the start and end of first, second and third cycles of the acetate fed-batch reactor were taken for HPLC to determine the substrate utilisation. Figure 4-14 shows the different concentrations of VFAs in the reactor at the start and end of the different cycles.

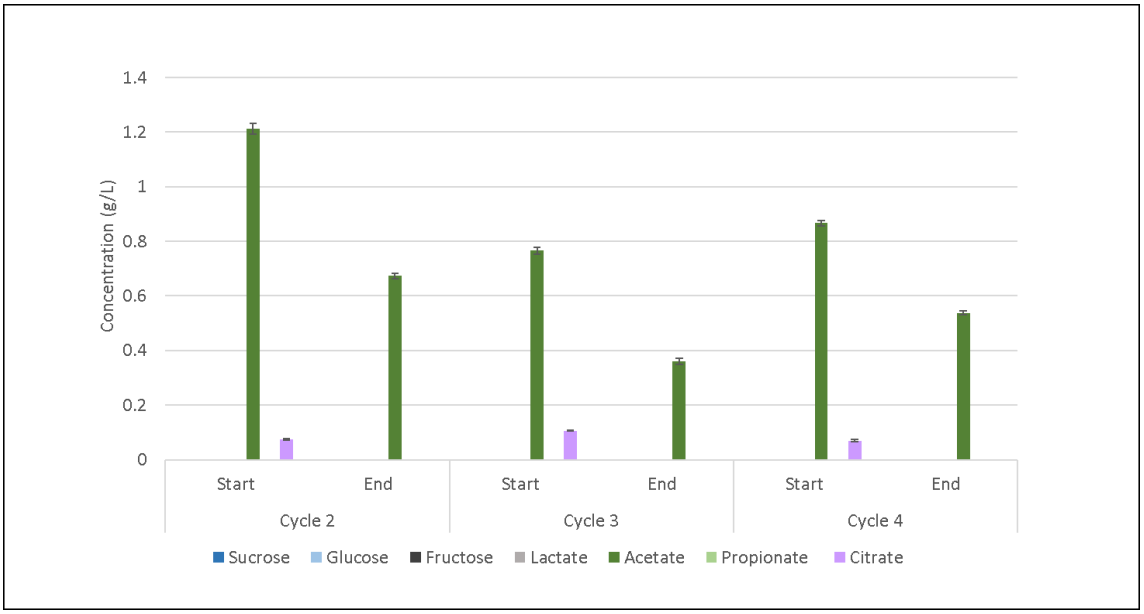


Figure 4-14: Sugar and VFA concentrations of the acetate fed-batch reactor at the start and end of three cycles.

The average acetate measured at the end of the second, third and fourth cycle is 0.524 mg/L and no citrate was measured before subculturing showing it had been completely utilised. Presence of the acetate at the end of cycles indicated that there was poor utilisation of the acetate substrate within the fed-batch reactor. The acetate concentration after subculturing was the highest at 1.21 mg/L at the start of the second cycle. A high acetate concentration at the beginning of the second cycle could explain the cycles low sulphate conversion. As discussed by Oyekola *et al.*, (2009, 2012) SRB activity is favoured in limiting concentrations of acetate as SRB are known to be organic carbon scavengers. The increased concentration of acetate in the second cycle could have resulted in poor SRB activity resulting in poor sulphate reduction. In the third and fourth cycle less, acetate was present at the start thus limiting acetate which may have resulted in improved SRB activity resulting in better sulphate reduction. The citrate measured at the beginning of each cycle with an average of 0.0833 mg/L comes from the synthetic, sulphate-laden feed.

Although acetate was the sole carbon source added to the acetate fed-batch reactor, there was incomplete utilisation of the acetate substrate. This may have been due to the slow reaction kinetics of acetate utilising SRB and the increased toxicity to sulphide of acetate utilising SRB (Maillacheruvu and Parkin, 1996; Celis *et al.*, 2013). Thus, the acetate fed-batch reactor, when compared to the lactate and molasses fed-batch reactor, had lower sulphate conversions, lower sulphide concentrations and poor substrate utilisation.

Honey

The fed-batch reactor on honey ran for 5 cycles, almost 250 days, subculturing in between each cycle when sulphate reduction and the sulphate concentration remained relatively constant.

The honey fed-batch reactor performed poorly as shown in Figure 4-15. It had the lowest overall sulphate conversion of 7.36% and lowest maximum sulphate conversion of 11.1 % among all the fed-batch reactors fed with a natural organic substrate. Honey is known to have anti-microbial properties which may have contributed to the low sulphate conversion observed in the honey fed-batch reactor (Mandal and Mandal, 2011).

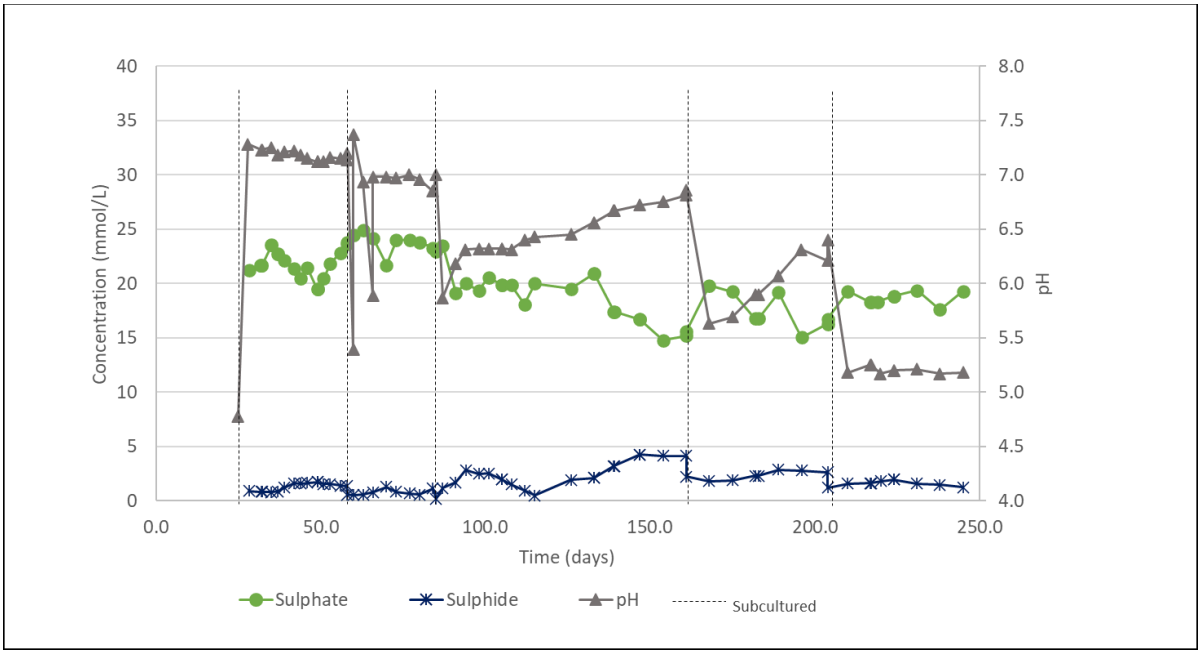


Figure 4-15: Sulphate and sulphide concentrations and pH time trends of the honey fed-batch reactor

It had a maximum sulphide concentration and overall average, highest 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 fed-batch reactor. A possible explanation for the high sulphide concentrations could be that there is sulphur in the honey. Sometimes honey contains sulphur which can possibly be reduced to sulphide in a BSR system (Samarghandian *et al.*, 2017).

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, with a pH ranging between 3.2 and 4.5 (Mandal and Mandal, 2011). SRB thrive in conditions with a pH range between 7.5-8 (Brahmacharimayum *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 conversion 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 act as a buffer, the pH rose on its own to a pH between 6 and 7 but this did not improve sulphate conversion. 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 fed-batch reactor was, however, an exception as the pH did not increase on its own and remained below 5.5 throughout the entire cycle. This could have indicated that the reactor had failed, there was little to no sulphate reduction and sulphide formation, due to the low pH and characteristics of the honey.

Honey was thus classified as a poor carbon source and electron donor for BSR systems, owing to the low conversions of sulphate. However, an analysis of sugars and VFAs in the honey fed-batch samples at the beginning and end of each cycle was conducted to determine substrate utilisation and to reveal potential inhibitions linked to sugars and VFAs in the system. Figure 4-16 shows the sugar and VFA concentrations of the honey fed-batch reactor.

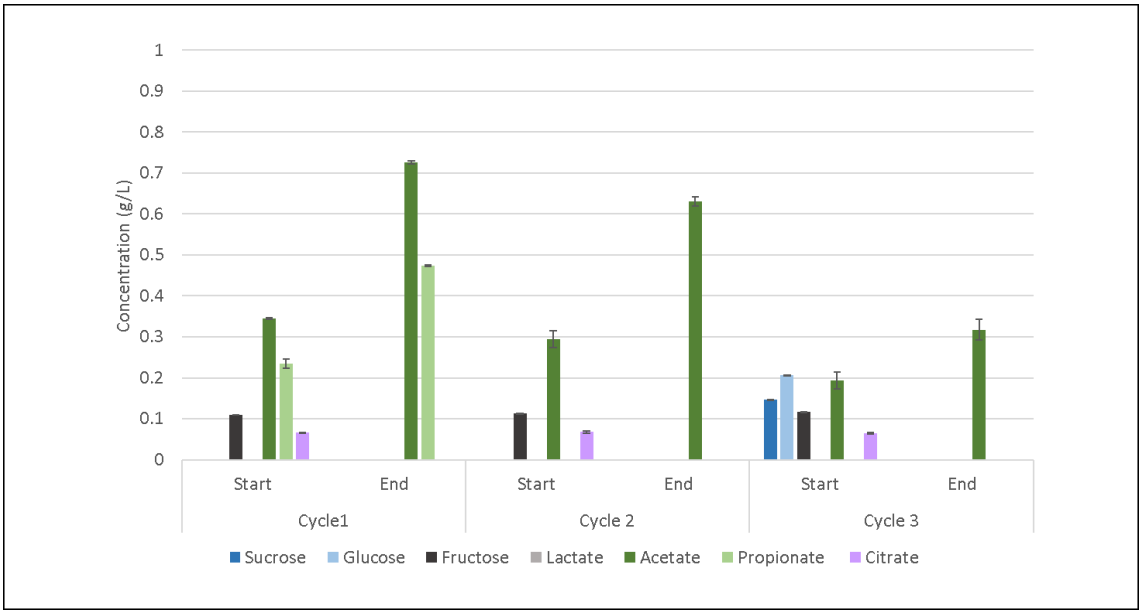


Figure 4-16: Sugar and VFA concentrations of the honey fed-batch reactor at the start and end of 3 cycles.

There were no sugars in the honey fed-batch reactor at the end of the three measured cycles. VFAs present at the end of the three measured cycles were acetate and propionate where propionate was only present in the first cycle. Presence of these VFAs indicate the incomplete oxidation of the usable sugars and VFAs in honey. The acetate concentration decreased after each subculture which could have been due to the removal of some of the acetate during subculture and some may have been utilised by acetate utilising SRB, which are known to have a slower rate compared to lactate utilising SRB. The main sugar present after each subculture was fructose across all cycles, at an average concentration of 0.112 mg/L. Sucrose and glucose were only detected in the third cycle after subculturing at concentrations of 0.148 mg/L and 0.206 mg/L respectively. Their presence may support eventual reactor failure when little to no sulphate reduction was observed in the final honey cycle. Samples for HPLC in the three cycles were taken a few hours after subculturing and so while the other cycles managed to break down the sucrose and glucose completely in the few hours before reactor sampling for HPLC, the third cycle failed to breakdown these sugars. Citrate was present after each subculture due to the addition of sodium citrate to the synthetic feed.

Overall, the honey fed-batch reactor had poorest sulphate conversions and lowest sulphide concentrations amongst the complex organic carbon sources: molasses, honey, and algal lysate. The acidity of the honey was detrimental to reactor operation as SRB prefer a more neutral pH (Brahmacharimayum *et al.*, 2019). Low sulphate conversion may have also stemmed from antimicrobial phenolic compounds and elevated osmotic pressure, both of which inhibit SRB metabolic activity (Brahmacharimayum *et al.*, 2019). Honey as a substrate for SRB also contained residual COD in the form acetate after treatment which is a disadvantage in BSR systems as the effluent from this system will be polluting to the environment if discharged.

Algal lysate

The sulphate concentration trend in the algal lysate fed-batch reactor differs from those in the fed-batch reactors fed with molasses and acetate, as shown in Figure 4-17. 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 fed-batch reactor was intermittently 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.

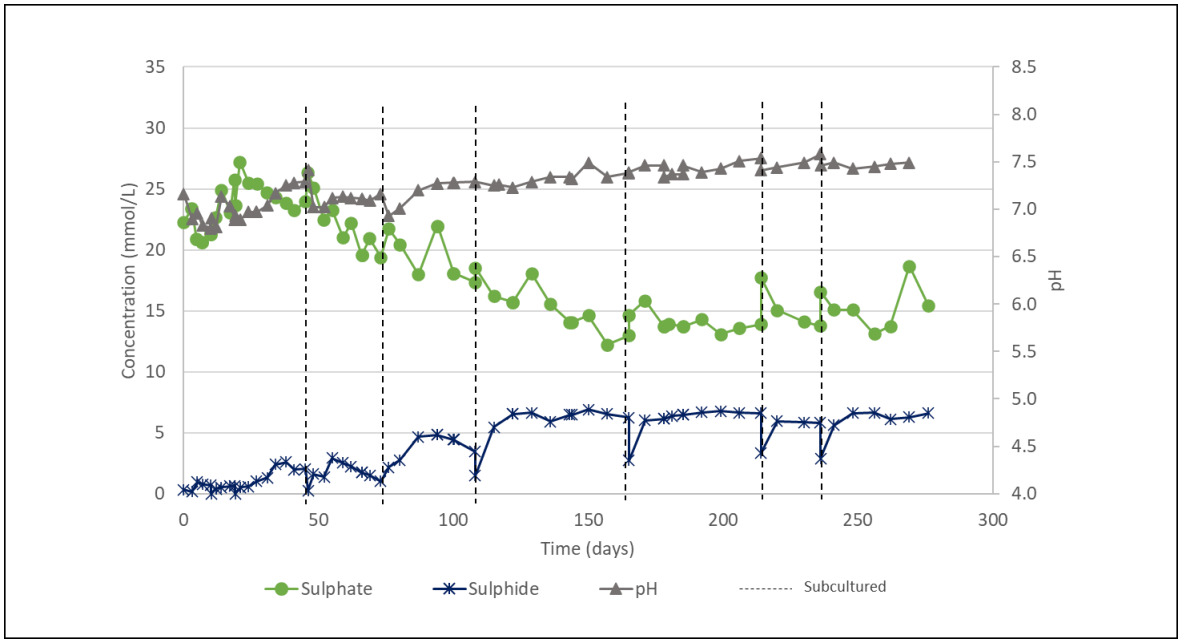


Figure 4-17: Sulphate and sulphide concentrations and pH time trends of the algal lysate fed-batch reactor.

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 after the instant decrease in sulphide caused by subculturing. The sulphide concentration then proceeded to remain constant for the rest of the cycle until the next subculture.

The reactor may have performed poorly during the first three runs due to the COD:sulphate ratio being below 0.67. The ratio was adjusted to a COD:sulphate ratio of 1 after the 3rd cycle. The performance of the reactor improved from an average sulphate conversion of 8.3% to 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 4-1 does indicate 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 (Gouda *et al.*, 2022). Thus, a much lower usable COD was available to SRB as observed when HPLC was used to determine the COD content of the algal lysate.

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 fed-batch reactors which had been running previously before the fed-batch reactor study started, the algal lysate fed-batch reactor was started on day zero of the substrate fed-batch study. The algal lysate fed-batch reactor experiment lasted for almost 300 days which would have given it enough time to adapt to the new substrate from lactate. Thus, the performance observed in Figure 4-17 most likely shows the optimum performance of the SRB culture using algal lysate as substrate with a COD:sulphate ratio of 1.

Analysis of the sugars and the VFAs at the beginning and end of the algal lysate fed-batch reactor cycles was performed using HPLC to determine the utilisation of the usable sugars and VFAs in the algal lysate substrate. The sugar and VFA analysis revealed that for both the samples taken before and after subculturing there were no measurable concentrations of sugars and VFAs except for citrate

detected after subculturing which is present due to the addition of sodium citrate to the synthetic. Some sugars and/or VFAs were expected to be present in the samples taken at the end of cycles (before subculturing), however, there were no sugars detected.

This showed that all sugars and VFAs that were formed from the breakdown of complex molecules such as long chain carbohydrates, proteins or lipids may have all been used up. The COD analysis revealed that there was high COD content in the algal lysate, however, HPLC analysis of the algal lysate substrate revealed a much lower usable COD content compared to the one measured through COD analysis. This may have been further diluted in the algal lysate fed-batch resulting in sugar and VFA concentrations which could not be detected through HPLC using the sugar and VFAs column.

Increasing the COD:sulphate ratio may be advantageous for the system using algal lysate as a substrate as this may increase the usable COD content in the reactor resulting in a higher sulphate conversion. 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 from the complex molecules such as the carbohydrates and proteins that would not have been broken down. This would make BSR systems, using algal lysate as a substrate, costly and impractical. Alternatively, the algal lysate can be anaerobically digested 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 more efficiently by the mixed cultures (Motleleng, 2020). Algal lysate was not a suitable substrate for large-scale and long-term treatment in the hybrid LFCR.

4.4.2 Conclusion on organic substrate fed-batch tests

Second to the lactate fed-batch reactor (base case), the molasses fed-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 fed-batch reactor cultures compared to those in the molasses and lactate fed-batch reactors. For the acetate fed-batch reactor, the acetate consuming SRB have slower reaction kinetics as discussed by Maillacheruvu and Parkin, (1996) and Celis *et al*., (2013). The honey fed-batch reactor was acidic which inhibited the function of the SRB. After increasing the pH, sulphate conversion remained low suggesting inhibition from the antimicrobial properties of honey. For the algal lysate fed-batch, the complexity of the substrate may have resulted in a lower usable COD content for the SRB. According to Inglesby, (2011) and Gouda *et al*., (2022), 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 conversion in the algal lysate fed-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). Focusing on the residual compounds at the end of each cycle, the molasses fed-batch reactor had most of its organic material used up as there were no detectable sugars or VFAs detected through HPLC conducted on samples taken after a cycle. All the other fed-batch reactors including the lactate fed-batch reactor had residual organic compounds at the end of each cycle and an effluent with a high organic content is harmful when discharged into the environment.

Thus, molasses was chosen as the alternative carbon source for the LFCR system as it performed the best in the substrate fed-batch reactor tests. Molasses outperformed other complex substrates, demonstrating high sulphate conversion (average conversion of 82.7%) and stable sulphide profiles. Acetate showed intermediate performance, hindered by slower SRB kinetics. Honey presented significant limitations due to pH sensitivity and antimicrobial activity, while algal lysate had low bioavailable COD. These findings reinforce molasses' suitability for scaling in BaH-LFCR systems. Molasses had shorter cycle times and non-detectable residual sugars and VFAs when using HPLC, resulting in an effluent stream with lower organic content when compared to the acetate, honey and algal lysate batches. It also had a higher average sulphate conversion than the lactate fed-batch reactor.

5 Linear flow channel reactor

5.1 Introduction

The hybrid LFCR, described by Marais *et al*. (2024), has been studied using a synthetic feed comprised of a defined media. This study investigates its performance with real AMD, which is complex and potentially contains inhibitors. The AMD's chemical composition was considered and potential treatment issues like metal precipitation and pH fluctuation were addressed. The research evaluates the ability of the baffled hybrid linear flow channel reactor (BaH-LFCR) to treat an AMD stream from a pilot study on treatment and disposal studies for coal waste and fines from a Mpumalanga, South Africa mine site (Kotsiopoulos *et al.*, 2022), using both lactate and the complex carbon source selected in the previous chapter, molasses, as both the carbon source and electron donor. Three experiments were conducted:

(1) synthetic feed with lactate carbon source as a base case,

(2) AMD feed with lactate carbon source, and

(3) AMD stream supplemented with molasses, a sugar industry by-product, as a potentially more cost-effective organic source as carbon source and electron donor.

The specific objectives addressed in this chapter are:

- Determine the chemical composition, physical qualities (pH, redox and conductivity) and trace elements of the supplied AMD to inform the suitable pre-treatment methods for the AMD before it is fed to the mixed culture.
- Setup and assess different AMD pre-treatment methods in small-scale fed-batch reactors (1 L Schott bottles) packed with PUF-BSPs as a support matrix using performance indicators such as sulphate reduction, sulphide concentration, sugar and VFA utilisation and pH.
- Select a suitable pre-treatment method which will be used to pre-treat the AMD fed to the LFCR system based on the AMD small-scale fed-batch reactor which performed the best.
- Evaluate the performance of the LFCR system on the synthetic Postgate media B (Postgate, 1963) supplemented with lactate as carbon source and electron donor to provide the base case.
- Evaluate the performance of the LFCR system on pre-treated AMD using a lactate substrate and compare it to the LFCR base case.
- Evaluate the performance of the LFCR system on pre-treated AMD and the alterative substrate and compare it to the LFCR fed with pre-treated AMD with a lactate substrate.

5.2 Demonstration of performance of the LFCR system on a synthetic, sulphate-laden feed and lactate substrate

This section examines the two-stage BaH-LFCR's performance using a synthetic feed containing 2 g/L sulphate concentration and lactate to provide a COD:sulphate ratio of 1.0 which equates to 23 mmol/L. Previous work by Marais *et al*. (2024) modified the LFCR to include baffles and a polyurethane foam (PUF) matrix for biomass retention in the primary reactor, then characterised its performance using a 1000 mg/L sulphate feed. anti. The current study increased the sulphate concentration to 2 g/L to match the higher sulphate levels (1500-2000 mg/L) produced from leaching of the coal mine discards and fine wastes. The increased sulphate loading aimed to adapt the mixed culture to higher concentrations and establish a base case for comparison with AMD-fed experiments. Initially the reactor was operated at a 5-day HRT for nine cycles on the 2000 mg/L (20.8 mmol/L) sulphate synthetic feed with lactate substrate, allowing the mixed sulphate-reducing culture to adapt. Following this adaptation period, an HRT study was conducted over HRTs decreasing from 5 to 2 days to determine the optimal HRT to

treat the 2 g/L sulphate feed concentration in the primary reactor of the two-stage system. The secondary reactor is fed with the effluent stream from the primary reactor.

Figure 5-1 A and B show the residual sulphate and dissolved sulphide concentrations measured during the HRT study. A general trend was observed between floating sulphur biofilm (FSB) harvests: post harvesting, the average sulphate concentration increased, and average sulphide concentration decreased temporarily in the primary reactor, between the 4-day and 2-day HRT. This was due to greater oxygen ingress caused by the disruption and removal of the FSB; the FSB is recognised to impede the rate of oxygen entering the liquid phase, thereby ensuring only partial sulphate oxidation. After these initial changes post-harvesting, sulphate concentration decreased as the FSB started to re-establish and stabilised while sulphide concentration increased till the system reached a pseudo-steady state.

In the primary reactor, the lowest sulphate concentrations and highest sulphide concentrations were mostly observed at the first sampling port (sample port 1, see Figure 3-3). Sulphate concentrations lower than the rest of the reactor suggest a localized region of high sulphate reduction around this point. Samples drawn from this port tended to contain a thick sludge, suggesting a localized region with a longer HRT compared to other sample points. Data from this port was therefore not included in the averages calculated. At a 5-day HRT, harvesting the FSB did not immediately affect sulphate and sulphide concentrations, suggesting that at a high HRT, the effect of oxygen ingress after harvesting into the bulk liquid is minimised.

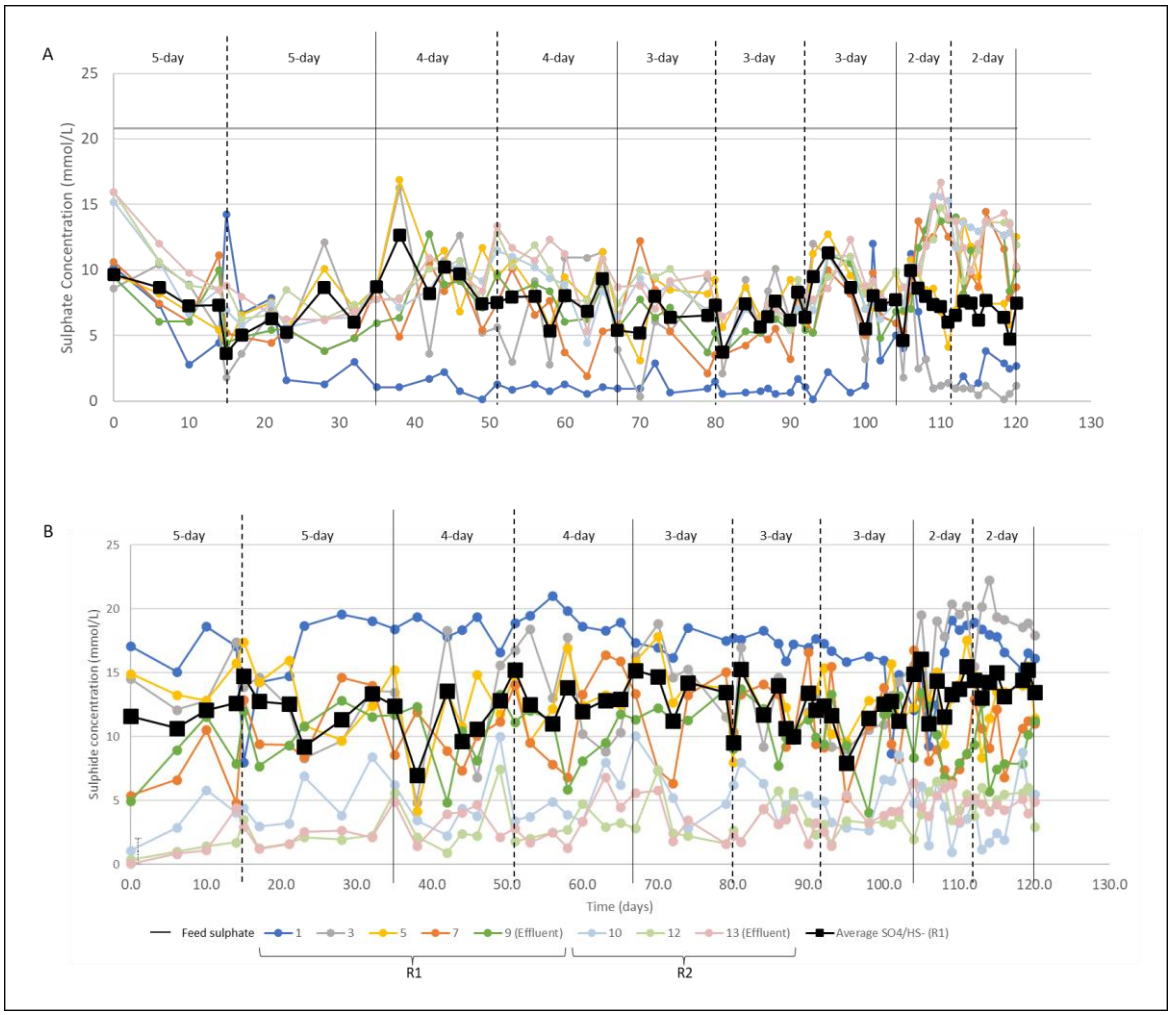


Figure 5-1: Time trends for A) sulphate and B) sulphide concentrations for the LFCR system fed with synthetic feed and a lactate carbon source at different hydraulic residence times. Average SO4 and HS- is the average of sample ports 3, 5, 7 as there was localised sulphate reduction at sample point 1. The vertical straight lines indicate biofilm harvest (solid lines indicate the start of a study).

Most sulphate reduction occurred in the primary reactor with slight increases from below 5 mmol/L to 5-10 mmol/L at sample points 5 and 7, indicating slight re-oxidation. The highest average sulphate concentration of 10.3 mmol/L occurred in the first cycle of the 2-day HRT. At 5-day HRT, average residual sulphate concentrations in the primary reactor were at 6.4±2.1 mmol/L at pseudo-steady state, representing 65-75% sulphate conversion. This concentration increased to 7.3±1.4 mmol/L at 4-day HRT, remained constant at 3-day HRT (7.3±0.9), and increased to 9.1±1.4 mmol/L at 2-day HRT. High dissolved sulphide concentration ranging between 12.0-16.5 mmol/L accompanied these changes.

The primary reactor's effluent sulphate and sulphide concentrations generally remained within the observed average range within the reactor, except at the 2-day HRT. Here, a significant increase in residual sulphate and decrease in residual sulphide concentrations were observed at the effluent port, indicating re-oxidation at a lower HRT. At sample points 5 and 7, increase in sulphate was not proportional to the decrease in sulphide, suggesting both re-oxidation and partial oxidation of sulphide to elemental sulphur was occurring.

In the secondary reactor, residual sulphate concentrations remained fairly similar across the three sampling ports. The decrease in sulphide concentration across the secondary reactor from the 10th to the 12th and 13th sampling ports, while sulphate concentration remained mostly constant, indicates partial sulphide oxidation to sulphur in the FSB.

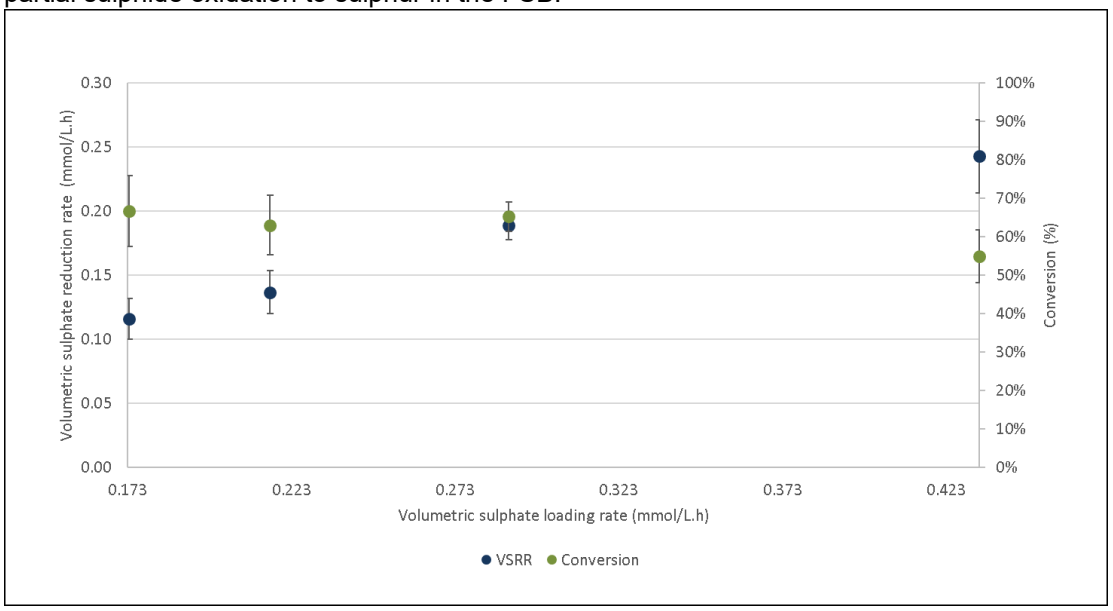


Figure 5-2 shows that with an increase in the volumetric sulphate loading rate (VSLR), there is an increase in volumetric sulphate reduction rate (VSRR). The highest VSRR of 0.243 mmol/L.h was observed at the 2-day HRT corresponding to a sulphate conversion of 56%. Sulphate conversions between 5 and 3-day HRTs were comparable ranging from 63% to 67% while VSRRs increased from 0.116 to 0.190 mmol/L/h.

The work done here can be compared to similar work carried out by Marais (2020) in a LFCR with a 10.4 mmol/L feed sulphate concentration in which cell retention was provided by presence of carbon fibres. Notably, the current study operated at 2-fold the VSLR at equivalent HRTs due to the higher feed concentration of 20.8 mmol/L.

For a direct performance comparison at matching VSLRs, the 4-day HRT results from this study can be examined against the 2-day HRT data from Marais (2020). Operating at a 4-day HRT,this study achieved a VSRR of 0.137 mmol/L.h with 56% sulphate conversion. For the same VSLR, Marais (2020) attained a comparable VSRR and a slightly lower conversion rate of 61% at a 2-day HRT. The BaH-LFCR at a 3-day HRT shows sustained performance at double the loading rate compared to the same HRT in Marais (2020). Sulphate conversions remained similar and VSRR doubled from 0.095 mmol/L.h in Marais (2020) to 0.189 mmol/L.h in this study.

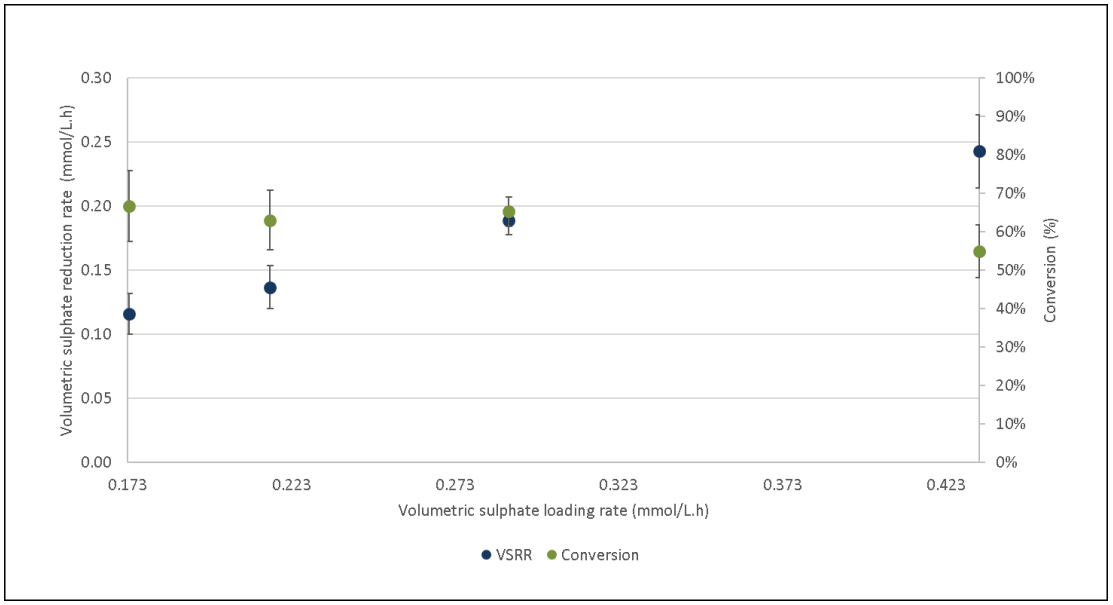


Figure 5-2: Steady state kinetics in the primary reactor of LFCR system fed with synthetic feed and a lactate carbon source.

HRT optimization is crucial for reaching a compromise between sulphate conversion, VSLR and VSRR. Higher HRTs allow more contact time between the sulphate-rich stream and the active SRB culture, maximising conversion. However, lower HRTs can achieve better reaction kinetics resulting in higher VSRR while compromising on sulphate conversion when VSLR is much higher than the VSRR. The 2-day HRT achieved the highest VSRR but lower conversions due to shorter residence time which caused the VSLR (0.433 mmol/L.h) to be almost double the VSRR 0.243 mmol/L.h). As expected, the 3-day HRT had a higher VSRR than the 4-day HRT due to a higher VSLR. The sulphate conversion at the 4-day and 3-day HRT were fairly similar.

Figure 5-3 A and Figure 5-3 B show expected sulphide concentration (calculated based on the sulphate converted), average residual, and effluent sulphide concentration trends for the primary and secondary reactors. In the primary reactor, at higher HRTs, 5-day and 4-day, expected sulphide exceeded average and residual sulphide concentrations, indicating the formation of sulphur. At lower HRTs (3-day and 2-day), average residual sulphide approached expected sulphide, suggesting less partial oxidation to sulphur at shorter HRTs. This could be due to longer HRTs allowing time for a more mature FSB to form.

Effluent sulphide in the primary reactor was generally lower than the average residual sulphide across all HRTs, indicating ongoing sulphide oxidation at the effluent port of the reactor. As cycles progressed, residual and effluent sulphide concentrations approached the expected sulphide concentration, attributed to gradual FSB formation impeding oxygen ingress and thereby sulphide oxidation (partial or complete). The sharp decrease in sulphide concentration at the effluent port after some harvesting events implies re-oxidation to sulphate as the port is close to the reactor surface.

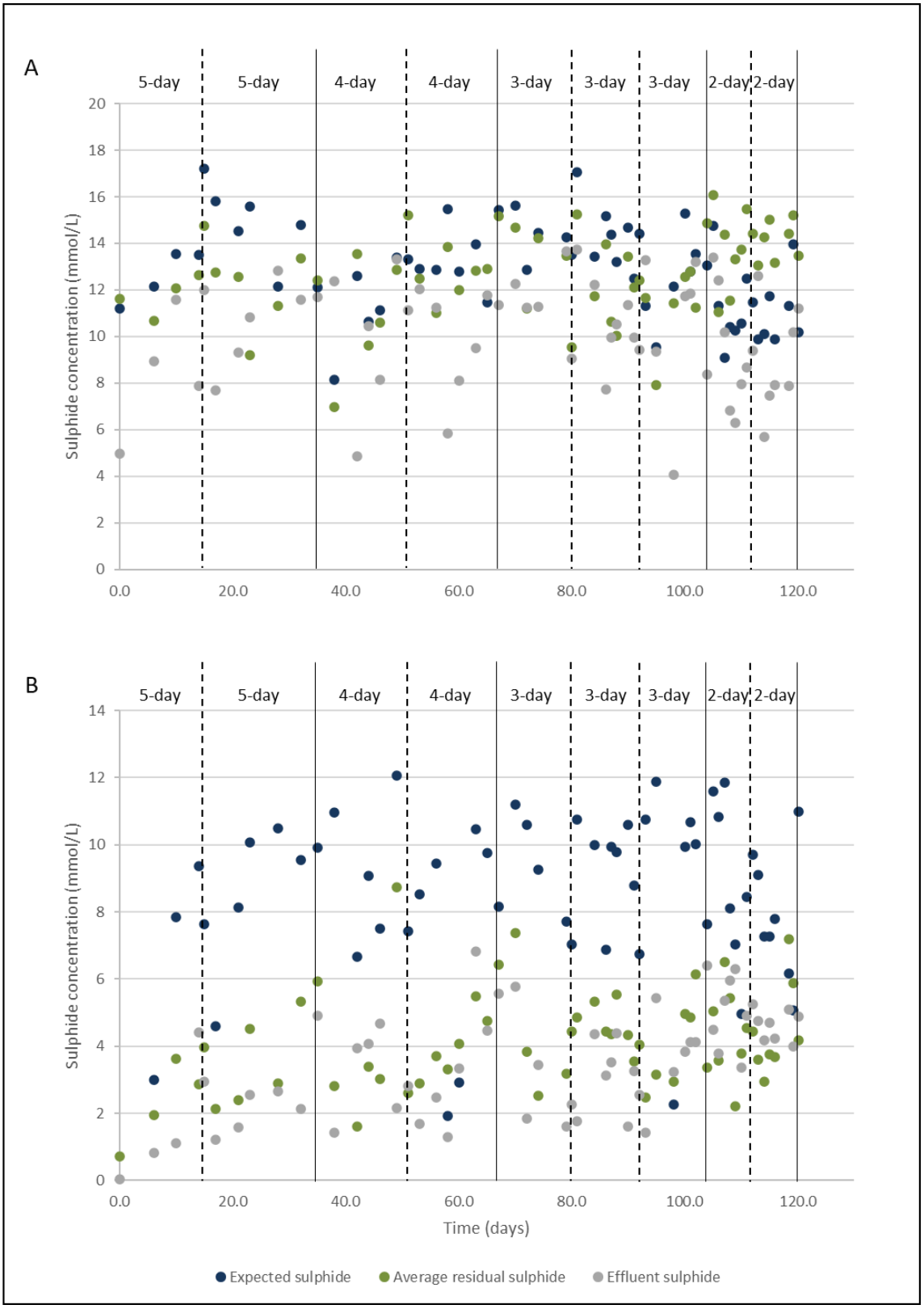


Figure 5-3: Expected, average and residual sulphide time trends in the A) primary reactor(average residual sulphide is the average of sample ports 3, 5, 7 as there was localised sulphate reduction at sample point 1 and reoxidation at the effluent port of each reactor) and B) secondary reactor of LFCR system(average of sample ports 10 and 12) fed with synthetic feed and a lactate carbon source at different hydraulic residence times. The vertical straight lines indicate biofilm harvest (solid lines indicate the start of a study).

The secondary reactor showed a greater difference between expected and average residual sulphide concentrations compared to the primary reactor, indicating increased partial oxidation of sulphide to elemental sulphur, as the decrease in sulphide was not matched by an increase in sulphate. The difference between expected and residual sulphide concentrations was larger at higher HRTs. At lower HRTs, particularly during the second 2-day HRT cycle, expected and residual sulphide concentrations began to converge, suggesting poor partial sulphide oxidation, less mature biofilm formation, and increased susceptibility to re-oxidation.

In comparing the primary and secondary reactors, the secondary reactor's effluent and residual sulphide concentrations were closer together across all HRTs, suggesting less oxidation at its effluent port. The primary reactor exhibited higher sulphide concentrations at the effluent port. Lower HRTs promoted higher VSRRs but resulted in lower conversions of both sulphate and sulphide and higher effluent sulphide concentrations. The secondary reactor proved important in providing sulphur area for partial sulphide oxidation to elemental sulphur in the biofilm, thereby avoiding high residual sulphide concentrations at lower HRTs.

The pH and redox were both within optimal range with pH ranging between 6-8 and the redox well below -250 mV signifying the environment was reducing (Appendix A.10).

VFA time trends provided insight into SRB metabolic pathways. Propionate concentrations were not determined through HPLC and so were calculated from residual lactate, acetate and sulphate concentrations, with an assumption of negligible propionate utilisation. Sulphate reduced was assumed to be linked to incomplete lactate oxidation, forming acetate. Any excess acetate over the acetate concentrations expected from lactate oxidation associated with BSR was estimated to be due to lactate fermentation. Thus, using the acetate produced by fermentation, a propionate concentration was determined stoichiometrically.

On average, 36.8% of lactate was incompletely oxidised to acetate, with the highest percentage occurring at the 5-day HRT. Near-complete utilization of lactate substrate was observed, with residual lactate below 1.50 mmol/L on day 15 at 5-day HRT and 1.04 mmol/L at 3-day HRT in the primary reactor.

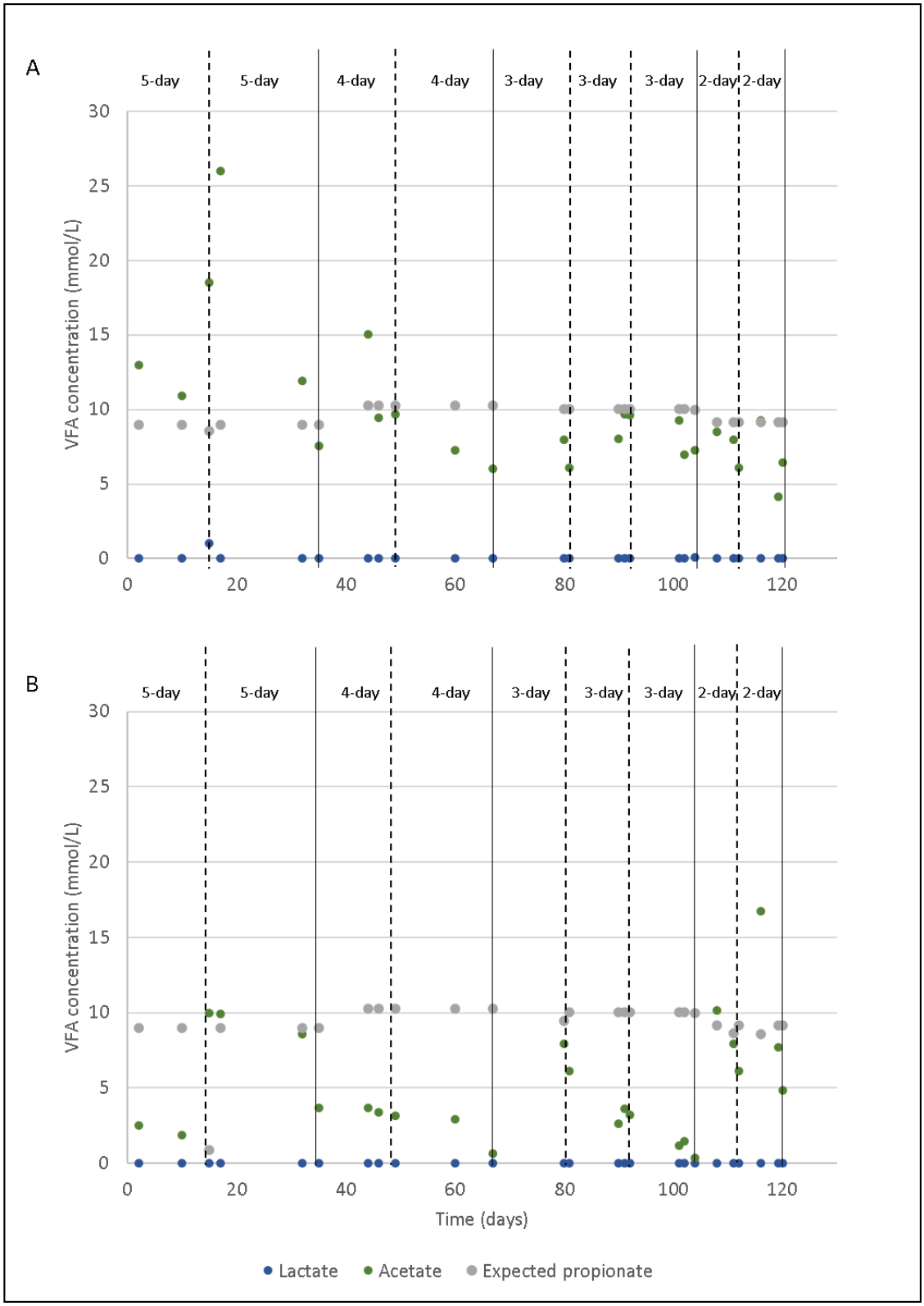


Figure 5-4: VFA concentration time trends in the A) primary reactor and B) secondary reactor of the LFCR system fed with synthetic feed and a lactate carbon source at different hydraulic residence times. The vertical straight lines indicate biofilm harvest (solid lines are the start of a study). Lactate and acetate concentrations were determined through HPLC as described in Section 3.7.7 while propionate concentrations were determined stoichiometrically from acetate concentrations.

High residual acetate concentrations were attributed to incomplete lactate oxidation. Incomplete SRB oxidisers have faster rates than complete oxidisers (Celis *et al.*, 2013). Unoxidized lactate was assumed

to ferment to propionate and acetate at 2:3 and 1:3 ratios respectively, according to Equation 5-1 (Liamleam and Annachhatre, 2007).

$$3\ Lactate \rightarrow Acetate + 2\ Propionate + HCO_3^- + H^+ \qquad \text{Equation 5-1}$$

Fernandes (2020) demonstrated poor propionate utilization by SRB, supporting the assumption of minimal propionate use in the primary reactor, resulting in an average propionate concentration of 9.60 mmol/L across all HRTs.

In the secondary reactor, residual acetate concentrations were below 5 mmol/L in the last 1.5 HRTs for the 4-day and 3-day HRTs. Given the limited sulphate reduction observed in the secondary reactor, acetate was likely used by heterotrophic bacteria necessary for the formation of the FSB (Molwantwa, 2007).

In order to estimate acetate use, available acetate for use was calculated as 'expected acetate' in the primary reactor; it is calculated stoichiometrically based on the assumption that all sulphate reduced was via the incomplete oxidation of lactate to acetate and that the remaining lactate was fermented to acetate and propionate. Expected acetate in the secondary reactor was the acetate concentration measured in the primary reactor effluent. As shown in Figure 5-5, at all HRTs, expected acetate exceeded residual acetate, indicating acetate utilization in both the primary and secondary reactor. Given the findings of poor utilization of propionate as a carbon source and electron donor for BSR (Fernandes, 2020), acetate was assumed to be utilised over propionate in the primary and secondary reactors.

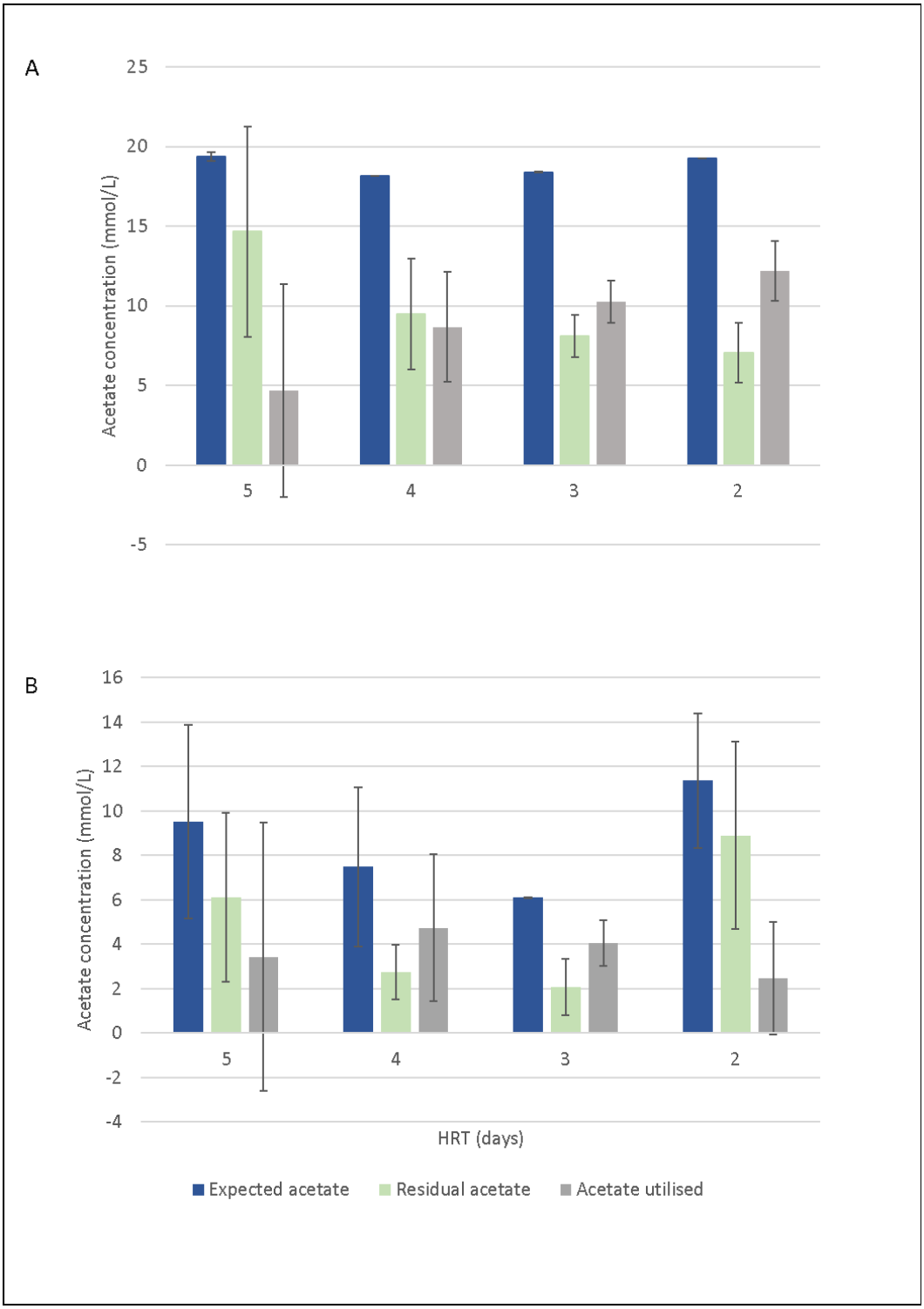


Figure 5-5: VFA concentration balance in the A) primary reactor and B) secondary reactor of the LFCR system fed with synthetic feed and a lactate carbon source at different hydraulic residence times. Lactate was completely utilised and so is not shown in the figure. Expected acetate in the primary reactor is the acetate concentration stoichiometrically calculated based on the assumption that all sulphate was reduced via the incomplete oxidation of lactate to acetate and the remaining lactate was fermented to acetate and propionate. Expected acetate in the secondary reactor, was the acetate concentration measured in the primary reactor effluent.

Figure 5-6 shows the concentration of sulphide converted to sulphur, calculated from the difference between expected and residual sulphide in the different reactors. Results indicate higher concentrations of sulphide converted to sulphur in the secondary reactor when compared to the primary reactor. Peak average sulphide converted to sulphur data was at 5.24 mmol/L (5-day HRT) in the primary reactor and 8.76 mmol/L (3-day HRT) in the secondary reactor. The negative values of sulphide converted to

sulphur at the 2-day HRT were a result of having higher residual sulphide concentrations compared to the expected sulphide concentrations. Higher residual sulphide concentrations were attributed to the release of sulphide from the localised region of high sulphate reduction at sample point one as seen in Figure 5-1. A faster flowrate at the shortened HRT could have disturbed the localised region resulting in the release of sulphide. This was supported by Figure 5-1 which showed an increase mostly at point 3 and at point 5. This increase in sulphide was not carried over to the other ports after port 5. An analysis across the two compartments in the primary reactor from Figure 5-1 shows that the extra sulphide was mostly re-oxidised to sulphate, consistent with the high sulphate concentration at port 7 which was then carried over to the secondary reactor.

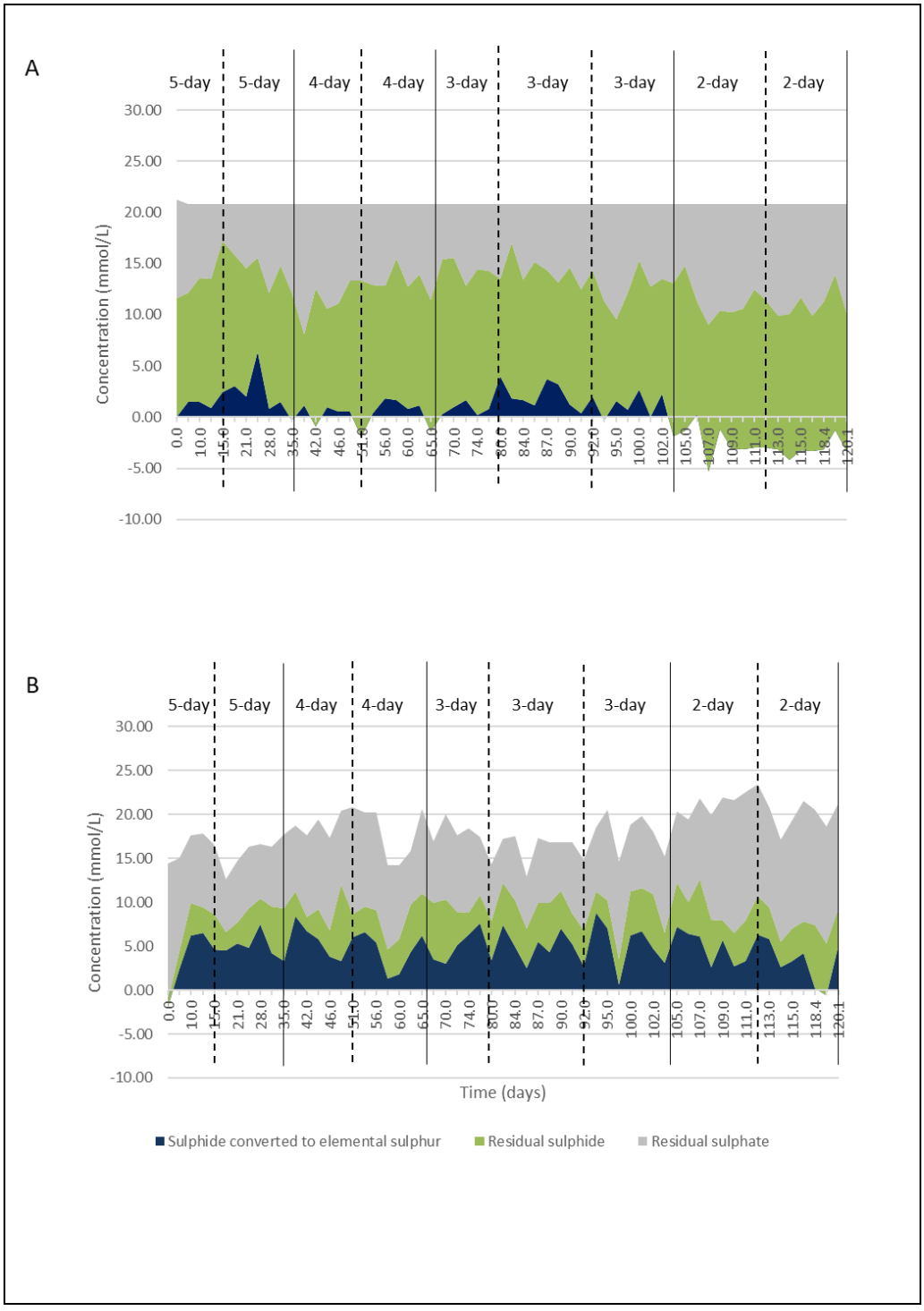


Figure 5-6 : Sulphide converted to sulphur, residual sulphide and residual sulphate concentration time trends in the A) the primary reactor and B) secondary reactor of the LFCR system fed with synthetic feed and a lactate carbon source at different hydraulic residence times, as indicated at the top of the graph. Feed sulphate concentration was at 20.8 mmol/L. The vertical straight lines indicate biofilm harvest (solid lines indicate the start of a study).

The residual sulphide concentration in the primary reactor ranged between 6.98 to 16.07 mmol/L while the secondary reactor ranged between 0.72 and 8.75 mmol/L. Values of residual sulphide entering the negative in both the primary and secondary reactor were due to the residual sulphide being higher than the expected sulphide which was unexpected. Due to the 2-day HRT having the fastest flowrate, there was possible mobilisation of dead spaces such as the one observed at sample point 1 (Figure 5-1) causing increase in the residual sulphide.

Figure 5-7 A and B shows the amount of expected sulphide, residual sulphide and residual sulphate in the LFCR system fed with approximately 670 mmol of sulphur in the form of sulphate over 4 HRTs. The 3-day HRT yielded the highest expected sulphide and one of the lower residual sulphide amounts, indicated by a high amount of sulphide converted to sulphur of 40.3 mmol second to the 5-day HRT (45.3 mmol) in the primary reactor (Figure 5-7 A). The 2-day HRT yielded the highest residual sulphate of 311 mmol. This was attributed to the biofilm at the 2-day HRT not fully maturing resulting in increased oxygen ingress into the system which in turn favours re-oxidation of sulphide over partial oxidation.

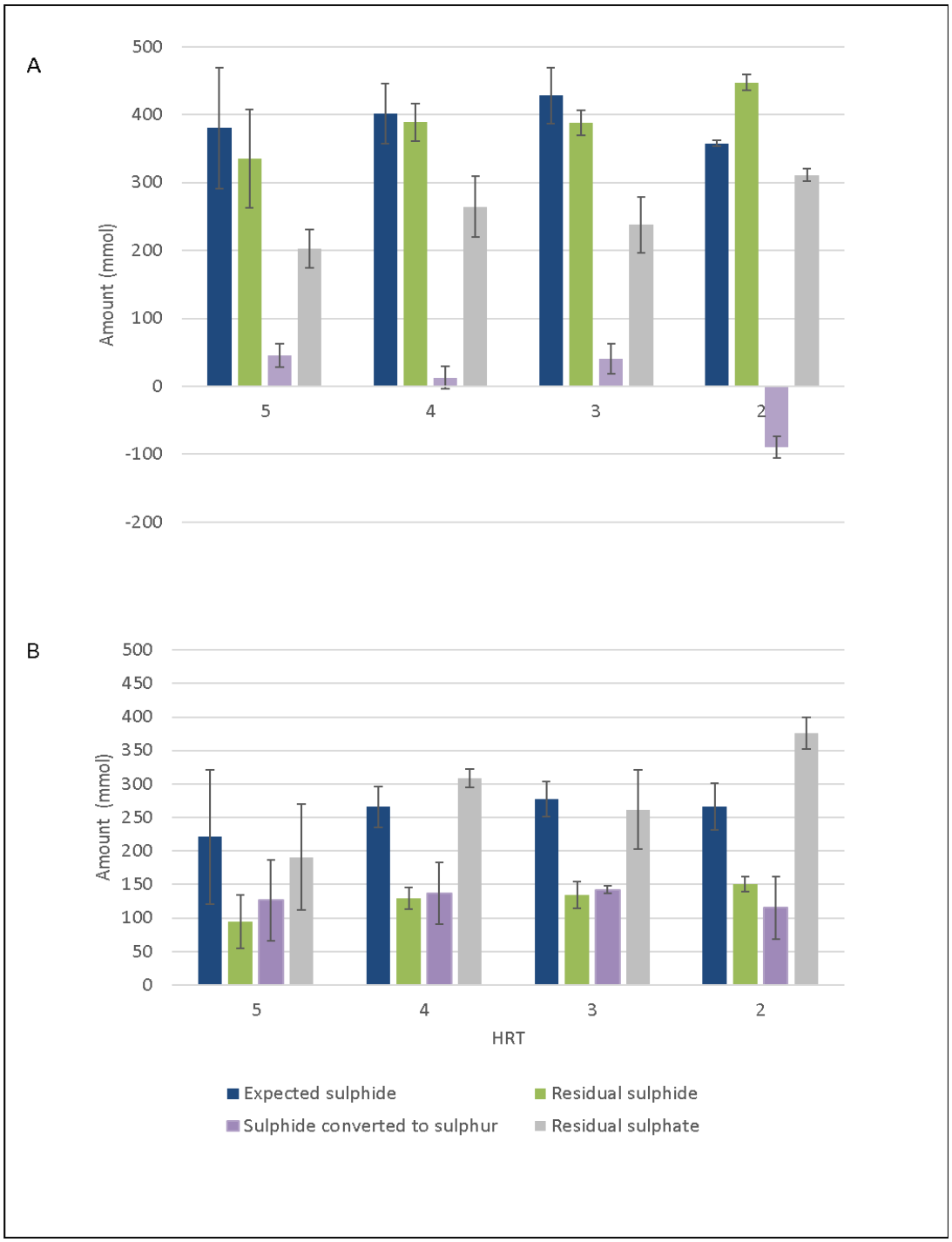


Figure 5-7: LFCR system fed with synthetic feed and a lactate carbon source expected sulphide, effluent sulphide, residual sulphate and sulphide converted to sulphur in the A) primary reactor and B) secondary reactor at different hydraulic residence times. Approximately 670 mmol of sulphate fed into the reactor in 4 HRTs.

Much higher amounts of sulphide were converted to sulphur in the secondary reactor (Figure 5-7 B) than in the primary reactor, confirming that the secondary reactor was necessary to improve sulphur recovery. High amounts of sulphide converted to sulphur with little to no sulphate conversion supported the assumption that acetate was mostly used by heterotrophic bacteria in the formation of the FSB. The 3-day HRT had the highest amount of sulphide converted to sulphur at 142 mmol while the 2-day had the lowest at 115 mmol.

Table 5-1: Reaction kinetics of the LFCR system fed with synthetic feed and a lactate carbon source at different hydraulic residence times. Sulphate conversion in the primary reactor is the feed sulphate concentration

		Primary reactor (R1)					Secondary reactor (R2)				
HRT (days)	VSLR mmol/L.h	Dilution rate h-1	VSRR (mmol/ L.h)	Sulphate Conversion [a] (%)	Sulphide Conversion [b] (%)	VSFR (mmol/ L.h)	Dilution rate h-1	VSRR (mmol/ L.h)	Sulphate conversion [c] (%)	Sulphide conversion [b] (%)	VSFR (mmol /L.h)
5	0.173	0.0083	0.116	66.7%	11.9%	0.0094	0.027	-0.047	-39.6%	57.3%	0.143
4	0.217	0.0104	0.137	63.0%	3.2%	-0.0016	0.033	-0.040	-15.1%	51.3%	0.149
3	0.289	0.0139	0.189	64.8%	9.4%	0.0095	0.044	-0.062	-24.7%	51.4%	0.216
2	0.433	0.0208	0.243	56.0%	-25.1%	-0.0579	0.066	-0.089	-14.4%	43.4%	0.189

[a] Sulphate conversion in the primary reactor is the percentage of feed sulphate converted

[b] Sulphide conversion in the primary reactor is the percentage of expected sulphide converted to sulphur

[c] Sulphate conversion in the secondary reactor is the percentage of influent sulphate from the primary reactor converted

The 3-day HRT showed moderate sulphate conversion, high VSRR (0.189 mmol/L.h), and significant sulphide conversion in the secondary reactor. In the primary reactor it also achieved a sulphate conversion of 9.4%, higher than the 3.2% achieved at the 4-day HRT. It was also noted in these runs that due to the bulk of the sulphate reduction occurring in the first compartment of the primary reactor, spanning from the inlet port to the first top baffle (including sample ports 1-4), a potential for reactor optimization is presented: either by reducing reactor length to cut material costs or by increasing sulphate loading to process higher volumes or more concentrated sulphate-rich wastewater.

Volumetric sulphur formation rate (VSFR) generally increased with decreasing HRT, peaking at 3-day HRT in the secondary reactor; a decrease at the 2-day HRT was possibly due to re-oxidation and shortened sulphur deposition time. In the primary reactor the VSFRs are much lower showing poor sulphur formation. The negative VSFR at the 2-day HRT was due to negative sulphide conversions as explained above.

Sulphate conversion remained fairly constant between the 3 and 5-day HRTs while the VSRRs at these residence times increased when the HRT was shortened. High VSLRs which are not matched by increase in the VSRR result in decreased sulphate conversion, leaving behind unprocessed sulphate as seen at the 2-day HRT which had the lowest sulphate conversion. A compromise was necessary to achieve high VSRR and sulphate conversion. The 3-day HRT was chosen as optimal, achieving high sulphate conversion, VSRR, and VSFR while also maintaining a high VSLR in the primary reactor. One of the highest amount of sulphide converted to sulphur in the secondary reactor was also achieved at the 3-day HRT.

5.3 Demonstration of performance of the LFCR system on an AMD and lactate substrate

The study examined the LFCR's performance using real AMD rather than the synthetic feed used above, to observe the additional considerations required for implementation into a real-world scenario. The chemical composition of the AMD, and potential treatment challenges like metal precipitation and pH fluctuation were considered. The research investigated the BaH-LFCR system's ability to treat AMD obtained from a pilot study of the accelerated leaching of sulphide-bearing coal waste and fines gathered from a mine site in Mpumalanga, South Africa (Tambwe *et al.*, 2020).

After selecting the necessary AMD pretreatment through fed-batch tests (Section 5.3.2), the hybrid reactor was fed pre-treated AMD. Initial experiments used lactate as the carbon source and electron

donor (Section 5.3.3). Following performance assessment, lactate was replaced with molasses (Section 5.4).

5.3.1 Characterisation of real AMD

AMD was produced in the CeBER greenhouse from coal discards and fine waste tailings from Mpumalanga. Pretreatment was assessed to remove metals and increase pH, as high heavy metal content and acidic pH were expected to inhibit SRB (Koschorreck, 2008).

Conventional AMD treatments typically address acid and heavy metal components but fail to remove sulphates to required standards, necessitating expensive polishing steps (Fernando *et al.*, 2018; Torres *et al.*, 2018). The LFCR system may offer potential for a cost-effective treatment capable of handling significant sulphate loads while achieving required water quality and partly reclaiming the sulphur for re-purposing.

Table 5-2 compares the compositions of the (untreated) AMD produced from studies by CeBER with AMD from Mpumalanga mines, as quoted in literature (Alegbe *et al.*, 2019).

Table 5-2: Composition, pH and conductivity comparison of AMD from the Mpumalanga region and untreated and treated AMD produced from coal discards sourced from the Mpumalanga region (Alegbe *et al.*, 2019)

Description	Units	Mpumalanga Region AMD	Untreated Greenhouse AMD	Greenhouse AMD treated with lime
Sulphate	mg/L	2 500 – 20 000	1701	1696
pH		2.0 – 3.0	2.43	8.42
Conductivity	mS/m	0.4 – 1.6	2.98	1.92
Al	mg/L	55.0 - 700	8.44	0.0616
As	mg/L	0.05 – 3.50	0.0029	0.0012
B	mg/L	0	0.0909	0.104
Ba	mg/L	0.001 - 0.035	0.0209	0.024
Be	mg/L	0.050 – 0.150	ND	ND
Ca	mg/L	500 -900	512	683
Cd	mg/L	0.050 – 0.310	0.00024	0
Co	mg/L	0.100 – 2.00	0.0071	0.0008
Cr	mg/L	0 – 0.300	0.0119	0.0006
Cu	mg/L	0.050 – 0.500	0.139	0
Fe	mg/L	100 - 4200	52.3	0.0037
Hg	mg/L	0 – 5.00	0	0
K	mg/L	2.00 – 20.0	0	12.8
Mg	mg/L	100 -500	8.57	87.6
Mn	mg/L	50.0 -200	0.928	0.199
Mo	mg/L	0 – 0.300	0.0002	0.0002
Na	mg/L	100 - 900	9.61	0.0102
Ni	mg/L	0 – 4.00	0.0198	0.0062
Pb	mg/L	0 – 0.200	0	0
Se	mg/L	0 – 2.00	0.0071	0.0029
Si	mg/L	9.00 – 60.0	25.1	13.7
Sr	mg/L	0.500 – 5.00	2.52	2.58

Description	Units	Mpumalanga Region AMD	Untreated Greenhouse AMD	Greenhouse AMD treated with lime
Th	mg/L	0.01 – 2.00	ND	ND
Ti	mg/L	0 – 0.200	ND	ND
V	mg/L	0 – 0.06	0.00058	0.00008
Y	mg/L	0.05 – 3.0	ND	ND
Zn	mg/L	0.1 – 20.0	0.190	0.0091

Both Mpumalanga and CeBER-produced AMD had acidic pH between 2 and 3. SRB are highly sensitive to pH as toxins and inhibitors are more potent at low pH ranges (Koschorreck, 2008). At pH 4 SRB and methanogens are inhibited in mixed cultures (Koschorreck, 2008;Watson-Craik and Senior, 1996). The ideal pH range for SRB was reported to be 6-8 (Koschorreck, 2008).

Low pH can cause protein denaturation and macromolecule destabilization (Fink *et al.*, 1994; Koschorreck, 2008). SRB 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 highly 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 so, though SRB produce alkalinity when they reduce sulphate, it is not enough to combat the fast diffusion of protons (Koschorreck, 2008).

Table 2-5 in Section 2.4.8 summarised toxic concentration levels for SRB. All metals in the untreated AMD presented in Table 5-2 were below these toxic levels. Calcium concentrations exceeded WHO potable water limits (100-300 mg/L). This concentration of calcium may not affect the efficiency of the mixed community's performance but should be considered when evaluating outlet water quality. Mg, K, and Si were in high concentration (>4 mg/L) in the CeBER-produced AMD. Cao *et al* (2009) showed increased Mg positively affected SRB, improving sulphate conversion even at concentrations as high as 100 g/L. Potassium (12 mg/L) was lower than that in the SRB Postgate media B, where it is 121 mg/|L (Postgate, 1963).

After lime pre-treatment, sulphate concentration remained fairly constant (Table 5-2). Conductivity decreased from 2.98 to 1.92 mS/m, remaining below the 2.5 mS/m limit for human consumption (Loock *et al.*, 2015). There was a pH increase from 2.43 to 8.42, causing metal precipitation. Table 5-3 shows removal efficiencies for metals above 0.001 mg/L, including Fe which had a concentration of 52.3 mg/L before treatment. Metal removal through pH increase created suitable conditions for sulphate removal by SRB.

Table 5-3: Metal removal efficiency from AMD produced from coal discards from the Mpumalanga region after lime pretreatment

Metal	Metal concentration before treatment (mg/L)	Metal concentration after treatment (mg/L)	Percentage removed
Al	8.44	0.0616	99%
Co	0.01	0.0008	89%
Cr	0.01	0.0006	95%
Cu	0.14	0	100%
Fe	52.34	0.0037	100%
Mn	0.93	0.1986	79%
Ni	0.02	0.0062	69%
Si	25.13	13.74	45%
Sr	2.52	2.578	0%
Zn	0.19	0.0091	95%

5.3.2 AMD fed-batch reactor results

Initial fed-batch experiments assessed the SRB culture's ability to perform sulphate reduction on real AMD pre-treated in various ways. These tests were conducted before introducing AMD into the baffled reactor system.

Fed-batch cultures were performed in 1 L Schott bottles at 30°C, using either pre-treated or untreated AMD from the CeBER greenhouse. Only lactate was added as the organic substrate (carbon source and electron donor), with no other nutrients or components from the modified Postgate B media included.

This initial fed-batch culture study provided a comprehensive assessment of the SRB culture's effectiveness in treating AMD and its need for pretreatment. These results are presented in Figure 5-8.

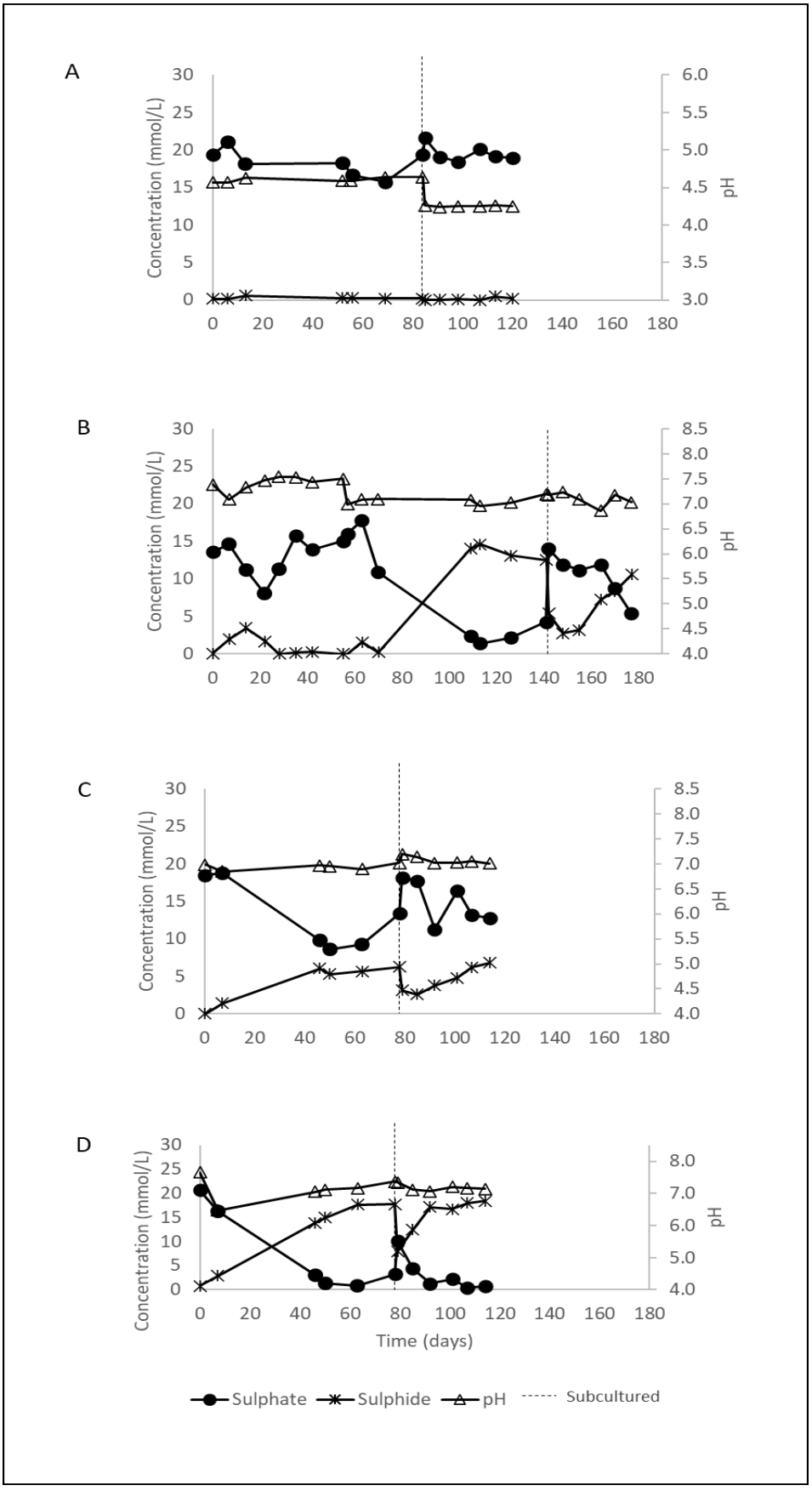


Figure 5-8: Trends in sulphate, sulphide and pH with time in the fed-batch reactor supplied with A) untreated AMD, B) AMD pre-treated with lime, C) AMD treated with lime and sodium sulphide and D) AMD pre-treated with lime and sterilised.

Figure 5-8 A) shows the performance of fed-batch reactor fed with untreated AMD from the CeBER greenhouse. Despite a 110-day adaptation period, SRB could not function in the harsh conditions, resulting in low sulphate reduction. The fed-batch reactor fed with untreated AMD had an average sulphate conversion of 9% across two cycles, the lowest among the four fed-batch reactors. Despite metal concentrations being below toxic levels (Table 5-2), poor performance was attributed to low pH.

Elliott *et al.*, (1998) reported SRB adaptation to acidic conditions typically took less than 50 days. However, this culture did not adapt sufficiently over 100 days, likely lacking acidophilic SRBs. At pH levels below 3.5, adaptation periods exceed 20 days, with unsustained sulphate reduction at pH 3 (Elliott *et al.*, 1998). Koschorreck, (2008) noted the importance of substrate type in acidic conditions, with non-ionic substrates like methanol and glycerol improving sulphate reduction over ionic substrates like lactate.

Figure 5-8 B shows time trends for sulphate, sulphide and pH in the lime-pre-treated AMD fed-batch reactor. This reactor ran for 180 days after a 110-day adaptation period. The pre-treated AMD fed-batch experiment underwent two cycles. The first cycle lasted about 140 days, with initial sulphate decrease in 20 days, followed by sulphide re-oxidation. Significant sulphate reduction resumed on day 60, reaching maximum conversion of 89.8% at day 113.The second cycle was shorter, with significant sulphate reduction occurring after 20 days, unlike the 60-day delay in the first cycle. An average sulphate reduction of 75.8% was reached. Initial delays in sulphate reduction might have been caused by metal sulphide inhibitors, alkaline pH, or competition from indigenous microorganisms. Despite feed pH above 8, the bulk fluid pH in the reactor was 7.5.

Two additional fed-batch reactors were set up to test potential inhibition by residual metal sulphides or competition from indigenous microorganisms. One used AMD pre-treated with lime and sulphide; the latter added to precipitate metals. The other used autoclaved, lime-pre-treated AMD to eliminate indigenous microorganisms. Both used lactate as the carbon source and electron donor. The reactor testing metal sulphide inhibition was fed with AMD treated with lime and sodium sulphide. Metal sulphide precipitates were removed by filtration before feeding. Figure 5-8 C) showed the concentration profiles for this reactor. This fed-batch reactor was inoculated with a culture adapted to lime-treated AMD. It achieved a maximum sulphate conversion of 54% in the first cycle around day 50, and an average of 29% across two cycles.

Sulphate reduction decreased compared to the lime-only treated AMD reactor. This could be due to increased sodium concentration, which can hinder sulphate reduction in BSR systems (Vinícius and Vallero, 2003).

The fed-batch reactor investigating competition between SRB and indigenous microorganisms used lime-treated, autoclaved AMD; this ensured all organisms were killed before treatment. The sterilisation step was a requirement for the set-up rather than a planned scale-up measure. Time trends are shown in Figure 5-8 D). It was also inoculated with a mixed culture from the fed-batch reactor receiving sterilised synthetic feed and a lactate carbon source. This was to avoid carry over of the indigenous microorganisms from the other three AMD fed-batch reactors which were fed a non-sterilised AMD feed. This reactor achieved the highest conversion among the four AMD fed-batch reactors: 96% maximum in the first cycle and 89% average across two cycles.

Improved performance was likely due to the removal of indigenous microorganisms. Coal discard leachates typically contain iron and sulphur oxidisers (Tambwe *et al.*, 2020; Li *et al.*, 2023). Conversion of the sulphide back to sulphate decreases BSR system efficiency (Suzuki *et al.*, 1990; Tambwe *et al.*, 2020; Li *et al.*, 2023). However, these acidophiles were unlikely competitors and other possible competitors were not characterised. While sterilization allowed SRB activity to thrive, it does not represent a practical approach but rather provides understanding of the system.

Table 5-4 shows a summary of the performance of the AMD fed-batch reactors, the average and maximum sulphate conversion and the maximum sulphide concentration reached in each. As expected, the reactor receiving AMD treated with lime and sterilised gave the highest average conversion of 89% and the highest sulphide concentration of 18.4 mmol/L.

Table 5-4: The summary of performance of AMD fed-batch reactors, given in terms of average and maximum sulphate conversion and maximum sulphide concentration

Fed-batch reactor	Untreated AMD	AMD treated with lime	AMD treated with lime and sulphide	AMD treated with lime and sterilised
Average sulphate conversion (%)	9%	76%	29%	89%
Maximum sulphate conversion (%)	19%	90%	54%	96%
Maximum sulphide concentration (mmol/L)	4.62	14.6	6.81	18.4

Sulphate reduction in the sterilised, pre-treated AMD also exceeded that observed in the lactate fed-batch reactor fed with synthetic AMD (Figure 4-1). This was unexpected given the residual heavy metals in pre-treated AMD compared to the nutrient dense synthetic AMD. Utgikar *et al*., (2002) indicated that low metal concentrations can improve SRB activity. Thus, trace metals in the sterilised, pre-treated AMD might have enhanced overall sulphate reduction.

Figure 5-9 shows the VFA content at the beginning and end of cycles in the AMD fed-batch reactors. Complete VFA utilisation occurred in AMD fed-batch reactors B and C (Figure 5-9). This was expected due to possible VFA consumption by native bacteria from coal discards and fine wastes as they both received unsterilised AMD feed. Reactor B's high sulphate conversion was attributed to maximum VFA utilisation. However, reactor C showed poor conversion despite maximum VFA use, suggesting VFA consumption primarily by native bacteria rather than SRB.

AMD fed-batch reactor A exhibited poor lactate utilization (17%). Incomplete oxidation was evident from increased acetate which rose from 0.93 to 1.21 g/L and propionate (0.46 to 0.54 g/L). Poor VFA utilisation was expected due to low pH, unsuitable for SRB function and possibly also reducing native bacterial activity.

The fed-batch reactor D), fed with pre-treat samples and from which the indigenous microbes had been eliminated, achieved the highest sulphate conversion, but with incomplete lactate oxidation. Lactate was fully utilised, with acetate increasing from 1.25 to 2.48 g/L. Incomplete VFA utilisation suggested the absence of native bacteria due to feed sterilisation, contrasting with AMD fed-batch reactor B where native micro-organisms likely oxidised residual VFAs.

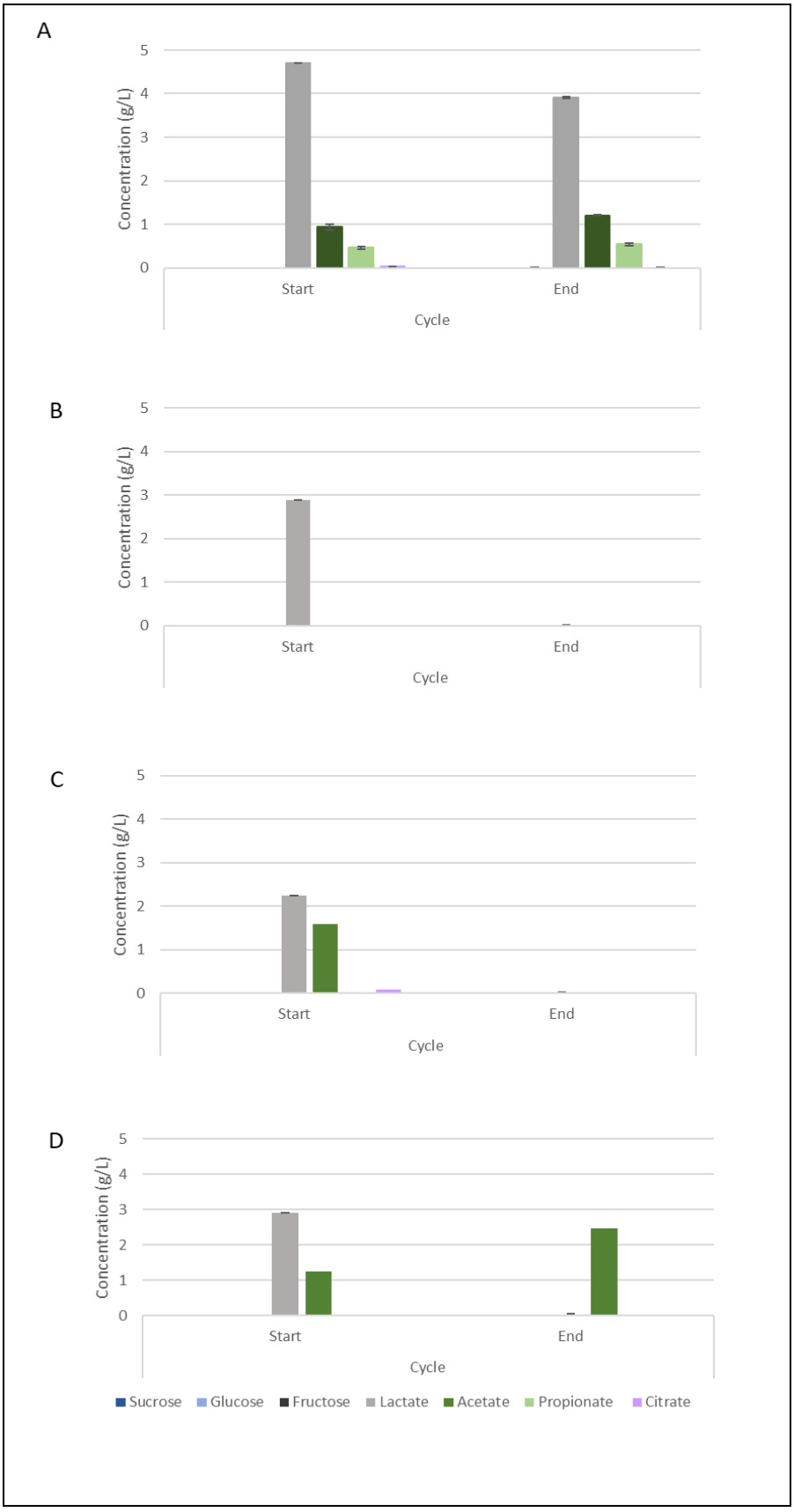


Figure 5-9: VFA time trends for A) untreated AMD, B) AMD pre-treated with lime, C) AMD treated with lime and sodium sulphide and D) AMD pre-treated with lime and sterilised in fed-batch reactors.

Both sterilisation and lime pretreatment were necessary for the LFCR system. Lime pretreatment increased pH to circum-neutral conditions required by the SRB and precipitated heavy metals. Lime treatment was chosen as the desired pre-treatment process due to its efficiency in metal removal, neutralisation and the low cost of lime, the active ingredient, compared to other alternatives (Aubé, 2003). Sterilisation eliminated indigenous microorganisms, which were expected to be at higher levels in the pilot study AMD than traditional environmental AMD (coal discards and fines were inoculated in the pilot study). Native bacteria reduced carbon source availability for SRB, as seen in reactors B and C. Sterilisation also prevented feed fermentation after carbon source addition.

5.3.3 LFCR studies with partially treated AMD and lactate as the feed

The AMD fed-batch reactor study assessed the mixed culture's ability to treat AMD and pre-treated AMD versus synthetic feed. The pre-treatment method used in the AMD fed-batch reactor that yielded best performance in terms of sulphate reduction was chosen as the pretreatment method for the LFCR system. Using pre-treated AMD with lactate as carbon source and electron donor, the LFCR system's capability to treat actual AMD with a simple, well-studied carbon source proven efficient in BSR was evaluated.

The mixed culture used in the LFCR in prior studies was adapted to pre-treated AMD by incrementally increasing AMD concentration in the feed from 20 to 100%, in increments of 20%. Postgate media components were also eliminated over time allowing only the carbon source to be supplemented. Stagewise adaptation was conducted, increasing AMD concentration after each FSB harvest at a 3-day HRT. All runs in which the ratio of AMD to synthetic feed were increased, including the 100% AMD test, were conducted at a 3-day HRT, previously determined optimal from the study on synthetic feed containing 2 g/L sulphate concentration and lactate at a COD:SO_4^{2-} ratio of 1 (Section 5.2). For 20-80% AMD runs, duration depended on reaching pseudo steady state before FSB harvesting. 100% AMD runs lasted 4 HRTs (12 days) for comparability with synthetic AMD studies.

Figure 5-10 A shows the sulphate concentration profiles of the LFCR sequence system fed with pre-treated AMD and lactate substrate. Sulphate reduction in the primary reactor increased with an increase in the real AMD fraction, peaking at a 92% average in the 80:20 AMD:Synthetic run. The lowest reduction (82.8%) occurred in 20:80 AMD:Synthetic run. Most sulphate reduction happened in the first compartment (sample points 1 and 3) of the primary reactor, maintaining low concentrations at points 5 and 7.

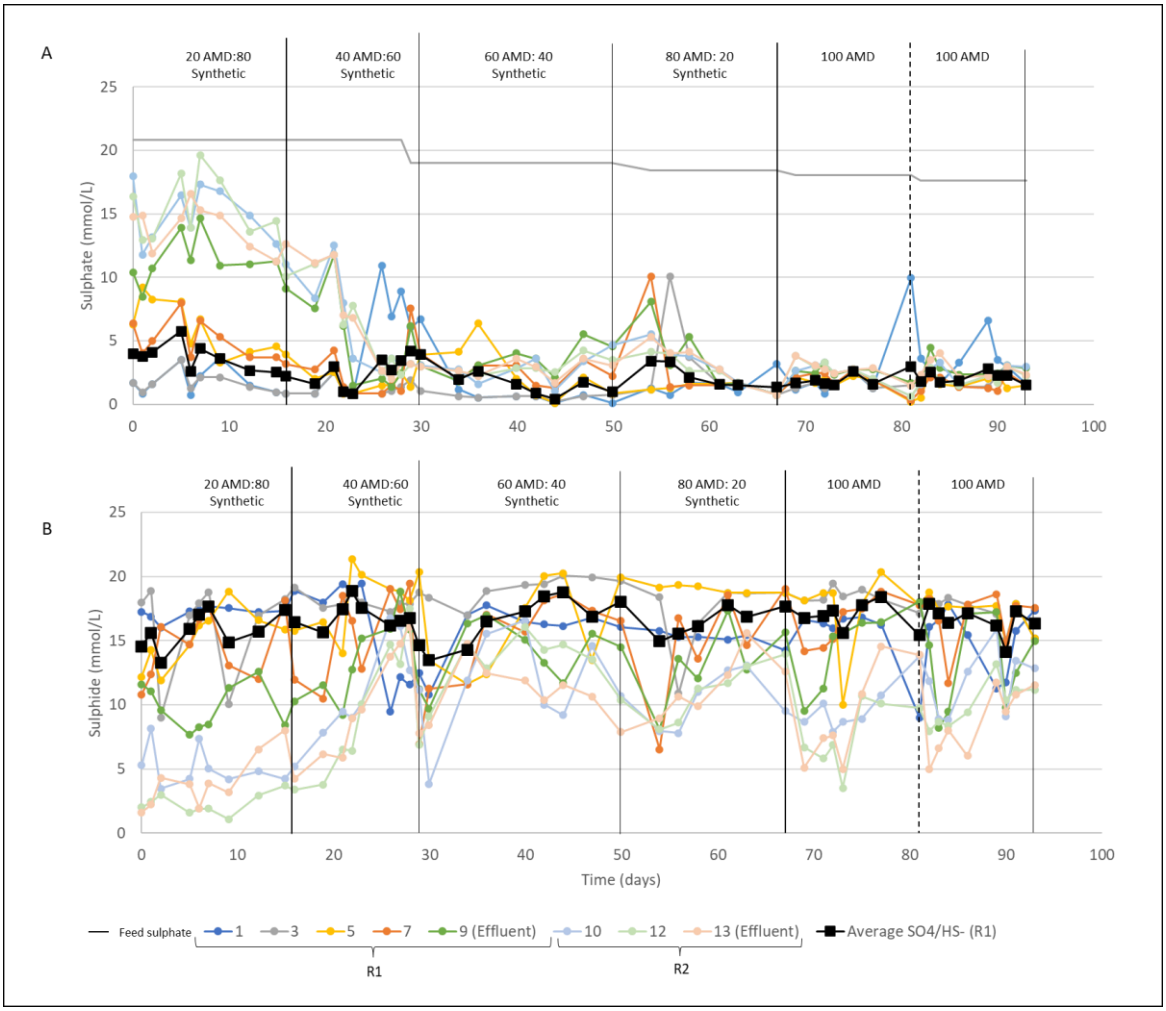


Figure 5-10: Time trends for A) sulphate and B) sulphide concentrations for the LFCR system fed with AMD and a lactate carbon source at different AMD feed ratios. Average SO4 and HS- is the average of sample ports 1, 3, 5, 7. The vertical straight lines indicate biofilm harvest (solid lines indicate the change in the AMD feed ratio).

Re-oxidation occurred at sample point 9 (primary reactor effluent) in the 20:80 AMD:Synthetic run, with increased sulphate concentration persisting through the secondary reactor. Average residual sulphate concentrations were 2-6 mmol/L in the primary reactor but exceeded 15 mmol/L (feed sulphate concentration was between 17 – 21 mmol/L) in the secondary reactor for the 20:80 AMD:Synthetic ratio due to re-oxidation.

As AMD concentration in the feed increased, re-oxidation decreased at the primary reactor effluent port and in the secondary reactor. Anomalies occurred between days 50-60 (sulphate spikes at sample points 7 and 3) and days 75-100 (spikes at point 1). The lowest sulphate concentration, 0.42 mmol/L, was observed in the 60:40 AMD:Synthetic run. After the 20:80 and 40:60 AMD:Synthetic runs, sulphate ranged between 0.4-4.0 mmol/L, averaging 2.20 mmol/L in the 100% AMD run.

Sulphate reduction remained high despite removing Postgate B media nutrients. The 100% AMD run achieved an average 87.4% sulphate conversion compared to a sulphate conversion range of 82% to 92% in the primary reactor. This conversion was higher than the 64.8% conversion in the synthetic feed at 2 g/L (20.8 mmol/L) sulphate concentration and 3-day HRT run. Unlike in the synthetic feed experiment (Section 5.2), biofilm harvesting had little effect on sulphate concentration. This was attributed to the high sulphide concentrations.

Figure 5-10 B shows sulphide concentration time trends for pre-treated AMD with lactate substrate. Sulphide concentrations remained high throughout all AMD runs in the primary reactor, averaging 16.5 mmol/L (546 mg/L). In the secondary reactor, sulphide concentrations were low for the 20:80 and 40:60

AMD:Synthetic runs. Figure 5-10 A shows increased sulphate concentration in the secondary reactor for 20:80 and 40:60 AMD:Synthetic runs, indicating re-oxidation. Low sulphate concentrations in subsequent runs, coupled with decreased sulphide, indicated elemental sulphur formation. The 80:20 AMD:Synthetic run showed minimal decrease in the sulphide concentration while maintaining low sulphate concentrations, suggesting little to no elemental sulphur formation. This was noteworthy since elemental sulphur formed deposits into a FSB that impedes oxygen from entering the bulk liquid. Instead some other form of film was observed at the surface which was visually different from the FSB but appeared to be impeding oxygen allowing for high sulphide concentrations to be maintained.

Figure 5-11 displays VSRR and sulphate conversions for increasing AMD:synthetic feed ratios. Increasing the AMD component tended to decrease VSRR though the change was slight. This corresponded to the slight decrease in the VSLR owing to the slightly lower sulphate concentration in the AMD stream than in the synthetic feed. The lowest VSRR (0.216 mmol/L.h) occurred during 100% AMD run and corresponded to a VSLR of 0.247 mmol/L.h, while the highest VSRR (0.257 mmol/L.h) was at 20:80 AMD:Synthetic ratio, corresponding to a VSLR of 0.289 mmol/L.h. The slightly reduced VSRR reduction at 100% AMD was largely attributed to fluctuations in feed sulphate concentration, and thereby VSLR, rather than decreased reactor performance.

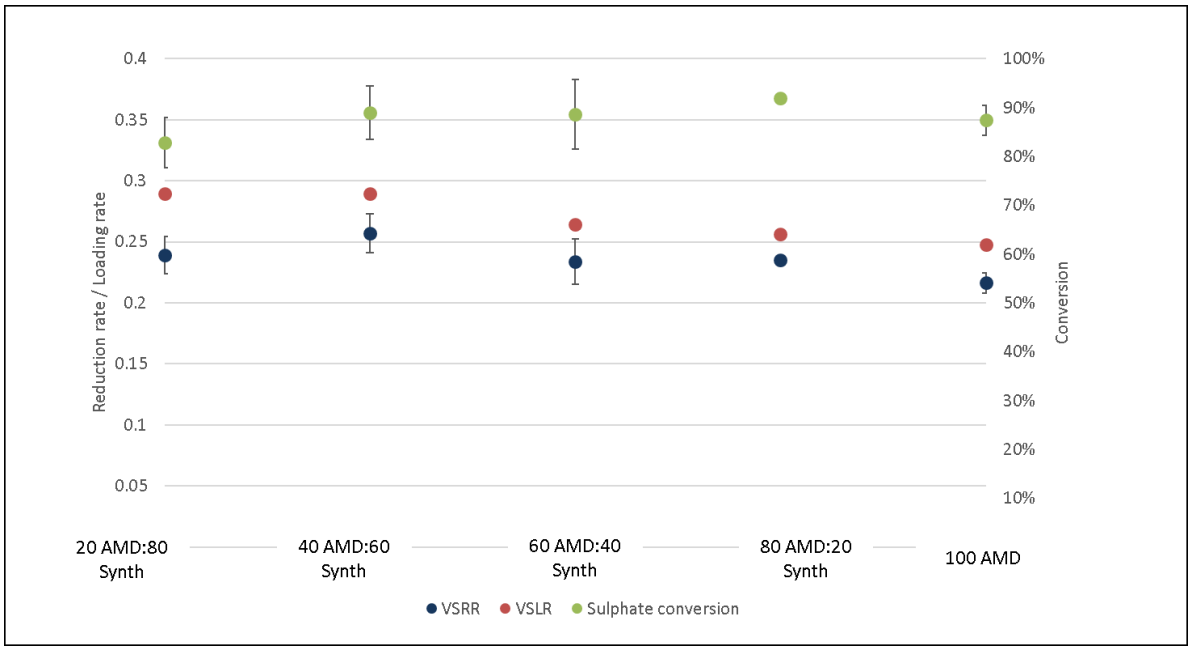


Figure 5-11: Steady state kinetics of sulphate reduction in the primary reactor of the LFCR system fed with AMD and a lactate carbon source at different AMD feed ratios.

Sulphate conversion tended to increase with AMD ratio, peaking at 92.0% for 80:20 AMD:Synthetic, however changes were within error. The lowest conversion was 82.8% at 20:80 AMD:Synthetic, with an average of 87.9% across all concentrations. The slight decrease from 80:20 AMD:Synthetic to 100% AMD may have been due to the complete removal of additional Postgate media nutrients. At a 3-day HRT, 100% AMD feed achieved an 87.9% sulphate conversion, significantly higher than the 64.8% with synthetic feed.

Figure 5-12 A shows the expected, average residual, and effluent sulphide concentrations in the primary reactor. Between the 20 AMD:80 Synthetic and the 40 AMD:60 Synthetic runs, expected sulphide was higher than the average residual sulphide which indicated conversion of sulphide to sulphur. This is supported by the low sulphate concentrations in the primary reactor in Figure 5-10. During the 60 AMD:80 Synthetic and 80 AMD:60 Synthetic runs, the expected and average residual sulphide concentrations converged showing less sulphur formation during these runs. The 100% AMD runs had slightly higher average residual sulphide concentrations than the expected sulphide and thus little to partial oxidation of sulphide was occurring in the primary reactor. Throughout the runs across all the AMD ratios, the effluent sulphide concentration was lower than the average residual and expected

sulphide concentrations indicating sulphide oxidation was occurring. Sulphide concentrations typically decreased post-harvest of the FSB, then increased with time until the next harvest, again due to gradual biofilm formation that impeded oxygen entry. Expected and effluent sulphide concentrations throughout the AMD+ lactate experiment mostly remained between 15 and 20 mmol/L.

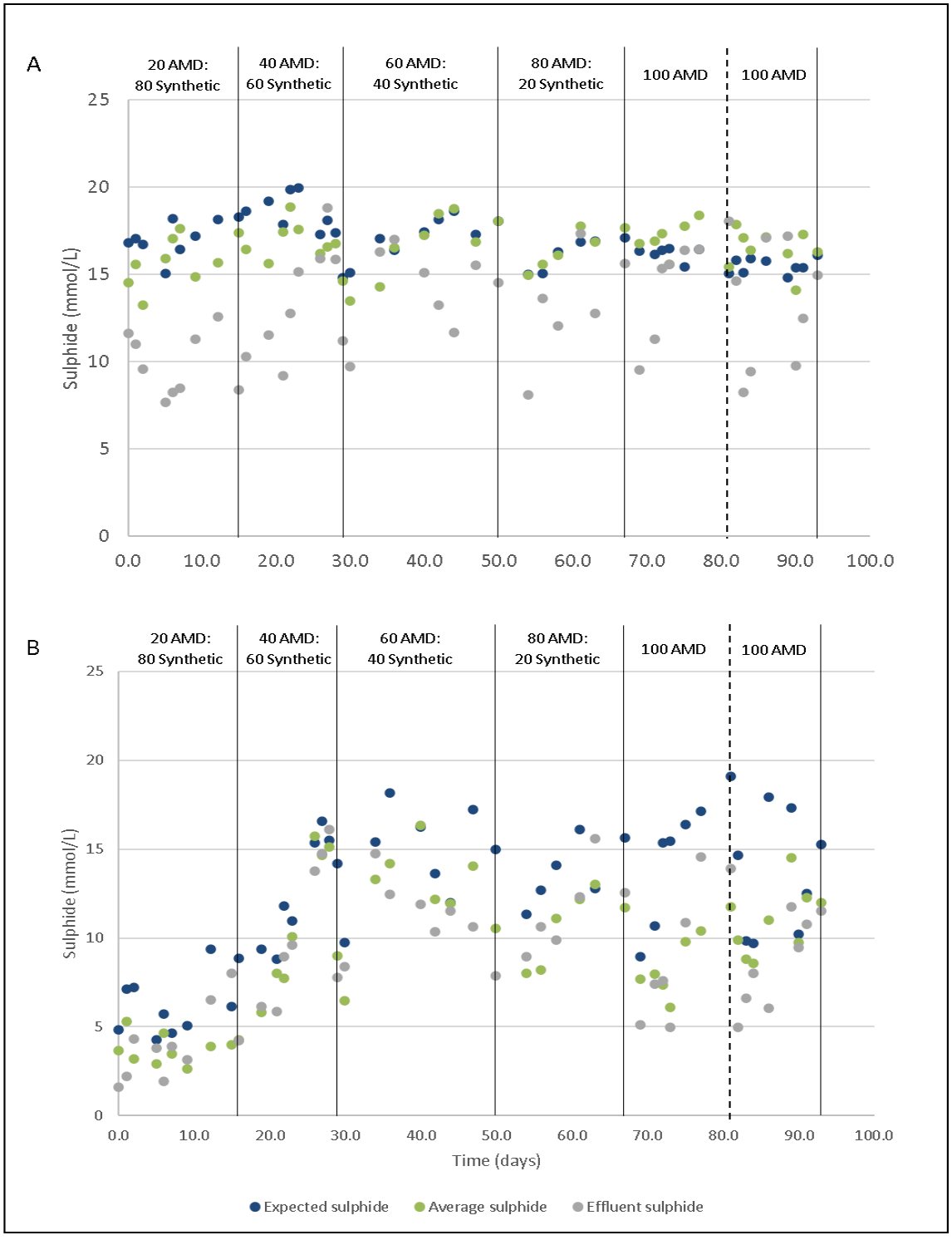


Figure 5-12: Expected, average and residual sulphide time trends in the A) primary reactor (average residual sulphide is the average of sample ports 1,3, 5, 7 in the primary reactor as there was reoxidation at the effluent port of the reactor) and B) secondary reactor (average of sample ports 10 and 12 in the secondary reactor) of the LFCR system fed with synthetic feed and a lactate carbon source at different AMD feed ratios. The vertical straight lines indicate biofilm harvest (solid lines indicate change in the AMD feed ratio).

Figure 5-12 B shows sulphide concentrations (expected, effluent, and average residual) increased in the secondary reactor with increase in AMD concentration from 20:80 to 60:40 AMD:Synthetic feed before stabilising. Up until the 100% AMD run, the three datasets show less spread amongst each other showing less partial oxidation of sulphide in the secondary reactor. At 100% AMD, expected sulphide was between 15-20 mmol/L after reaching pseudo-steady state, while average and effluent concentrations were lower at around 5 and 10 mmol/L. This suggests that more sulphur formed in the secondary reactor of the 100% run compared to the other ratio runs.

As seen in Appendix A.11, the pH and redox in both reactors were within optimal ranges for SRB function between 7.00-8.5 and below -300 mV respectively. This was similar to the synthetic feed + lactate experiment across all HRT except for the 2-day HRT in which the redox exceeded -300 mV most likely due to re-oxidation.

Figure 5-13: VFA time trends in the A) primary reactor and B) secondary reactor of the LFCR system fed with 100% AMD and a lactate (23 mmol/L) carbon source. The vertical line indicates biofilm harvest.Figure 5-13 A displays VFA concentration profiles of the primary reactor with 100% AMD feed and lactate as carbon source and electron donor, providing insight into SRB metabolic pathways. Measured lactate, acetate, and propionate concentrations indicated both incomplete oxidation and fermentation of lactate. Equation 5-2 shows the pathway for sulphate reduction through the incomplete oxidation of lactate to acetate.

$$2\,Lactate + SO_4^{2-} \rightarrow 2\,Acetate + 2HCO_3^- + HS^- + H^+ \qquad \text{Equation 5-2}$$

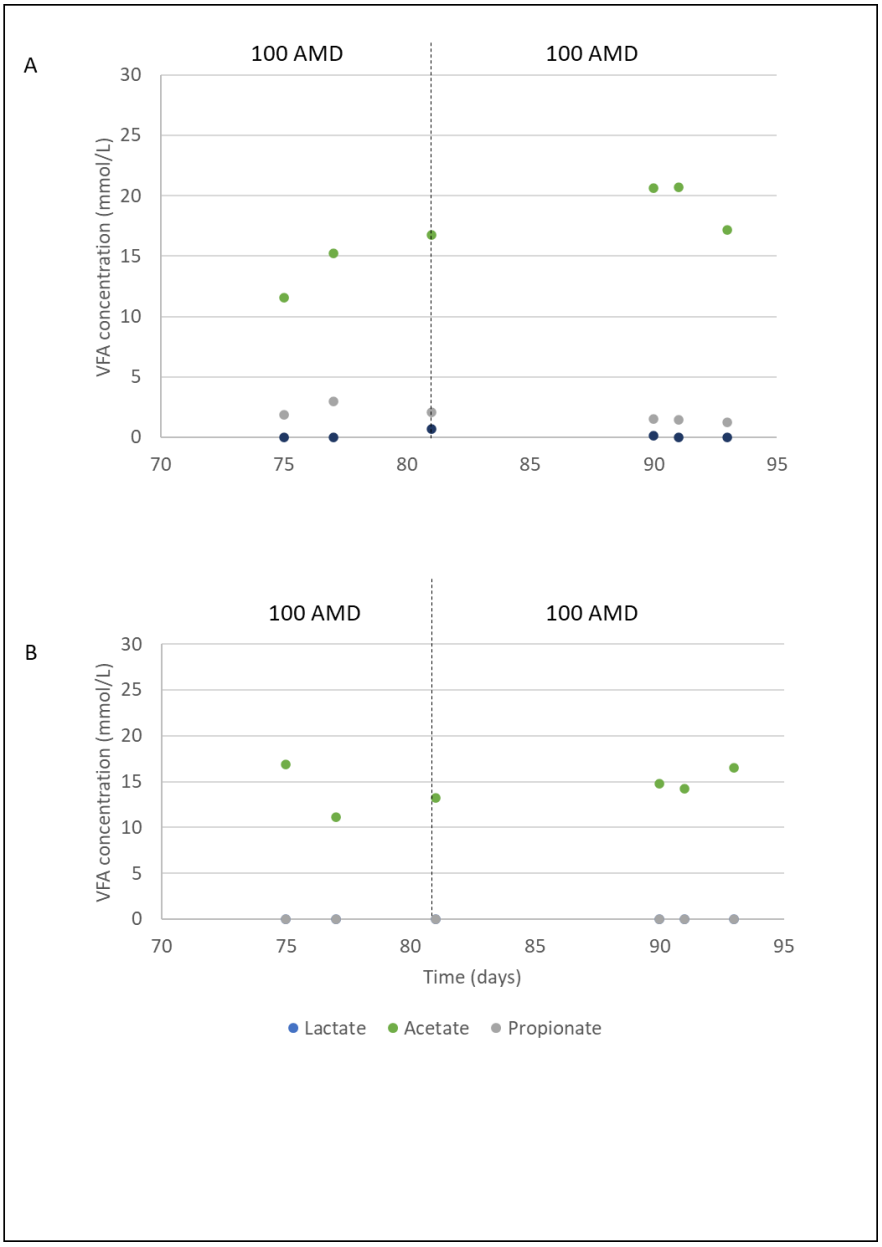


Figure 5-13: VFA time trends in the A) primary reactor and B) secondary reactor of the LFCR system fed with 100% AMD and a lactate (23 mmol/L) carbon source. The vertical line indicates biofilm harvest. Lactate, acetate and propionate concentrations were determined through HPLC as described in Section 3.7.7.

Lactate was mostly incompletely oxidised in the primary reactor as evidenced by high acetate accumulation (close to 20 mmol/L, nearly equal to feed lactate concentration in accordance with Equation 5.3) and low propionate concentrations indicating limited fermentation. Assuming all sulphate reduction was from incomplete lactate oxidation with concomitant acetate formation and with no propionate utilisation and that the remaining lactate metabolised underwent fermentation, then approximately 6.4% of lactate underwent fermentation, while 94.6% underwent incomplete oxidation.

Figure 5-13 B shows complete utilisation of propionate and a decrease in residual acetate between the primary reactor and secondary reactor. This indicated further sulphate reduction or sulphur formation in the secondary reactor. Acetate concentration decreased from around 20 mmol/L to below 16 mmol/L.

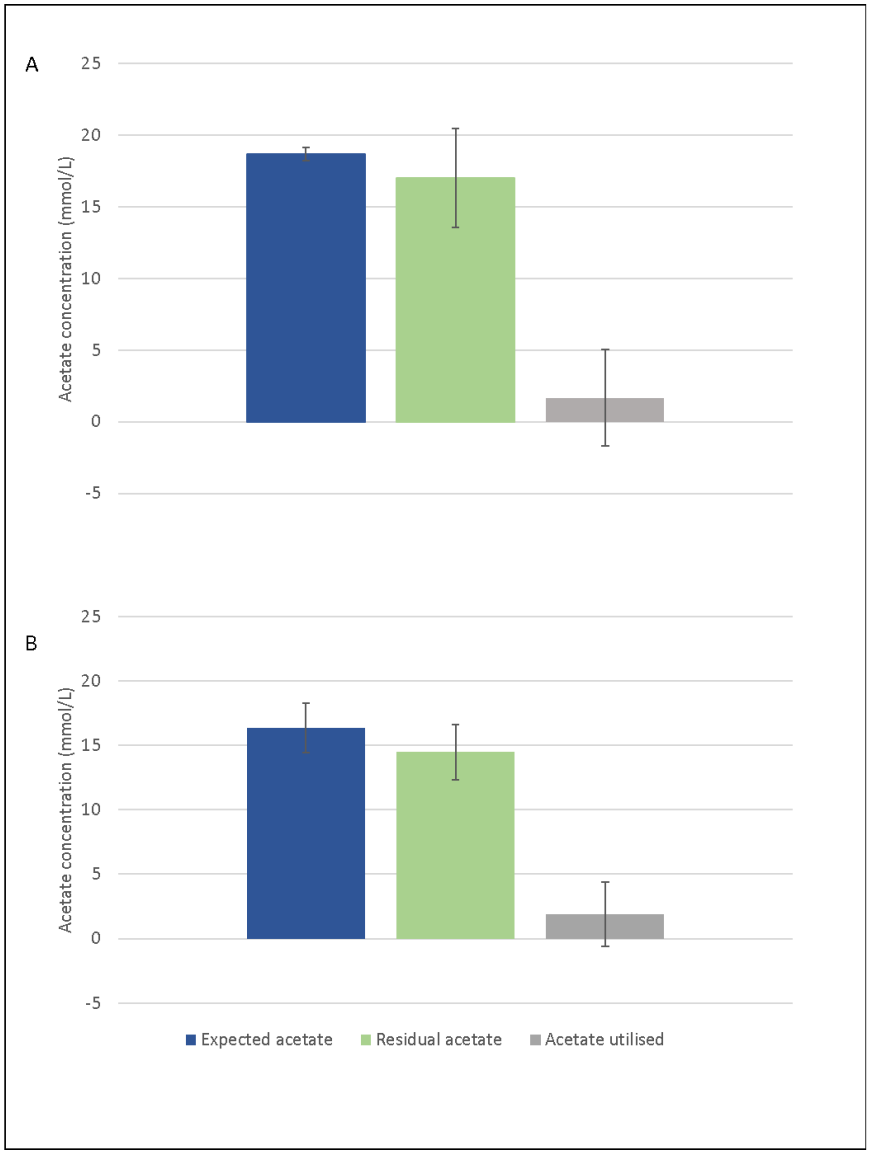


Figure 5-14: VFA concentration balance in the A) primary reactor and B) secondary reactor of the LFCR system fed with 100% AMD and a lactate carbon source. Lactate was completely utilised and so is not shown in the figure. Expected acetate in the primary reactor is the acetate concentration stoichiometrically calculated based on the assumption that all sulphate was reduced via the incomplete oxidation of lactate to acetate and the remaining lactate was fermented to acetate and propionate. Expected acetate in the secondary reactor, was the acetate concentration measured from the primary reactor effluent.

In the primary reactor, an expected acetate concentration of 18.7 mmol/L from the oxidation of lactate was calculated to account for sulphate reduction and 17.0 mmol/L of residual acetate was measured. This indicated that only 1.66 mmol/L of acetate was utilised (Figure 5-14). A small difference in the expected acetate compared to the residual acetate in both the primary and secondary reactor indicate minimal utilisation of acetate.

Figure 5-15 shows a stacked chart of sulphide converted to sulphur, residual sulphide and sulphate. In the primary reactor (Figure 5-15 A), most of the sulphate was converted to sulphide which remained at a high average concentration around 16 mmol/L even after biofilm harvest. Increase in the pre-treated fraction of AMD showed a decrease in the amount of sulphide converted to sulphur with little to no sulphur produced in the 80 AMD:20 Synthetic and the 100% AMD run. The highest concentration of sulphide converted to sulphur was 3.6 mmol/L at the 40 AMD:60 Synthetic run.

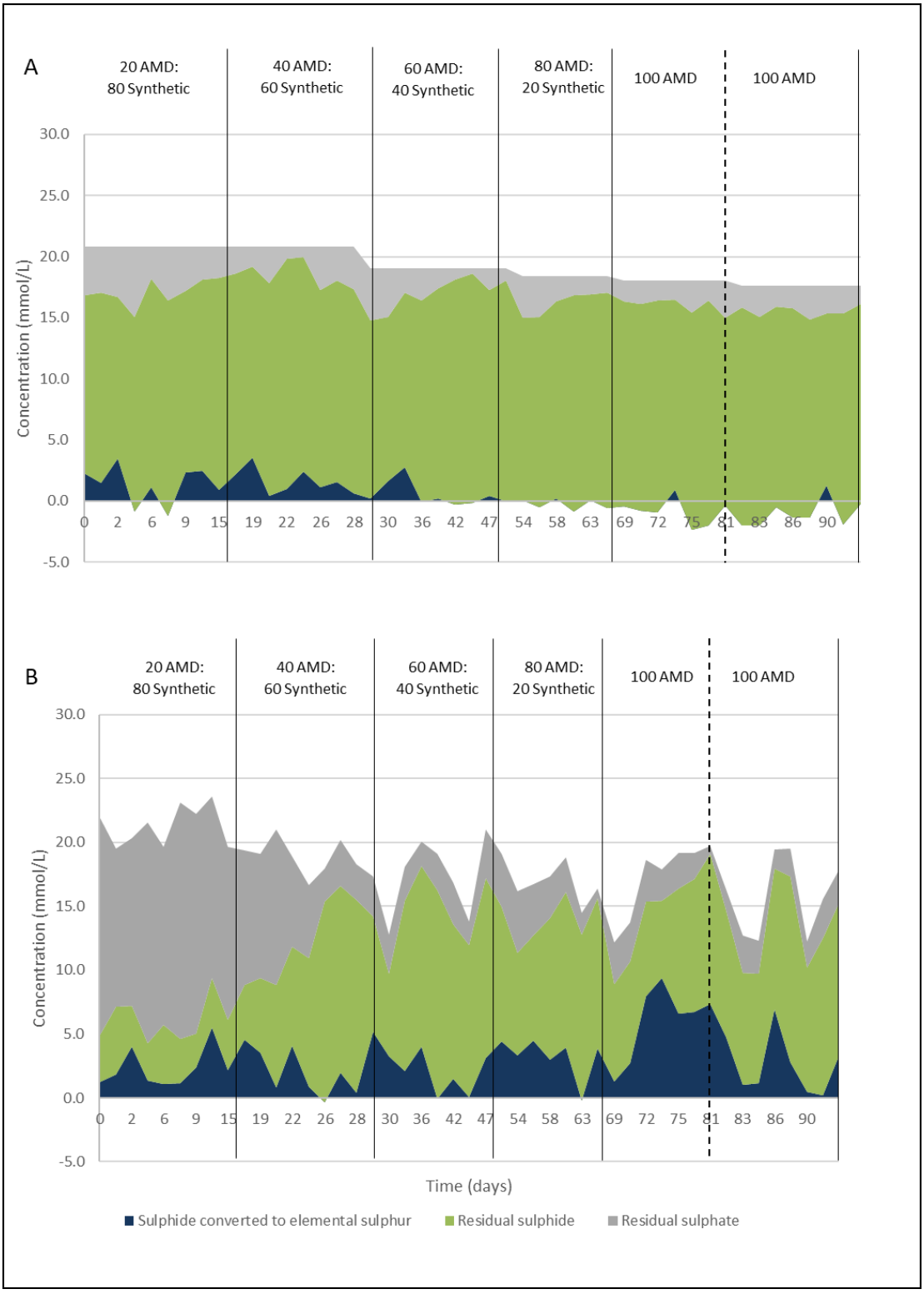


Figure 5-15: Sulphide converted to sulphur, residual sulphide and residual sulphate concentration time trends in A) the primary reactor and B) secondary reactor of the LFCR system fed with pre-treated AMD and a lactate carbon source at different AMD ratios, as indicated at the top of the graph. Feed sulphate concentration ranged between 17 - 21 mmol/L. The vertical straight lines indicate biofilm harvest (solid lines indicate change in the AMD feed ratio).

In the secondary reactor, an increase in the fraction of pre-treated AMD in the feed showed a decrease in residual sulphate resulting in an increase in residual sulphide and sulphide converted to sulphur. High residual sulphate concentrations in the secondary reactor at the low AMD ratios indicates high re-oxidation at the primary reactor's effluent port. Increasing the AMD fraction decreased re-oxidation.

Concentrations of sulphide converted to sulphur were much higher in the secondary reactor compared to the primary reactor, reaching a maximum of 9.4 mmol/L in the first 100% AMD run.

Figure 5-16 A shows that very little sulphide was converted to sulphur with most of the sulphur in the system existing as sulphide. In the secondary reactor (Figure 5-16 B), more sulphide was converted to sulphur (a maximum of 157 mmol) with significant re-oxidation to sulphate seen in the 20 AMD:80 Synthetic run. Low amounts of acetate utilised as seen in Figure 5-14 may be connected to the low amounts of sulphide converted to sulphur.

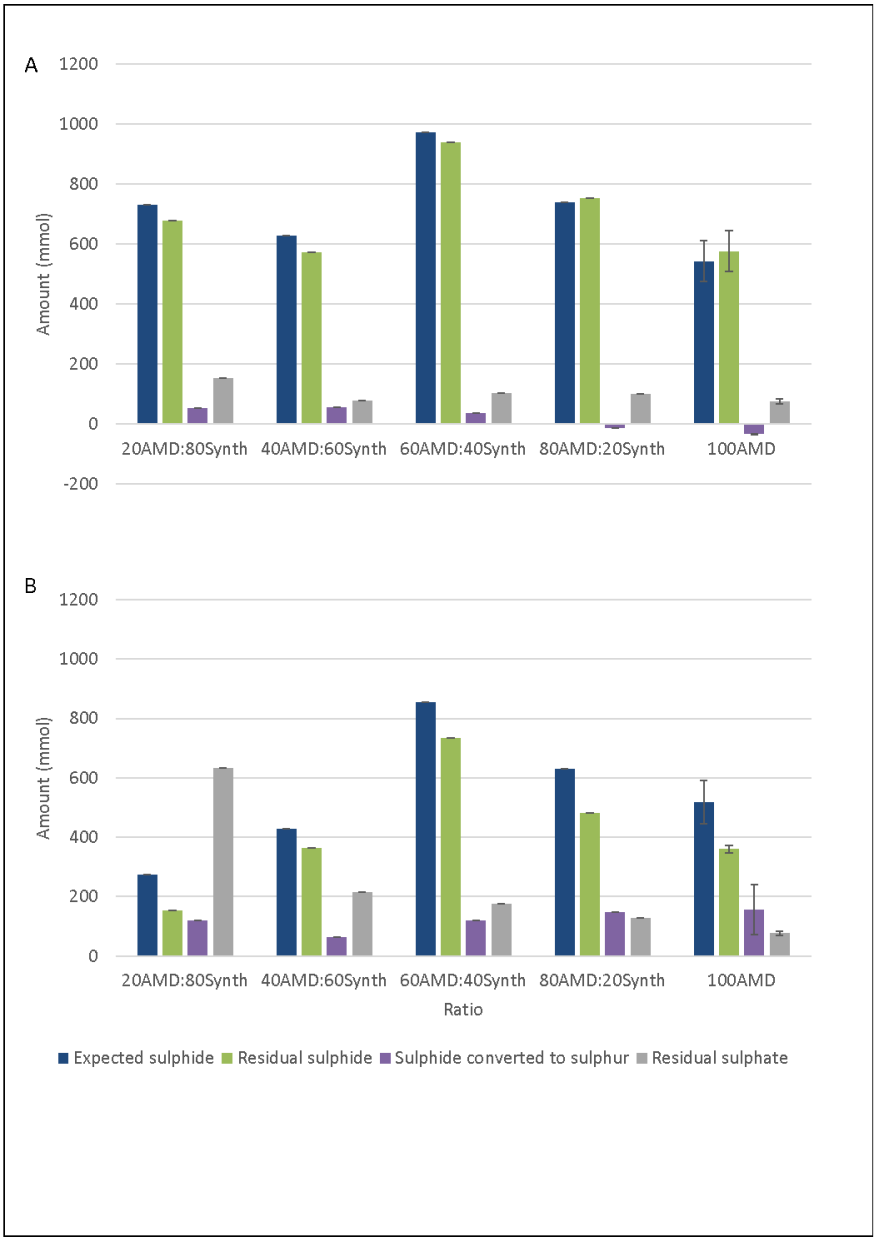


Figure 5-16: Expected sulphide, effluent sulphide, sulphide converted to sulphur and residual sulphate amounts in the A) primary reactor and B) secondary reactor of the LFCR system fed with pre-treated AMD feed and a lactate carbon source at different AMD ratios. Between 550 – 670 mmol of sulphate were fed into the reactor in 4 HRTs.

Table 5-5 summarises the LFCR system's overall performance on AMD and lactate. The 80:20 AMD:Synthetic run had the highest sulphate conversion (92.0%). Sulphide conversion however remained low in the primary reactor, below 10% across all the runs. In the AMD + lactate experiment, the sulphate feed concentrations may have been underestimated due to the fluctuating sulphate concentration in the AMD giving rise to some negative sulphide conversion values.

The highest sulphide conversion in the secondary reactor of 43.5% occurred at the 20 AMD:80 Synthetic run. VSRR tended to decrease with increasing AMD in alignment with decreasing VSLR due to the AMD having a slightly lower sulphate concentration than the synthetic feed; however, sulphate conversions remained fairly consistent. VSFR decreased with increasing AMD feed concentration, with the highest expected VSFR achieved in the primary reactor at the 20:80 AMD:Synthetic run.

Table 5-5: Reaction kinetics of the LFCR system fed with AMD and a lactate carbon source at different AMD feed concentrations

	Primary reactor (R1)					Secondary reactor (R2)		
HRT	VSLR	VSRR	Sulphate Conversion [a]	Sulphide Conversion [b]	VSFR	Sulphate conversion [c]	Sulphide conversion [b]	VSFR
(days)	(mmol/L.h)	(mmol/L.h)	(%)	(%)	(mmol/L.h)	(%)	(%)	(mmol/L.h)
20:80	0.289	0.239	82.8%	7.1%	0.095	-0.4	43.5%	0.111
40:60	0.289	0.257	88.9%	8.8%	0.022	-17.8%	14.8%	0.128
60:40	0.264	0.234	88.6%	3.7%	0.039	-27.3%	14.1%	0.228
80:20	0.256	0.235	92.0%	-1.9%	0.026	-26.3%	23.5%	0.060
100	0.247	0.216	87.4%	-6.1%	0.021	18.1%	30.3%	0.131

[a] Sulphate conversion in the primary reactor is the percentage of feed sulphate converted

[b] Sulphide conversion in the primary reactor is the percentage of expected sulphide converted to sulphur

[c] Sulphate conversion in the secondary reactor is the percentage of influent sulphate from the primary reactor converted

The VSRR and conversions at all AMD ratios were greater than what was observed at the 3-day HRT of the synthetic feed + lactate experiment (0.187 mmol/L.h). The 20 AMD: 80 Synthetic run and the 40 AMD: 60 Synthetic A ratio had similar VSLR to the 3-day HRT. Decrease in the VSLR after the 40 AMD: 60 Synthetic run was due to an increase in the AMD fraction which had a lower sulphate concentration. The 100% AMD feed VSRR and conversion were also higher than the 3-day synthetic feed HRT although the VSLR was slightly lower.

Figure 5-17 shows aerial pictures of the different LFCR system experiments, visually evaluating FSB formation in three different cases. Figure 5-17 A shows an opaque, rigid FSB in both reactors fed with synthetic feed and lactate at 3-day HRT, confirming partial oxidation of sulphide to elemental sulphur. A mature FSB was formed.

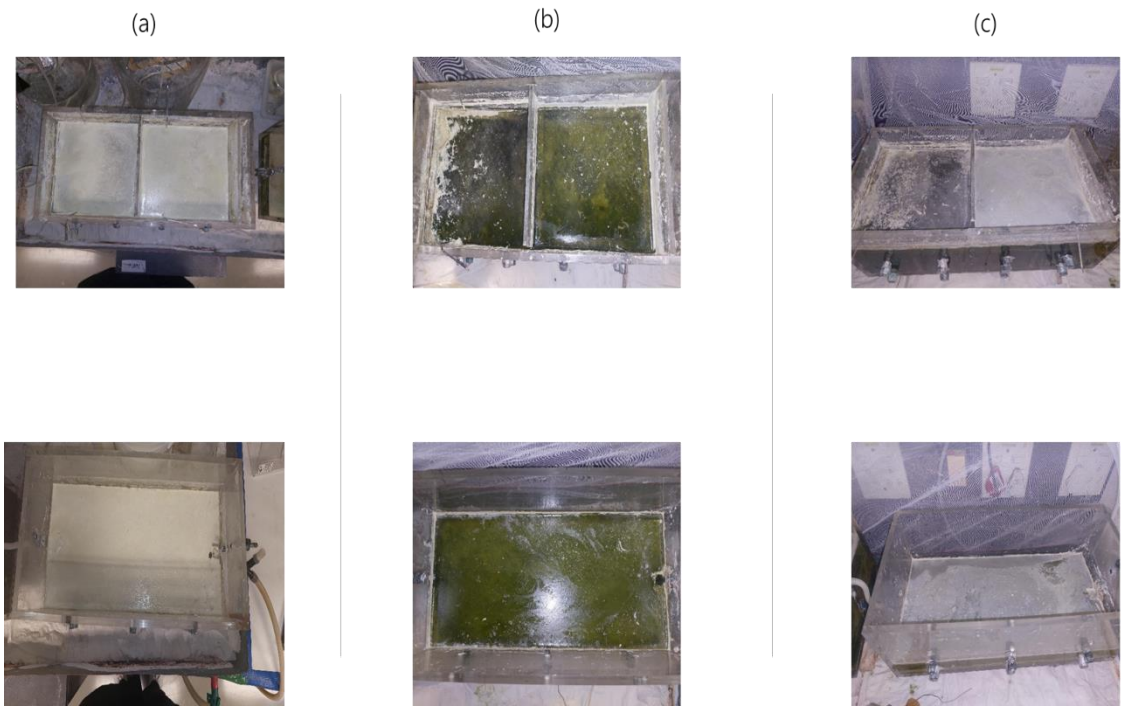
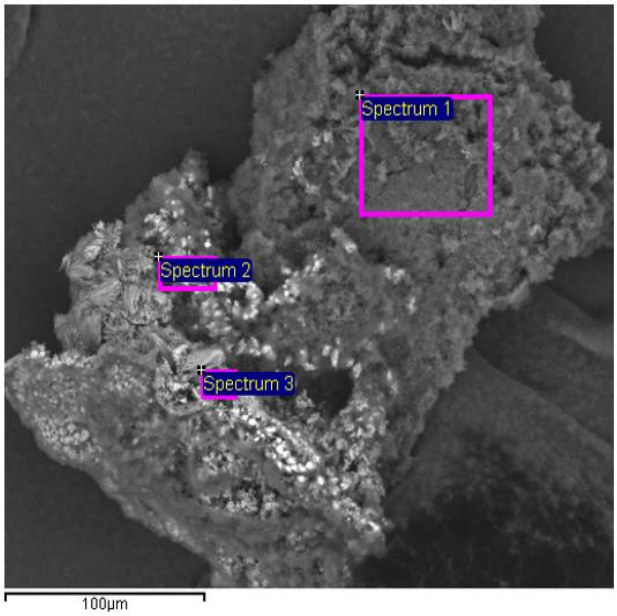


Figure 5-17: Aerial view of the primary and secondary reactor showing (a) FSB formed during a 3-day HRT experiment with a 100% synthetic feed and lactate carbon source (b) thin film formed during a 3-day HRT experiment with a 80% AMD and 20% synthetic AMD feed and lactate carbon source (c) FSB and thin film mixture formed during a 3-day HRT experiment with a 100% AMD feed and a lactate carbon source. In each case the primary reactor (top) and secondary reactor (bottom) are shown just before harvesting.

Figure 5-17 B, depicting the system fed with 80:20 AMD:Synthetic and lactate, showed little to no sulphur biofilm after four 3-day HRTs. A lustrous, transparent coat on the liquid surface was observed, presumably maintaining an anaerobic or anoxic environment in the bulk of the reactor. The poor FSB formation in the 80:20 AMD:Synthetic run could be attributed to AMD introduction which could contain SOB inhibitors, or the SOB are possibly more fastidious with respect to nutrient requirements, no longer supplied by Postgate media. A third possibility is that the transparent cover is impervious to oxygen, thus limiting partial sulphide oxidation.

Figure 5-17 C, showing the 100% AMD run with lactate after four 3-day HRTs, displayed a forming FSB. This FSB was not yet mature, being sticky when harvested rather than brittle as observed in the synthetic run (Figure 5-17 A).Thus, it was theorised that compounds in the synthetic feed interacted with substances in the AMD to form a thin, transparent film that impeded FSB formation. This would explain the immature but recovering FSB formation in the 100% AMD run where minimal Postgate nutrients remained in the system.

A sample containing both the transparent film and pale-yellow opaque FSB from the 100% AMD run was taken from the primary reactor for scanning electron microscopy (SEM) with elemental analysis. Figure 5-18 shows the SEM image and a table showing elemental analysis percentages from different image spectrums.



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 5-18: SEM image of a biofilm sample from the primary reactor of the 100% AMD with a lactate run containing both the thin, transparent film (spectrum 1 and 3) and the opaque FSB (spectrum 2) together with a table showing the elemental percentage composition of the sample. Spectrums are representative of the area immediately around them.

Spectrum 2 showed the highest sulphur content, corresponding to bright spheres or circles seen in the image, likely elemental sulphur deposits in the FSB. High carbon content in this spectrum was attributed to microorganisms or exopolymeric substances (EPS) present in the FSB.

All spectra contained calcium, with spectrum 3 having the highest concentration. Unlike previous synthetic AMD studies conducted by Fernandes (2020) where no calcium was reported, calcium was present in this study, however this was unsurprising due to a combination of the lime pre-treatment and calcium present in the AMD. This suggested the transparent, thin biofilm might be a calcium complex containing a compound from Postgate media B. The presence of 0.31 element% magnesium might indicate its role in the calcium complex formation; however it is very low in comparison to the percentage of calcium.

Spectrum 1, dominated by carbon and oxygen with little sulphur and no nitrogen, was likely mostly biomass, similar to what was seen by Fernandes (2020). Spectrum 3 was part of the transparent film and it did have sulphur as part of the elements detected but it was in lower percentages (11.68 element%) compared to spectrum 2. It was concluded that the translucent biofilm had low concentrations of sulphur when compared to the FSB. FSB has a high sulphur content, >20 element% and is typically an opaque, pale-yellow biofilm (Fernandes, 2020; Marais, 2020). Another possibility is that spectrum 3 may have been an aggregation of the translucent film and small amounts of FSB.

Figure 5-19 shows a magnified view of spectrum 3 from Figure 5-18, revealing crystal structures. Magnification of the spectrum showed a combination of petal-like crystals (a) and bright sphere-like clusters (b). The structures in sections labelled (b) closely resembled the bright spheres in Figure 5-18 (spectrum 2), which showed high sulphur. Thus, the petal-shaped crystals were likely the calcium complex as while the spheres were sulphur.

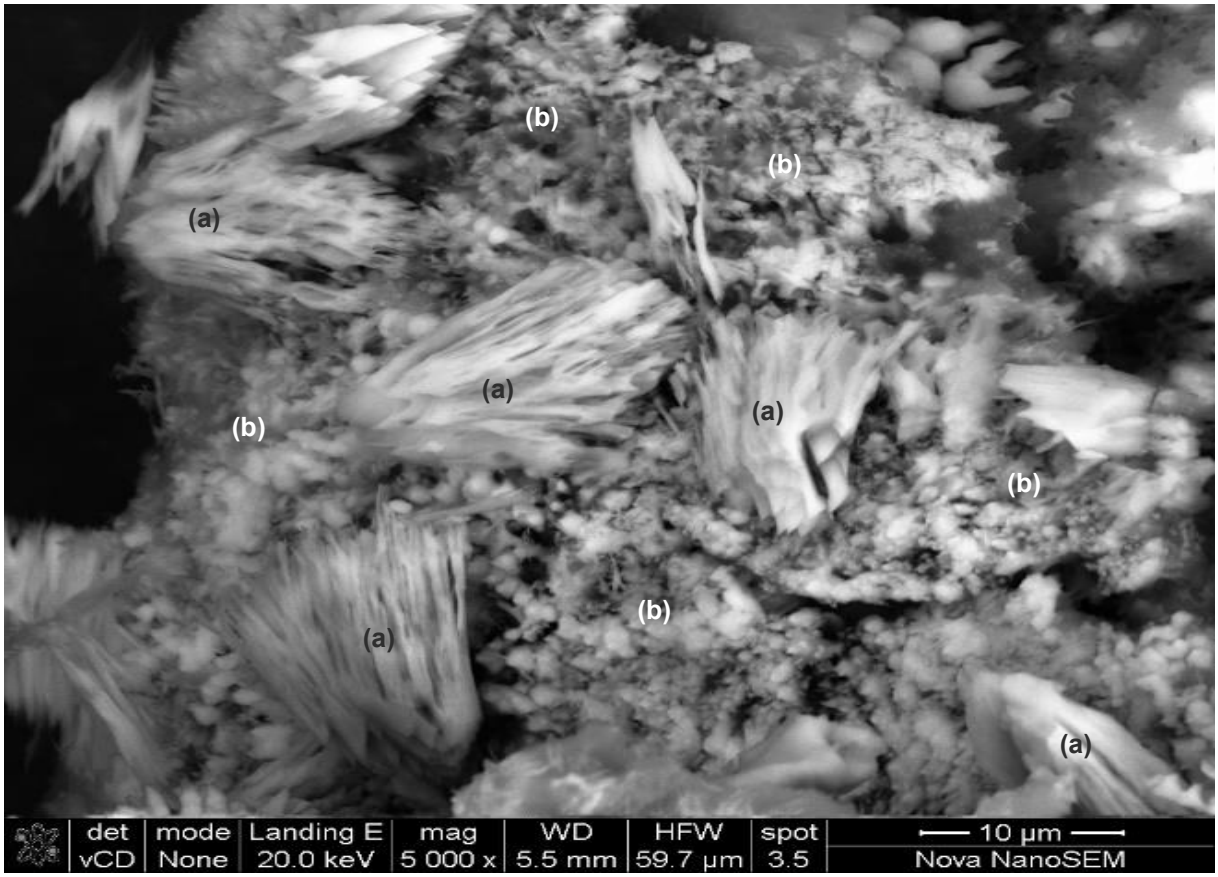
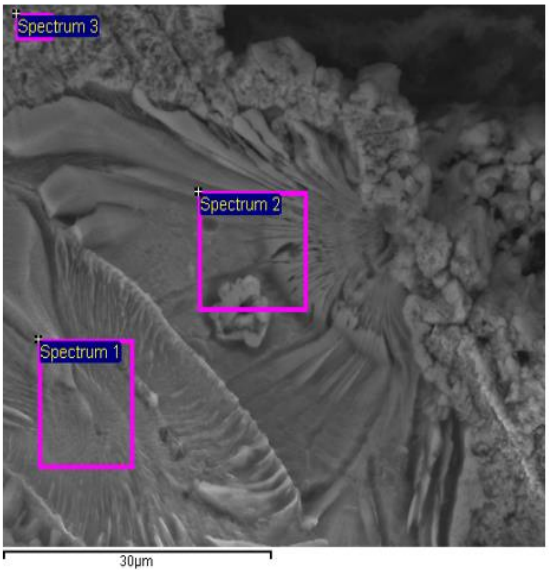


Figure 5-19: Magnified view of spectrum 2 from Figure 5-18 with a composition of (a) crystal-like formations and (b) bright spherical clusters

Figure 5-20 displays what was assumed to be an intact transparent, thin film, along with its elemental analysis 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 5-20: SEM image of thin, transparent film from the LFCR system fed with AMD and a lactate carbon source

Spectrum 3 in Figure 5-20 had the highest calcium content (29.6 element%), appearing as a folded area of the film. The transparent, thin film was concluded to be a calcium-magnesium complex containing trace Postgate B media substances in crystal form. Its lustrous appearance (Figure 5-17 B) was attributed to these crystals, contrasting with the opaque, dull FSB composed of clustered sulphur

spheres. The uniform, flat crystal structure of the thin film (Figure 5-20) likely impeded oxygen more effectively than the irregular, textured FSB structure (Figure 5-18), which potentially allowed more oxygen ingress through breaks or pores.

Figure 5-20 showed no bright spheres, with sulphur content below 1 element% in all spectrums. Magnesium was present throughout, supporting the assumption of its inclusion in the calcium complex, albeit in very low concentrations. High carbon and oxygen concentrations were again attributed to microbial presence in the biofilm or the presence of a calcium carbonate. Elemental analysis of the biofilm by Fernandes (2020) showed a carbon to oxygen ratio of 10:3 whereas the ratio in Figure 5-20 is 4:10. Excess oxygen measured in the translucent film supports the presence of a calcium carbonate.

SRB are known to participate in microbially-induced calcium carbonate precipitation (MICP), potentially explaining the high oxygen and calcium-containing crystals (Wang *et al.*, 2023). MICP crystals have also been described to form flower, petal-like structures (Dikshit *et al*, 2020) which could explain the petal-like crystals seen in Figure 5-19.

The petal shaped crystals in Figure 5-19 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 the Postgate B media components (K_2HPO_4), there could be interaction with the calcium in the pre-treated AMD resulting in the formation of calcium phosphate crystals which are forming the thin, transparent layer in the LFCR system. However, potassium element% was not shown.

Another assumption for higher sulphate conversion in the AMD run with lactate could be attributed to calcium forming stable biofilms through extracellular polymeric substance (EPS) crosslinks (Körstgens *et al.*, 2001). Fernandes (2020) also noted that lower magnesium concentrations increased sulphide to elemental sulphur conversion. The hydrated lime pretreatment, containing magnesium (Section 3.2), might have decreased this conversion due to an increased magnesium concentration.

CHNS analysis was conducted on biofilm samples from the 3-day HRT study with synthetic feed and the 100% AMD run. Table 5-6 shows percentage concentrations of C, H, N, and S in these samples. The remaining percentage was assumed to be inorganics, previously reported as a crystalline structure mainly composed of magnesium and phosphorus (Marais, 2020). Marais noted that this crystalline structure formation decreased sulphur deposition in the FSB, attributing its presence to the modified Postgate media feed composition.

Table 5-6: FSB composition of the FSB from the 3-day HRT runs fed with a synthetic feed and AMD feed both using a lactate carbon source

Run	Experiment	N [%]	C [%]	H [%]	S [%]	Inorganics (%)
2nd run	Synthetic feed + lactate 3-day HRT (R1)	4.0	4.4	4.4	29.8	57.5
	Synthetic feed + lactate 3-day HRT (R2)	3.8	5.3	4.1	36.3	50.6
3rd run	Synthetic feed + lactate 3-day HRT (R1)	4.3	6.7	4.5	32.0	52.4
	Synthetic feed + lactate 3-day HRT (R2)	4.2	5.7	4.7	29.3	56.1
1st run	AMD feed + lactate 3-day HRT (R1)	0.7	13.0	0.7	11.7	73.8
	AMD feed + lactate 3-day HRT (R2)	0.2	9.8	1.1	16.9	71.9
2nd run	AMD feed + lactate 3-day HRT (R1)	0.6	10.6	1.0	20.5	67.2
	AMD feed + lactate 3-day HRT (R2)	0.4	9.4	1.6	19.7	68.9

Sulphur compositions in 100% AMD runs were lower across the LFCR system compared to the 3-day HRT with synthetic feed. Synthetic feed runs showed 25-40% sulphur composition, aligning with Marais (2020). In AMD + lactate runs, sulphur composition dropped to 11-21%, with the lowest (11.7%) in the primary reactor of the first 100% AMD run.

The first 100% AMD run had the highest inorganics content, likely due to more lustrous film formation in the primary reactor's first compartment (Figure 5-17 C). Carryover of Postgate media components

from the 80:20 AMD:Synthetic run could have reduced sulphur formation, resulting in 73.8% inorganics concentration.

Sulphur composition improved in the second 100% AMD run (20.5% primary, 19.7% secondary) due to fewer Postgate media components interacting with AMD compounds. Nitrogen and hydrogen compositions decreased when AMD feed was used instead of synthetic, while carbon compositions increased. Compositions for synthetic feed and lactate experiments aligned with Marais' (2020) findings, supporting reliability.

However, these compositions could not be used to calculate observed sulphur due to biofilm loss during harvesting and adherence to reactor components. Instead, calculated sulphur values were used for all LFCR system experiments.

The introduction of pre-treated AMD with lactate into the LFCR sequence system resulted in higher sulphate conversion to sulphide but decreased sulphide conversion to elemental sulphur. A thin, lustrous film formed, impeding oxygen better than the FSB and maintaining high sulphide concentrations. This reduced re-oxidation in the secondary reactor, a problem observed in synthetic AMD-lactate experiments.

Decreased sulphide conversion was attributed to factors including increased magnesium concentration from hydrated lime pretreatment, 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 pre-treated AMD forming a calcium complex crystal. Formation of this crystal is postulated to depend on a substance found in Postgate B media and magnesium.

5.4 Demonstration of performance of the LFCR system on an AMD and molasses substrate

Lactate is not cost-effective for large-scale, long-term AMD treatment in the LFCR system and so a third experiment using pre-treated AMD and molasses, a cheaper sugar industry byproduct, was conducted based on fed-batch reactor substrate tests.

The LFCR system's feed was switched to molasses while using the same pre-treated AMD from the CeBER greenhouse. To prevent the Maillard reaction and loss of usable sugars at high temperatures (Van Boekel, 2001), a concentrated 500 g/L molasses solution was pasteurized at 106°C for 30 mins separately before aseptic addition to sterilised AMD, maintaining a COD:Sulphate ratio of 1. The reactor was given 10 days (3.3 residence times) to adapt to the new carbon source and replace the entire reactor volume with the new molasses-containing feed.

Figure 5-21 shows sulphate and sulphide concentration profiles for the LFCR system fed with molasses and pre-treated AMD feed. The average feed sulphate concentration was 18.0 mmol/L and all runs were conducted at 3-day HRT with biofilm harvesting after 4 residence times, similar to the AMD-lactate study.

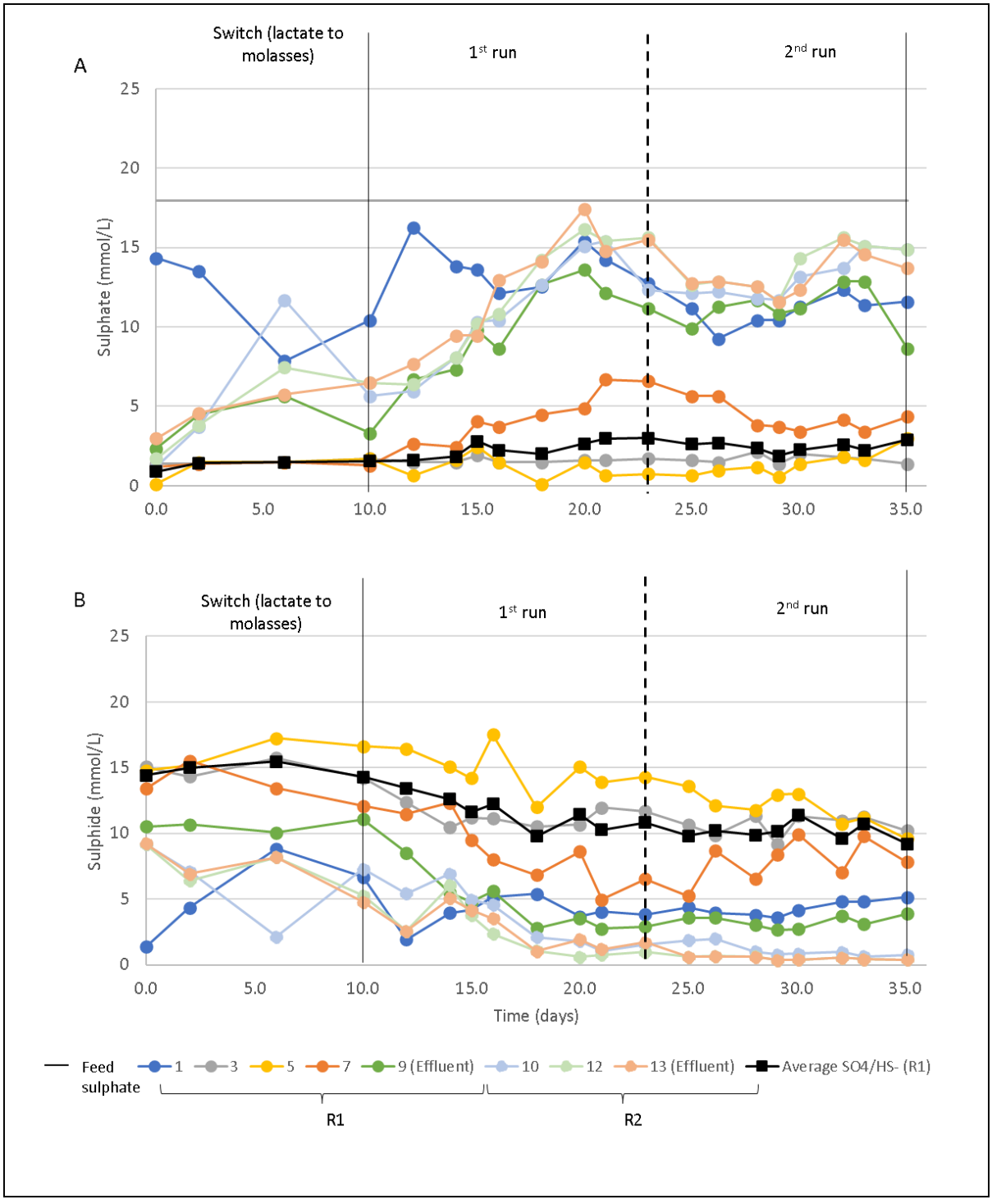


Figure 5-21: Time trends for A) sulphate and B) sulphide concentrations for the LFCR system fed with pre-treated AMD and a molasses carbon source. Average SO4 and HS- is the average of sample ports 3, 5, 7 as there was little sulphate reduction at sample point 1, not representative of the bulk concentration. The vertical straight lines indicate biofilm harvest (solid line indicates the start of a study).

Unlike the AMD-lactate study, high sulphate concentrations were observed at sample point 1 of the primary reactor, indicating low initial sulphate reduction. This was attributed to molasses being a more complex substrate than lactate, containing a mixture of sugars (Palmonari *et al.*, 2020). The delay in sulphate reduction likely resulted from the need for substrate breakdown by fermenters and acidogens before SRB utilisation of the VFAs formed.

Sample points 3, 5, and 7 showed low average sulphate concentrations (<2.5 mmol/L). However, re-oxidation occurred at the primary reactor effluent port (sample point 9) and in the secondary reactor, with sulphate concentrations exceeding 12.0 mmol/L in the first post-adaptation cycle. This differed from the AMD-lactate study, which maintained low sulphate concentrations throughout. Lower sulphide re-oxidation during switch period was attributed to residual lactate from the previous run.

The average sulphate conversion across both runs after adaptation was 84.8% in the primary reactor and -28.6% in the secondary reactor. The negative value in the secondary reactor indicated re-oxidation. Sulphate conversion with the molasses carbon source nearly matched conversion achieved using lactate to treat AMD, both of which were higher than the conversion with synthetic feed + lactate in the primary reactor. This demonstrated that molasses, despite its complexity, was suitable for treating sulphate-rich wastewaters.

Figure 5-21 B showed that the sulphide trends complemented sulphate trends, confirming low sulphate conversion at the primary reactor's feed entry side and re-oxidation at the primary reactor effluent and in the secondary reactor. High sulphide concentrations at sample points 3, 5, and 7 indicated significant sulphate reduction in the primary reactor volume away from the entry point. The highest sulphide concentration after the switch period was 13.4 mmol/L at day 12 and an average of 10.3 mmol/L across both runs at pseudo-steady state. Over time, sulphide concentration in the secondary reactor decreased to below 4.0 mmol/L in the first run and below 2.0 mmol/L in the second, suggesting an oxidizing environment unsuitable for SRB function.

VSRR decreased to 0.212 mmol/L.h and sulphate conversion to 85.2% during the first pure molasses run, likely due to system adjustment (Figure 5-22). Molasses, though having a high COD content, also contains other nutrients and compounds that can be both beneficial and inhibitory to SRB. Other substances such as heavy metals and non-biodegradable compounds can accumulate and become inhibitory to SRB (Annachhatre and Suktrakoolvait, 2001). In the second run, sulphate conversion and VSRR remained fairly constant at 86.1% and 0.214 mmol/L.h respectively, suggesting successful adaptation to the new carbon source.

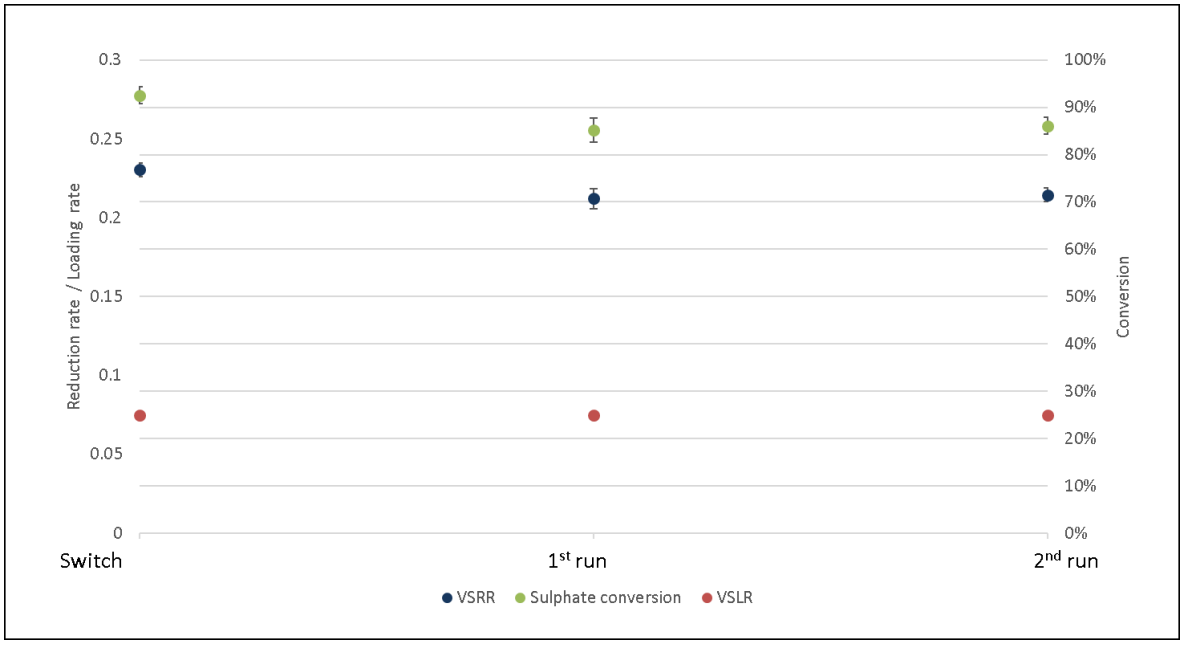


Figure 5-22: Steady state kinetics of sulphate reduction in the primary reactor of the LFCR system fed with pre-treated AMD and a molasses carbon source.

Figure 5-23 A shows expected, average, and effluent sulphide concentration trends in the primary reactor. The highest average sulphide concentrations (14-16 mmol/L) occurred during the lactate-to-molasses switch, corresponding with higher sulphate conversions. In the first run after adaptation, sulphide concentrations dropped, averaging around 10 mmol/L. Re-oxidation was observed at the primary reactor effluent port and carried over to the secondary reactor. Expected sulphide

concentrations were higher than average in both runs in the primary reactor, indicating that there was some sulphur formation occurring.

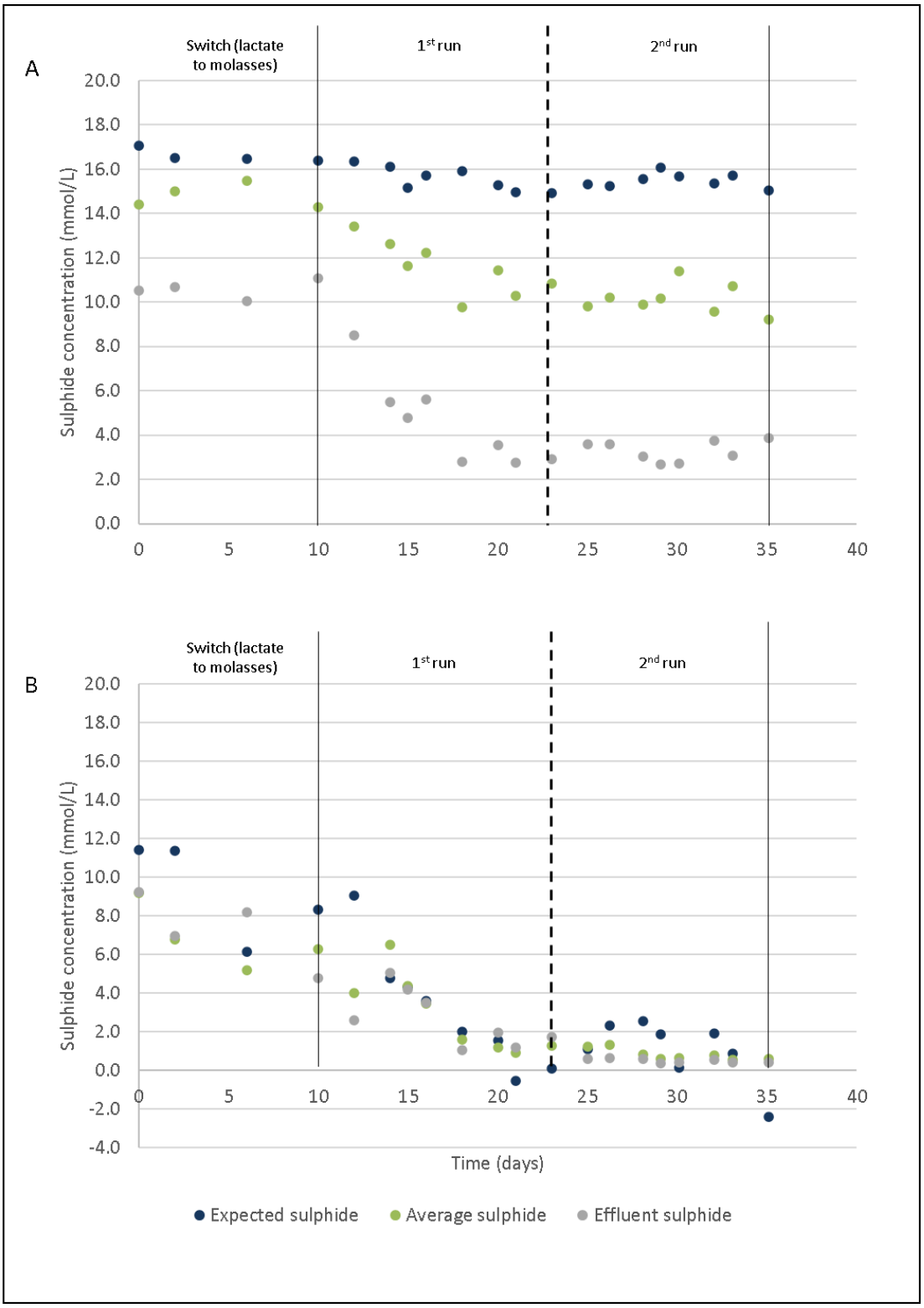


Figure 5-23: Expected, average and residual sulphide time trends in the A) primary reactor(average sulphide is the average of sample ports 3, 5, 7 as there was low sulphate reduction at sample port 1, not representative of the bulk concentration and reoxidation at the effluent port) and B) secondary reactor(average sulphide is the average of sample ports 10 and 12) of LFCR system fed with pre-treated AMD and a molasses carbon source at a 3-day HRT. The vertical straight lines indicate biofilm harvest (solid lines indicate the start of a study).

In the second molasses run, average sulphide concentrations were between 9-11 mmol/L while expected concentrations were higher averaging around 15 mmol/L.

Effluent sulphide was mostly equal to the average residual sulphide in the secondary reactor. This was different to the primary reactor which had effluent sulphide concentrations much lower than the residual average sulphide. This indicated that re-oxidation mostly occurred at the effluent port and the low sulphide concentrations were carried over into the secondary reactor. In the secondary reactor during the molasses runs, the low sulphide concentrations were maintained but there was less re-oxidation compared to the primary reactor effluent port. Expected sulphide concentrations were equal to the average and effluent sulphide concentrations showing there was little sulphur formation occurring.

The pH was within optimal range for SRB function however the redox was higher than seen in the lactate fed experiments (appendix A.12). It ranged between -300 to -100 mV with the secondary reactor having higher redox potential due to re-oxidation caused by the poor formation of FSB resulting in more oxygen ingress.

Across the switch, no detectable sugars were measured, showing that they were all fermented to VFAs or directly used up by SRB to reduce sulphate to sulphide (Figure 5-24). Acetate was the only VFA detected post switching to molasses, with concentrations below 5mmol/L in the primary reactor and was completely utilised in the secondary reactor.

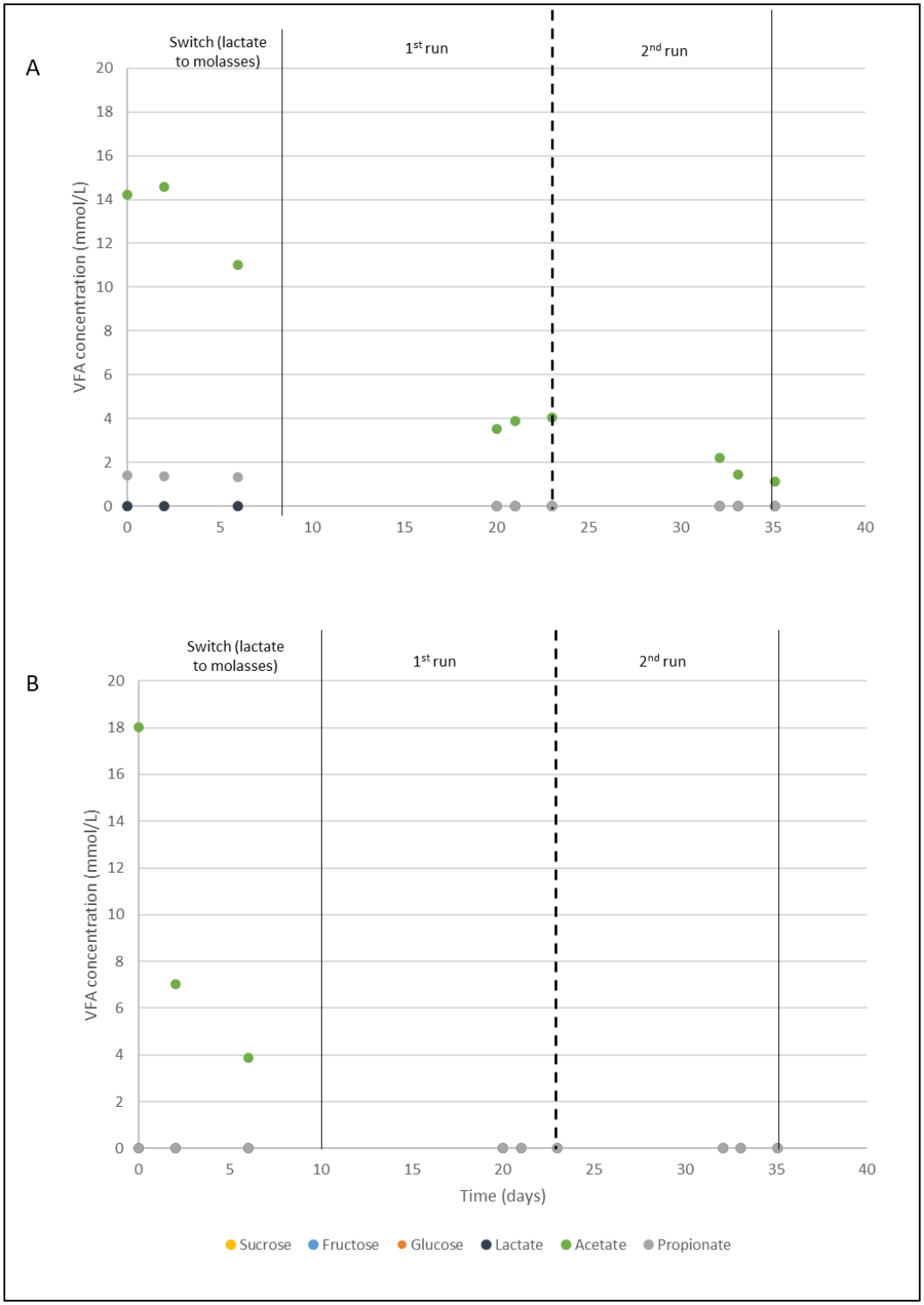


Figure 5-24: VFA time trends in the A) primary reactor and B) secondary reactor of the LFCR system fed with pre-treated AMD and a molasses (0.51g of COD in 1 g of molasses) carbon source. The vertical lines indicate biofilm harvest (solid lines indicate the start of a study). Propionate concentrations were determined through HPLC as described in Section 3.7.7.

The presence of propionate during the switch was likely from the fermentation of lactate. As discussed in Section 4.4.1, the molasses used in this study appeared to have undergone a certain level of fermentation to lactate by the following Equation 5-3.

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4C_3H_6O_3 \qquad \text{Equation 5-3}$$

Therefore, with the COD:sulphate ratio of 1:1 having been maintained, there was a potential for full fermentation of molasses to yield a lactate concentration between 19 mmol/L and 22 mmol/L. Unlike previous experiments which used lactate as the carbon source, the concentration of residual acetate is low averaging below 4 mmol/L and 2 mmol/L in the 1st and 2nd run respectively, in the primary reactor; especially considering that the expected amount of lactate from molasses is within the range of what was in the lactate fed runs. This could indicate better utilisation of substrate in the molasses fed LFCR system than the lactate fed LFCR system. However, it is more likely that the COD measured was not all readily available to the microbial community and was not picked up via HPCL. Nearly matching conversion in the primary reactor supported this. However, significant re-oxidation at the effluent port which resulted in low sulphide concentrations and high sulphate concentrations in the secondary reactor could indicate that the measured COD in the molasses is not all usable COD resulting in less VFAs available to the SRB.

The amount of acetate utilised in the secondary reactor had an average of 25% across the last two data points during the switch. The high acetate concentration measured at day zero in the secondary reactor was probably carried over from the primary reactor and the decrease across time could have been due to utilisation or washout. Across the first and second run of the molasses fed LFCR system, in the secondary reactor, there were no VFAs measured indicating that there was complete utilisation of all measured VFAs. The VFAs were either oxidised which simultaneously resulted in the reduction of sulphate or fermented. The low concentration of acetate entering the secondary reactor may have meant limited COD available for heterotrophic bacteria which are essential for the formation of the FSB.

The stacked chart in Figure 5-25 shows that similar to the AMD + lactate experiment, most of the sulphate was converted to sulphide as seen by the consistent high concentrations of sulphide that ranged between 9-16 mmol/L in the primary reactor. However, there was much higher concentrations of sulphide converted to sulphur in the primary reactor of the AMD experiment using a molasses carbon source than when using lactate. The highest concentration of sulphide converted to sulphur was in the second run at 6.2 mmol/L (day 16) as opposed to the AMD + lactate experiment which had little to no sulphur forming but maintained high sulphide concentrations. Concentrations of sulphide converted to sulphur in the primary reactor of the AMD + molasses experiment also exceeded those measured in the synthetic feed + lactate experiment (highest concentration was 3.8 mmol/L).

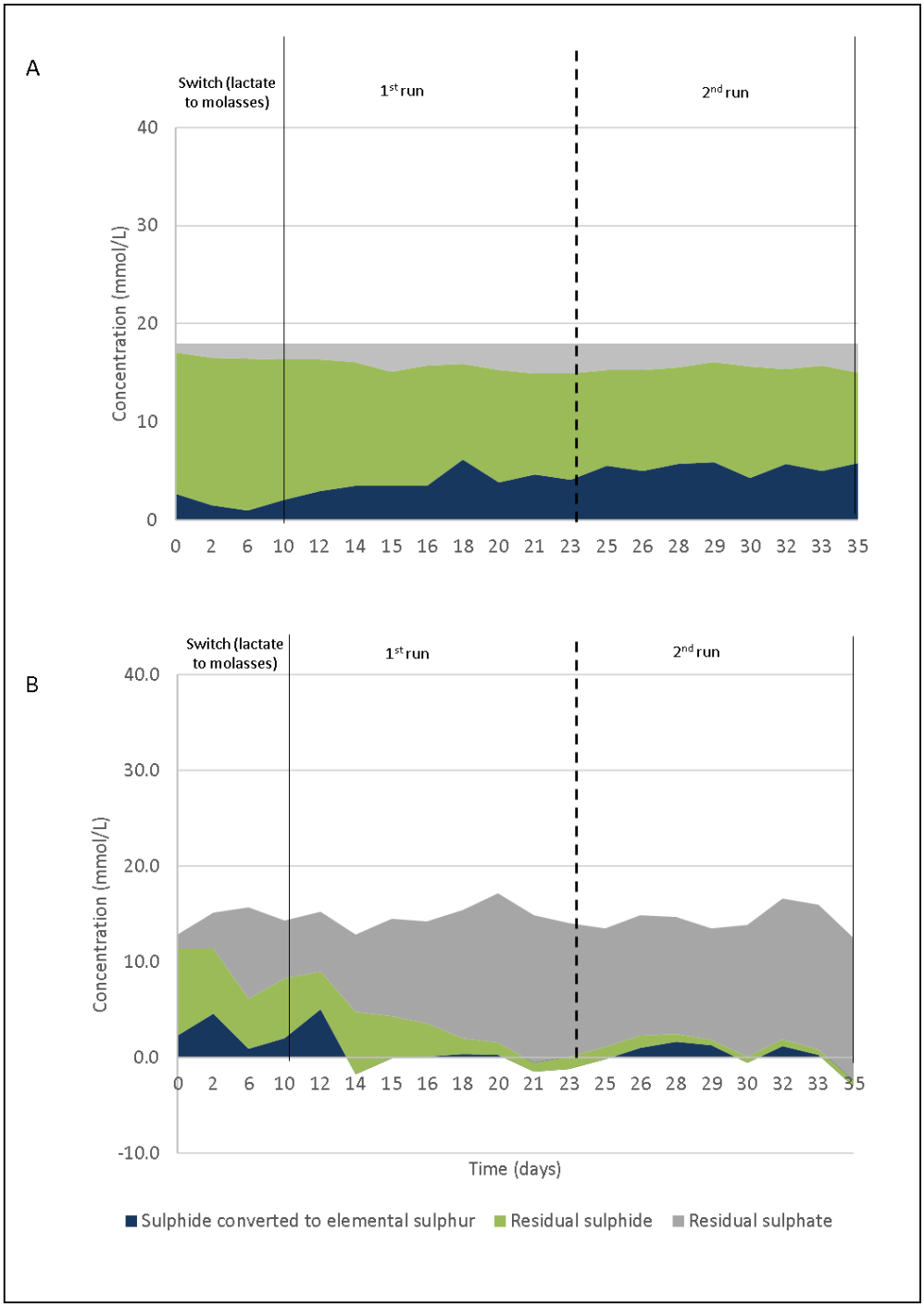


Figure 5-25: Sulphide converted to sulphur, residual sulphide and residual sulphate concentration time trends in A) the primary reactor and B) secondary reactor of the LFCR system fed with pre-treated AMD and a molasses carbon source. Feed sulphate concentration was at 18 mmol/L. The vertical straight lines indicate biofilm harvest (solid lines indicate the start of a study).

In the secondary reactor, a significant drop in the sulphide converted to sulphur is observed (Figure 5-25). Significant re-oxidation of sulphide to sulphate occurred. The lack of sulphide led to poor formation of sulphur in the secondary reactor. The highest concentration of sulphide converted to sulphur in the secondary reactor was observed in the first 2 days of the first run at 5.0 mmol/L before it significantly dropped to below 1 mmol/L for the second half of the first run. Higher concentrations of sulphide converted to sulphur at the start of the first run were attributed to carry over from the switch period. Overall, when comparing lactate to molasses, molasses has a higher concentration of sulphide converted to sulphur in the primary reactor whereas lactate tends to form more sulphur in the secondary reactor. This could indicate a preference to acetate by the SOB as more acetate was used up in the

primary reactor when molasses was used while in the lactate experiment acetate was mostly used up in the secondary reactor. Low acetate and sulphide concentrations entering the secondary reactor resulted in negligible sulphate reduction and less FSB forming; high concentrations of sulphate from the primary reactor effluent port were maintained in the secondary reactor.

Figure 5-26 A shows the amount of expected sulphide, residual sulphide and sulphide converted to sulphur. There was much less sulphide converted to sulphur in the switch period compared to the runs.

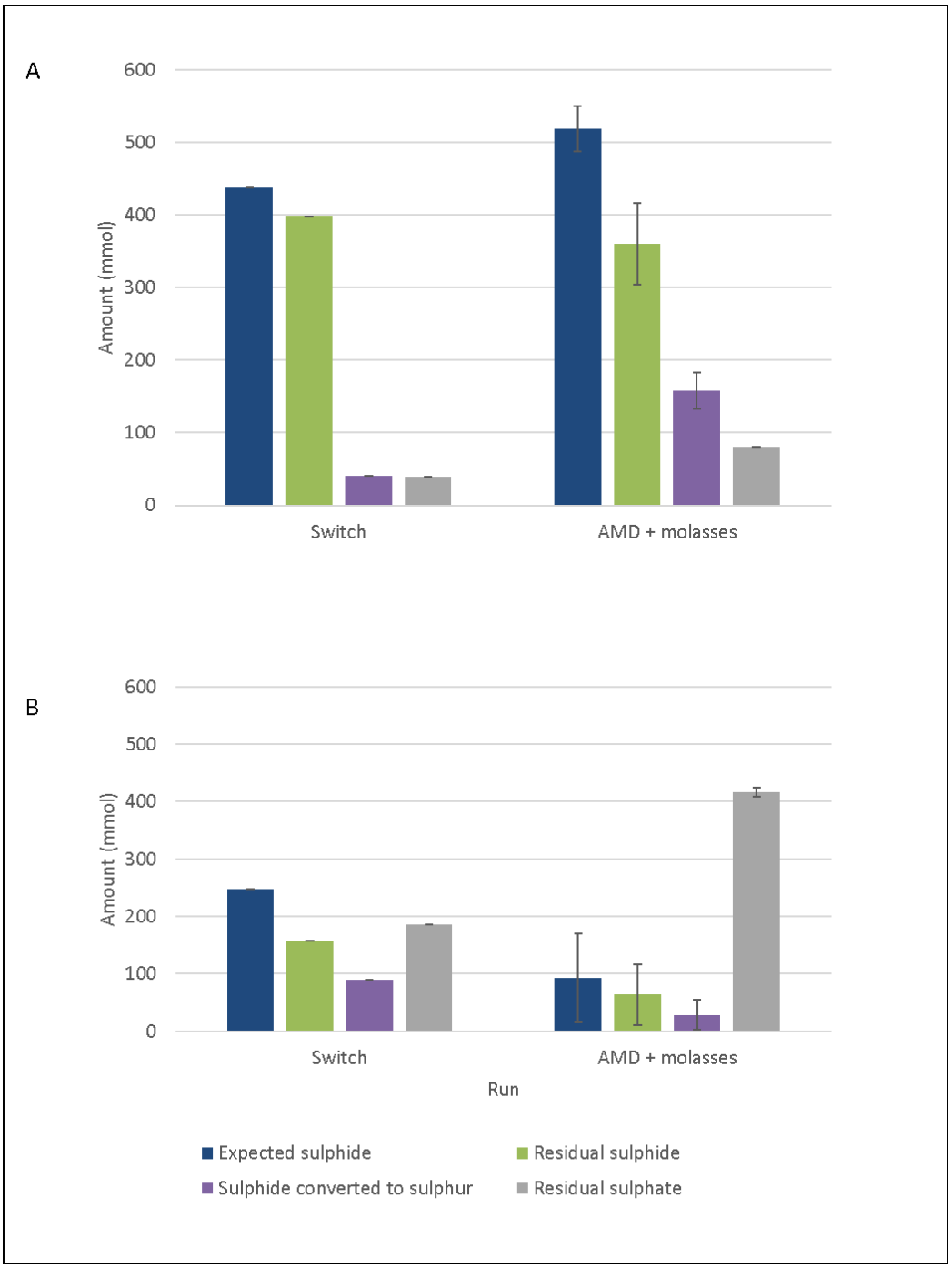


Figure 5-26: Expected sulphide, effluent sulphide, sulphide converted to sulphur and residual sulphate amounts in the A) primary reactor and B) secondary reactor LFCR system fed with pre-treated AMD feed and a lactate carbon during the switch period and the averaged two molasses runs. Approximately 575 mmol of sulphate fed into the reactor in 4 HRTs.

In the secondary reactor after the switch period, the amount of residual sulphate increased from 81 mmol to 417 mmol showing significant re-oxidation had occurred between the primary and secondary reactor. The sulphide converted to sulphur also decreased significantly by 129 mmol while residual

sulphide decreased by 297 mmol. Expected sulphide was high (519 mmol) in the primary reactor during the molasses runs ranging but decreased to around 93 mmol in secondary reactor due to re-oxidation at the effluent port.

Poor sulphur formation in the secondary reactor of the AMD + molasses LFCR experiment prompted further investigation. The FSB forming in the secondary reactor was not impeding oxygen well enough to prevent sulphide re-oxidation and maintain an anoxic environment that promotes sulphate reduction. Figure 5-27 shows the FSB formed in A, the first run and, B the second run of the AMD + molasses experiment.

It can be seen from Figure 5-27 that an FSB was forming in both runs when the LFCR was fed with pre-treated AMD and molasses as a carbon source. However, in the secondary reactor, the FSB was not consistent, and the reactor had areas on the liquid surface absent of FSB. A combination of the inconsistent biofilm which had areas with no biofilm and the low concentrations of sulphide entering from the effluent port of the primary reactor could have led to poor sulphate reductions and re-oxidation in the secondary reactor; lower sulphide concentrations resulted in less sulphide ions to mop up oxygen entering the bulk liquid from the surface resulting in a less reducing environment.

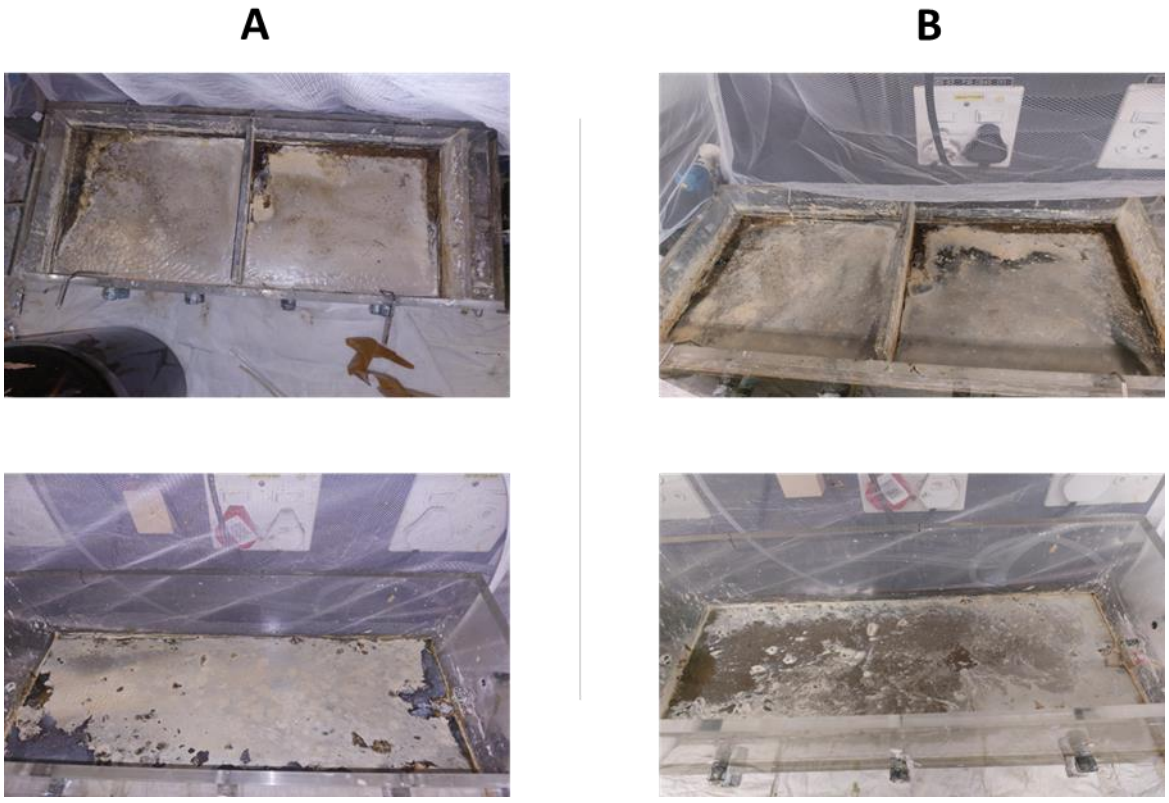


Figure 5-27: Aerial view of the primary (top images) and secondary (bottom images) reactor units running on pre-treated AMD and molasses carbon source showing A) the first run FSB formation B) the second run FSB formation

It was postulated that the amount of usable COD in molasses after pasteurisation decreased and so the COD:Sulphate ratio of 1 was not met. This would have resulted in a lower concentration of usable COD for the mixed culture and SOB. Available COD was probably used up in the primary reactor and less was available to the secondary reactor causing poor FSB formation in the secondary reactor as seen with the low concentrations of acetate in Figure 5-24. Fernandes, (2020) observed poor biofilm formation when carbon source was limited and(Van Hille and Mooruth, 2014) also reported that in the sticky phase of biofilm formation, heterotrophic bacteria are necessary for EPS excretion; in conditions where the carbon source is limited there is poor biofilm formation. In this scenario, the sticky phase would have been compromised, affecting subsequent incorporation of SOB.

To test whether there was a carbon limitation in the system, a preliminary experiment was performed where two 50 ml volumes of bulk liquid were taken from the secondary reactor in areas where there was no biofilm formation. These were placed in glass sample bottles, with and without the addition of molasses as extra carbon source. After a week, with the bottles partially closed and left at ambient conditions, the bottles were observed for any changes. Figure 5-28 shows an image of the sample bottles after a week. Evidence of biofilm of formation could be seen in the bottle with extra molasses. This suggests that carbon source limitation may have been responsible for poor formation of the FSB and negligible sulphate reduction.

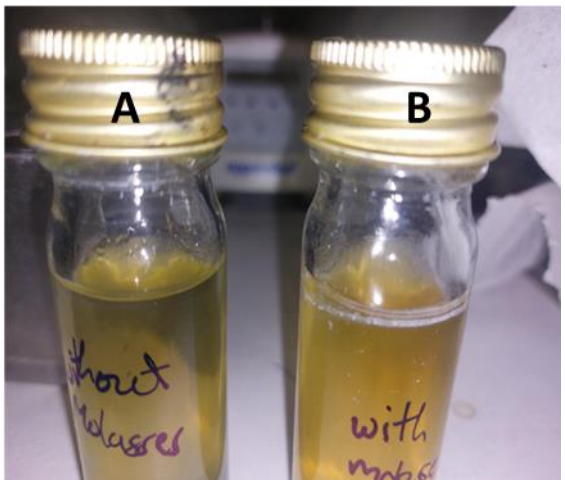


Figure 5-28: Testing for FSB formation when additional molasses carbon source is added to a carbon depleted SRB system which cannot form a FSB: A) control with no additional carbon source added and B) with additional molasses, carbon source added showing evidence of FSB formation

The compositions of the biofilms harvested after each run are shown in Table 5-7. There was less sulphur in the biofilm harvested from the primary reactor during the switch period compared to when the reactor was fed with a molasses carbon source. The proportion of sulphur for the first and second run in the primary reactor was higher than that obtained for the synthetic feed + lactate 3-day experiment and the 100% AMD + lactate experiment. Thus, molasses is not only a suitable substrate but also allows for higher concentrations of sulphur to be deposited in the FSB. However, in the secondary reactor there was less sulphur in the biofilm. This could have been due to the reoxidation that occurred at the primary reactor effluent port decreasing sulphide concentrations which were carried over to the secondary reactor. Poor sulphur formation could have also been caused by depletion of the carbon source, affecting heterotrophic bacteria that are essential for biofilm formation.

Table 5-7: FSB composition of the FSB from the 3-day HRT runs fed with an AMD feed with a molasses carbon source

Run	Experiment	N [%]	C [%]	H [%]	S [%]	Inorganics (%)
Switch period	AMD + molasses 3-day HRT (R1)	1.1	13.8	1.4	15.3	68.4
	AMD + molasses 3-day HRT (R2)	0.3	10.6	0.5	17.1	71.4
1st run	AMD + molasses 3-day HRT (R1)	2.0	14.4	2.1	40.9	40.6
	AMD + molasses 3-day HRT (R2)	1.6	12.7	2.4	8.9	74.4
2nd run	AMD + molasses 3-day HRT (R1)	2.8	16.3	3.1	39.3	38.5
	AMD + molasses 3-day HRT (R2)	2.4	16.3	2.5	20.1	58.8

Switching from lactate to molasses decreased performance in the primary reactor as can be seen by the switch period having a higher VSRR and sulphate conversion at 0.230 mmol/L.h and 92.6% respectively (Table 5-8). However, use of the molasses substrate increased sulphide conversion in the primary reactor suggesting that heterotrophic bacteria involved in FSB formation, prefer molasses to lactate in AMD treatment. This may be due to the extra nutrients found in molasses when compared to lactate. In the secondary reactor, the sulphide conversion was matched in the 1st run and then decreased in the 2nd run.

Table 5-8: Reaction kinetics of the LFCR system fed with AMD and a molasses carbon source. During the transition (switch) period, the system switched from lactate to molasses as the primary carbon source. For both the first and second runs, it was assumed that molasses, along with all its fermentation and oxidation products, had completely replaced the previous carbon source throughout the entire reactor volume.

		Primary reactor (R1)				Secondary reactor (R2)		
	VSLR (mmol/L.h)	VSRR (mmol/L.h)	Sulphate Conversion [a] (%)	Sulphide Conversion [b] (%)	VSFR (mmol/L.h)	Sulphate conversion [c] (%)	Sulphide conversion [b] (%)	VSFR (mmol/L.h)
Switch	0.249	0.230	92.6%	9.3%	0.083	-24.9%	36.1%	0.168
1st run	0.249	0.212	85.2%	26.0%	0.122	-18.4%	31.7%	-0.068
2nd run	0.249	0.214	86.1%	35.4%	0.144	-31.9%	14.4%	0.012

[a] Sulphate conversion in the primary reactor is the percentage of feed sulphate converted

[b] Sulphide conversion is the percentage of expected sulphide converted to sulphur

[c] Sulphate conversion in the secondary reactor is the percentage of influent sulphate from the primary reactor converted

In the primary reactor, the AMD + molasses runs had similar performance to the AMD + lactate experiment in terms of the VSRR, sulphate conversion and sulphate residual concentrations (Table 5-9). The AMD + molasses experiment outcompeted the synthetic feed + lactate experiment's sulphate conversion by around 21% in the primary reactor. It also had highest sulphide conversion in the primary reactor across the three experiments. Sulphide conversion in the secondary reactor at a 100% AMD feed with lactate (30.3%) was lower than the 51.4% observed in the synthetic study at 3-day HRT (Section 5.2), while sulphate conversion was higher in the primary reactor with AMD present.

Table 5-9: Comparison of the three LFCR system experiments' reaction kinetics (primary and secondary reactor. Residual concentrations are the average concentrations for each experiment in the last 1.5 HRT.

	Primary reactor					Secondary reactor			
Experiment	VSRR (mmol/L.h)	Residual sulphate (mmol/L)	Sulphate Conversion	Residual sulphide (mmol/L)	Sulphide Conversion	Residual sulphate (mmol/L)	Sulphate Conversion	Residual sulphide (mmol/L)	Sulphide Conversion
Synthetic feed + lactate	0.189	7.2	64.8%	12.5	9.4%	7.62	-24.7%	4.2	51.4%
AMD + lactate	0.216	2.2	87.4%	17.0	-6.1	2.2	18.1%	11.0	30.3%
AMD + molasses	0.213	2.7	85.6%	10.3	30.7%	15.1	-25.2%	0.9	23.1%

[a] Sulphate conversion in the primary reactor is the percentage of feed sulphate converted

[b] Sulphide conversion is the percentage of expected sulphide converted to sulphur

c Sulphate conversion in the secondary reactor is the percentage of influent sulphate from the primary reactor converted

Looking at the residual concentrations revealed sulphate concentration in the AMD + molasses increased significantly from 2.7 mmol/L to 15.1 mmol/L while the synthetic feed and lactate experiment had constant sulphate concentrations across the reactors. Significantly lower sulphate concentrations at the effluent port compared to the bulk liquid in the primary reactor of the AMD + molasses experiment were carried over to the secondary reactor. The AMD + lactate experiment was the only LFCR system experiment which had a positive sulphate conversion of 18.1% in the secondary reactor indicating additional sulphate reduction.

Overall system performance saw the highest sulphate conversion obtained in the AMD + lactate experiment which was expected as it had the highest conversion in the primary reactor and no re-oxidation in the secondary reactor (Table 5-10). The AMD + molasses experiment had the lowest overall sulphate conversion in the system of 16.8% which was caused by extensive re-oxidation at the effluent port of the primary reactor; high sulphate concentrations carried over to the secondary reactor resulting in poor sulphur formation. This resulted in the AMD + molasses experiment having the lowest amount of feed sulphate converted to sulphur. Most of the sulphide was converted to sulphur in the primary reactor prior to re-oxidation at the effluent port.

Table 5-10: Summary of the overall system performance across the primary and secondary reactors.

	Overall					
Experiment	VSRR (mmol/L.h)	Sulphate conversion	Expected sulphide	Residual sulphide	Sulphide converted to sulphide	Feed sulphate converted to sulphur [a]
Synthetic feed + lactate	0.187	64.8%	562	177	385	57.9%
AMD + lactate	0.217	87.8%	712	474	237	41.6%
AMD + molasses	0.042	16.8%	240	83.9	156	27.2%

[a] Percentage of total sulphate fed into the system across a run that was converted to elemental sulphur

5.5 Conclusion on the studies conducted on the hybrid LFCR system

The introduction of pre-treated AMD to the LFCR system, using lactate as a substrate, resulted in a high sulphate reduction of 87.4% in the primary reactor. This was higher than the 64.8% sulphate reduction obtained when the LFCR system was fed with synthetic AMD made of Postgate media B and lactate as a carbon source at a 3-day HRT. Pretreatment of the AMD was necessary due to its low pH, which was not conducive for SRB growth and function, as observed in the AMD fed-batch reactor studies, where different treatment methods were explored. The untreated fed-batch reactor failed due to the detrimental effects of low pH (4.0-5.0) on SRB. Sterilisation of the AMD was also required due to the presence of native bacteria that competed with the SRB, which in turn reduced sulphate reduction, as demonstrated in the AMD fed-batch reactor studies. The initial fed-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 the need to pre-treat the AMD first.

The pre-treated AMD did not compromise the reactor's performance, even when lacking the additional nutrients provided by the Postgate media B, as seen in the 100% AMD run of the LFCR system on pre-treated AMD and lactate. Instead, a higher sulphate conversion was achieved when a 100% pre-treated AMD and lactate feed was used. This higher conversion was assumed to have been caused by the presence of low concentrations of metals, which have been reported to positively influence SRB activity. However, the overall sulphide converted to sulphur recovery in the 100% pre-treated AMD with lactate as a carbon source was the lowest among the LFCR system experiments at a 3-day HRT. This was an interesting observation, as low sulphate concentrations were maintained in both the primary and secondary reactors of the pre-treated 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 reactors of the AMD and lactate study. It was suggested that this thin transparent film impeded oxygen from entering the liquid phase more effectively 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 BaH-LFCR system resulted in a sulphate conversion (85.6%) similar to that observed in the AMD + lactate experiment in the primary reactor. This showed that molasses was a suitable alternative which maintained high sulphate conversion. However, FSB formation in the secondary reactor of the AMD + molasses experiment was very limited. There was extensive re-oxidation at the primary reactor effluent port which caused high concentrations of sulphate to be carried over to the secondary reactor. Thus, the overall sulphate conversion for the AMD + molasses experiment was low at 16.8%. Limited carbon source also perpetuated the high sulphate concentrations and poor FSB formation.

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 and VSRR in the primary reactor. These findings demonstrated the LFCR system's effectiveness in treating circum-neutral, sulphate-laden mining impacted waters cost-effectively, albeit while compromising elemental sulphur recovery.

6 Conclusions and Recommendations

Traditional active and passive treatment methods incompletely address the problems associated with AMD by only treating acidity and heavy metal content while leaving a high sulphate concentration in the treated stream. Active technologies available tend to have high chemical demand and are typically not cost effective for the treatment of low-volume, circum-neutral streams while passive technologies have tended to lack predictability. There is typically need for an additional polishing step to reduce high sulphate concentrations remaining in partially treated AMD streams. The Linear Flow Channel Reactor (LFCR), developed in previous work, addresses the treatment of high sulphate concentrations biologically and is a cost-efficient way to treat circum-neutral AMD. It was designed to overcome challenges faced by biological sulphate reducing (BSR) systems, including enhancing the rate of BSR through biomass retention and avoiding high sulphide effluents with a tendency for re-oxidation by promoting the partial oxidation of sulphide to elemental sulphur, a value-added product that enables easy recovery by phase separation. Here, it was further modified to include polyurethane foam (PUF) as an improved support structure to increase biomass retention and thereby rate of sulphate reduction, and baffles were inserted to allow controlled, directional flow. This promoted contact with active biomass and aimed to enhance performance by promoting stratified colonization with different SRB becoming more relevant along the length of the reactor while decoupling HRT and biomass retention. In this study, the modified LFCR is referred to as the baffled hybrid linear flow channel reactor (BaH-LFCR).

Previously, the BaH-LFCR was fed with a synthetic feed (Postgate media B) at a 1 g/L sulphate concentration with lactate as a carbon source and electron donor (Marais *et al.*, 2024). Lactate is a well-studied and efficient carbon source commonly used for sulphate reduction by the SRB. However, lactate is not readily available at the scale required for large scale application over extended periods. The synthetic feed also contained additional nutrients specific for the function of SRB and had a neutral pH. AMD generated in the field varies in composition from site to site and does not contain the full suite of macro- and micro- nutrients found in the synthetic feed. AMD also typically contains heavy metals. Thus, this study focused on exploring the effectiveness of the BaH-LFCR in the treatment of AMD substituted with an alternative, readily available and cost-effective carbon source.

Four carbon sources were tested in small-scale reactors. Preliminary small-scale reactor tests were conducted to select the most appropriate of three reactor types for small scale assessment of carbon source: continuous mini-columns, continuous Schott bottle reactors and fed-batch Schott bottle reactors. Fed-batch reactors were chosen over small-scale continuous reactors owing to improved exclusion of oxygen. The work done here led to observations on the effect of different scales of BSR experiments. Small-scale continuous reactors proved unreliable as they were prone to failure from excessive oxidation of sulphide back to sulphate. The need for frequent sampling to monitor the system and gaps at inlet and outlet ports, caused oxygen to enter the system. Due to their small volume, the effect of small amounts of oxygen ingress are exaggerated. This is detrimental to BSR systems which need to maintain an anaerobic environment to allow for the continual reduction of sulphate to sulphide by obligately anaerobic SRB. Fed-batch reactors used in this study were larger and offered better oxygen ingress control which promoted consistency allowing for better carbon source comparison. However, batch systems have limitations such as toxin build-up which is inhibitory to SRB. This slows down reactions significantly or decreases the conversion rates. In continuous reactors, there is continual removal or dilution of inhibitory compounds due to the presence of a constant steady flow of feed which improves reactor performance. Hence, while initial choice of carbon source and electron donor was made in small-scale fed-batch reactors, the performance of the selected carbon source required validation. Testing of the carbon source and its comparison to lactate in LFCRs provided a good next step from initial smaller scale tests as they are large enough to produce enough sulphide to mop up oxygen entering the system and through partial oxidation, develop a FSB which impedes oxygen ingress through the surface of the reactor. However, they are not easy to set up and take a long time to colonise well; hence are not appropriate for initial assessment of multiple carbon sources.

The preliminary carbon source tests showed molasses to be the most effective at sulphate reduction. Molasses is relatively affordable and produced in large quantities as a byproduct of the sugar industry.

A 0.51 g COD per 1 g molasses was used in both the fed-batch and the linear flow channel reactors. To achieve a 1:1 COD:sulphate ratio, about 2.2 g of molasses was needed for one litre of AMD with a 2 g/L sulphate concentration which is similar to 2.1 g of lactate needed to meet the same ratio. In South Africa, 1 kg of molasses costs less than 0.1% of 1 kg of lactate making molasses a much more suitable choice for large-scale, long-term treatment. Similarly to lactate, molasses would not be produced onsite and transport costs relative to transport distance need to be factored into carbon source decisions.

Poor performance of other complex substrates such as honey and algal lysate demonstrates the multiplicity of using a complex, organic substrate as an alternative carbon source. In the case of algal lysate, the algal biomass and resulting useable COD content was low resulting in a low sulphate conversion. Algal lysate may contain long chain carbohydrates, proteins and lipids which are not easily accessible to the SRB. Anaerobic digestion of the algal lysate could help in breaking down these long chain molecules allowing for more COD to be available to SRB; this requires further testing. Honey, though containing simple sugars accessible to SRB, has antimicrobial properties and an acidic pH which led to poor sulphate reduction.

Successful use of molasses, a readily available and more cost-effective option for an alternative carbon source, supported the first hypothesis:

> *"Locally available, organic substances such as molasses, honey or algal biomass with high sugar content can be used as an alternative carbon source to lactate in BSR systems. The high COD content of complex and simple sugars in these substrates will result in readily available and accessible carbon sources for efficient sulphate reduction in a mixed culture which contains SRB and fermenters."*

The high COD content of molasses allowed for efficient sulphate reduction in both the small-scale fed-batch reactor and the continuous LFCR reactor system. However, due to its complex nature it may introduce an array of different compounds which could affect the quality of the effluent stream. Thus, a trade off needs to be made to achieve fit-for-purpose water. AMD treated with molasses produced a coloured effluent which may have been caused by the non-organic components in molasses.

The proposed second hypothesis:

> *"Characterisation and preliminary testing of different AMD pre-treatment methods in BSR systems will enable identification of the AMD pre-treatment method required to enable the use of established optimal conditions similar to those used on the synthetic feed in the BaH-LFCR which allow for effective sulphate reduction by SRB and sulphide oxidation by SOB"*

was supported by successful reduction of sulphate from AMD achieved in the lactate fed-batch reactor and the LFCR experiments. However, pre-treatment and sterilisation of the AMD was necessary to allow for efficient sulphate reduction. This presents a cost block when considering large-scale and long-term treatment. Although lime treatment is one of the more cost-effective pre-treatments with a high efficiency in metal removal and neutralisation, it produces precipitates requiring removal before the AMD is further treated in the LFCR system. Alternatives to this could be the introduction of acidophilic SRB into the mixed culture. This may allow for build-up of alkalinity from sulphate reduction by the acidophilic SRB resulting in an increase in pH allowing fast acting mesophilic SRB to function. Treated AMD can also be fed back to the reactor which would increase the pH. Sterilisation was performed to prevent native bacteria from the AMD competing with SRB for substrate. At a large-scale, sterilisation of the AMD would result in high energy costs which are not feasible at scale. Thus, it would be better to adapt the SRB mixed culture to an unsterilised feed allowing for them to outcompete the native bacteria found in AMD.

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 the complex waste stream, molasses, as a carbon source maintained a high sulphate reduction and VSRR in the primary reactor. Introduction of pre-treated and sterilised AMD to the BaH-LFCR system, using lactate as a substrate, resulted in a sulphate conversion of 87.8%, higher than that achieved in the BaH-LFCR system fed with synthetic feed and lactate (66.8%). These findings demonstrated the LFCR system's effectiveness in treating circum-neutral, sulphate-laden mining impacted waters, albeit while

compromising elemental sulphur recovery in both the lactate and molasses-fed AMD LFCR experiments. Sulphur recovery decreased due to limited carbon availability in the molasses-fed LFCR and FSB inefficiency.

Although sulphate reduction performance was good and there was minimal re-oxidation demonstrated by the sulphide concentration remaining high, there was poor conversion of sulphide to sulphur through partial oxidation by SOB in the AMD + lactate experiment. This may be attributed to lack of availability of oxygen owing to a complete translucent biofilm forming over the surface. In the AMD + lactate experiment, a calcium crystal complex formed at the surface of the reactors as a thin film. This thin transparent film was presumed to impede oxygen from entering the liquid phase more effectively than the FSB formed during the synthetic feed + lactate experiment thus reducing the partial oxidation of sulphide to elemental sulphur. It was theorised that the formation of the calcium crystal complex was dependent on the high concentrations of calcium from the lime pretreatment step of the AMD.

Sulphate conversion of 85.2 to 86.1% in the primary reactor of the BaH-LFCR experiment on AMD + molasses was similar to that achieved in the AMD + lactate experiment, outcompeting the synthetic feed + lactate experiment. This experiment also achieved the highest percentage conversion of available sulphur in the feed sulphate to elemental sulphur in the primary reactor (30.7%), however of the total amount of sulphur available overall sulphide produced in the system was lower compared to the other two LFCR experiments. FSB formation in the secondary reactor, was very poor when molasses was used for AMD treatment in the LFCR system due to extensive re-oxidation at the effluent port of the primary reactor which fed into the secondary reactor. This resulted in AMD + molasses experiment having the lowest overall conversion. It was hypothesised that not enough carbon source was entering the secondary reactor and so there was limited carbon source for continued sulphate reduction and for the heterotrophic bacteria essential in the formation of the FSB. Preliminary investigations supported this hypothesis. Further addition of molasses or other carbon sources such as acetate to the secondary reactor could improve sulphur formation in the secondary reactor and decrease re-oxidation

To conclude, development of cost effective, sustainable treatment solutions for circum-neutral, low-volume wastewater is crucial in preventing the continuous pollution of freshwater streams and soils in South Africa. The LFCR system, utilising molasses as a carbon source, offers a viable solution both as a primary treatment method and as a polishing step to remove residual sulphate from existing treatment processes. Its dual functionality aligns with SDG goals such as ‘clean water and sanitation’ and ‘responsible consumption and production’. Water treated through this process could potentially be used for irrigation or could be refunneled to mines where there is need for large volumes of water, decreasing the strain on unpolluted water sources. Additionally, optimising sulphur formation and recovery can also yield a by-product that can be used as fertilizer in farming or to produce essential chemicals needed for industrial processes. This system presents a practical approach to water treatment that will deliver both economic and environmental benefits.

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Analytical Methods

A.1 Sulphate Assay Standard Curve

The sulphate stock solution was a 1 g/L solution made from sodium sulphate and deionised water.

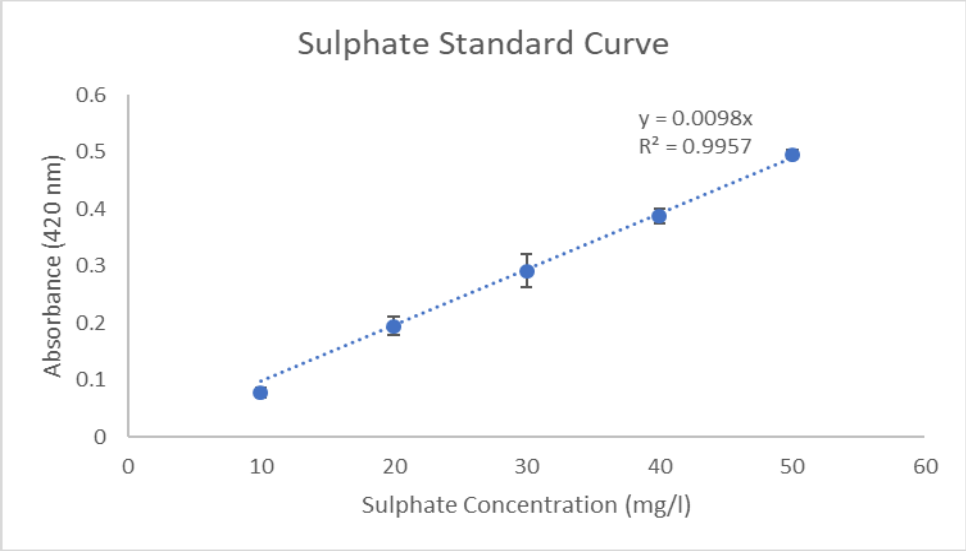

Figure A.1 1: Sulphate concentration standard curve generated using a 1 g/L sulphate solution (concentration range between 0-50 mg/L). Equation of the line and R^2 value are shown on the graph and the error bars show the mean ± one SD.

A.2 Sulphide Assay Standard Curve

The sulphide stock solution was a 100 mg/L solution made from sodium sulphide nonahydrate and deionised water.

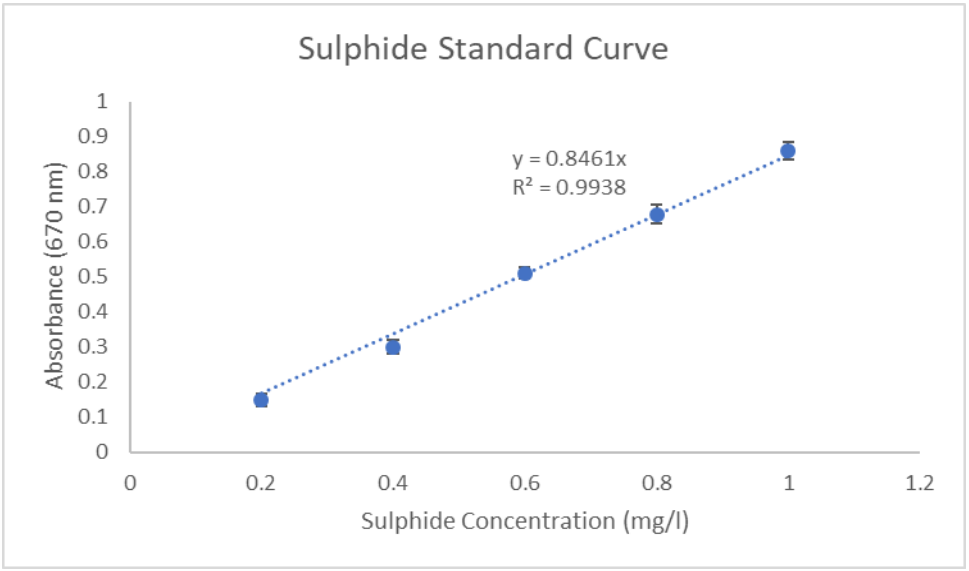

Figure A.2 1: Sulphide concentration standard curve generated using a 100 mg/L sulphide solution (concentration range between 0-1 mg/L). Equation of the line and R^2 value are shown on the graph, and the error bars show the mean ± one SD

A.3 Chemical Oxygen Demand Standard Curve

The COD stock solution was a 2 g/L solution of potassium hydrogen phthalate. Table A.3 1 shows the corresponding COD concentrations for the standard concentrations of potassium hydrogen phthalate used.

Table A.3 1: Potassium hydrogen phthalate concentrations with equivalent COD concentrations

Phthalate Concentration (mg/L)	100	300	600	900	1200
COD Concentration (mg/L)	118	353	706	1059	1412

The COD concentration standard curve obtained from the potassium hydrogen standard concentration is shown in Figure A.3 1.

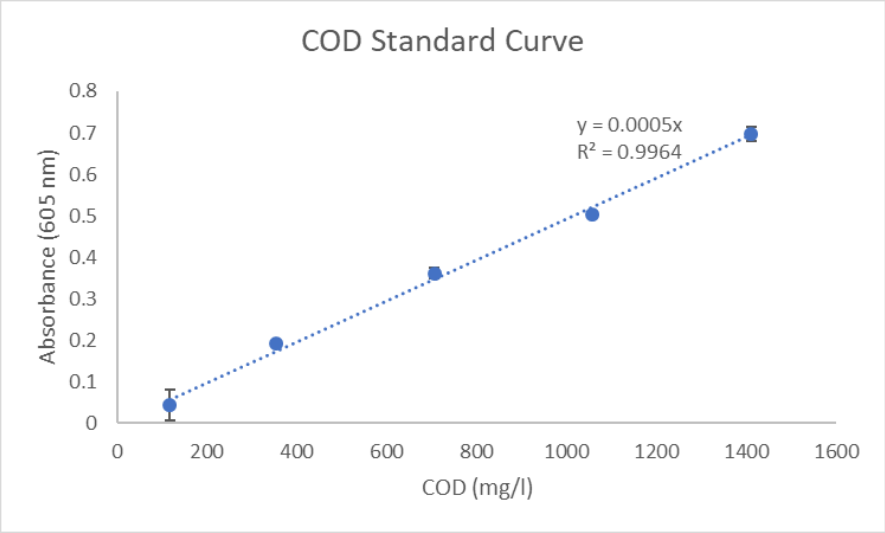

Figure A.3 1: COD concentration standard curve generated using a 2 g/L potassium hydrogen phthalate solution (COD concentration range was between 100-1500 mg COD/l. Equation of the line and R^2 value are shown on the graph and the error bars show the mean ± one SD.

A.4 Sugar, alcohol and VFA retention times

Table A.4 2: Sugar, alcohol and VFA retention times in HPLC using a 5 mM H_2SO_4 mobile phase

Sugar/ alcohol/ VFA	Glucose	Fructose	Sucrose	Ethanol	Acetate	Citrate	Propionate	Lactate
Retention time (minutes)	11.21	12.02	9.28	25.81	18.49	9.79	22.27	15.90

A.5 Total Iron Standard Curve

The iron stock solution of 100 mg/L was made of a mixture of sulphuric acid, deionised water, ferrous sulphate heptahydrate. The iron standard curve used to calculate the total iron concentrations is shown in Figure 5 1 and was taken from (Leslie Mostert, 2020).

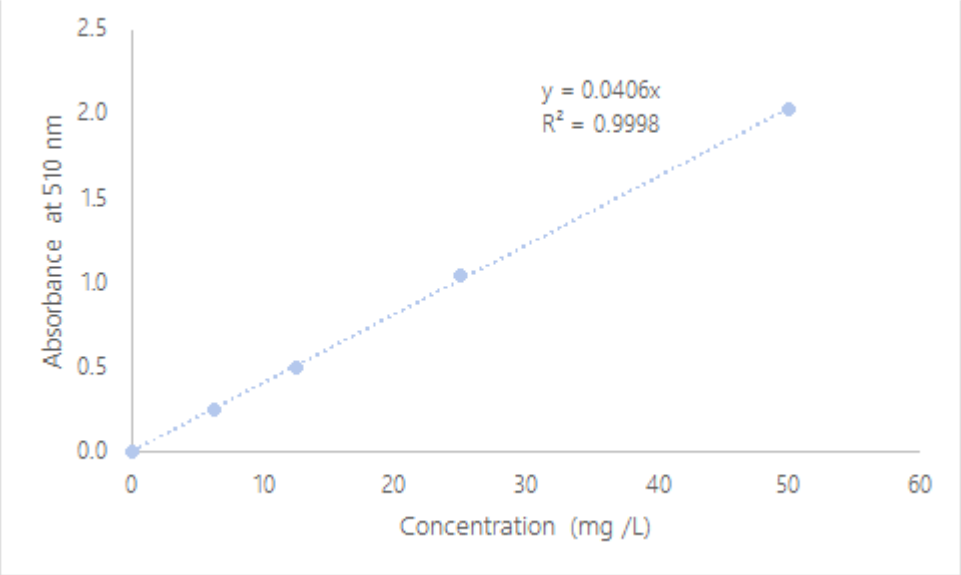

Figure A.5 1: Total iron concentration standard curve generated using a 100 mg/L ferrous solution (concentration range between 0-50 mg/L). Equation of the line and R^2 value are shown on the graph.

Preliminary substrate tests

A.6 COD calculations

A.6.1 Literature derived COD sample calculation

Molasses

Composition (wt%) of molasses from literature (Palmonari *et al.*, 2020):

Table B.1.1 1: Sugar and VFA compositions in molasses and the molecular formulae and molar masses of the sugar and VFAs in molasses

Sugar/VFA	Composition (wt%)	Molecular formula	Molar mass (g/gmol)
Sucrose	48.8%	$C_{12}H_{22}O_{11}$	342
Glucose	5.3%	$C_6H_{12}O_6$	180
Fructose	8.1%	$C_6H_{12}O_6$	180
Starch	0.3%	$C_6H_{10}O_5$	162
Lactic acid	6.1%	$C_3H_6O_3$	90

Theoretical COD calculation:

$$COD\left(\frac{molO_2}{mol_{sugar\ or\ VFA}}\right) = (\frac{1}{4})(4x + y + 2z)$$

Where: $C_xH_yO_z + aO_2 \rightarrow bCO_2 + cH_2O$

The COD in g O_2/g substrate was obtained by converting the mol O_2/mol substrate using molar masses.

Each sugar and VFA was then multiplied by its composition to obtain the mass of COD contributed by the specific sugar and VFA in the substrate. Each COD (g COD/g substrate) value from each sugar and VFA was summed up to give a total g COD/g substrate.Table B.1.1 2 shows the individual COD contributions of each sugar and VFA and the final total COD in the molasses substrate (total g COD/g substrate).

Table B.1.1 2: COD contribution of each sugar and VFA in molasses to the total COD in molasses

Sugar/VFA	Maximum COD (g O2/g substrate)	COD contribution (g O2/g substrate)
Sucrose	1.12	0.548
Glucose	1.07	0.0564
Fructose	1.07	0.0861
Starch	1.18	0.00391
Lactic acid	1.06	0.0651
Total		**0.759**

A.7 Sugar and VFA concentration of honey and molasses determined through HPLC analysis

Table B.2 1: Sugar concentration of honey and molasses determined through HPLC analysis

	Sucrose	Fructose	Glucose
Honey (g/g substrate)	0.144	0.152	0.200
Molasses (g/g substrate)	0.183	0.101	0.185

Chromatogram and SST Results

Injection Details

Injection Name:	**Molasses (1:10)**	*Run Time (min):*	**30.00**
Vial Number:	**A21**	*Injection Volume:*	**10.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**Nyasha_IM**	*Bandwidth:*	**n.a.**
Processing Method:	**Nyasha_PM**	*Dilution Factor:*	**10.0000**
Injection Date/Time:	**02/Aug/23 16:43**	*Sample Weight:*	**1.0000**

Chromatogram

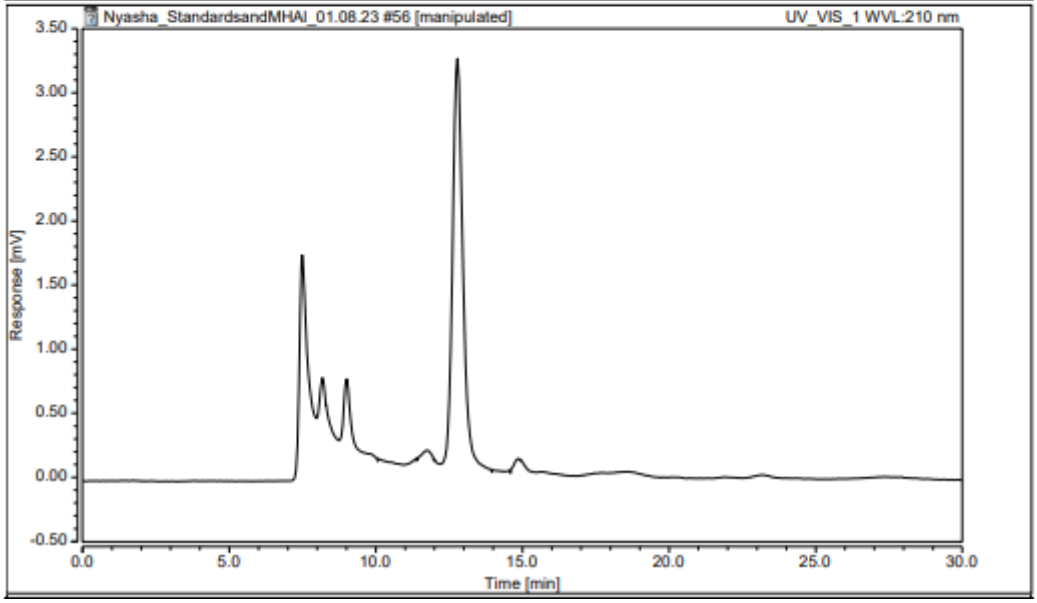

Figure B.2 1: Molasses UV chromatogram showing a peak around 15 minutes indicating the presence of lactate in molasses before it is fed to the batch or continuous reactors

A.8 Continuous reactor results

In the continuous reactors, only molasses, algal lysate, acetate, and lactate were tested. Honey was not tested in the continuous reactors because of its antimicrobial properties and high acidity which resulted in poor sulphate conversion which was evident from the fed-batch reactors.

A.8.1 Mini column reactors

Molasses

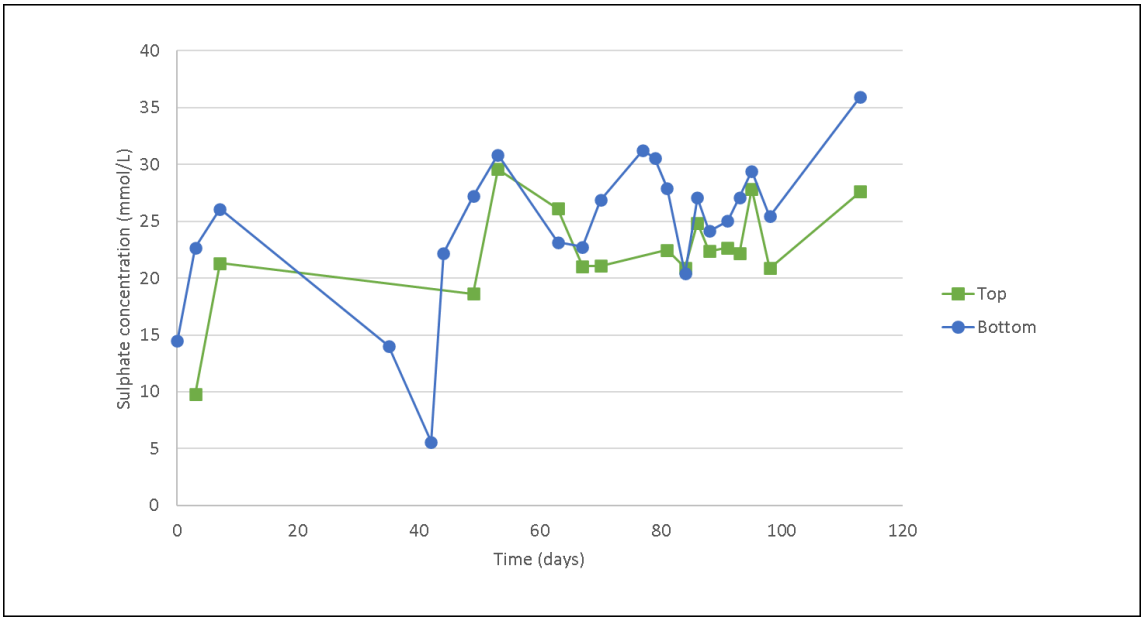

Figure B.3 1: Sulphate concentration time trends for the molasses-fed continuous mini column reactor.

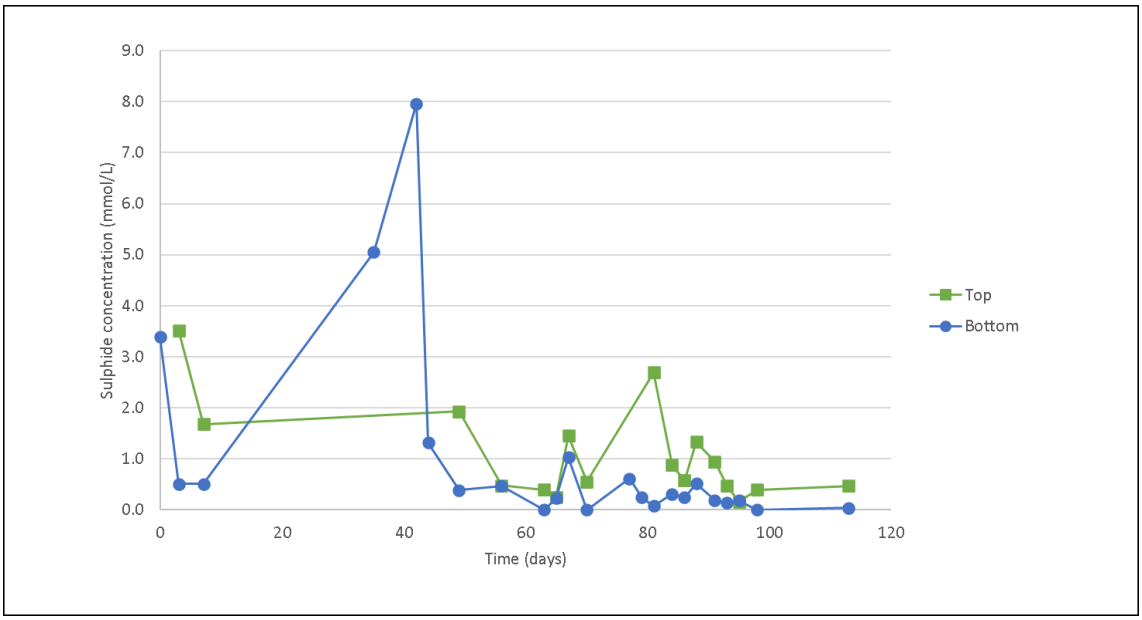

Figure B.3 2: Sulphide concentration time trends for the molasses-fed continuous mini column reactor.

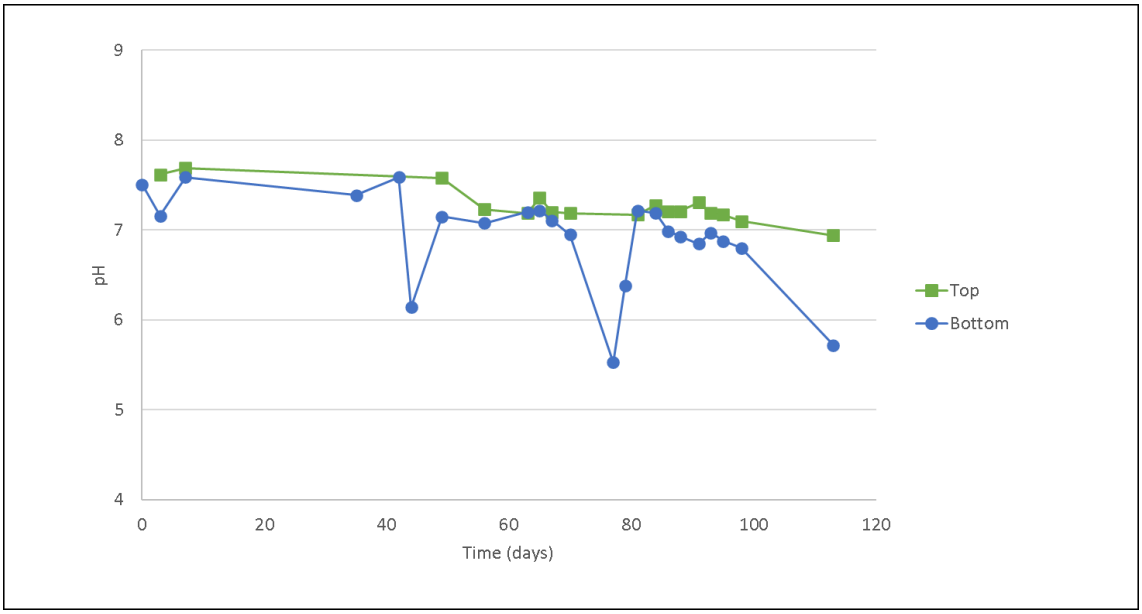

Figure B.3 3: pH time trends for the molasses-fed continuous mini column reactor.

Acetate

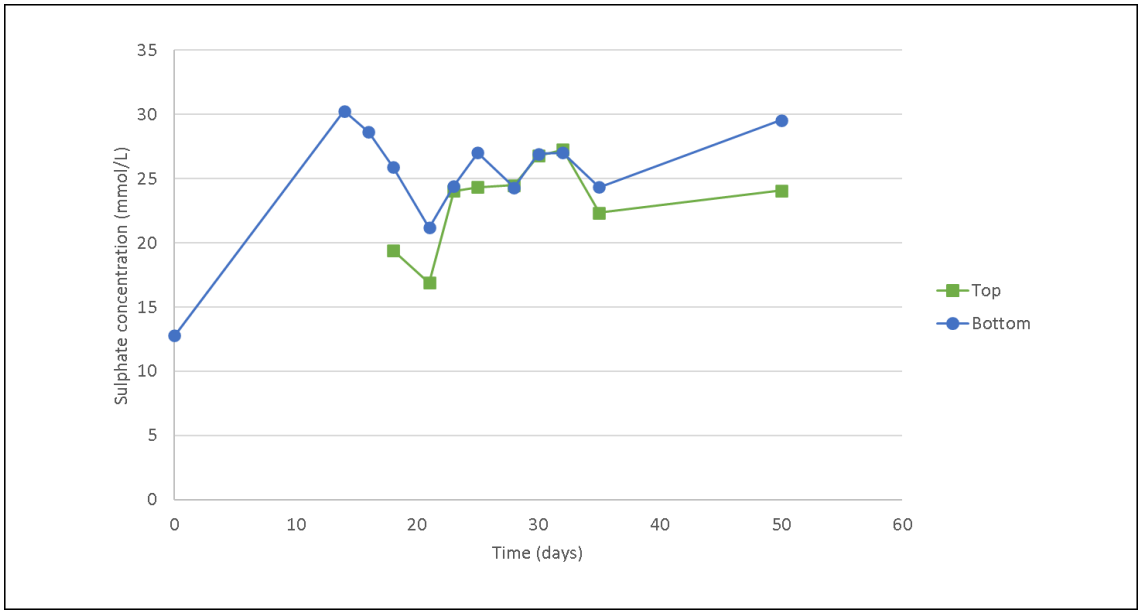

Figure B.3 4: Sulphate concentration time trends for the acetate-fed continuous mini column reactor.

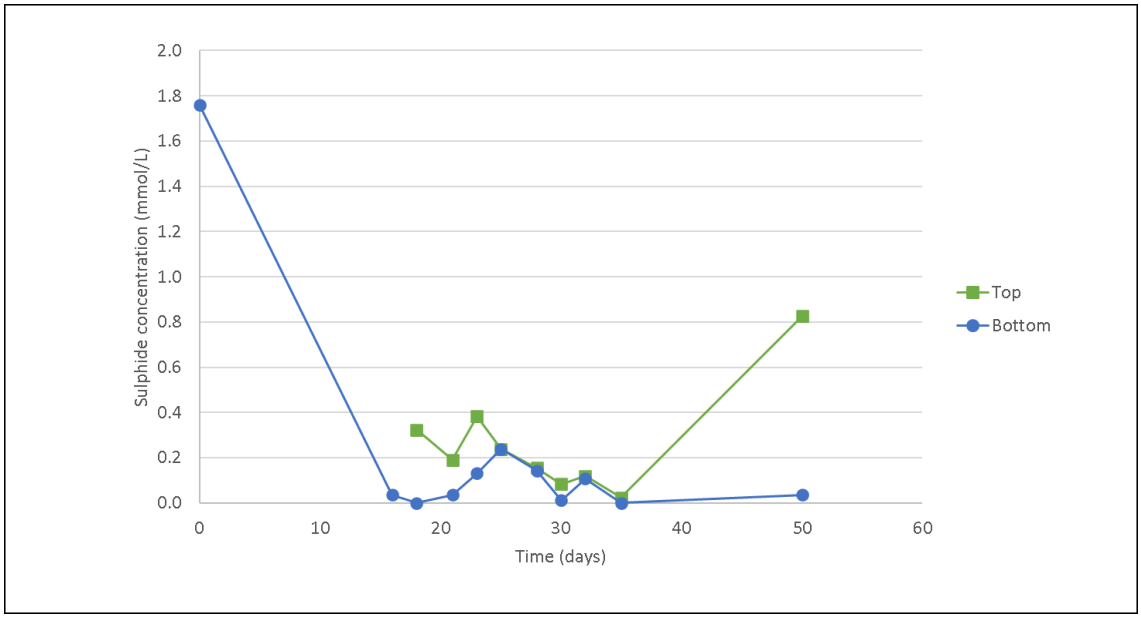

Figure B.3 5: Sulphide concentration time trends for the acetate-fed continuous mini column reactor.

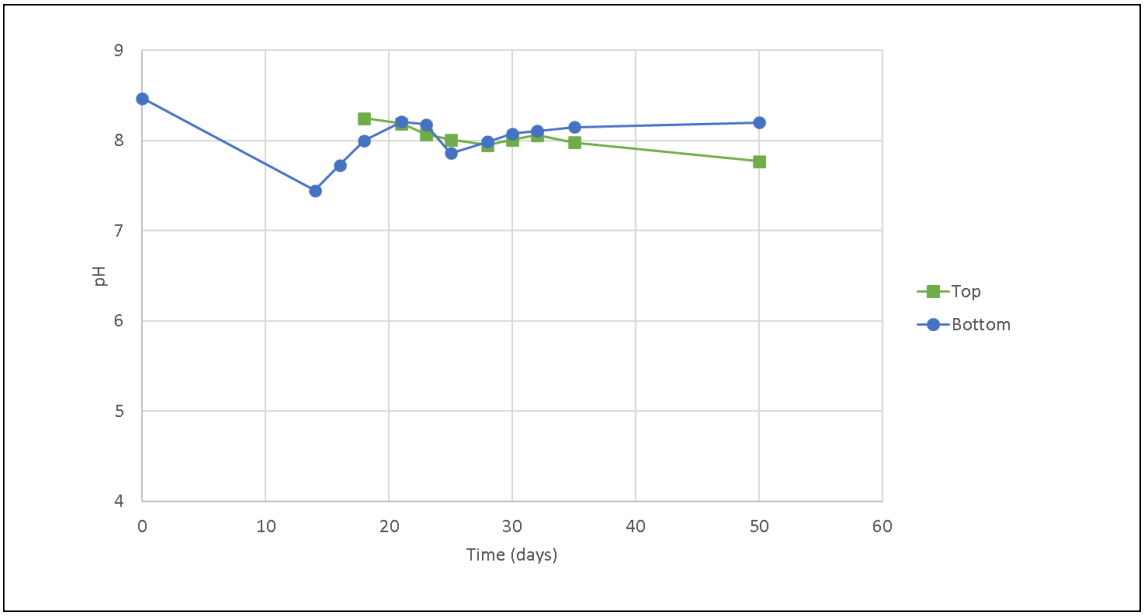

Figure B.3 6: pH time trends for the acetate-fed continuous mini columns reactor

A.8.2 Schott bottle reactors

Molasses

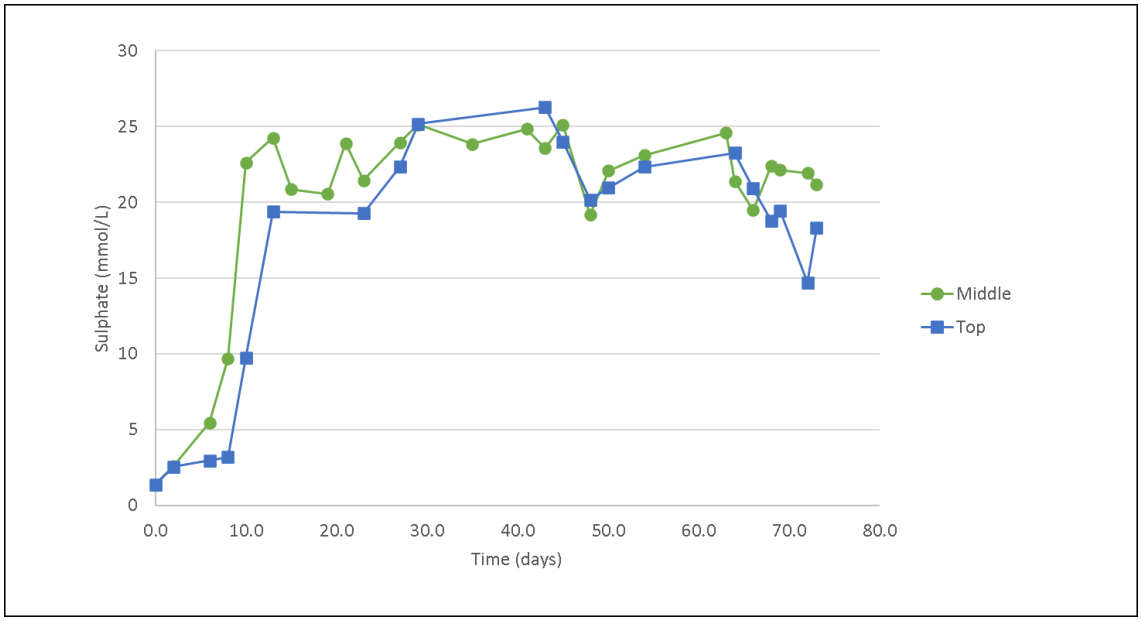

Figure B.3 7: Sulphate concentration time trends for the molasses-fed continuous Schott bottle reactor.

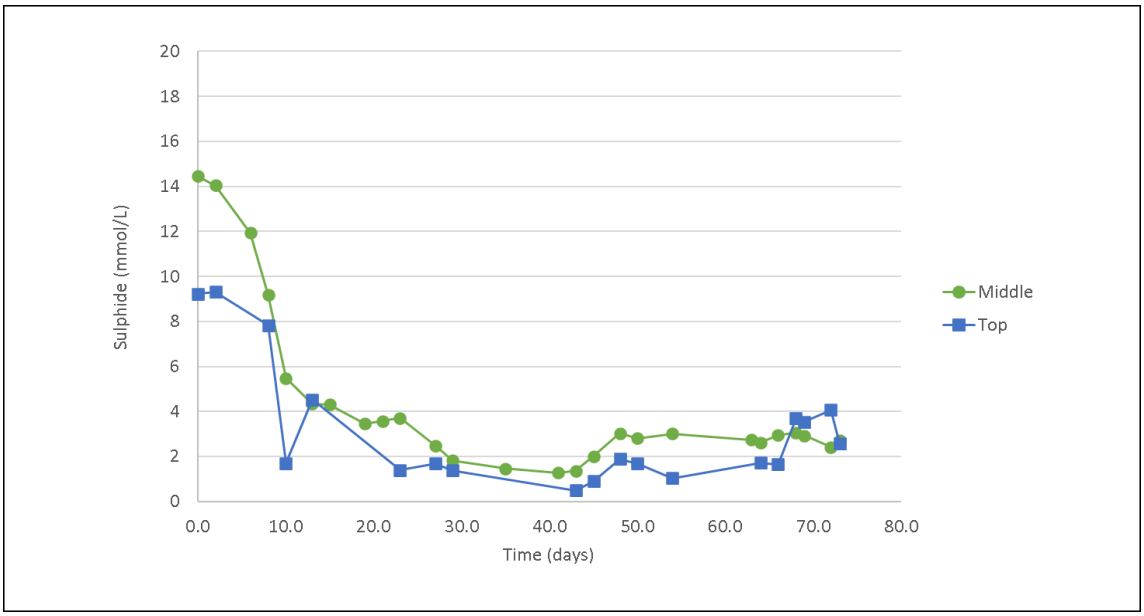

Figure B.3 8: Sulphide concentration time trends for the molasses-fed continuous Schott bottle reactor.

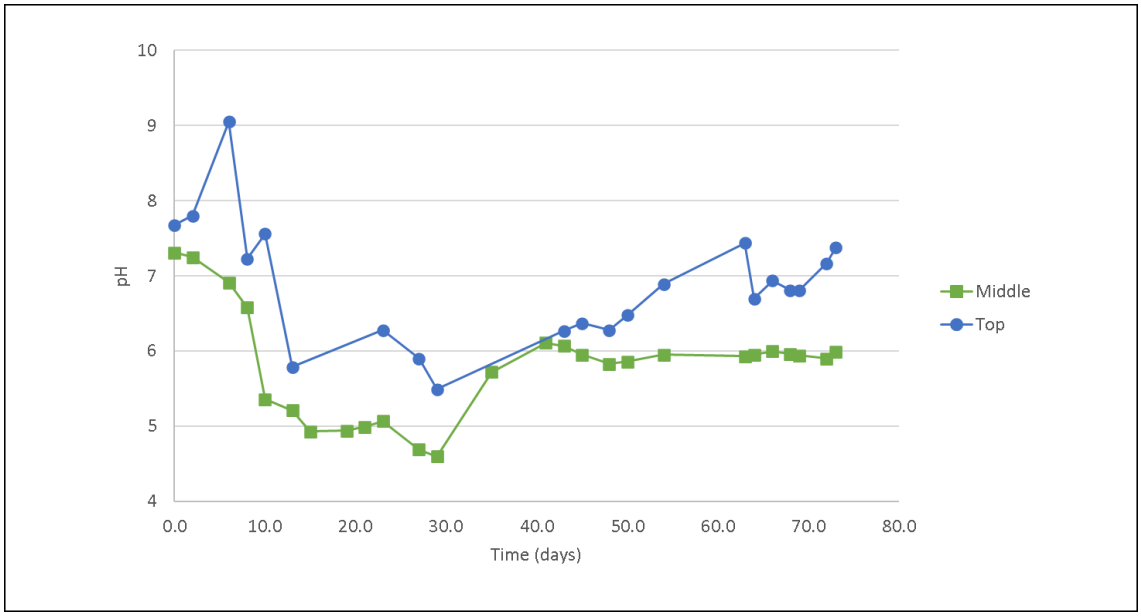

Figure B.3 9: pH time trends for the molasses-fed continuous Schott bottle reactor.

Acetate

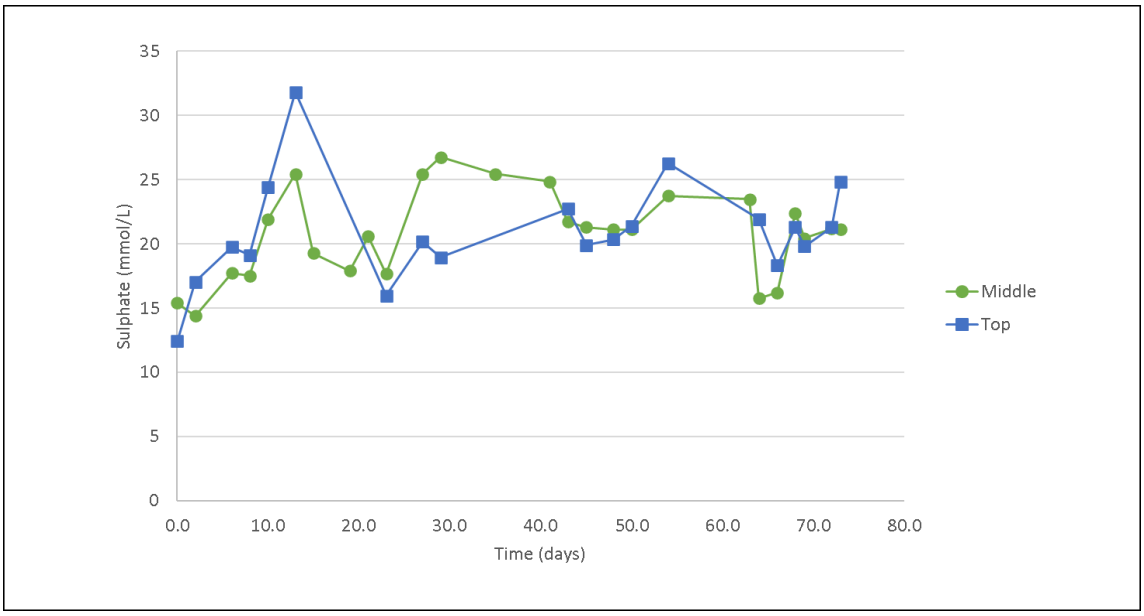

Figure B.3 10: Sulphate concentration time trends for the acetate-fed continuous Schott bottle reactor.

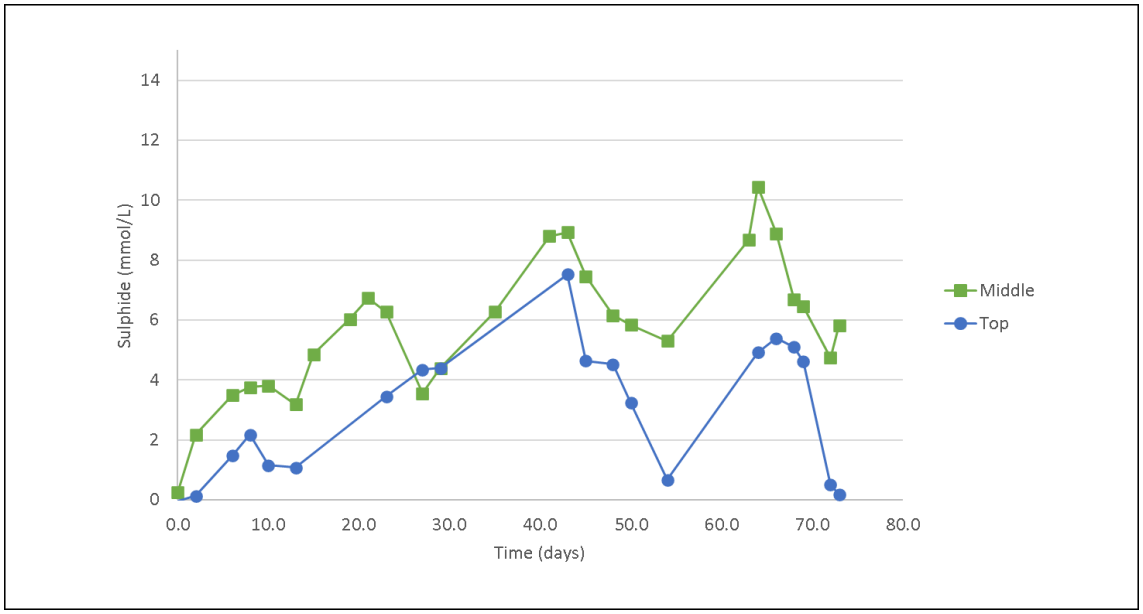

Figure B.3 11: Sulphide concentration time trends for the acetate-fed continuous Schott bottle reactor.

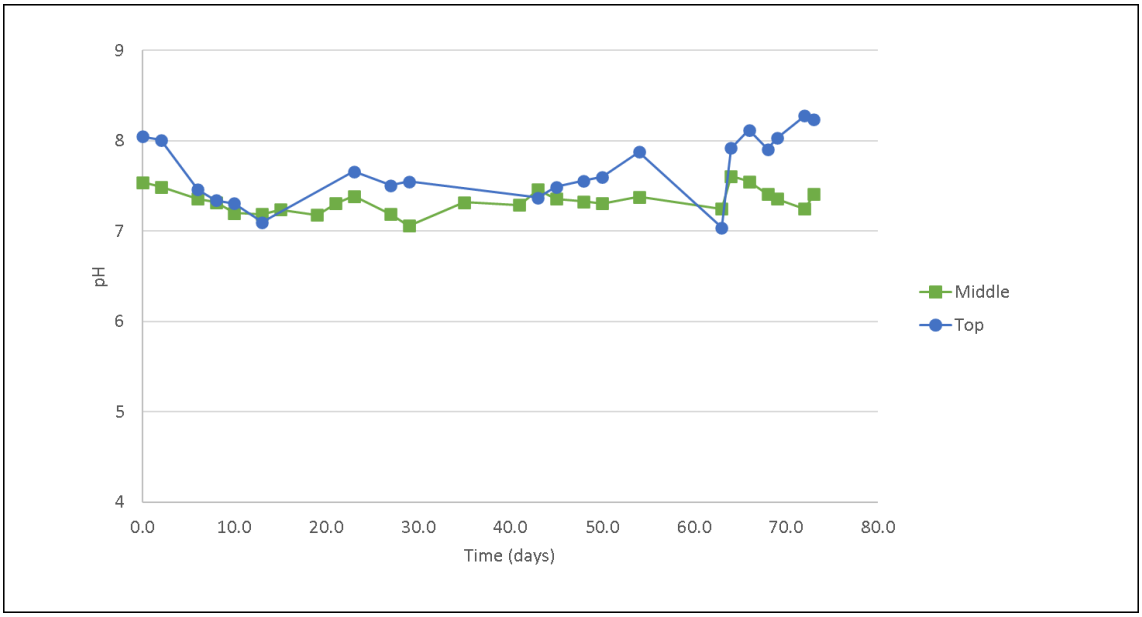

Figure B.3 12: pH time trends of the acetate-fed continuous Schott bottle reactor.

Algal lysate

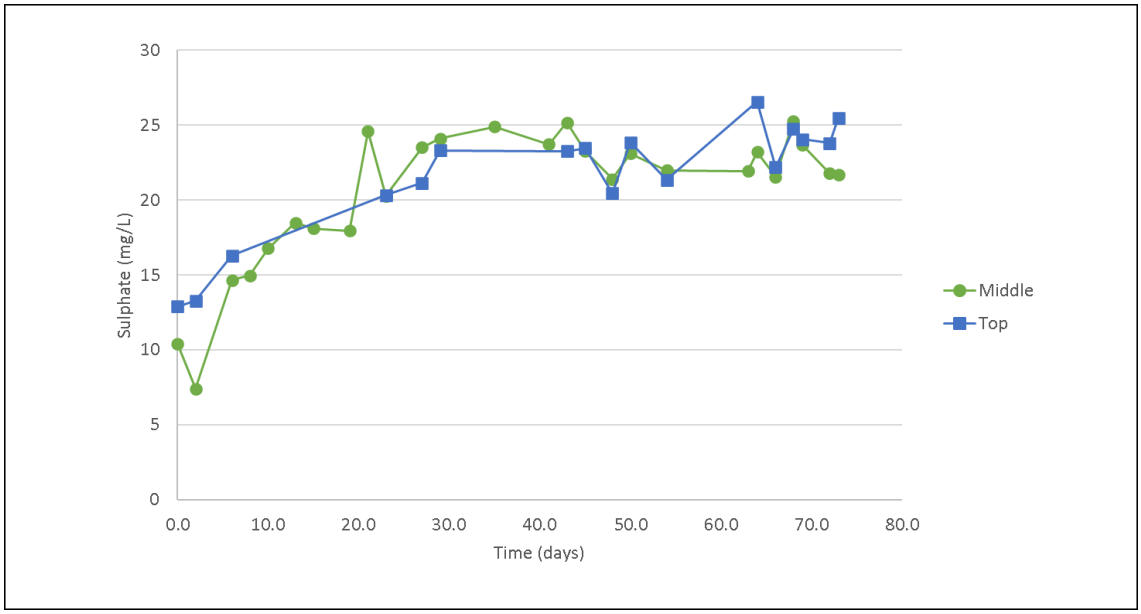

Figure B.3 13: Sulphate concentration time trends for the algal lysate-fed continuous Schott bottle reactor.

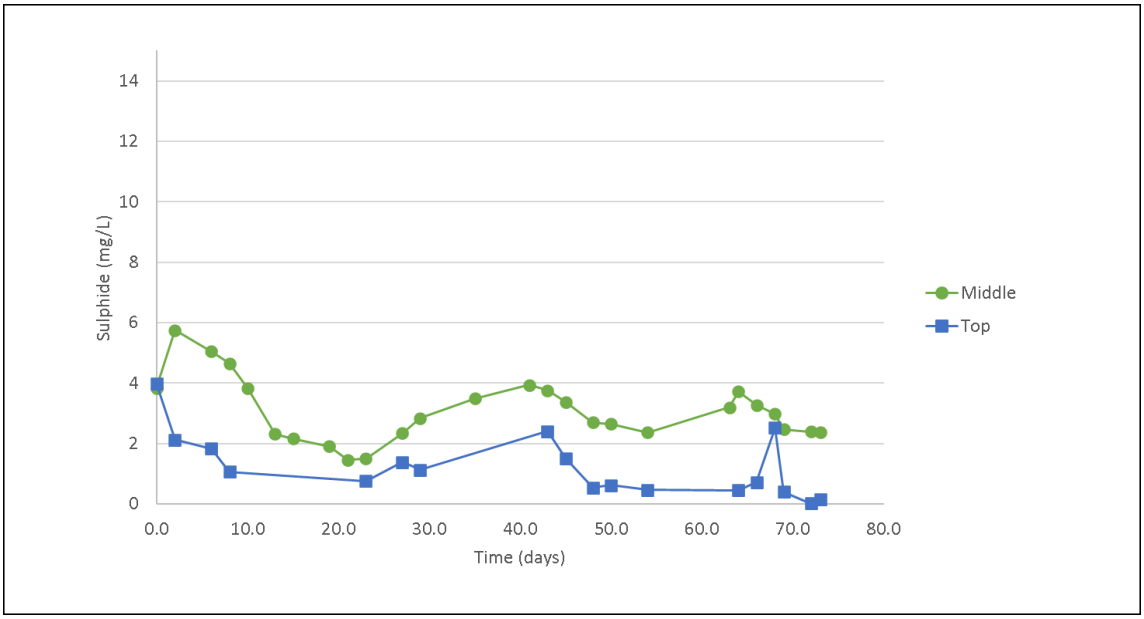

Figure B.3 14: Sulphide concentration time trends of the algal lysate-fed continuous Schott bottle reactor.

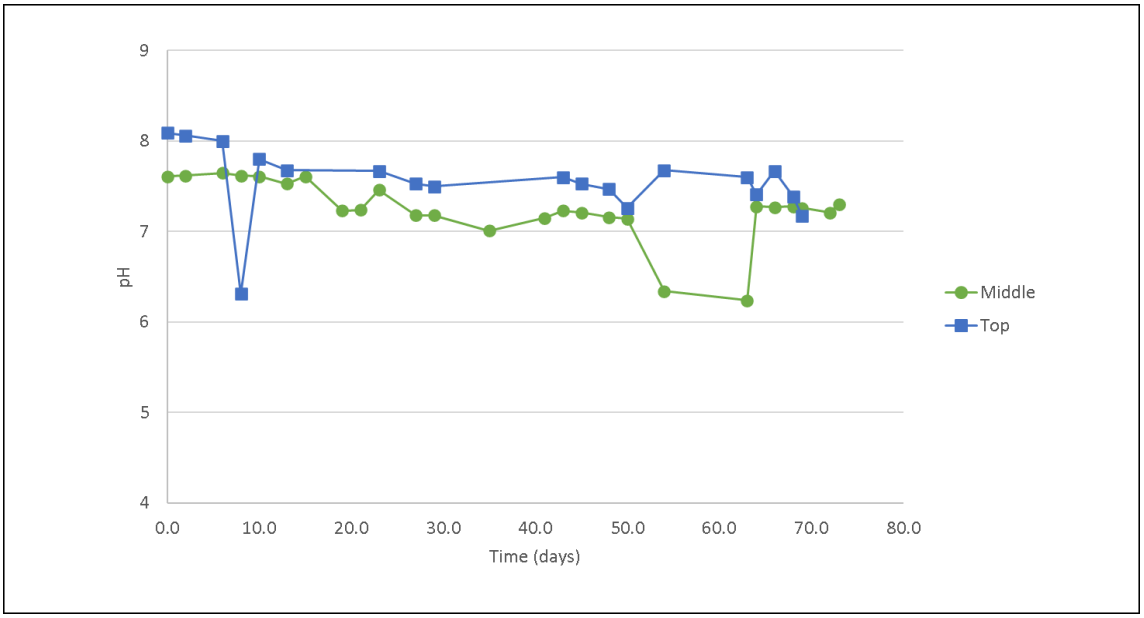

Figure B.3 15: pH time trends of the algal lysate-fed continuous Schott bottle reactor.

A.9 HPLC results

A.9.1 Molasses fed-batch Schott bottle reactor chromatograms

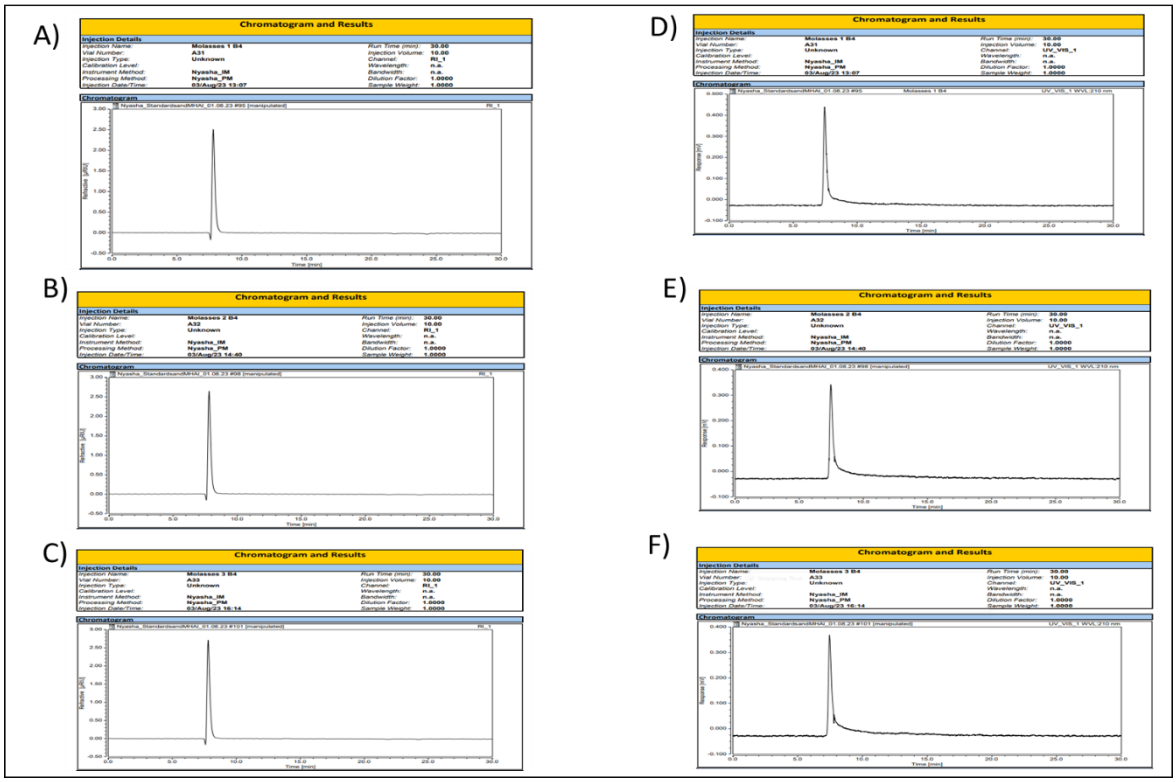

Figure B.4 1: Molasses fed-batch reactor RI(A), B), C)) and UV(D), E), F)) chromatograms for samples taken at the end of 3 cycles with no peaks except for the solvent peak between 7 and 9 minutes, showing that all sugars and VFAs were used up.

The only peak visible is the solvent peak which appears between 7 and 9 minutes for both the RI and UV chromatograms.

BaH-LFCR experiments supplementary results

A.10 Synthetic feed and lactate substrate

A.10.1 Primary and secondary reactor average pH time trends

The feed's neutral pH, as seen in Figure C.1 1, provided an optimal environment for SRB activity and ensured sulphide remained in the aqueous phase, as gaseous hydrogen sulphide is toxic (Brahmacharimayum *et al.*, 2019).

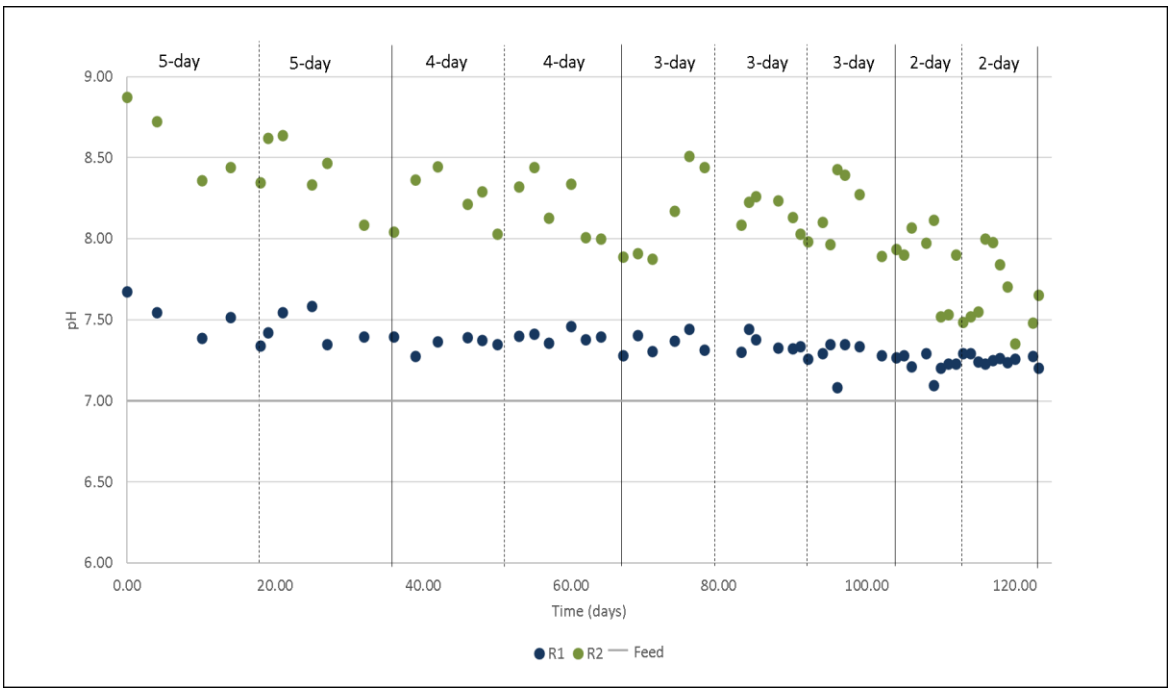

Figure C.1 1: LFCR system fed with synthetic feed and a lactate carbon source pH time trends at different hydraulic residence times. The vertical straight lines (dotted and solid) indicate biofilm harvest.

Higher pH in the reactors compared to the feed was attributed to bicarbonate alkalinity from sulphate reduction, and hydroxyl ions produced by partial oxidation of sulphide to elemental sulphur. The primary reactor's range of pH (7.00-8.00) was lower than the secondary reactor's (7.50-9.00). This higher pH in the secondary reactor resulted from increased partial sulphide oxidation combined with alkalinity produced from sulphate reduction in the primary reactor.

Partial oxidation of sulphide caused of the secondary reactor's pH to be higher. In both reactors, pH remained generally constant between the 5-day and 3-day HRTs but decreased at the 2-day HRT. This decrease was likely due to increased re-oxidation at the 2-day HRT, with oxidation of sulphide to sulphate producing protons that lowered pH.

A.10.2 Primary and secondary reactor effluent port redox time trends

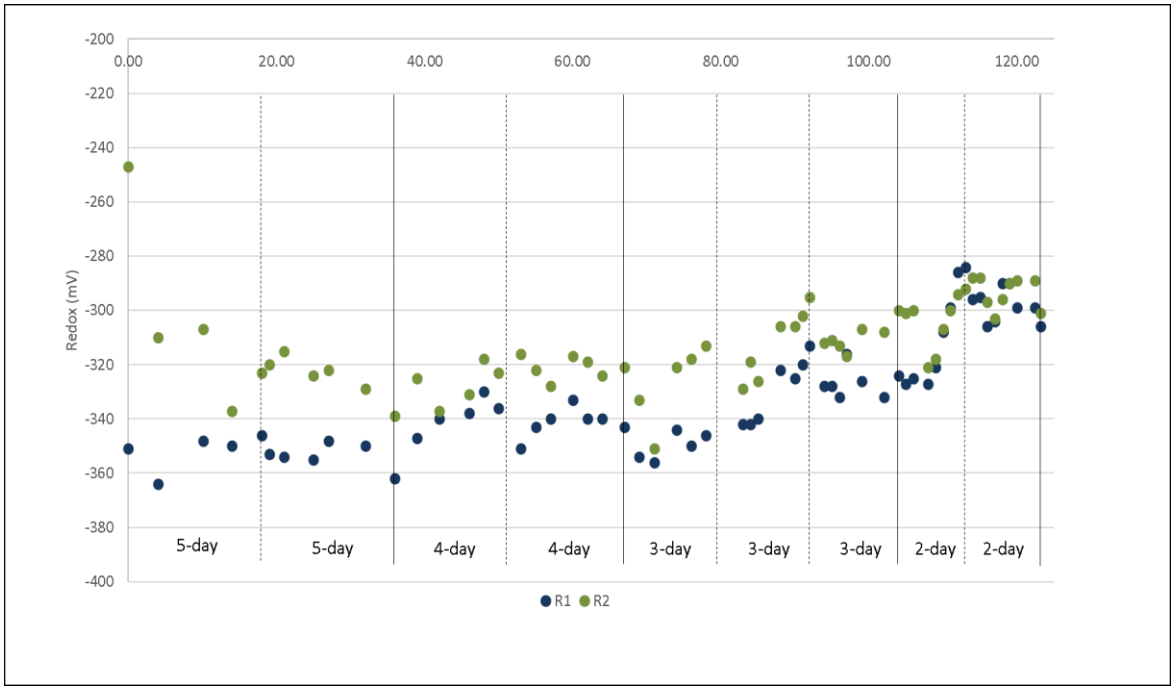

Figure C.1 2: LFCR system fed with synthetic feed and a lactate carbon source redox time trends at different hydraulic residence times. The vertical straight lines (dotted and solid) indicate biofilm harvest.

The redox potential remained negative in both reactors, ranging between -400 and -250 mV (Figure C.1 2), indicating a reducing and anoxic environment essential for optimal SRB performance.

The primary reactor's average redox potential was lower than the secondary reactor's across the 5-day, 4-day, and 3-day HRTs, converging at the 2-day HRT. Higher redox values in the secondary reactor suggested a less reducing environment. At the 2-day HRT, redox potentials in both reactors increased, indicating increased susceptibility to re-oxidation at lower HRTs.

A.10.3 Sulphate and sulphide time trends at different sampling points

Analysis of samples from ports distributed across the reactor provided insight into the LFCR system's process and reaction dynamics Figure C.1 3.

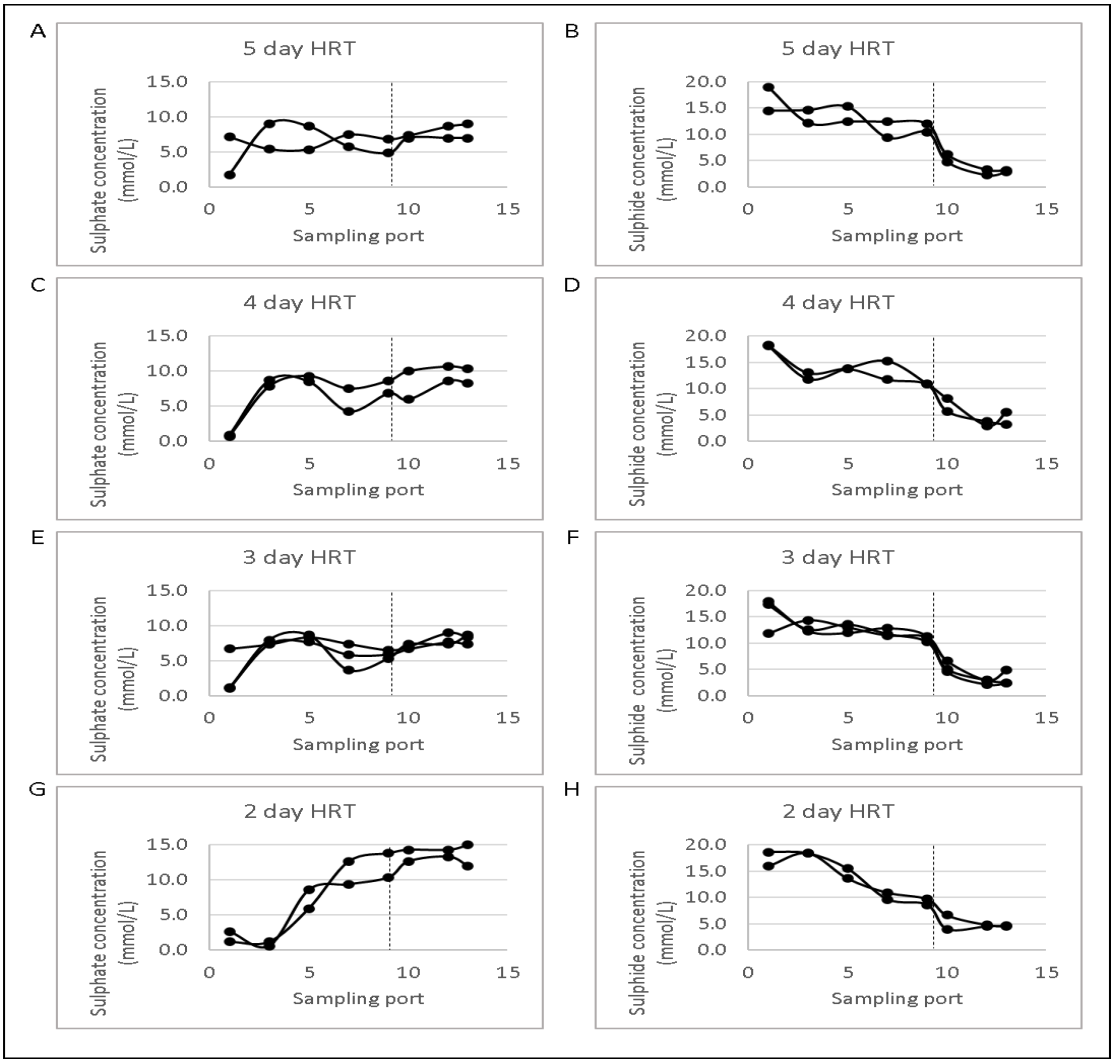

Figure C.1 3: LFCR system fed with synthetic feed and a lactate carbon source sulphate and sulphide time trends at different sampling ports over different hydraulic residence times. The vertical dotted lines indicate split between the primary and secondary reactor sample points.

Figure C.1 3 showed localized sulphate reduction at sample point 1, with extremely low sulphate concentrations. Sulphate levels increased from point 2 onwards, except for the 2-day HRT, better representing overall reactor conditions. Sulphate concentrations remained fairly constant in the primary reactor, with negligible sulphate increases in the secondary reactor compared to sulphide decrease, suggesting partial oxidation to elemental sulphur as the dominant process.

The primary reactor, fed with 20.8 mmol/L sulphate, provided sufficient time for reduction and handled a higher sulphate loading effectively. Sulphate concentrations remained between 5.00 and 10.0 mmol/L for 3-day to 5-day HRTs, exceeding 10.0 mmol/L at 2-day HRT, consistent with lower conversion.

From 5-day to 2-day HRT (except 3-day), sulphide concentrations decreased along the length of both reactors, indicating significant oxidation. At 3-day HRT, sulphide levels remained constant in the primary reactor before decreasing in the secondary. Consistent sulphate concentrations coupled with sulphide decrease across both reactors suggested partial oxidation to elemental sulphur as the primary mechanism.

A.11 Treated AMD feed and lactate substrate

A.11.1 Primary and secondary reactor average pH time trends

Figure C.2 1 displays average pH values for primary and secondary reactors over time. The pH trends were similar to those observed in the synthetic AMD study with 2 g/L sulphate feed concentration.

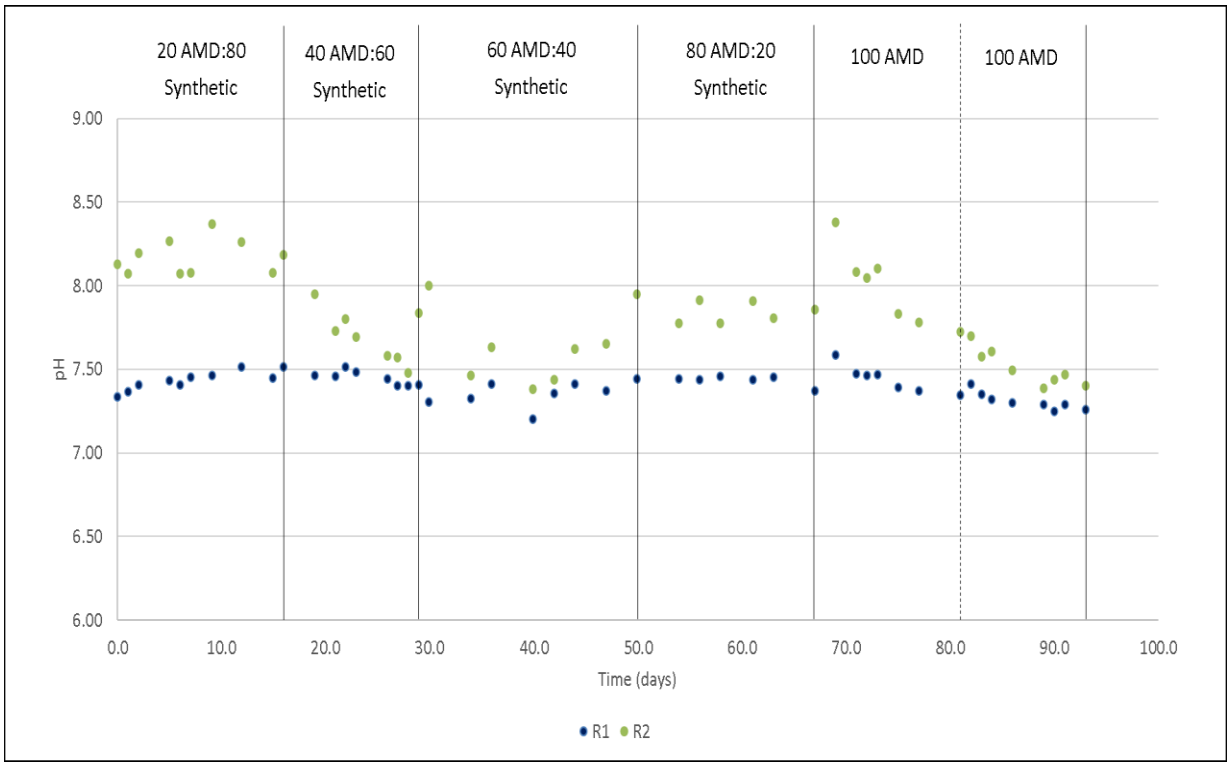

Figure C.2 1: LFCR system fed with AMD and a lactate carbon source pH time trends at different AMD feed concentrations. The vertical straight lines (dotted and solid) indicate biofilm harvest.

pH in the primary reactor was consistently lower than in the secondary reactor, ranging between 7.0 and 8.5 for both. Generally, pH increased from the first sampling point of the primary reactor to the last point of the secondary reactor across all ratio runs.

Figure C.2 2 displays redox time trends for the reactor running on AMD and lactate. In the 80:20 AMD:Synthetic run, bulk liquid in both reactors remained anoxic and reducing, with negative redox potential. Redox potential in both reactors stayed below -300 mV, which is ideal for SRB activity (Hessler, 2020; Marais, 2020). For runs after 40:60 AMD:Synthetic, redox in the secondary reactor was mostly equal to that in the primary reactor.

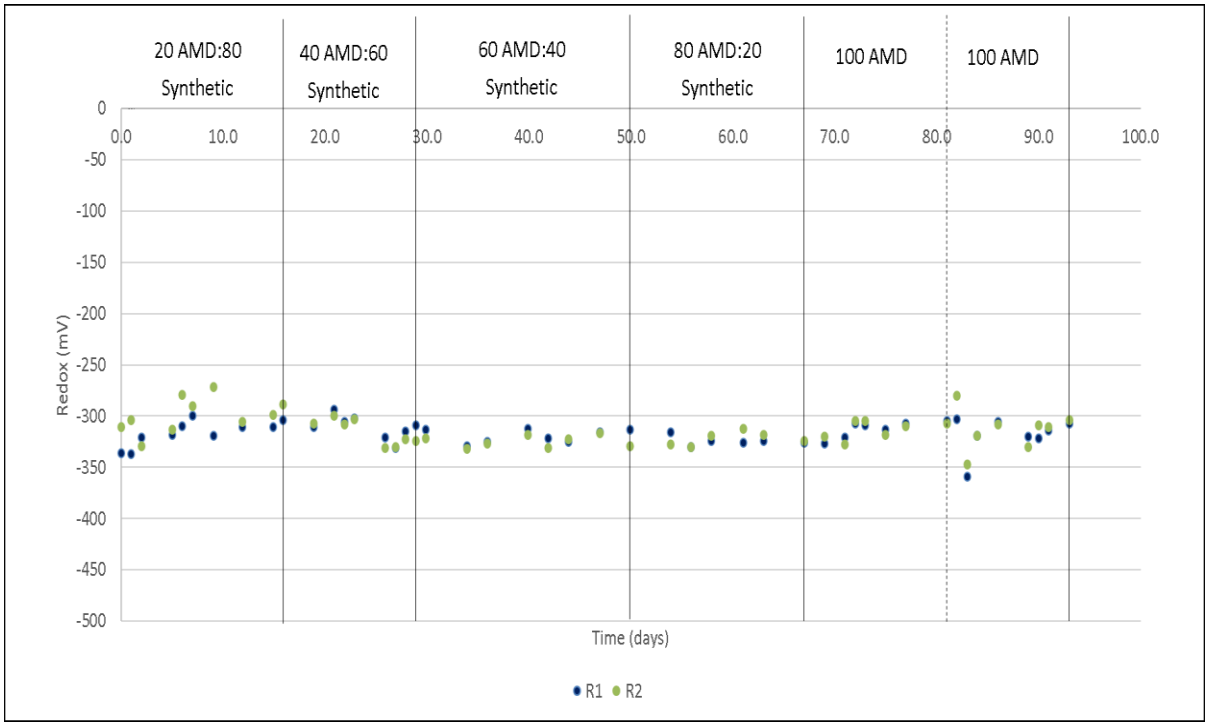

Figure C.2 2: LFCR system fed with AMD and a lactate carbon source redox time trends at different AMD feed concentrations. The vertical straight lines (dotted and solid) indicate biofilm harvest.

Figure C.2 2, showing redox for the synthetic AMD experiment with 2 g/L sulphate loading and lactate as carbon source, indicated higher redox in the secondary reactor compared to the primary reactor at 3-day HRT. This suggested more re-oxidation in the secondary reactor for the synthetic AMD study compared to the pre-treated AMD and lactate study.

A.11.2 Sulphate and sulphide time trends at different sampling points

Analysis of samples from ports across the reactor at 100% AMD concentration (Figure C.2 3) revealed a decrease in sulphate concentration between sample points 1 and 7. Sulphide concentration remained relatively constant between 15 and 20 mmol/L for these same sample points.

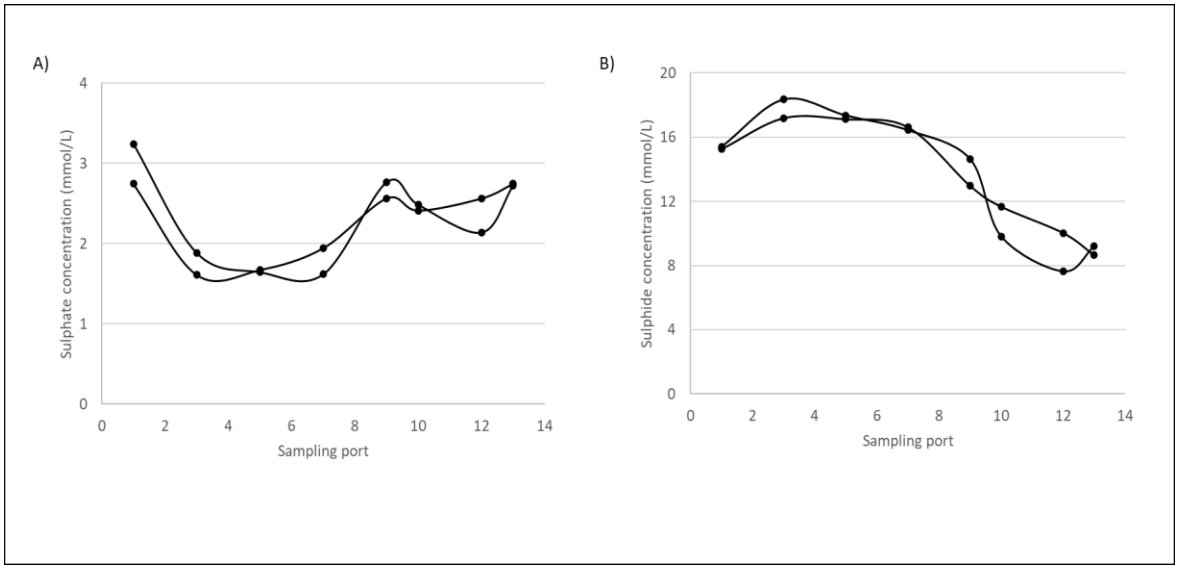

Figure C.2 3: LFCR system fed with AMD and a lactate carbon source sulphate and sulphide time trends at different sampling ports on a 100% AMD feed.

Sulphate concentration increased between sample points 7 and 10, then remained fairly constant at 2-3 mmol/L. Low sulphate concentrations indicated significant conversion. Sulphide concentrations decreased between points 7 and 13, possibly due to both partial and complete oxidation. Low sulphate concentrations suggested less sulphide re-oxidation and more partial oxidation.

A.12 Treated AMD feed and molasses substrate

A.12.1 Primary and secondary reactor average pH time trends

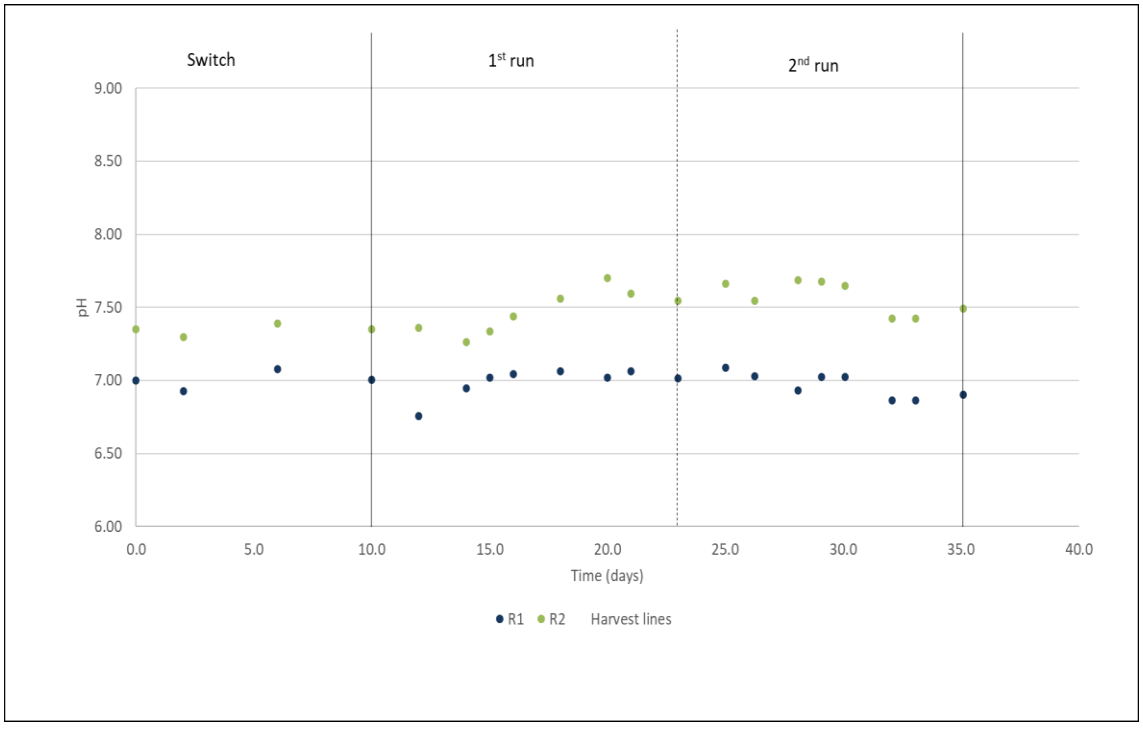

Figure C.3 1: LFCR system fed with AMD and a molasses carbon source pH time trends. The vertical straight lines (dotted and solid) indicate biofilm harvest.

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 C.3 1. 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.

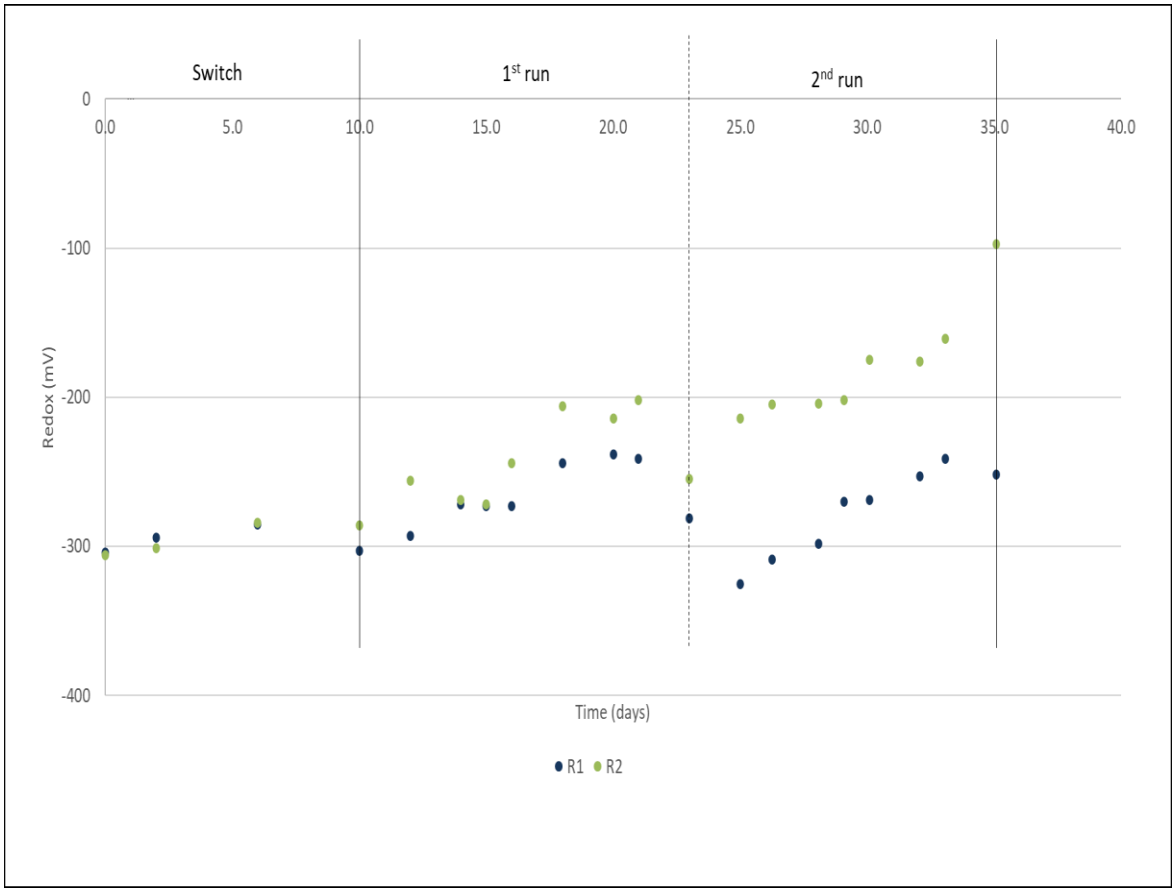

Figure C.3 2 LFCR system fed with AMD and a molasses carbon source redox time trends. The vertical straight lines (dotted and solid) indicate biofilm harvest.

Figure C.3 2 shows the redox time trends in the primary and secondary reactor when the LFCR system was running on molasses as its carbon source and had pre-treated AMD as the feed of sulphate laden wastewater. As can be seen in Figure C.3 2, 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 showed that the environment was becoming less reducing which could have been caused by the re-oxidation that occurred at the effluent port of the primary reactor and was carried over and maintained in the secondary reactor.

A.12.2 Sulphate and sulphide time trends at different sampling points

Analysis of the samples taken from the ports distributed across the reactor, Figure C.3 3, with a pure molasses carbon source showed a decrease in the sulphate concentration between sample point 1 and 3; sulphate concentration decreased from above 10 mmol/L to below 2 mmol/L. This supported by the sulphide analysis graph that there was less sulphide occurring at the first sample point. This could have been due to the complex nature of molasses which needed to be fermented to usable VFAs that can be used by the SRB to reduce sulphate to sulphide.

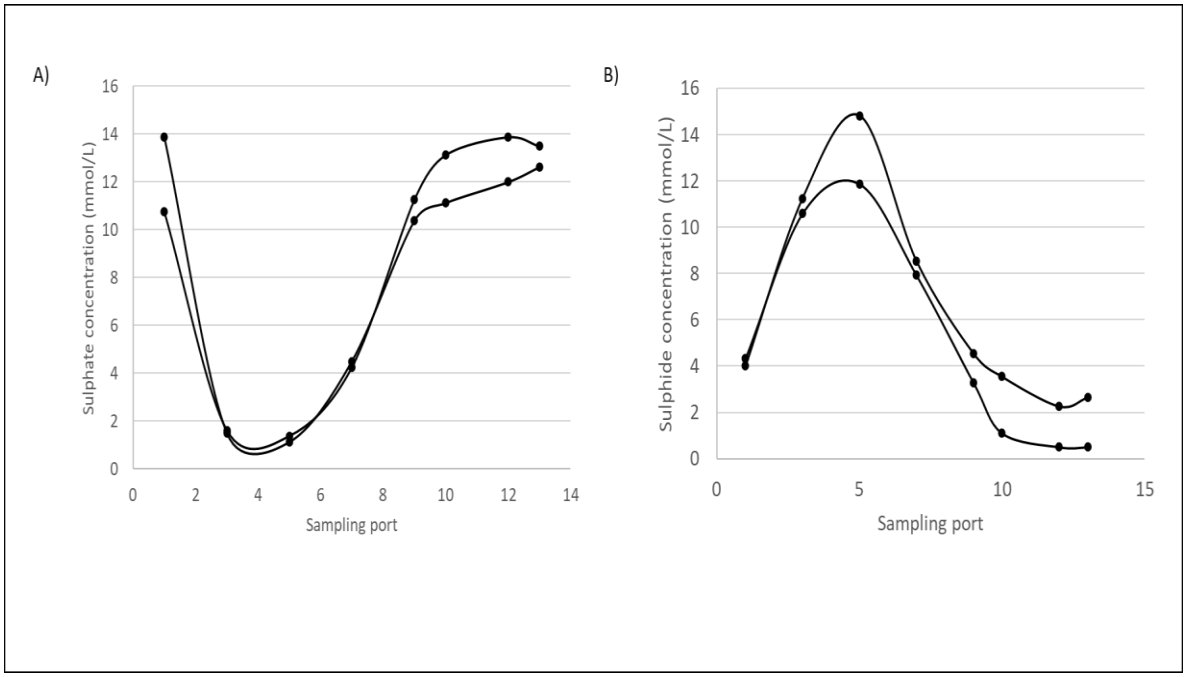

Figure C.3 3: LFCR system fed with AMD and a molasses carbon source sulphate and sulphide time trends at different sampling ports

After sample point 3, the sulphate concentration remained below 2 mmol/L until sample point 5 and then increased to above 10 mmol/L as seen in Figure C.3 3 A. The high concentration of sulphate was mostly due to the re-oxidation at point 9. Analysis of the sulphide graph shows that it complements the sulphate graph with low sulphide concentrations at the first sample point, increase in sulphide concentration to its highest point at sample point 5 with a value above 14 mmol/L and then a decrease to concentrations below 2 mmol/L at the ports in the secondary reactor. Complimentary trends for the sulphate and sulphide analysis indicate that very little sulphur was formed when molasses was used as a carbon source.