Optimisation of a linear flow channel reactor for semi-passive, simultaneous biological sulphate reduction and partial sulphide oxidation

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Declaration

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Lastly, to my Creator, from whom all the strength has come.

AMDG

Abstract

Acid rock drainage (ARD) is a growing concern, particularly in South Africa, as a country already classified as water scarce. ARD is defined as water that has been impacted by mining activities and typically has high levels of sulphate and heavy metals, at acidic pH. Similarly, high sulphate neutral rock drainage is of increasing concern. High sulphate content increases water salinity leading to adverse effects on human health as well as agriculture. Types of ARD and neutral rock drainage can be categorised into those that are produced in high volumes from groundwater rebound, and those that are generated from diffuse sources, as low-flow ARD. Low-flow ARD and neutral rock drainage are amenable to biological treatment of the sulphate component using sulphate reducing bacteria (SRB). Biological sulphate reduction (BSR) generates sulphide, which requires further treatment to remove it from the stream in gaseous form or as a solid sulphur-containing compound. Alternatively, it can also be used, in part, to precipitate metals present within ARD waters. Key challenges associated with SRB-based bioremediation include the cost of the supplemented electron donor needed for SRB to reduce sulphate, as well as the downstream management or treatment of the excess sulphide remaining. This investigation aimed to optimise a semi-passive treatment process which integrates BSR, and concomitant partial oxidation, by sulphur oxidising bacteria (SOB), of the sulphide produced to elemental sulphur. This is generated as a floating sulphur biofilm (FSB). These processes occur simultaneously within a linear flow channel reactor (LFCR), facilitating both treatment of the water stream to a fit-for-purpose water product, and recovery of the sulphur for use within fertilisers or fungicides. The work focused on the effective utilisation of electron donors and a sustainable option thereof, as well as the optimisation of partial sulphide oxidation and sulphur recovery.

In addressing the cost of a supplemented electron donor, the use of a waste product is of interest. To explore this and build on earlier work within the Centre for Bioprocess Engineering Research (CeBER) labs, this study investigated the efficient use of the volatile fatty acids (VFAs) acetate, propionate and lactate. Firstly, the study investigated propionate, a common fermentation product of waste organic sources. It is found as a component of the effluent or digestate, of anaerobic digestion (AD) processes such as algal AD. Propionate was proposed as an attractive option for a sustainable source of electron donor. An LFCR fed with synthetic propionate showed sulphate reduction occurring via the utilisation of both propionate as well as acetate produced from propionate metabolism. Fermentative bacteria were seen to work syntrophically within the system availing a significant amount of acetate to the SRB community. This acetate was the preferred electron donor over propionate. Maximum volumetric sulphate reduction rates (VSRRs) of 190 mg/L/day were achieved in the reactor. However, detailed analysis showed few propionate-utilising SRB in the community. It was concluded that a more diverse inoculum was needed to investigate the potential of propionate more fully.

Secondly, the study investigated lactate as a means to explore the efficiency of electron donors that are incompletely oxidised to acetate. Higher chain VFAs such as lactate are partially oxidised to acetate under biosulphidogenesis; however, acetate oxidation by SRB appears to be a rate-limiting step in most systems. Simultaneous incomplete oxidation of the more complex VFAs with complete oxidation of

acetate are rarely reported. Acetate accumulates in the effluents of these processes and results in high chemical oxygen demand (COD) remaining which can lead to environmental impacts such as eutrophication if released into river systems. Further, utilisation of the electron donor is inefficient. In order to address this, the study presents a sequential LFCR system to increase utilisation efficiency of the incompletely oxidised VFA feed. The sequential system was developed by coupling a second reactor unit, specifically colonised with acetate-utilising SRB, to a primary reactor unit utilising lactate. The SRB community in the secondary reactor was able to oxidise acetate from the primary reactor resulting in further sulphate reduction and lower residual COD levels. Residual acetate decreased from 475 mg/L in the primary reactor to 275 mg/L in the secondary reactor. Similarly, residual sulphate decreased from 533 mg/L in the primary reactor to 150 mg/L in the secondary reactor, on a 1 g/L sulphate feed, achieving a much improved effluent water quality. A VSRR of 213 mg/L/day across the sequential system, an overall conversion of 85% and a two-fold increase in sulphate reduced / lactate consumed from 0.45 to 0.85 (g/g) were achieved.

Lastly, this study investigated sulphide removal via the incorporation of elemental sulphur into a floating sulphur biofilm (FSB). The generation of an FSB within a sulphate reducing bioreactor is a passive and sustainable method of sulphide remediation, producing a value-added product of elemental sulphur. The sequential reactor system resulted in an increase of two to three-fold in the amount of elemental sulphur recovered, with an improved conversion of sulphide formed to elemental sulphur. Further, the effect of the feed inorganic ions, magnesium and phosphate, on sulphur yield and sulphide removal was studied using the sequential LFCR system. It was found that a decrease of magnesium in the media supplied resulted in an increase in sulphide conversion to sulphur from 21 to 39%, while a concomitant reduction in feed phosphate resulted in a further increase to 50% of the same. In both cases the sulphur concentration in the FSB was substantially increased.

Overall, the thesis addresses the need to decrease the cost associated with the supply of a suitable electron donor, as well as improves the water quality of a BSR effluent both in terms of residual sulphate, sulphide and COD. The LFCR system studied was improved both by a sequential reactor system, allowing greater sulphate reduction, sulphide removal via elemental sulphur recovery, and substrate utilisation efficiency. Additionally, changes to the inorganic component of the feed led to further sulphide removal and elemental sulphur recovery. Propionate was concluded to have been only partially used as an electron donor for SRB, however fermentative bacteria present in the mixed community degraded propionate to acetate which was then used for BSR. The work presented here contributes towards the broader research into a semi-passive, environmentally sustainable and economically viable treatment solution for low-flow, circumneutral ARD.

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Abbreviations

ARD	Acid rock drainage
BESA	Bromoethanesulphonate
BSO	Biological sulphide oxidation
BSR	Biological sulphate reduction
COD	Chemical oxygen demand
CSRT	Continuously stirred tank reactor
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DPBR	Degrading packed bed reactor
EPS	Extracellular polymeric substance
FSB	Floating sulphur biofilm
HPLC	High pressure liquid chromatography
HRT	Hydraulic residence time
ICP-MS	Inductively coupled plasma mass spectrometry
LFCR	Linear flow channel reactor
OTU	Operational taxonomical unit
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
SOB	Sulphur oxidising bacteria
SRB	Sulphate reducing bacteria
UAPBR	Up-flow anaerobic packed bed reactor
VAUR	Volumetric acetate utilisation rate
VFA	Volatile fatty acid

VPUR	Volumetric propionate utilisation rate
VSLR	Volumetric sulphate loading rate
VSRR	Volumetric sulphate reduction rate
YE	Yeast Extract

Chapter 1 Introduction

1.1 Background

The cumulative effects of climate change and a growing global population have led to increased water scarcity in many areas of the world. Approximately two billion people now live in areas classed as water stressed and nearly eight million people lack access to drinking water (United Nations [UN], 2019). This is impacted by both the quantity of water available and its quality. Wastewater discharge has large consequences on the quality of fresh water supplies, with estimates of up to 80% of wastewaters being discharged without treatment (The UN World Water Development Report, 2017). Mining activities pose one of the greatest threats to fresh water supply (UN-Water, 2010) because the passage of water through mines, ore deposits and waste rock leads to water with altered solution chemistry, broadly categorised as mining-impacted water. In particular, acid rock drainage (ARD), with its heavy metal content, high sulphate levels and low pH's, is a large contributor to mining-impacted water and results in environmental, social, health and economic ramifications (Johnson and Hallberg, 2005; Taylor *et al.,* 2005; Simate and Ndlovu, 2014). Equally, neutral mine drainage leads to many similar ramifications.

South Africa is a country that is both drought-prone and has had a strong mining history which has contributed to a large proportion of its Gross Domestic Product (GDP) over the past 100 years. In 2012, South Africa was ranked seventh in global mining production (Dorin *et al.,* 2014). Even while losing ground in the mining production rankings over recent years, the mining legacy in South Africa demands that we focus on treatment of historic ARD. The water-related impacts of the country's mining history can largely be divided into those associated with groundwater rebound as well as those related to surface impoundments such as low-grade heaps and waste rock dumps (McCarthy, 2011; van Hille *et al.,* 2016). Beginning in the late 1800s, gold mining in the Witwatersrand Supergroup involved deep underground networks of tunnels and vertical shafts. Penetration of the water table meant that mines had to constantly pump out water to avoid flooding (Durand, 2012). Upon decommissioning of mines such as these, active pumping ceases and groundwater rebound causes decanting of ARD and the contamination of surrounding water (McCarthy, 2011). This ARD is typically high in volume and aggressive in nature, requiring active treatment, most commonly in the form of chemical neutralisation via high-density sludge (HDS) treatment processes (The International Network for Acid Prevention, 2018). Membrane separation processes involving ultrafiltration and reverse osmosis are also used (Aguiar *et al.,* 2016).

More diffuse and low-volume sources of mining-impacted water in South Africa is often associated with coal as well as sulphidic gold ore waste rock. Coal mining is prolific across the coalfields in the Mpumalanga and Limpopo provinces. Coal currently accounts for 70% of the country's total energy supply (The South African Energy Sector Report, 2019). While high volume discharges may receive attention with the implementation of large-scale remediation processes (Hutton *et al.,* 2009), low volume discharges often go less noticed with ARD discharge only beginning several years after mine closure in some cases (McCarthy, 2011). Following its onset, ARD generation can continue for decades or even

centuries (Gazea *et al.,* 1996; Pulles and Heath, 2009; Rose, 2013). Of particular concern in South Africa are the numerous abandoned mines as well as mines that are due to be decommissioned in the near future.

This form of low-flow ARD does not warrant the highly technical and expensive treatments employed for more voluminous ARD. Rather a low cost, passive or semi-passive process for water from these geographically scattered mining locations is required (van Hille *et al.,* 2016). Much of the mining impacted water is formed in catchment areas which drain into the Vaal River. Sulphate salinity has been a challenge for potable water in this region, and is on the rise (McCarthy, 2011). Analysis of contaminated waters in the Witbank Coalfield (Mpumalanga) have also shown consistently high sulphates (Bell *et al.,* 2002), and variable pH's dependent on the associated gangue materials. Biological sulphate reduction (BSR) via the action of sulphate reducing bacteria (SRB) is a sustainable treatment option for the sulphate component of ARD and additionally offers a degree of neutralisation potential from generated hydrogen carbonate, while sulphide produced from the reduction of sulphate can in part be used to precipitate heavy metals. Excess sulphide generated can be managed by the action of sulphur oxidising bacteria (SOB) forming elemental sulphur within an easily harvestable floating sulphur biofilm (FSB; van Hille and Mooruth, 2011; Van Hille and Mooruth, 2014; Van Hille *et al.,* 2016). The elemental sulphur produced can be used in fertilizer and fungicide production (Benschop *et al.,* 2002). This thesis focuses specifically on BSR treatment for sulphate-laden waters and the partial oxidation of the produced sulphide to elemental sulphur as a value-added product. The work described is applicable both to circumneutral mining-impacted water rich in sulphate as well as partially treated, neutral ARD.

BSR has been extensively studied over the past few decades with high sulphate removal rates being demonstrated (Dries *et al.,* 1998; Silva *et al.,* 2002; Papirio *et al.,* 2013; Bertolino *et al.,* 2014; Harrison *et al.,* 2014; Zhang and Wang, 2016). The studies presented here are an extension of previous work carried out within the Centre for Bioprocess Engineering Research (CeBER) at the University of Cape Town (UCT). This work has surrounded BSR with particular regards to process efficiency, operational parameters, electron donor utilisation, management of the sulphide produced in the process, as well as the microbial communities and metabolisms involved (Moosa *et al.*, 2002, 2005; Moosa and Harrison, 2006; Icgen *et al.*, 2007; Oyekola *et al.*, 2009, 2012b; van Hille and Mooruth, 2011; van Hille *et al.*, 2011, 2012, 2016; T. S. Marais *et al.*, 2017; Hessler *et al.*, 2018; Hessler, 2020; Marais *et al.*, 2020; Motleleng, 2020). The central theme of the work carried out here is focussed on optimising a semi-passive, hybrid linear flow channel reactor (LFCR) that facilitates sulphate reduction and sulphide oxidation via the passive generation of an FSB within a single reactor unit. The reactor system has previously shown proof of concept success at a laboratory scale (Marais *et al.,* 2020).

A major set-back however of BSR is the associated cost which is largely attributed to the provision of a suitable electron donor needed for the sulphate reducing metabolism of SRB (Gopal, 2005). This is considered in two ways in this thesis. Firstly, the use of propionate is investigated. As a component of organic fermentation, propionate could be generated from organic waste streams to provide a sustainable carbon source for a BSR system. Secondly, the efficiency of electron donor usage with

regards to the extent of substrate utilisation is explored. Incomplete oxidation to acetate of carbon sources such as higher chain VFAs is typical. Here a sequential reactor system was designed to incorporate incomplete and complete oxidation of a lactate feed source in one system to attain more sulphate reduced per substrate supplied.

Additionally, the extent of sulphur incorporation into the FSB and resulting yield has been limited in the hybrid reactor system (Marais, 2019) and optimisation of this process is thus investigated here, particularly with regard to the effect of inorganic ion accumulation in the FSB. The thesis, therefore, aims to provide insight into alternative electron donor options and the optimisation of their usage to minimise electron donor requirement and maximise resultant water quality, as well as increased sulphur yields, with associated sulphide removal through focus on the FSB.

1.2 Thesis structure

The thesis begins with a literature review (Chapter 2). First this gives an overview of ARD and its causes and effects. Thereafter, treatment technologies are outlined with a focus on BSR, its development as a treatment method and its potential for application. Electron donor options for SRB are discussed, drawing attention to sustainable carbon sources and their potential for metabolism. An overview of biological sulphide oxidation (BSO) and the passive generation of an FSB follows, with a description of hybrid LFCR used in this study, and the work leading up to its development. The literature review is concluded by an outline of this study, positioning its relevant hypotheses and associated objectives in terms of the current knowledge base. Chapter 3 details the experimental approach and associated materials and methods used in this thesis. In Chapter 4, the use of propionate as an electron donor is explored owing to its availability in digestates. The characteristics of propionate-utilising SRB are explored through a hydraulic residence time (HRT) study using the LFCR. Chapter 5 investigates a novel sequential LFCR reactor system to address the accumulation of acetate from incomplete oxidation of higher chain volatile fatty acids – in this case lactate. A primary LFCR fed with lactate, is linked in series to a secondary reactor specifically colonised with acetate-utilising SRB. The performance of this sequential reactor is compared to that of each independent reactor operated on lactate or acetate. This investigates the more efficient utilisation of the lactate feed for sulphate reduction. The ratio of carbon source to sulphate is also investigated and brings the chapter to a close. Both Chapters 4 and 5 focus solely on BSR within the hybrid LFCR. BSO and the FSB structure and formation are the focus of Chapter 6. Specifically, the extent of elemental sulphur recovery and the effect of the inorganic ions, magnesium and phosphate, on the composition of the FSB and associated sulphur recovery are considered. Finally, conclusions and further recommendations drawn from this study are discussed in Chapter 7.

Chapter 2 Literature review

2.1 Acid rock drainage

2.1.1 Generation

Acid rock drainage (ARD) forms when sulphide minerals, most commonly pyrite (FeS_2), become exposed to oxygen and water. Gold and coal deposits in South Africa are predominantly associated with pyrite and therefore contribute largely to the formation of ARD in the country. This process occurs naturally as weathering but at an extremely slow rate. However, mining operations leave vast amounts of rich mineral rocks exposed to moisture and air, and disaggregated in shafts, quarries and tailings dams (McCarthy and Pretorius, 2009). The chain of reactions that typically occur are shown by Equation 2-1 through to Equation 2-3 (Akcil and Koldas, 2006).

$$FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad \text{Equation 2-1}$$

$$Fe^{2+} + 1/4O_2 + H^+ \rightarrow Fe^{3+} + 1/2H_2O \quad \text{Equation 2-2}$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2+} + 16H^+ \quad \text{Equation 2-3}$$

At first, iron sulphides are abiotically oxidised upon reaction with water and oxygen resulting in ferrous iron and sulphate production, along with acid generation (Equation 2-1). Acidophilic iron-oxidising bacteria greatly accelerate oxidation rates converting the ferrous iron back to ferric iron (Equation 2-2). Ferric ions then proceed to further attack the pyrite producing more ferrous, sulphate and hydrogen ions (Equation 2-3), thus exacerbating the process and allowing an iterative cycle of accelerated mineral weathering. Ferric ions also attack other heavy metal minerals which dissolve into solution at low pH (Singer and Stumm, 1970). The result is an acidic runoff that contains high sulphate levels and often a range of heavy metal ions. Some of the ferric ions can precipitate as iron hydroxide which results in the orange-red coloured contamination associated with mining impacted water shown in Figure 2-1 (Akcil and Koldas, 2006).

Figure 2-1 ARD river contamination in the Rio Tinto River, Spain. Red colouring of the water by iron hydroxide is evident. (Image used with permission).

2.1.2 ARD Sources

As well as the severity and type of ARD, the most defining characteristic is related to the depth of the mining activities from which it is generated and thereby the source of the water. In South Africa, ARD can be divided into two categories termed 'high-' and 'low-flow' ARD (Harrison *et al.,* 2014). High-flow ARD is the result of groundwater rebound where deep mining operations, such as those employed for gold mining in the Witswatersrand basin, have penetrated the water table. In many cases, the formation of ARD is mitigated by active pumping and dewatering when mines are in operation. When dewatering of underground workings is discontinued, shafts and tunnels give rise to large volumes of mining impacted water at high flow rates (Johnson and Hallberg, 2005; McCarthy, 2011; Moodley *et al.,* 2018). Low-flow ARD encompasses diffuse sources of acidic and sulphate rich mine water run-offs, such as from tailings dumps, waste rock piles and the dewatering of open pits. In South Africa this type of ARD is largely associated with the coal mines of the Mpumalanga, KwaZulu Natal and Limpopo provinces. Coal mining in South Africa dates back to the mid-1800s and therefore has left in its wake numerous abandoned mines, along with their potential to keep generating ARD for decades after mine closure (Gazea *et al.,* 1996; McCarthy and Pretorius, 2009).

2.1.3 Environmental and health impacts

The impacts of ARD are broad since the type and severity of the ARD varies greatly depending on the mining activities, geology, topography and weather patterns of the area as well as the particle size and permeability of the exposed minerals and rock (McCarthy, 2011; Moodley *et al.,* 2018). In river networks, low pH waters, with high dissolved solids and precipitates can bring about the formation of a rust-like layer along the bottom of riverbeds; these adversely affect benthic macroinvertebrate abundance and diversity (DeNicola and Stapleton, 2002). In extreme cases, near complete destruction of aquatic life can result (Gazea *et al.,* 1996; Sánchez *et al.,* 2006).

In the case of heavy metals, ecosystems can suffer lasting effects as these are environmentally persistent pollutants and are readily assimilated into aquatic life and passed up the food chain. Accumulation in animals and humans can cause acute and chronic illness via the disruption of metabolic function (Simate and Ndlovu, 2014).

The case of ARD in South Africa is particularly exacerbated by the relationship between the geology and climate across the country, well detailed by McCarthy (2011). Figure 2-2 shows that the country's main water catchment areas are situated within an extensive coalfield. The local river networks which feed into the major rivers - most notably the Vaal river - are thus contaminated by the expansive mining operations of the area. The Vaal river drains across the country from east to west along a gradient of decreasing rainfall and increasing evapotranspiration. Therefore, this river system runs across the country into increasingly water scarce land. Accumulation of sulphate to levels largely ranging between 200 – 700 mg/L have been observed in major dams within the broader coal mining area and are on a trajectory to keep increasing (McCarthy, 2011).

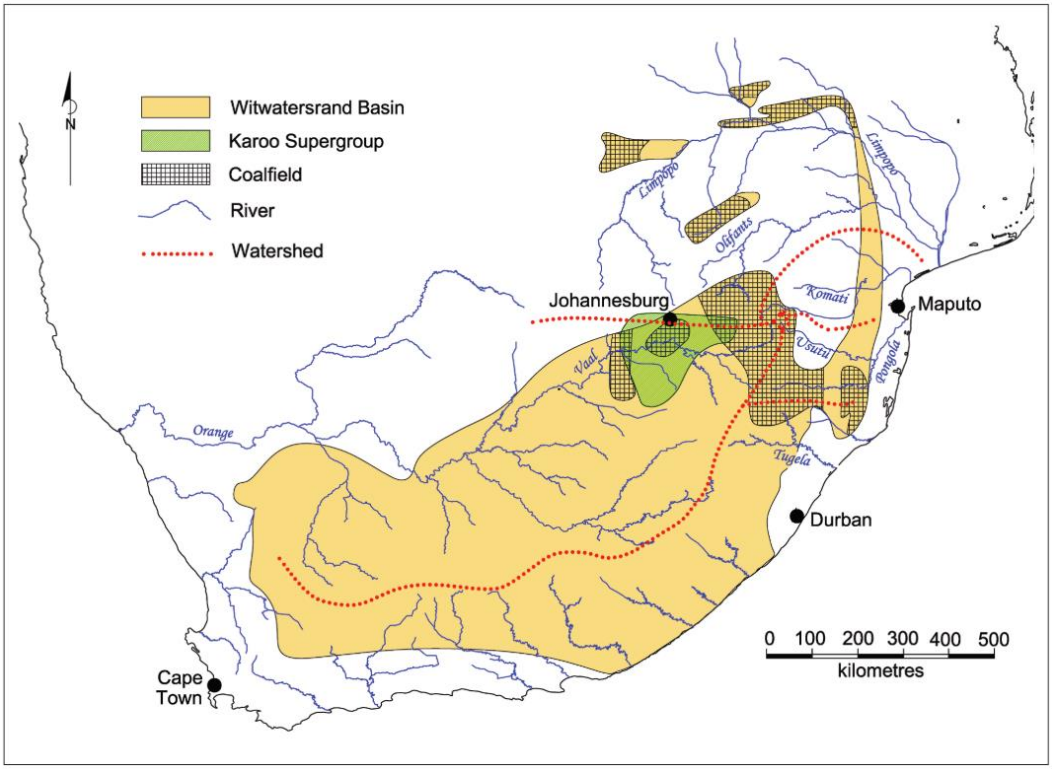


Figure 2-2 The overlay of the geology, water catchment areas and river networks in South Africa. The Witwatersrand Basin gold deposits and the dominant coal fields of the country are shown. Taken from McCarthy (2011).

Sulphate contamination is often given less attention as the effects of acidity and heavy metals are more acute (Arnold *et al.,* 2016). However, sulphate levels are recommended to be below 200 mg/L according to South African water quality guidelines for domestic use, and the maximum permissible concentration is 600 mg/L. Above this most individuals will start to experience gut disturbance such as diarrhoea (Department of Water Affairs and Forestry, 1996). Further, there is need to consider the adverse effects of high salinity when downstream water is used for crop irrigation. Salinity stress is already a major

concern in agriculture and is one of the most serious threats to plant productivity and yield (Parihar *et al.,* 2015).

2.2 Treatment options

Ideally, the prevention of ARD is preferred but is often practically difficult to implement or only achieves partial prevention. Therefore, when generation is not avoided, treatment strategies must be considered (Gazea *et al.,* 1996; Johnson and Hallberg, 2005; Taylor *et al.,* 2005). Treatment methods can be categorised into active and passive options, each of which can utilise abiotic or biotic means. A brief overview of active and passive treatments will be provided in this section. Biological sulphate reduction (BSR) and related processes will then be the focus of the remainder of the chapter.

2.2.1 Active treatment

Traditional methods of ARD treatment are generally active processes requiring the constant input of chemicals and energy. Regardless of whether an active or passive strategy is being employed, neutralisation via alkaline chemicals such as lime or soda ash is generally a necessary first step for acidic streams, and in some cases can be the sole treatment method used (Johnson and Hallberg, 2005). This is because it has the added result of lowering the solubility of the contaminating metals often contained in ARD. Most of these metals are amphoteric, meaning that they are least soluble at neutral pH's and thereby can be precipitated out of solution (Taylor *et al.,* 2005). Lime is the often utilised neutralising agent, but its hydrophobic nature means high energy inputs are required for mixing for it be effective (Skousen *et al.,* 2000). When lime or other calcium containing neutralisers are used, sulphate may also be removed via the formation of gypsum (Johnson and Hallberg, 2005). However, deposition of gypsum, along with other precipitates into the limestone pore spaces can result in decreased porosity of lime (Skousen *et al.,* 2000). More complex technologies such as reverse osmosis and ion exchange exist but are employed less frequently due to expense (Gopi Kiran *et al.,* 2017). Overall, active treatments are generally costly, both in implementation and maintenance, and pose further disposal problems as sludge produced from such treatment must be encapsulated and sent to landfill (Johnson and Hallberg, 2005; Kefeni *et al.,* 2017). However, active treatments are necessary particularly in cases of high-flow ARD, where passive treatments would be too slow.

2.2.2 Passive and semi-passive treatment

Passive treatments are more suited to waters with low acidity loads and low flow rates where residence times can be longer compared to those achieved in active treatment (Taylor *et al.,* 2005). They focus on providing low-cost, long-term solutions for mining sites that can be sustained long after mine closure, with regular but infrequent maintenance. Generally, these treatments are applicable to abandoned mines as opposed to those still in operation and have shown some success in the management of ARD post mine closures (Taylor *et al.,* 2005; Kefeni *et al.,* 2017). Passive systems do not involve the constant input of chemical or energy requiring processes. They use a range of physical, chemical and biological processes which can range from the ability of a large wetland to dilute salinity, to the direct action of

plants up-taking heavy metals and sulphate reducing bacteria (SRB) activity (Gazea *et al.,* 1996). SRB-mediated treatment is an environmentally friendly, low energy requiring option particularly for diffuse, long lasting sources of ARD. Passive treatment processes include biological sulphate reducing reactors, anaerobic and aerobic wetlands, infiltration beds and permeable reactive barriers where SRB processes are incorporated alongside alkaline generating material, anoxic limestone drains and open limestone channels (Gazea *et al.,* 1996; Skousen *et al.,* 2000; Gopi Kiran *et al.,* 2017). However, failure of some systems due to precipitate build-up can occur in the long term and cause reduced performance or complete treatment failure (Skousen *et al.,* 2000; Johnson and Hallberg, 2005). Other notable drawbacks of passive treatment technologies revolve around the large areas needed to allow effective treatment and their inability to guarantee consistent, acceptable effluent levels of ARD contaminants, especially with changing flow rates and chemical composition (Gazea *et al.,* 1996). The pay-offs of passive treatment are the significantly lower operational and maintenance costs as compared to active treatment.

As a compromise between the expense of active treatment technologies and the limited process control and consistent performance of passive systems, there has been a move towards semi-passive technologies. The economic and environmental advantages of passive systems remain but some degree of active management – for example periodic carbon source addition for biological sulphate reducing systems and intermittent parameter control – allows increased performance, predictability and longevity (Nielsen *et al.,* 2018). A semi-passive sulphate reducing bioreactor used in this work is described in detail in Section 2.6.3.

2.3 Biological sulphate reduction

Biological sulphate reduction (BSR) is carried out by sulphate reducing bacteria (SRB), obligate anaerobes that fulfil their energy needs by the oxidation of an electron donor alongside which sulphate is concomitantly reduced to sulphide and hydrogen carbonate is produced (Johnson and Hallberg, 2005):

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

BSR offers a multifaceted treatment option as it addresses sulphate levels of ARD but also has the potential to mitigate acidity and metal content. Metal sulphides have extremely low solubilities and thus the sulphide generated can be cycled back to precipitate heavy metals out of solution upstream of BSR processes (Liamleam and Annachhatre, 2007). Similarly, the hydrogen carbonate produced results in effluents with a degree of neutralisation capacity (Johnson and Hallberg, 2005).

2.3.1 Sulphate reducing bacteria

Whereas most microorganisms can only follow the assimilatory sulphate reduction pathway and obtain sulphur for protein synthesis and cellular growth, SRB also carry out dissimilatory sulphate reduction whereby they utilise sulphate as their final electron acceptor in cellular respiration (Rückert, 2016).

Sulphate is extremely stable and therefore generally persistent in the environment except for the activity of SRB, highlighting their key role in the sulphur cycle. The reduction of sulphate to the intermediate bisulphite, has a very low redox potential, E°' = -526 mV, making it energetically unfavourable (Rabus *et al.,* 2015). SRB overcome this by the action of the enzyme sulphate adenyl transferase which converts the energy storing molecule of cells - adenosine triphosphate (ATP) - to adenosine phosphosulphate (APS), thus activating the sulphate and allowing further reduction to sulphide (Muyzer and Stams, 2008; Rabus *et al.,* 2015).

Since ATP is required for the reduction of sulphate, the process occurs intracellularly. Investigation of the importation of sulphate into the cell has shown that two membrane transport systems which couple sulphate uptake to hydrogen or sodium ion uptake (Cypionka, 1995). The two systems allow either a high or low level of accumulation which operate under limiting or excess sulphate levels respectively. This is interesting as it correlates to the observation of SRB that can and cannot scavenge sulphate due to differences in sulphate affinity (Rabus *et al.,* 2015).

As strict anaerobes, major habitats of SRB are marine environments rich in sulphate, however, they are ubiquitous across both aquatic and terrestrial anoxic environments (Rabus *et al.,* 2015). They are a diverse group of organisms containing both bacteria and some archaeal species, with the most dominant lineages being Deltaproteobacteria and Clostridia (Figure 2-3). SRB are largely mesophilic and operate optimally at pH's of 5 – 9, though both thermophiles and acidophiles exist (Visser, 1995; Kolmert and Johnson, 2001; Lens *et al.,* 2002).

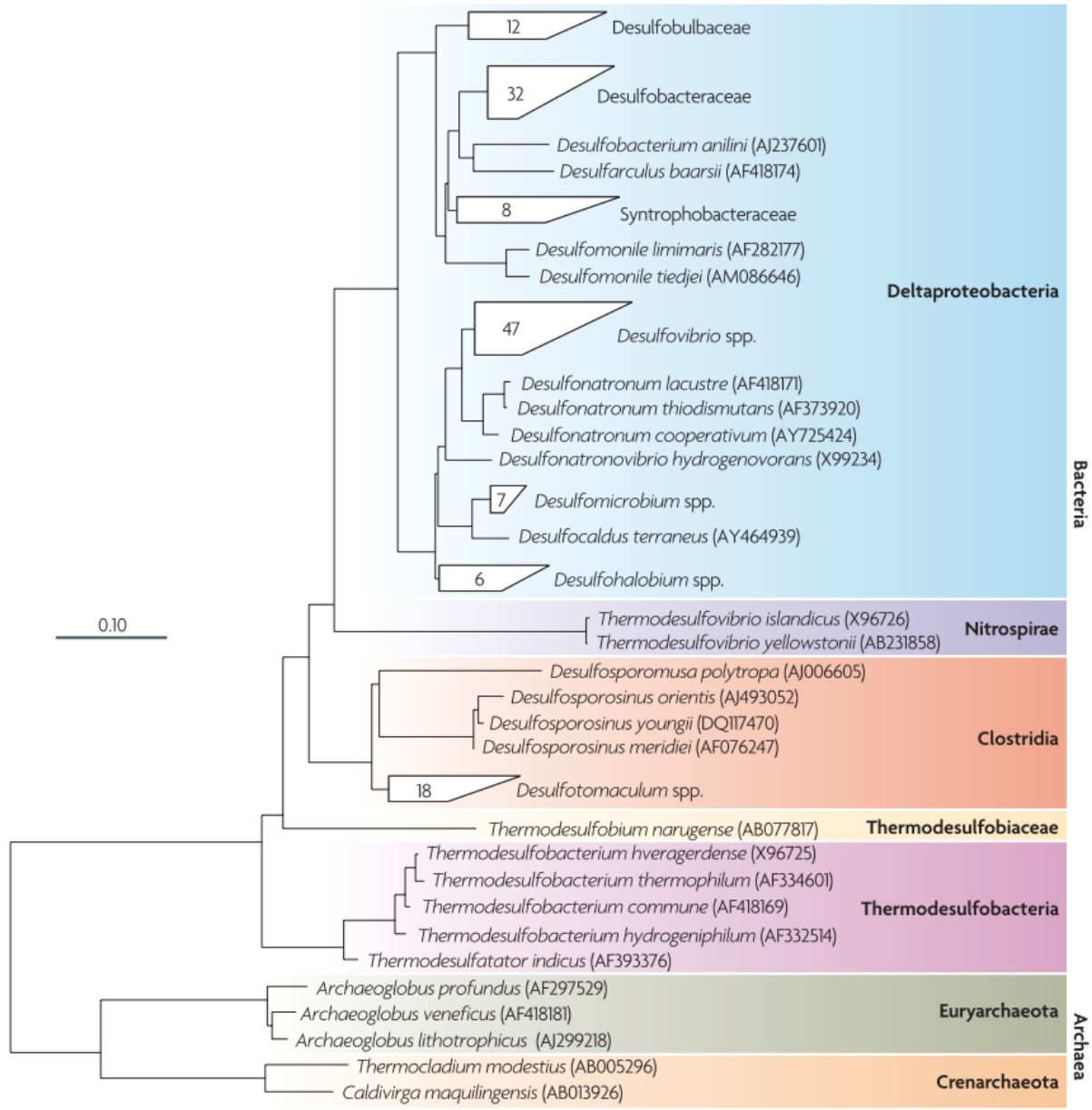


Figure 2-3 Phylogenetic tree showing SRB lineages, constructed using 16S rRNA gene sequencing data. Numbers within collapsed clusters represent the number of species within that group. The scale bar represents 10% sequence difference. Taken from Muyzer and Stams (2008).

SRB are more frequently categorised, however, according to whether they oxidise their carbon substrate completely to carbon dioxide, or incompletely which generally results in acetate production. Incomplete oxidisers are generally known to have faster growth rates (McCartney and Oleszkiewicz, 1993; Oude Elferink *et al.,* 1994; Colleran *et al.,* 1995). This means that in an environment of an organic source more reduced than acetate – such as higher chain VFAs - incomplete oxidisers will outcompete complete oxidisers and produce acetate, as has been observed (Oude Elferink, Luppens, *et al.,* 1998; Oyekola *et al.,* 2010). Therefore, in many systems the most relevant complete oxidisers are those that oxidise acetate. The dominant SRB genera found in sulphate reducing bioreactors are *Desulfomicrobium*, *Desulfobulbus* and *Desulfovibrio* - all incomplete oxidisers, as well as *Desulfobacter* - the most common acetate-oxidising SRB (Hao *et al.,* 2014).

Considerations associated with the structure and dynamics of mixed SRB microbial communities

Although SRB have been isolated and studied to gain understanding at a genus or species level, multiple SRB species will generally coexist in complex microbial communities, together with fermentative bacteria and methanogenic archaea, amongst other microbial groups. An understanding of the interplay between different organisms, their respective growth rates, affinities for sulphate and for different carbon sources, as well as tolerance to sulphide are all crucial to determine the diversity and competition that may result. This in turn can inform how best to run sulphate reducing bioreactors with respect to reactor configurations, electron donor choice and concentration, sulphate concentration and dilution rates.

2.3.2 Challenges associated with BSR

Generally, three major challenges are recognised with regards to BSR treatment technologies. Firstly, the slow growth rates associated with anaerobic organisms such as SRB; secondly, the identification or generation of an electron donor that is both efficient and cost-effective; and thirdly, management of the sulphide produced which, if untreated, poses a greater environmental and health concern than the original sulphate (Harrison *et al.,* 2014). The first challenge mentioned has demanded methods of cell retention to be implemented, to avoid cellular washout and allow uncoupling of hydraulic and cell retention times. These include support structures for cell adhesion, anaerobic sludge granulation and membrane bioreactors (Omil *et al.,* 1998; Mohan *et al.,* 2005; van Hille *et al.,* 2015; Hessler *et al.,* 2018; Marais *et al.,* 2020). SRB naturally occur in sediments and are prone to forming biofilms (Labrenz and Banfield, 2004). Biofilms are structured communities of sessile microorganisms within a matrix, that adhere to surfaces and interfaces. The formation of a biofilm gives the microbial communities therein increased robustness to withstand environmental perturbations (De Beer and Stoodley, 2014). Tested support structures include carbon microfibres, sand, glass beads, polyvinyl alcohol and polyurethane foam (Baskaran and Nemati, 2006; Hsu *et al.,* 2010; van Hille *et al.,* 2015; Hessler *et al.,* 2018; Marais *et al.,* 2020). Carbon microfibres offer a support structure with a large surface area whilst taking up minimal reactor volume and have shown high SRB retention ability (Harrison *et al.,* 2014; Marais *et al.,* 2020). They provide a completely passive method of retention which requires minimal to no upkeep. This compares with other active or maintenance-requiring retention techniques such as centrifugation and sedimentation and avoids fouling problems associated with cell membrane reactors (Harrison *et al.,* 2014). The second and third challenges mentioned will be discussed in more detail below and are closely interlinked with the foci of this thesis.

2.4 Electron donors for BSR

The identification of an electron donor that gives satisfactory performance whilst being cost-effective has been, and remains, a major challenge in the implementation of BSR in industry. Generally, electron donors that can be utilised by SRB for sulphate reduction are low molecular weight organic molecules which in many cases can also serve as a carbon source for growth. These include several volatile fatty

acids (VFAs), formate, methanol, ethanol, glycerol and glucose (Gibert *et al.,* 2002; Liamleam and Annachhatre, 2007). There is great variation in the source of these substrates – from synthetically produced lactate or ethanol, to waste streams and complex organic matter which are degraded down to a mix of these short chain molecules (Cao *et al.,* 2012; Moodley *et al.,* 2018). Research into the latter two seeks to provide low-cost electron donor options. Hydrogen is also an effective option however it is rarely used due to expense and safety concerns (Liamleam and Annachhatre, 2007). A detailed review of efficiencies achieved across various studies utilizing a rage of electron donors is given in Hessler (2020).

The choice of electron donor naturally affects the microbial ecology that can be supported. This, in turn, can determine what rates of biomass growth and sulphate removal can be achieved as well as the extent of competition that may arise from other microbes present (Liamleam and Annachhatre, 2007; Cao *et al.,* 2012). Several factors should be considered for the selection of a good electron donor: i) the cost per unit sulphate – this takes into account the stoichiometry governing the ratio of sulphate reduced per electron donor supplied; ii) the extent of oxidation of the substrate – incomplete oxidation leads to remaining chemical oxygen demand (COD) in effluents; iii) other microbial groups that could be supported and could compete with SRB for the substrate; iv) the availability of the substrate in the area and v) in the case of complex organics, the biodegradability of the substrate. However, not simply the choice of electron donor, but its loading rate and ratio to sulphate loading must also be considered (McCartney and Oleszkiewicz, 1993; Dar *et al.,* 2008; Papirio *et al.,* 2013). A comprehensive study by Cao *et al.* (2012) showed that organic acids and their salts gave the best sulphate reducing activities. A few of these will be discussed here.

2.4.1 Lactate

Lactate is commonly seen as the classic electron donor of choice. The overwhelming majority of SRB can grow on lactate with a few exceptions, notably some *Desulfobacter* species (Rabus *et al.,* 2000). It has been used widely as an electron donor (Kaksonen *et al.,* 2004; Oyekola *et al.,* 2009; Bertolino *et al.,* 2012; Cao *et al.,* 2012; Cassidy *et al.,* 2017) and, in being able to support a high diversity of sulphate reducing bacteria, it has been suggested to lead to more robust communities (Oyekola *et al.,* 2007).

With all electron donors, it is important to understand the relevant metabolic pathways involved. The reactions shown by Equation 2-5 to Equation 2-7 are key in the possible pathways of lactate degradation under anaerobic biosulphidogenesis (Liamleam and Annachhatre, 2007; Bertolino *et al.,* 2012). As mentioned above, though complete oxidation is possible (Equation 2-6), lactate is typically incompletely oxidised to acetate via Equation 2-5 (Bertolino *et al.,* 2012; Rabus *et al.,* 2000). This means that effluents from systems where only incomplete oxidising SRB are present will contain significant residual COD. Equation 2-7 describes a pathway by which lactate is degraded by fermentative bacteria in a process not involving sulphate reduction.

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

$$2\ \text{Lactate} + 3\ SO4^{2-} \rightarrow 6\ HCO^{3-} + 3\ HS^- + H^+ \qquad \text{Equation 2-6}$$

$$3\ \text{Lactate} \rightarrow \text{Acetate} + 2\ \text{Propionate} + HCO_3^- + H^+$$ Equation 2-7

The minimization of fermentative activity in sulphate reducing bioreactors is crucial to maintain efficient usage of the electron donor. Studies by Oyekola *et al.* (2009, 2010) showed that fermentative activity became more dominant at higher lactate concentrations. Kinetic modelling of lactate utilisation clarified this observation as lactate oxidisers were shown to have a lower K_s as compared to lactate fermenters, with the fermenters displaying a greater μ_{max} (Oyekola *et al.,* 2012a). Compared to other SRB, lactate-utilisers are generally associated with higher growth rates (Kaksonen and Puhakka, 2007). With lactate being a relatively expensive electron donor (Martins *et al.,* 2009; Bertolino *et al.,* 2014; Hao *et al.,* 2014) it is necessary to determine strategies to allow complete utilisation to take place as well as methods to minimise lactate fermentation.

2.4.2 Acetate

Acetate is a major product of organic fermentation, making it an attractive electron donor option (Oude Elferink *et al.,* 1998; Rabus *et al.,* 2000; Rotaru *et al.,* 2015). Furthermore, as previously mentioned, it is the final product of incomplete oxidation of several other electron donors (Liamleam and Annachhatre, 2007; Muyzer and Stams, 2008). This means that effective use of acetate for BSR is highly desired. As alluded to in Section 2.3.1, acetate utilisers are not typically found to be active alongside incomplete oxidisers due to their slower growth rates, and therefore acetate accumulation is observed in these situations (Lens *et al.,* 2002; Celis *et al.,* 2013; Hao *et al.,* 2014). However, numerous studies have shown promising results when acetate is used as a sole electron donor, (Dries *et al.,* 1998; Moosa *et al.,* 2002; Marais, 2019; Hessler, 2020). The most common acetate utilisers belong to the *Desulfobacter* genus, with some species utilising it exclusively (Rabus *et al.,* 2000). Key competition in acetate systems involves methane producing microorganisms such as methanogenic archaea (MA) and methane producing bacteria (MPB). The relevant reactions are shown below (Liamleam and Annachhatre, 2007):

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

$$\text{Acetate} + H_2O \rightarrow CH_4 + HCO_3^{--}$$ Equation 2-9

Competition between methanogens and SRB for acetate has been widely studied (Parkin *et al.,* 1990; O 'Flaherty *et al.,* 1998; Oude Elferink *et al.,* 1998a; Oude Elferink *et al.,* 1998b; Scholten *et al.,* 2000; Rotaru *et al.,* 2015). Yoda et al. (1987) found SRB to outcompete methanogens at low acetate loading whilst the opposite was observed at higher acetate loading. This was attributed to higher growth rates of methanogens but a greater affinity for acetate by SRB, also observed by Harada et al. (1994). This agrees with work done by Choi and Rim (1991) which showed that at a COD/sulphate ratio below 1.7 SRB were found to predominate over MPB. However, work by Oude Elferink *et al.* (1998b) showed two acetate utilising SRB to have higher or comparable growth rates to the methanogens present, indicating that the competition is not so clear cut. As shown by the Gibb's free energy above, acetate oxidation by SRB is more favourable.

Key in the ultimate dominance of one community over another is their susceptibility to sulphide toxicity. MPB have been reported to have a greater sensitivity to both sulphide toxicity as well as sulphate levels as compared to SRB (Choi and Rim, 1991). Further, methanogenic activity can be potently inhibited by bromoethane sulfonate (BESA; Gunsalus *et al.,* 1978).

2.4.3 Propionate

Along with acetate, propionate is a key product of the fermentation of organic biomass (Widdel and Pfennig, 1982). These two molecules often represent the final breakdown products of fermentation since even-chain fatty acids are broken down to acetate and odd-chain fatty acids to both acetate and propionate (Rabus *et al.,* 2000). This makes propionate an attractive option for a sustainable electron donor as a low-cost carbon source. It has been shown to contribute to the electron donor supply for sulphate reduction in marine sediments, along with acetate and butyrate (Sørensen *et al.,* 1981). However, work on propionate has been sporadic over the past few decades, and more limited as compared to several other electron donors. Therefore, a more detailed review of the literature with regards to this under-utilised electron donor will be given here.

Between the 1930s – 1950s a few studies reported propionate utilising SRB, one of which was reportedly a complete oxidiser (reviewed in Widdel and Pfennig, 1982). However, none of these isolates were reported on again and appear to have been lost with no other propionate oxidising SRB being known at the time (Widdel and Pfennig, 1982). Two decades later, Rabus et al. (2000) reviewed several propionate utilising SRB genera. *Desulfobulbus* species are by far the most dominant species in the literature and Hao et al. (2014) reports only one species – *Desulfobulbus propionicus* - as prevalent in sulphate treatment bioreactors.

As with lactate, partial oxidation of propionate is typical (Ghigliazza *et al.,* 2000). Propionate can be utilised for sulphate reduction via Equation 2-10 (Muyzer & Stams, 2008; Zhao *et al.,* 2008) and Equation 2-11 (Liamleam and Annachhatre, 2007), or can be degraded by fermentative bacteria (Equation 2-12; Muyzer and Stams, 2008). All three reactions result in the production of acetate.

$$\text{Propionate} + 0.75\ SO_4^{2-} \rightarrow \text{Acetate} + 0.75\ HS^- + HCO_3^- + 0.25\ H^+ \quad \text{Equation 2-10}$$

$$\text{Propionate} + SO_4^{2-} + H_2 \rightarrow \text{Acetate} + HS^- + HCO_3^- + H_2O \quad \text{Equation 2-11}$$

$$\text{Propionate} + 3H_2O \rightarrow \text{Acetate} + HCO_3^- + H^+ + 3H_2 \quad \text{Equation 2-12}$$

The various microbial groups important in a propionate fed anaerobic environment therefore include propionate utilising SRB, acetate utilising SRB and propionate fermenters. Competition between these microbes is a matter of much discrepancy in the literature. In assessing the possible pathways involved, Maillacheruvu *et al.* (1993) considered of the relevant free energies (ΔG) as a function of the partial pressure of hydrogen and the concentration of sulphide. They suggested that the incomplete oxidation of propionate by SRB is more favourable than either SRB-mediated acetate oxidation or propionate fermentation. Later, Maillacheruvu and Parkin (1996) showed that propionate degrading fermenters out competed propionate-utilising SRB, but this occurred at low sulphate concentrations. They predicted

that SRB would dominate at high sulphate concentrations. Visser *et al.* (1993) confirmed this, with propionate-utilising SRB displaying a faster doubling time compared to propionate fermenters at higher sulphate concentrations, with the opposite occurring at lower sulphate levels.

D. propionicus was shown by Widdel and Pfennig (1982) to have a relatively fast doubling time (10 hours), with two other strains of the same species reported as slightly slower. This SRB was also studied by O 'Flaherty *et al.* (1998) and shown to have faster growth rates than acetate, ethanol and some hydrogen utilising SRB, as well as a higher affinity for sulphate as compared to the acetate SRB. However these data were for pure cultures, and growth rates dropped significantly in adapted anaerobic sludges, to well below those of the other SRB (O'Flaherty *et al.,* 1998). Oude Elferink *et al.* (1998a) showed preferential utilisation of propionate over acetate by a *Desulfobulbus*-like species and showed that more than half of the propionate utilised was channelled towards sulphate reduction, and the rest to propionate fermentation.

Despite some disagreement on the effectiveness of propionate as an electron donor and carbon source, successful implementation of a propionate-fed sulphate reducing bioreactor was demonstrated by Ghigliazza *et al.* (2000). They achieved a sulphate removal rate of 98% at an influent sulphate concentration of 2000 mg/L and a 2-day hydraulic residence time (HRT), albeit after a significant adaptation time post reactor start-up.

2.4.4 Complex organics and waste streams

An array of complex organics and waste streams have been trialled for BSR, particularly in passive systems (Gibert *et al.,* 2002; Moodley *et al.,* 2018). As alluded to previously, the biodegradability of complex organic substrates is key. The use of plant waste matter can however be difficult due to the presence of structural compounds such as lignin, resins and waxes which are not easily degraded (Dill *et al.,* 2001). Biodegradability has been shown to correlate with lower lignin or lignocellulosic contents (Gibert *et al.,* 2004; Li *et al.,* 2013). Processes making use of complex organics rely on the partial degradation of the organic polymers present, by other bacterial communities, to products that SRB can utilise. The fermentation of such substrates involves acidogenesis, resulting in the production of VFAs, which are commonly combinations of acetate, propionate and butyrate (Speece, 1996; Elferink *et al.,* 1998; Scholten *et al.,* 2000; Liamleam and Annachhatre, 2007).

Complex organic substrates that have been studied include animal manure, wood chips, leaf mulch, grass cuttings, compost and peat moss (Waybrant *et al.,* 1998; Gibert *et al.,* 2004; Zagury *et al.,* 2006; Matshusa-Masithi *et al.,* 2009). However, sulphate conversion rates are often low. For example, sheep manure has been shown to facilitate the complete removal of sulphate over 60 days in batch cultures fed with 1000 mg/L sulphate (Gibert *et al.,* 2004). In continuous systems conversion levels dropped by two thirds at a 9-day HRT. Mixes of different organic waste substrates have given better sulphate reducing and metal removing activity as compared to single substrates (Waybrant *et al.,* 1998; Zagury *et al.,* 2006). Batch experiments fed with simulated mine drainage water containing between 3000 to 5000 mg/L sulphate have shown 100% sulphate removal over periods of 30 – 60 days on substrates such as wood chips, leaf mulch, sawdust and sewage sludge (Waybrant *et al.,* 1998). The long time

periods taken for sulphate depletion demonstrated by these studies can be associated with slow rates of soluble carbon production. The hydrolysis of complex lignocellulose has been previously noted as a rate-limiting step in such systems (Pulles and Heath, 2009). Varying successes have been shown with waste streams which often have more readily available carbon, such as winery waste with its ethanol content, and even landfill leachate which is often high in soluble carbon and also displays neutralising capacity (Moodley *et al.,* 2018).

Microalgal digestate has been proposed as a source of VFAs for SRB (Harrison *et al.,* 2014). High productivity and lipid content mean that algae are an attractive option for biomass generation (Williams and Laurens, 2010). As with plants, microalgae pose problems with resistance to degradation due to cell wall or high cellulose content (Williams and Laurens, 2010; Inglesby *et al.,* 2015). A study carried out by Harrison *et al.* (2014) investigated the potential of the digestate from the anaerobic digestion of *Spirulina* sp., as a feed for BSR. *Spirulina* lacks a cell wall and is much easier to harvest than unicellular microalgae due to its filamentous structure that makes solid liquid separation easier. VFA profiles constructed for the digestate showed varying ratios of acetate, propionate and butyrate across the course of digestion. This work was furthered by Motleleng (2020), where continuously stirred tank reactors (CSTRs) fed with algal digestate achieved maximum volumetric sulphate reductions rates (VSRRs) comparable to, or higher than those attained in similar studies which used lactate-fed CSTRs (Oyekola *et al.,* 2010). Algae would preferably be cultivated on-site using treated ARD. Growth of *Spirulina* on ARD effluent has been attempted but proved unsuccessful (Harrison *et al.,* 2014). Interestingly, *Spirulina* has previously been tested for sulphide sensitivity and found to be able to grow with minimal inhibition in the presence of 250 – 300 mg/L sulphide (Rose *et al.,* 1998).

Near complete removal of a range of heavy metals with various cellulosic organic substrates together with animal manure has also been demonstrated (Zagury *et al.,* 2006). However, it has been noted that metal removal efficiencies are often over attributed to sulphide precipitation from SRB action and a significant amount may instead adsorb onto the substrate (Moodley *et al.,* 2018).

2.4.5 COD/sulphate ratio

In addition to the choice of electron donor and its concentration, the amount of electron donor to sulphate loaded, i.e. the COD/sulphate ratio is an important parameter to consider in BSR systems and is briefly mentioned here. At minimum, enough COD needs to be supplied to allow reduction of all sulphate present. It can therefore be somewhat informed by the stoichiometry of the metabolic reaction. Choi and Rim (1991) calculated the theoretical minimum COD/sulphate ratio as 0.67 for sulphate reduction to occur. However, in many cases, for example where a carbon source is incompletely oxidised by SRB to acetate, a higher ratio is required. An optimum ratio for different electron donors often exists that is dependent on the metabolisms involved, and can allow for selection of SRB over other microorganisms (Ghigliazza *et al.,* 2000; Bertolino *et al.,* 2012). A ratio that is too high can lead to undesirable COD remaining in the effluent. The COD/sulphate ratio chosen must often be a compromise between maximum sulphate reduction and maximum COD removal.

2.5 Sulphide: chemistry, toxicity and management

The fundamentals of BSR relies on converting sulphate, a very stable pollutant in an aerobic environment, into sulphide - a more reactive form of sulphur which can then be more easily removed. However, sulphide produced by BSR poses an even greater concern as a pollutant than does sulphate and appropriate removal is therefore crucial (Rückert, 2016). Corrosive action of sulphide on submerged steel structures or pipes can cause significant damage (Barton and Fauque, 2009), and gaseous hydrogen sulphide is highly toxic and can be fatal at concentrations upwards of 800 mg/L (Liamleam and Annachhatre, 2007). Though predominantly present as dissolved sulphide at neutral pH, gaseous sulphide becomes the dominant sulphide species at lower pH's (Stefess *et al.,* 1996; also see Figure 1-4). For these reasons the discharge of sulphide laden waters is prohibited in many countries (Greben *et al.,* 2005).

The reactivity of sulphide means there are various methods for the removal of sulphide, such as precipitation with metal ions, air stripping and reaction with chemicals such as hydrogen peroxides, chlorine gas and potassium permanganate (Cadena and Peters, 1988; van Hille *et al.,* 2012). These are generally high energy demand active processes and in the case of precipitation, can lead to further disposal problems (Díaz *et al.,* 2011; van Hille *et al.,* 2012; Gopi Kiran *et al.,* 2017). Though precipitation can allow the co-removal of heavy metal contamination, ARD in most cases forms from the oxidation of pyrite (FeS_2) whereas iron sulphide precipitates as amorphous FeS. Therefore, due to its chemical make-up there will usually still be an excess of sulphide to be removed (van Hille *et al.,* 2011).

2.5.1 Biological sulphide oxidation

Biological sulphide oxidation (BSO), catalysed by sulphide oxidising bacteria (SOB), offers a cleaner alternative for the treatment of sulphide. SOB are a diverse group of chemoautotrophic organisms largely belonging to *Proteobacteria.* In non-oxygen limiting conditions, SOB will preferentially oxidise sulphide completely to sulphate, via Equation 2-13. This is more energetically favourable than partial oxidation to elemental sulphur, shown in Equation 2-14 (Visser *et al.,* 1997; Krishnakumar *et al.,* 2005). However, elemental sulphur formation can be forced by low dissolved oxygen (DO) concentrations or high sulphide loading (Figure 2-4; Stefess *et al.,* 1996; Visser *et al.,* 1997).

$$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+ \qquad \Delta G° = -733 \text{ kJ/mol} \qquad \text{Equation 2-13}$$

$$2HS^- + O_2 \rightarrow 2S^0 + 2OH^- \qquad \Delta G° = -169 \text{ kJ/mol} \qquad \text{Equation 2-14}$$

Buisman *et al.* (1990) found the oxygen concentration at which maximal elemental sulphur production occurred to be dependent on the sulphide concentration supplied. Sulphur formation occurs as sulphide loading approaches or exceeds the maximum sulphide-oxidative capacity of the SOB culture, indicated by the oxygen consumption rate reaching a maximum (Stefess *et al.,* 1996; Visser *et al.,* 1997). Figure 2-4 shows the narrow window of pH and redox within which partial oxidation to sulphur can occur. Processes that exploit this partial oxidation must therefore be able to finely control and monitor the relevant variables. The THIOPAQ™ process is an example of an industrial process that employs the

activity of SOB. Developed by Shell Global Solution International BV, UOP and Paques BV, it monitors and controls the DO and pH levels to achieve sulphur production. The sulphur is produced as a suspension in the bulk liquid and must be harvested via settling tanks (Ayangbenro *et al.,* 2018).

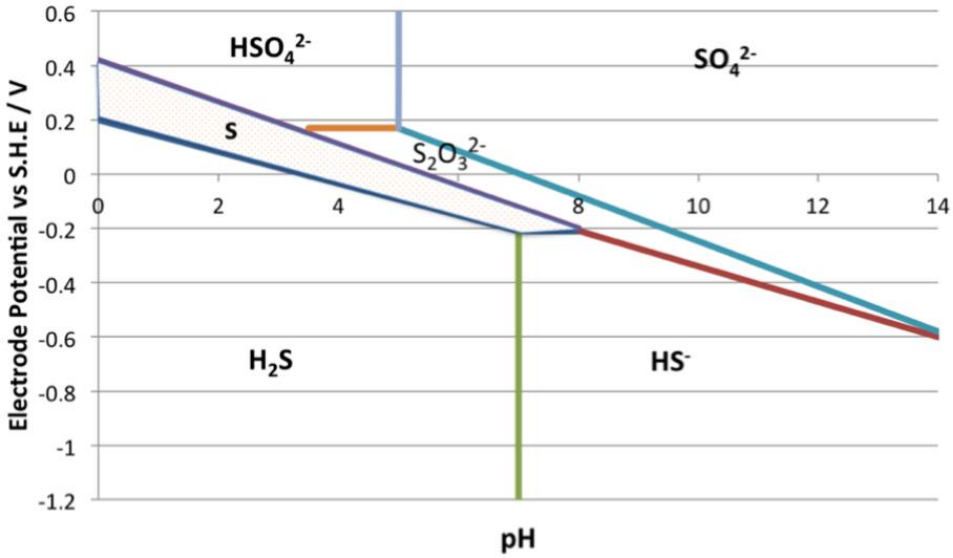


Figure 2-4 Pourbaix diagram of dominant sulphur species in water. Image taken from Mooruth, (2013).

Visser *et al.* (1997) propose that partial sulphide oxidation is a survival strategy of the microorganisms to mitigate the toxic effects of increasing sulphide concentration; i.e. to use more sulphide and generate less energy rather than having sulphide accumulate. In this case the available oxygen is used to convert more sulphide via Equation 2-14 than would be possible via Equation 2-13. The deposition of sulphur has been observed on the membranes of *Thiobacillus* species (Stefess *et al.,* 1996). If recovered, the sulphur produced is a value-added product from a BSO process. Biogenic sulphur has the advantage of being more hydrophilic in nature, possibly due to the attachment of proteins to the hydrophobic surface of sulphur particles, forming an outer polymer layer (Rose and Rein, 2003). It would therefore be more easily absorbed in soil and it presents itself as a good material for fertilizer or sulphur fungicide production (Benschop *et al.,* 2002).

Floating sulphur biofilms

Biofilms usually form attached to a solid surface at a liquid-solid interface. Floating biofilms are less common and occur at liquid-air interfaces, where organisms benefit from access to both oxygen from the air above as well as nutrients that the liquid phase may provide (Spiers *et al.,* 2003). Floating sulphur biofilms (FSBs) were first noticed arising on the surfaces of sulphide-laden tannery waste ponds (Bowker *et al.,* 2000; Gilfillan *et al.,* 2000).

They have been applied as a passive means of sulphide removal, with high sulphur recoveries of up to 66% (Molwantwa and Rose, 2013) and 75% (van Hille and Mooruth, 2014) of the sulphide loaded. The elemental sulphur is incorporated into the biofilm and is easily harvested from the surface of the bulk fluid, as a value-added product.

The potential of SOB to treat sulphide from ARD via FSB formation was first investigated by Molwantwa (2008). Effluent from a sulphate reducing degrading packed bed reactor (DPBR) was fed into a non-agitated open linear flow channel reactor (LFCR) that allowed aeration at the surface of the liquid and FSB formation. As sulphide rich BSR effluents are anaerobic, a steep redox potential gradient exists at the air/water interface of these systems. This was reported as 0 to -200 mV across a depth of 260 – 380 µm. The formation of the biofilm itself generates oxygen limiting conditions as mass transfer of oxygen from the headspace of the reactor is thus inhibited (Van Hille & Mooruth, 2014). A microaerobic environment, suitable to partial oxidation, is therefore naturally generated. This is equivalent to the strictly monitored and controlled processes described in the section above. Additionally, this process allows easy harvesting from the surface of the liquid and does not require extra treatment such as centrifugation or sedimentation.

Generation of the biofilm has been observed in as little as 12 hours (van Hille and Mooruth, 2014). During its development, the biofilm has been reported to undergo characteristic and observable changes: firstly a “thin” and translucent phase, secondly a “sticky” phase and thirdly a “brittle” phase where the biofilm is hard to the touch (Molwantwa, 2008). In the first two phases, the biofilm is largely organic with elemental sulphur deposition beginning with the third phase (van Hille *et al.,* 2011). Typical FSB thickness is between 50 – 500µm (Molwantwa and Rose, 2013). FSBs are a complex array of different organisms with at least three layers of distinct microbial communities. EPS architecture is also complex with an extracellular polymeric substance (EPS) matrix along with channels and pores, and sulphur occurring both as small granules and as larger crystals within the biofilm (Molwantwa, 2008; van Hille *et al.,* 2011).

A model of FSB formation was proposed by Molwantwa (2008) where several stages are hypothesised to explain the structural changes observed in the biofilm. The thin phase is associated with aerobic and microaerophilic organisms which form the first redox gradient at the surface. Rapid biomass growth occurs during the sticky phase with the formation of an EPS matrix. The organisms associated with EPS excretion are likely heterotrophic, as poor biofilm formation under carbon limitation has been observed (van Hille and Mooruth, 2014). The brittle phase is laden with elemental sulphur signifying the incorporation and activity of SOBs and that sufficient oxygen limitation has been achieved to allow elemental sulphur production (Molwantwa, 2008). The aforementioned retardation of biofilm growth with insufficient organic carbon could also speak to the importance of heterotrophic sulphide oxidisers in SOB communities (van Hille *et al.,* 2011; van Hille and Mooruth, 2014).

Overall, the passive formation of FSBs provides a sulphide removal technology that requires neither energy inputs nor a stream of oxygen to be supplied and gives a value-added product of biogenic sulphur.

2.5.2 Microbial toxicity of sulphide: a selection strategy

Generally, SRB have a high tolerance to sulphide (Manilal *et al.,* 2000; Kuo and Shu, 2004) meaning that elevated sulphide concentrations in bioreactors can be exploited as a selective pressure to limit the growth of other groups of microorganisms (Oyekola *et al.,* 2009). In a lactate-fed system studied by

Oyekola *et al.* (2009), fermentative activity dominated at a sulphate feed concentration of 5 g/L, with sulphide concentrations between 0.02 – 0.3 g/L, while SRB activity dominated at a sulphate feed concentration of 10 g/L, where resulting sulphide levels were between 0.3 – 0.6 g/L. Further, their study found that in a solely fermentative reactor, lactate utilisation decreased by 70% on addition of 0.5 g/L sulphide. Laanbroek *et al.* (1983) compared the growth rates of a lactate oxidising SRB (*Desulfovibrio* spp.) and a lactate fermenter (*Veillonella* spp.) under sulphide concentrations of 0.0165 and 0.165. It was found that the growth rate of the fermenter was reduced by 50% under the higher sulphide concentration whereas that of the oxidiser remained unchanged. However, in a propionate environment there are some contradictory data. In one instance, sulphide IC50 values of propionate oxidising SRB and propionate fermenters were shown to be 0.41 and 0.83 g/L respectively (O'Flaherty *et al.,* 1998a). By contrast, Maillacheruvu and Parkin (1996) showed propionate utilising SRB to be far more tolerant of sulphide compared to propionate fermenters with respective K_i values of 0.68 and 0.05 g/L sulphide. However, no microbial ecology analyses were carried out in these studies and contradictory data may speak to the importance of overlaying microbial ecology data with performance data in order to achieve more comparable results. With the development of molecular techniques this has become significantly more feasible in recent years.

2.6 Development of passive and semi-passive BSR and BSO treatments in South Africa

This section details certain developments and implementations of BSR and BSO technologies in South Africa. The section leads to and culminates in a detailed description of a hybrid LFCR, the reactor system used in this thesis.

2.6.1 Rhodes BioSURE Process®

The development of the Rhodes BioSURE Process® began at Rhodes University and has been well reviewed by Rose (2013). During initial development, algal biomass was shown to serve well as an electron donor within a multistage ARD treatment process called the Integrated Algal Sulphate Reducing Ponding Process for Acid Metal Wastewater Treatment (ASPAM). This initial system demonstrated successful recycling of sulphide-rich, treated ARD to neutralise and precipitate metals in the incoming ARD stream. However algal biomass was deemed as unsuitable for larger volumes of ARD and the process was developed to incorporate primary sewage sludge (PSS) as the substrate, thus resulting in co-disposal of two waste streams. A pilot plant was established at Grootvlei Gold Mine, which consisted of a recycle sludge bed reactor followed by a multicompartment baffled reactor which allowed for immobilisation of SRB. Sulphide produced by the system was managed by the precipitation of iron as iron sulphide. The system showed successful removal of sulphate to levels below 250 mg/L (Rose, 2013). Drawbacks of the system were primarily associated with the availability of PSS in more remote locations.

2.6.2 Integrated Managed Passive Process

The Integrated Managed Passive (IMPI) process, developed by Pulles Howard and de Lange Inc. (PHD), was implemented as a pilot plant at the Vryheid Coronation Colliery. The system was centred firstly, on the use of DPBRs that were filled with a range of lignocellulosic substrate from the surrounding region, and secondly, on downstream BSO reactors (Pulles and Heath, 2009). In later years the sulphide oxidising reactors were developed into LFCRs for FSB formation (van Hille *et al.,* 2012). Additionally, slow dosing of molasses into the DPBRs was a key novel feature of this system. This was done as the DPBR did not produce sufficient soluble organic carbon. Further, in allowing increased SRB activity it was proposed that the resulting lower redox lead to a more suitable environment for the hydrolysis of the lignocellulosic carbon. The IMPI process was aimed particularly at treating sulphate laden waters at low pH's in the range of 2-3. The treatment plants typically showed a short period (eight months) of high performance (>60 g sulphate/m^3 carbon/d), followed by five years of a significantly lower but sustained level of sulphate reduction (29 g sulphate/m^3 carbon/d). After this performance dropped by approximately a third (Pulles and Heath, 2009; Molwantwa *et al.,* 2010). The target performance for economic viability was determined to be 60 g sulphate/m^3 /d (Molwantwa *et al.,* 2010). Initial performance that was in this range was explained by the early degradation of more readily available carbon sources in the DPBRs over the first period of steady state, with the more recalcitrant organics remaining. Supplementation of organics into the system is therefore required at this point to sustain sulphate reduction (van Hille *et al.,* 2011).

2.6.3 Laboratory scale development of sulphide oxidising and sulphate reducing LFCRs

Since the incorporation of sulphide oxidising LFCRs in the IMPI process mentioned above, further development has been carried out at the Centre for Bioprocess Engineering Research (CeBER) within the University of Cape Town. Rigorous studies using sulphide oxidising LFCRs have looked at process performance with regard to hydrodynamics, sulphide oxidation rates, sulphide loading, organic loading and optimal harvesting times (Mooruth, 2011, 2013; van Hille *et al.,* 2012; van Hille and Mooruth, 2014). Reactors were made from Perspex, allowing visualisation of the bulk liquid, and dye tracer studies showed laminar parabolic flow through the reactor. A density difference between the influent - laden with dissolved salts - and the bulk volume was shown to be a crucial factor for reactor mixing. Higher flow rates were shown to result in greater structural integrity of the biofilm with optimal residence times being one to two days. This may have been due to increased loading of organic carbon (1 g/L acetate) as opposed to hydrodynamic reasons. The system was fitted with a hydrogen sulphide scrubber which showed negligible losses of the toxic gas to the environment (Mooruth, 2013). Since the amount of oxygen supplied to such a system is not controlled and simply relies on exposure to the atmosphere, there is need to maintain sulphide levels above a minimum to promote sulphide oxidation (van Hille *et al.,* 2012).

Further developments at the University of Cape Town then led to a novel, hybrid LFCR which incorporated both sulphate reduction and sulphide oxidation (van Hille *et al.,* 2016; Marais, 2019; Marais *et al.,* 2020). SRB biomass was supported by the carbon microfibres, as demonstrated previously in a

sulphate reducing LFCR by Van Hille *et al.* (2015). The headspace of the reactor was kept open which allowed FSB formation, provided there was a high enough sulphide concentration present in the bulk liquid. A sulphide concentration of 250 mg/L was present at initial FSB formation carried out by van Hille *et al.* (2016). FSB formation generated a steep redox at the surface of the reactor liquid which in turn allowed an open reactor to remain sufficiently anaerobic to enable BSR (Marais *et al.,* 2017; Marais, 2019; Marais *et al.,* 2020). Regular harvesting of the FSB creates trends of sulphide accumulation from harvest to harvest while maintaining stable residual sulphate levels after a period of reactor start-up and stabilisation (Marais, 2019; Marais *et al.,* 2020). As the biofilm matures and thickens, oxygen mass transport becomes limiting and the rate of partial sulphide oxidation decreases leading to sulphide accumulation. Upon harvesting, a steep drop in sulphide is observed with oxygen influx at the surface of the system initiating biofilm growth (Marais *et al.,* 2020). This system does not involve active mixing, drains by overflow and passively generates an FSB. However, regular harvesting of the FSB is required to maintain optimal performance, and influent is pumped in at specified flow rates. The system is therefore described as a semi-passive process.

FSB harvesting strategies have evolved with development of the LFCR. In the solely sulphide-oxidising reactors, FSBs were collapsed to the bottom of the reactor and reactors were periodically drained to allow harvesting (van Hille *et al.,* 2012; Mooruth, 2013). However, in the hybrid LFCR this would not be practical with an active SRB culture in the bulk volume. Additionally, it was suggested that biofilm remaining at the base of the reactor would be prone to disintegration and could react with sulphide to produce polysulphides (van Hille *et al.,* 2012). A wire mesh was therefore inserted just below the surface of the bulk volume in the hybrid LFCR to allow the FSB to be collapsed onto this screen and be periodically harvested from the surface with minimal disruption to the rest of the reactor volume (Marais, 2019).

The hybrid reactor system achieved operational performance levels of up to 98% sulphate conversion at feed sulphate concentration of 1g/L with 82% sulphide removal (Marais, 2019). A large majority of this sulphide was not recovered as sulphur and was presumed to have been lost as colloidal sulphur or disintegrated biofilm. To the author's knowledge this is the only reactor system where BSR and passive BSO occur simultaneously within the same reactor unit.

2.7 Research motivation and rationale

The literature above demonstrates the applicability and potential of BSR, particularly within a water stressed country with a strong mining economy, such as South Africa. Several challenges typically associated with BSR have been overcome to certain extents, particularly those related to slow kinetics of SRB as well as sustainable methods of sulphide management (Harrison *et al.,* 2014). Proof of concept studies of the hybrid LFCR described in the above section have shown this system to be a promising semi-passive and low-cost solution for remote areas with low volumes of ARD generation (Marais, 2019). However, the identification of a cost-effective electron donor is still a key challenge. Additionally, the expense involved in transportation to remediation sites highlights the need to identify electron donor sources that may be readily available within mining areas. The treatment of ARD using

complex organics, that could be generated on site, or high organic content waste streams, has been attempted with varying success (Greben *et al.,* 2005; Zagury *et al.,* 2006; Martins *et al.,* 2009; Matshusa-Masithi *et al.,* 2009; Moodley *et al.,* 2018; Motleleng, 2020). The fermentation of organic material creates a mix of VFAs, of which acetate and propionate are the most dominant (Widdel and Pfennig, 1982; Rabus *et al.,* 2000). However, research surrounding propionate as an electron donor has been limited as compared to acetate. Despite this, various studies across the past few decades have shown the potential of propionate to compare with more “classic” electron donors such as lactate and acetate (Widdel and Pfennig, 1982; Maillacheruvu *et al.,* 1993; Visser *et al.,* 1993; Maillacheruvu *et al.,* 1996; O’Flaherty *et al.,* 1998a;b Ghigliazza *et al.,* 2000). The literature lacks a rigorous study to determine sulphate conversion and VSRRs across a range of sulphate loading rates. An understanding of the role propionate can play as an electron donor, and the rate of its utilisation, can inform what proportional mix of VFAs should be targeted from an anaerobic digester or fermentation reactor in order for the digestate to feed sulphate reducing bioreactors.

A related challenge is linked to the inefficiency of electron donor utilisation from partial oxidation of the substrate. Acetate accumulation within sulphate reducing reactors is a widely acknowledged problem and leads to effluents with high COD content (Lens *et al.,* 2002; Liamleam and Annachhatre, 2007; Celis *et al.,* 2013; Hao *et al.,* 2014). An explanation as to why acetate-utilising SRB do not develop in these reactors could lie with the growth rates involved since partial oxidisers are classified as faster growers than full oxidisers that utilise acetate (McCartney and Oleszkiewicz, 1993; Oude Elferink *et al.,* 1994; Colleran *et al.,* 1995) and presumably outcompete the complete the acetate utilising SRB. This has led to the idea of a sequential reactor system whereby the two separate communities colonise distinct compartments of a reactor system to allow both partial oxidisers and complete oxidisers to be incorporated in one system. This would lead to both a greater ratio of sulphate reduced per substrate supplied, reducing overall cost, as well as decreased COD contamination in the effluent.

This study specifically investigates treatment of the sulphate component of ARD, at a neutral pH. Influent ARD can be fairly neutral if it has had low oxygenation or if bicarbonates in the rock have caused natural neutralisation at source (Johnson and Hallberg, 2005). Alternatively, a neutralisation step can be incorporated upstream of BSR.

2.8 Research Scope

The reactor systems used in this study are based on the hybrid sulphate reducing, sulphide oxidising LFCRs described in Section 2.6.3. This is a low-cost reactor system which requires minimal technical maintenance or operation. The study presented here investigates the usage of the electron donor to inform further research in identifying an approach to cost-effective substrate (electron donor) provision for BSR and BSO. The efficiency of elemental sulphur incorporation into the FSB for sulphide removal and increased sulphur yield is also investigated.

2.8.1 Research hypothesis

A sequential LFCR, fed with lactate, will be set up whereby the first and second reactor units will be selectively colonised for lactate-utilising and acetate-utilising SRB respectively. It is hypothesised that this will result in a decoupling of the metabolisms involved, with partial oxidation of lactate predominantly occurring in the first reactor unit, while acetate oxidation will be predominant in the second. This will result in more complete utilisation of the substrate, with a concomitant increase in sulphate reduced, as compared to a single reactor unit fed either lactate or acetate.

2.8.2 Research Objectives

- Start-up a propionate-fed LFCR from a diverse SRB inoculum
- Evaluate performance of the propionate-fed LFCR with regards to sulphate conversion, VSRR and VFA utilisation over the course of a hydraulic residence time (HRT) study
- Set-up a sequential LFCR from lactate-fed and acetate-fed LFCR units
- Compare performance with regards to sulphate conversion, VSRR and VFA utilisation, as well as recovery of elemental sulphur from the single-stage reactor set-up and the sequential reactor set-up
- Determine the effect of reduced inorganic ions in the feed on elemental sulphur yields, sulphide removal and FSB structure

Chapter 3 Materials and Methods

3.1 Inoculum

The lactate- and acetate-fed reactors used in this study were originally inoculated, several years before the commencement of this study, with a mixed sulphate-reducing bacterial community that was obtained from Professor Peter Rose (Rhodes University). Subsequently, the inoculum was supplemented at the University of Cape Town (UCT) with sludge and river sediments from various sources to increase the microbial diversity present. Several different batch cultures, which had been maintained at UCT on a range of electron donors, were used to colonise the reactors. The propionate-fed reactor inoculum was collected from a range of these reactors and batch cultures, and is further described in Section 3.5.1. Sulphide oxidising bacterial communities were developed at the University of Cape Town (UCT) using native species present in SRB cultures (van Hille and Mooruth, 2014; van Hille *et al.,* 2016) and have been maintained in floating sulphur biofilms (FSBs) within the linear flow channel reactors (LFCRs) at UCT (Marais *et al.,* 2017; Marais *et al.,* 2020).

3.2 Reactor description: The Linear Flow Channel Reactor

LFCRs, designed at the University of Cape Town (van Hille *et al.,* 2016), were used for all experiments. Figure 3-1 i) and iii) show the reactor set up. Reactors were made from clear Perspex (12 mm thickness), with self-sealing sampling ports across the front of the reactor to enable routine sampling across lateral and vertical aspects of the bulk volume. Ports used in these studies were the front top (FT), front bottom (FB), back top (BT) and back bottom (BB) ports. Dimensions of the lactate- and acetate-fed reactors were as follows: 250 mm (l) × 100 mm (w) × 150 mm (h) with a total volume of 2.125 L bulk liquid. The propionate-fed reactor had a slightly different geometry with a total bulk volume of 2.4 L and dimensions of 275 mm (l), 123 mm (w) and 112 mm (h). A feed port was positioned at or a few millimetres above the surface of the bulk volume in each reactor and feed was continuously introduced via high precision peristaltic pumps. Carbon microfibres were used for microbial attachment, as previously described by Marais *et al.* (2020). These were approximately 10 cm long and were fixed between two aluminium rods which spanned the length of the reactor, allowing these microfibres to be suspended in the bulk volume (Figure 3-1 ii). An effluent port, positioned opposite the influent port, allowed for passive outflow from the reactor via overflow. A wire gauze was suspended approximately 1 cm below the surface to aid in harvesting of the FSB and prevent broken biofilm material from falling to the base of the reactor. A heating coil, connected to a water bath ran through each reactor, maintaining them at 25 °C during operation.

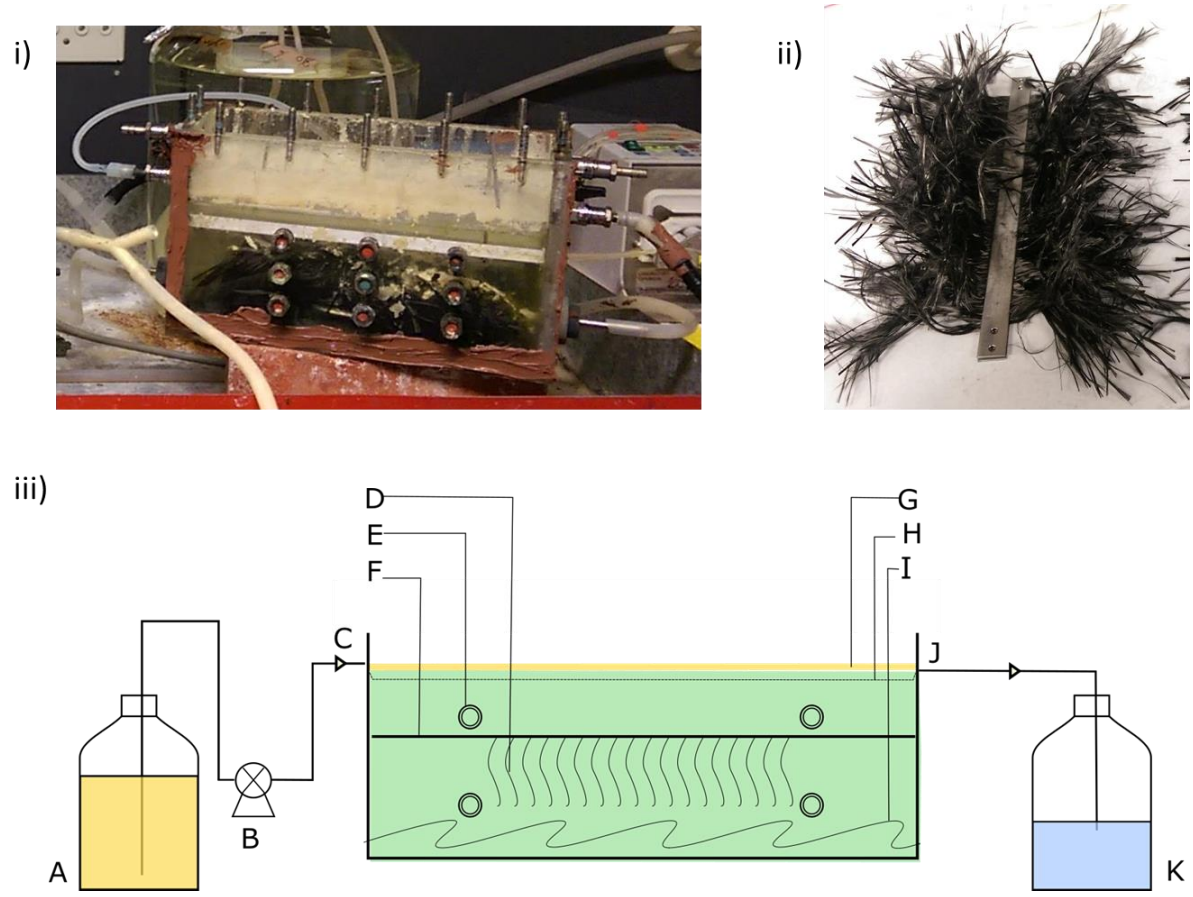


Figure 3-1 i) Photograph of one of the LFCRs used. ii) Carbon microfibres which are suspended in the LFCR for microbial attachment. iii) Schematic of the LFCR and set up. A: feed, B: peristaltic pump, C: feed inlet port, D: carbon microfibres, E: sampling port, F: metal support rod for carbon microfibres, G: floating sulphur biofilm (FSB), H: wire gauze to aid in harvesting of FSB and to prevent pieces falling to the base of the reactor, I: heating coil connected to a water bath, J: effluent, K: effluent drainage vessel.

3.3 Reactor media

Reactors were fed with modified Postgate B media (Hessler *et al.,* 2018). This was made up with the following basal constituents unless otherwise specified: 0.46 g/L K_2HPO_4, 1 g/L NH_4Cl, 1 g/L $MgSO_4.7H_2O$, 0.9 g/L Na_2SO4, 0.3 g/L sodium citrate and 0.4 g/L yeast extract (YE). The two sulphate salts resulted in an overall sulphate concentration of 1 g/L. The chosen sulphate concentration was informed by previously reported field data which showed sulphate levels of up 700 mg/L and rising in dams within coal mining areas in South Africa (McCarthy, 2011). The feed was then supplemented with the relevant electron donor: 0.92 g/L sodium acetate or 0.69 g/L sodium propionate. For the lactate reactor, two different lactate concentrations were used: 1.94 ml/L 50% (w/w) sodium lactate solution for the lower concentration and 4.30 ml/L 50% (w/w) sodium lactate solution for the higher concentration. With partial oxidation of lactate being assumed to be the sole BSR pathway occurring in the lactate reactor - the lower lactate concentration theoretically allowed for a maximum of 50% reduction of the total sulphate supplied, while the higher lactate concentration was in excess by 15%. These concentrations relate to volatile fatty acid (VFA) and associated COD concentrations of 1.0 g/L lactate (lower concentration, 1.0 g/L COD), 2.2 g/L lactate (higher concentration, 2.2 g/L COD), 0.66 g/L acetate

(0.63 g/L COD) and 0.52 g/L propionate (0.75 g/L COD). Media was made up with deionised water and sterilised by autoclaving at 120 °C for 20 minutes.

3.4 Lactate- and acetate-fed LFCRs

The reactors used for the lactate and acetate studies had been in continuous operation for approximately four and three years respectively, with the respective VFA feed and had been used for studies described by Marais (2020). These two reactors made up the two units of the sequential reactor system, with strongly selected lactate-utilising and acetate-utilising SRB communities for the primary and secondary reactors respectively. Both reactors were run and monitored for at least six months before the studies reported here commenced. This was to allow recover from previous stress studies, to allow adjustment to new parameters designated by the studies conducted here and to ensure stable sulphate reduction performance was achieved. Reactors were also 'desludged' before experiments were commenced. This involved the removal of accumulated biomass particularly at the front bottom corner of the reactor. A 2-day hydraulic residence time (HRT), determined as the optimum in the work of Marais (2020), was used for the single-stage, independent reactors (Study I as described in Section 3.4.2). For studies using the sequential reactor system (Studies II – V as described in Section 3.4.2), the HRT was kept at two days for each reactor unit, making the overall HRT four days. Reactors were maintained at 25 °C for all studies.

3.4.1 Sequential reactor setup

A sequential reactor was set up whereby the acetate LFCR was connected to the lactate LFCR to make one system with two reactor components (Figure 3-2). The lactate LFCR was raised up in order for the level of the bulk liquid to be approximately 4 cm higher than that of the acetate reactor. The effluent tubing from the lactate reactor was then connected to the feed inlet port on the acetate reactor becoming the feed for this reactor. Lactate supplemented media was fed into the lactate reactor.

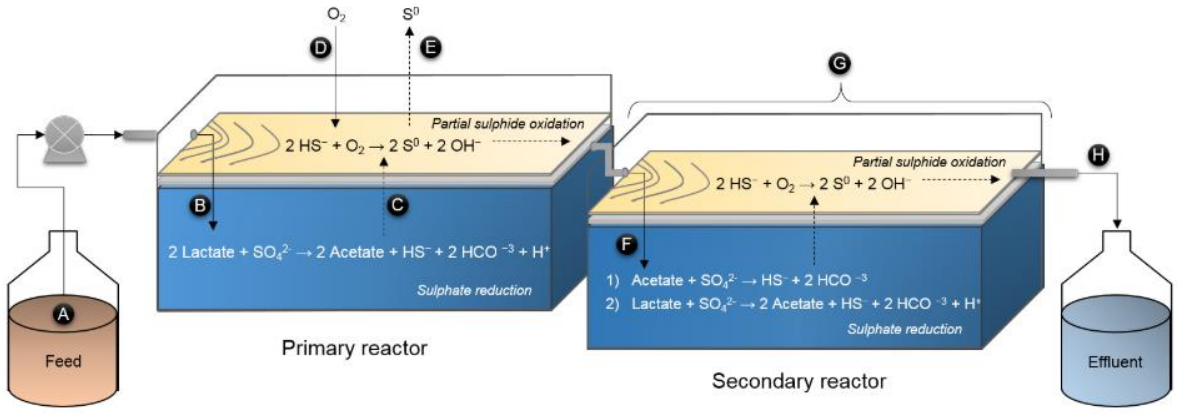


Figure 3-2 Schematic of the sequential reactor set-up. A higher chain electron donor feed (A) is pumped into the primary reactor where it facilitates BSR (B) producing acetate. Generated sulphide is channelled to the surface (C) where partial sulphide oxidation (D) takes place within an FSB. Elemental sulphur is easily harvested as the FSB (E). Acetate and residual lactate are carried through to the secondary reactor (F) where further BSR can occur as well as BSO (G) leading to an effluent further polished of sulphate, sulphide and COD (H). Image taken from Marais (2020).

3.4.2 Reactor studies

Five reactor studies were carried out in the lactate and acetate reactors. The first of these was carried out with the lactate- and acetate-fed reactors as independent reactors (Study I) and the remaining studies (Studies II – V) were carried out on the sequential lactate-acetate reactor system, as described in Table 3-1. Each study consisted of two experimental runs as defined in Section 3.6. For Studies IV and V, the sodium sulphate concentration in the feed was increased from 900 mg/L to 1190 mg/L to maintain sulphate levels at 1 g/L. as the fraction of sulphate supplied by magnesium sulphate was reduced in this study to achieve the desired magnesium concentrations.

Table 3-1 Description of studies carried out on the lactate / acetate reactor systems. Independent refers to the reactor operation as single lactate and acetate reactors, while sequential refers to the lactate-acetate system connected in series.

	Reactor orientation	Feed concentrations (mg/L)		
		Lactate	Magnesium	Phosphate
Study I	Independent	1000	100	250
Study II	Sequential	1000	100	250
Study III	Sequential	2200	100	250
Study IV	Sequential	2200	50	250
Study V	Sequential	2200	50	175

3.5 Propionate-fed LFCR

The propionate reactor was started up as a new system in this study. This involved first preparing an active SRB-containing microbial culture on propionate as the sole electron donor through a series of sub-cultures. Once inoculated, the reactor was run as a closed anaerobic system, as explained in Section 3.5.2, for a period of approximately 180 days to ensure anaerobiosis during the colonisation of the microfibres. Thereafter, the headspace of the reactor was opened up to allow floating sulphur biofilm formation.

3.5.1 Propionate-fed batch starter cultures

Batch cultures (2 x 2 L) for inoculation of the propionate reactor were maintained at 30 °C on modified Postgate medium as described in Section 3.3 albeit with 2.0 g/L propionate, 2.5 g/L sulphate, and 3.2 g/L 2-bromoethane sulphonic acid (BESA) as a methanogenic inhibitor (Gunsalus *et al.,* 1978). A starter culture was made up from a mix of sources to attain a diverse inoculum. These comprised of: the lactate and acetate reactors described in Section 3.4, two upstream anaerobic packed bed reactors, two continuously stirred tank reactors and two closed LFCRs – all maintained on a lactate or acetate supplemented feed (Hessler, 2020). Additionally included were the batch cultures described in Section 3.1, which had been maintained on a range of electron donors including lactate, acetate, ethanol and anaerobic digestate from an algal anaerobic digester. The batch cultures were monitored for SRB activity by analysing sulphate, sulphide, pH and redox via methods described in Section 3.7. Batch

cultures were sub-cultured using a 50% draw and feed scheme when the sulphate levels had been reduced to approximately half of that in the feed. After four rounds of sub-culturing, the batches were used to inoculate the propionate-fed LFCR at a five-day hydraulic residence time (HRT).

3.5.2 Propionate reactor start-up

One of the 2 L batch cultures was supplemented with 500 mL of propionate feed at 2.5 g/L sulphate as used in Section 3.5.1, before being added to an empty LFCR. The reactor was then sealed with an airtight lid and continuously operated at 25 °C, with a propionate feed of 1 g/L sulphate and 0.52 g/L propionate as detailed in Section 3.3, at a 5-day HRT. The reactor over this period of time is referred to as the 'closed system'. Once stable sulphate reduction was achieved in the closed reactor, the reactor lid was removed and the surface of the bulk volume was sprinkled with dry powdered biofilm from the lactate and acetate reactors to introduce the sulphide oxidising bacteria (SOB) needed for formation of the FSB. This system is referred to as the 'open system'.

3.5.3 Propionate reactor HRT study

An HRT study was then carried out with increasing flow rates beginning with a 5-day HRT followed by a 4-, 3- and finally 2-day HRT. Data are interpreted in this study with respect to both the HRT or the dilution rate (Table 3-2). Experimental runs were carried out as described in Section 3.6 with each run ending with the harvesting of the FSB.

Table 3-2 Desired and achieved HRTs and the corresponding dilution rates over which the propionate reactor was evaluated.

Desired HRT (days)	Actual HRT (days)	Dilution rate ($days^{-1}$)
5	4.9	0.21
4	4.2	0.24
3	3.0	0.33
2	2.0	0.50

3.6 Operating procedure for experimental reactor runs

Experimental runs began immediately following the harvesting of the previous FSB and ran until the FSB was again harvested. Unless specified otherwise, runs were held at a constant retention time (feed flow rate) for four residence times. Where studies compared discrete parameter changes, as was the case with all the studies using the lactate and acetate reactors, these runs were performed in duplicate for each study. At least four residence times were allowed to pass after a parameter change before a new study was commenced.

3.6.1 Pseudo-steady state

Across the studies in this work, the designation of a "pseudo-steady state" was necessary due to the cycles of FSB formation and harvesting which cause cyclical changes over each experimental run. Therefore, pseudo-steady state was taken as the last one and a half residence times of each run consisting of four residence times. Reactors were sampled within this time frame a minimum of three

times and these data were used to calculate the averages used for reactor performance analysis and stoichiometric calculations with regards to sulphate and VFA levels. However, for overall sulphur mass balances, data from across the duration of each study was used as this all contributed to FSB formation and sulphur incorporation. Standard deviations for all pseudo-steady state data represents the standard deviation of the averages from the relevant ports for each time point.

3.6.2 FSB harvesting

At the end of each run, the FSB was harvested by scraping off the surface of the bulk volume and removing any pieces trapped by the wire mesh. The broken biofilm was then kept at 37°C until it was visibly dry. Following this, it was dried thoroughly in an 80°C oven for 24 hours after which it was cooled in a desiccator before grinding to a powder for analysis.

3.6.3 Reactor sampling strategy

Reactors were sampled by drawing out a 2 mL sample from the FT, FB, BT and BB ports using a syringe fitted with a hypodermic needle (Mooruth, 2013). Effluent samples were collected by drawing up a sample a few millimetres below the surface of the bulk liquid immediately before the effluent port. Once collected, samples were immediately assayed for pH and an aliquot was removed for sulphide analysis before 40 µL of 10 % (w/v) zinc chloride was added to each sample to precipitate out the sulphides and prevent any oxidation of sulphide to sulphate. Sulphate could then be assayed. Samples were filtered (0.22 um filter) and frozen for subsequent VFA analysis.

3.7 Analytical methods

3.7.1 pH and redox

The FT and BT ports were used for pH measurements, taken timeously after sampling. These were measured using a Jenway 3510 pH Meter fitted with a XS Sensor 2-Pore T DHS pH probe. Redox readings of effluent samples were taken using a Metrohm Ion Analyser 827 pH lab meter fitted with a Pt-ring 3 M KCl electrode. The pH probe was calibrated using Accsen Instrumental standard buffering solutions of pH 4.0 and 7.0.

3.7.2 Sulphide analysis

The aqueous sulphide concentration was measured spectrophotometrically via reaction with the colorimetric reagent *N,N*-dimethyl-*p*-phenylenediamine which creates methylene blue when catalysed by a mild oxidising agent, in this case acidified ferric ions (Cline, 1969). Briefly, 20 µL of sample was added to 200 µL of 1% zinc acetate to stabilise the sulphide and prevent any loss as gaseous hydrogen sulphide with addition of the acidic reagents. Deionised water was added to a volume of 5 mL, following which 500 µL each of 4 g/L *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride and 16 g/L ferric chloride solution, both made up in 6 M hydrochloric acid, were added. Samples were briefly vortexed, and their absorbance read at 670 nm. Sulphide concentration was determined from a standard curve (Appendix

A.3). The range of detection for this assay is 0 – 1 mg/L and average percent standard deviation (SD) was 2.2 %. Where necessary, samples were therefore diluted further, and absorbance measured again.

3.7.3 Sulphate analysis

Sulphate concentrations were determined turbidometrically using the barium sulphate assay (American Public Health Association, 1975). Samples were briefly vortexed and then centrifuged (13 000 rpm for 10 minutes) to remove any particulate material. A 200 µL aliquot of the supernatant was added to a solution comprising 250 uL of a conditioning reagent (75 g sodium chloride, 30 mL hydrochloric acid (32%), 50 mL glycerol and 100 mL ethanol in 300 mL deionised water), a micro scoop of barium chloride crystals and deionised water to make up a final volume of 5 mL. This was vortexed for 20 seconds before reading absorbances at 420 nm. A standard curve was generated using sodium sulphate (Appendix A.1) and concentrations were determined from the equation of the line. The range of detection for this assay is 0 – 50 mg/L and the average percent standard deviation was 5.3 %.

3.7.4 Volatile fatty acids

Residual VFA concentrations were determined using a Waters high performance liquid chromatography (HPLC) system (Waters 717plus Autosampler, Waters 2487 Dual ƛ Absorbance Detector, Waters 1525 µ Binary Pump) fitted with a Bio-Rad Organic Acids ROA column, as has been used previously (Hessler *et al.,* 2018). The mobile phase used was a 0.01 M solution of sulphuric acid made using an analytical grade concentrated solution of the acid run. HPLC runs were carried out at a flow rate of 0.6 mL/min and a temperature of 60 °C. Waters Breeze 2 software was used in conjunction with this set-up. Standard solution mix of VFAs at 1 g/L each was made up using analytical grade propionic acid, analytical grade acetic acid, 50% sodium D-lactate solution and citric acid monohydrate. Standard curves were generated with each run using with concentrations of citrate, acetate, lactate and propionate ranging from 100 – 600 mg/L (Appendix A.2). Samples were filtered (0.22 µm syringe filter), diluted in deionised water to give concentrations within the linear range of the standard curve and re-filtered.

3.7.5 Cations

Magnesium and phosphate levels in effluent and FSB samples were determined via solution inductively coupled plasma mass spectrometry (solution-ICP-MS) on a Thermo ICap 6200 ICP-AES. Analysis was carried out by Central Analytical Facilities (CAF, Stellenbosch University, South Africa) and data was quantified with calibration solutions prepared from National Institute of Standards and Technology (NIST) traceable standards.

3.7.6 Sulphur analysis

Powdered FSB samples (prepared as described in Section 3.6.2) were analysed by CAF, Stellenbosch University, where sulphur levels were determined CHNS analysis was accomplished by combustion

analysis using an Elementar Vario EL Cube Elemental Analyzer. This method converts all reduced species of sulphur to sulphur dioxide gas and measures using a refractive index (RI) detector.

3.7.7 Scanning electron microscopy

FSB samples were prepared for scanning electron microscopy (SEM) analysis as has been reported previously (Marais *et al.,* 2020). Samples of fresh FSB were removed directly from the reactor and fixed in 2.5% (v/v) glutaraldehyde solution at 4°C overnight. Following this, samples were gently rinsed with 1x phosphate buffered saline (PBS, Appendix A.4) and treated with an alcohol dehydration series by immersing them in increasing concentrations of ethanol (30, 50, 70, 90, 95 and 100% (v/v)) for at least 10 minutes each. Samples were mounted with tape onto SEM stubs and treated with a few drops of hexamethyldisilazane (HMDS) to bring them to critical drying point. The stub-mounted samples were sputter-coated with carbon before analysis. A FEI NovaNano SEM was used to visualise the samples in conjugation with performing energy dispersive X-ray spectroscopy (EDS).

3.7.8 DNA extraction

Planktonic and 'attached' phases of the microbial community were sampled for DNA extraction. For attached samples, pieces of carbon microfibres were aseptically collected. For planktonic samples, an appropriate volume of bulk liquid (between 20 – 60 mL) was sampled equally from across the reactor and biomass was harvested by centrifugation (10 000 rpm for 10 minutes).

Nucleic acid was extracted using a Macherey-Nagal NucleoSpin® Soil kit as per manufacturer's protocol. A modification was introduced into the elution step to improve extraction; here samples were incubated with elution buffer for five minutes at 60°C before centrifugation. Quantification of DNA was carried out using a Thermo Scientific NanoDrop™ 2000 spectrophotometer. DNA samples were then stored at -20 °C.

3.7.9 16S rRNA gene sequencing

Extracted DNA from reactor samples was diluted to between 10 – 20 ng/L and sent Macrogen (South Korea) for polymerase chain reaction (PCR), sequencing, data processing and analysis of the 16S rRNA gene. Variable regions V3 and V4 of the 16S rRNA gene were targeted with a forward primer FwOvAd_341F:

5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG-3′

and a reverse primer ReOvAd_785R:

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′

giving an amplicon of approximately 460 bp. PCR denaturation, annealing and elongation occurred at temperatures of 95, 55 and 72 °C, respectively. An initial denaturation step at 95 °C was carried out for 3 min followed by 25 cycles of denaturation, annealing and elongation (each step lasting 30 s), before a final 4 min extension. The product was then sequenced using next generation Illumina MiSeq® sequencing. Reads of between 400 to 500 bp were filtered and were assembled using Fast Length

Adjustment of Short Reads (FLASH version 1.2.11, website: http://ccb.jhu.edu/software/FLASH/) software. Pre-processing and clustering of Operational Taxonomic Units (OTUs) was carried out using CD-HIT-OTU/ rDnaTools (http://weizhongli-lab.org/cd-hit-otu/ and https://github.com/PacificBiosciences/rDnaTool). Taxonomy and diversity analysis were determined with the Quantitative Insights into Microbial Ecology (QIIME) pipeline (http://qiime.org/).

3.8 Data handling

The following methods and equations were used to analyse reactor performance.

3.8.1 Bulk residual concentrations

Residual sulphate, sulphide and VFA concentrations reported refer to the average measurements from the four ports sampled (FT, FB, BT, BB). As an exception, for the acetate reactor unit in Studies II and III only the BT and BB ports were averaged as is detailed in Chapter 5.

3.8.2 Sulphate conversion

The percentage of sulphate converted was calculated as follows:

$$Sulphate\ converted = \frac{S_{feed} - S}{S_{feed}} \times 100$$

Equation 3-1

where S_{feed} represents the feed sulphate concentration and S represents the residual sulphate concentration measured in reactor samples.

3.8.3 Expected sulphide produced

Expected sulphide levels in the reactor bulk volume (mg/L) were determined by calculating the theoretical sulphide produced stoichiometrically from the amount of sulphate converted (mg/L) at the same time point based on a 1:1 molar ratio.

$$Expected\ sulphide = (S_{feed} - S)\ \times \frac{31.1}{96.1}$$

Equation 3-2

where S_{feed} and S represent the feed and bulk residual sulphate concentrations respectively in mg/L. The channelling of sulphide to elemental sulphur in the biofilm is not considered in the expected sulphide levels; therefore, the difference between the expected and observed sulphide is indicative of the sulphide expected to have been incorporated into the biofilm. The total expected sulphur in the biofilm was calculated as described in Section 3.8.7.

3.8.4 Feed to the acetate unit of the sequential reactor system

Influent sulphate, sulphide and VFA concentrations for the acetate reactor unit in the sequential reactor system were taken as the pre-effluent levels of the lactate unit. The influent sulphide was thus added to the expected sulphide produced in the acetate unit as calculated via Equation 3-2 to determine the total expected sulphide.

3.8.5 Volumetric sulphate reduction rate and volumetric VFA utilisation rates

Volumetric lactate, acetate and propionate utilisation rates (VLUR, VAUR and VPUR, respectively) as well as volumetric sulphate reduction rates (VSRR) were calculated as follows:

$$r_s = (S_{feed} - S)D$$

Equation 3-3

where r_s represents the respective rate, S_{feed} and S represent the feed and residual substrate levels, respectively, and D represents the dilution rate.

3.8.6 Recovered elemental sulphur in the FSB

The mass of elemental sulphur recovered from each biofilm harvested was determined with the mass of FSB recovered and the percentage sulphur of the FSB as determined by CHNS analysis (Section 3.7.6):

$$Mass_{elemental\ sulphur}\ (g) = Mass_{FSB} \times Percentage\ sulphur_{FSB}$$

Equation 3-4

3.8.7 Expected elemental sulphur in the FSB

The amount of sulphur which accumulates in the FSB is a function of the expected sulphide produced by sulphate reduction across the duration of each run together with the observed sulphide that exited the reactor in the effluent. As sulphide levels typically increased as the experimental run progressed, an average of all the time points taken across each run could not simply be used due to sampling times not being evenly spaced across each run. To account for somewhat unevenly spaced sampling times over a run, the time between data points was taken into account and the predicted amount of sulphur channelled to the biofilm was determined between each data point and summed as shown over the following equations. Equation 3-5 shows the amount of sulphide expected to have been produced between two given data points.

$$HS^-_{expected}\ (mg) = HS^-_{expected} \times (T_n - T_{n-1}) \times D \times V$$

Equation 3-5

where $HS^-_{expected}$ is the concentration of sulphide expected in the bulk from sulphate reduction (mg/L), T_n and T_{n-1} represent timepoint n and $n-1$ (days), D represents the dilution rate ($days^{-1}$) and V represents

the volume of the reactor (L). Equation 3-6 shows the amount of sulphide which has exited the reactor between two data points.

$$HS^-_{effluent}\ (mg) = HS^-_{effluent}\ \times\ T_n - T_{n-1} \times D\ \times V$$

Equation 3-6

where $HS^-_{effluent}$ is the concentration of sulphide leaving the reactor in the effluent (mg/L).

The amount of sulphide calculated via Equation 3-5 and Equation 3-6 for an experimental run gives the total expected sulphide produced and total sulphide having exited the reactor for a given run respectively (Note: when T_n is the first data point of the run, T_{n-1} is taken as the starting time point of the run). Therefore, the expected sulphur within the FSB was defined by:

$$Expected\ FSB\ sulphur\ (mg) = total\ HS^-_{expected}\ - total\ HS^-_{effluent}$$

Equation 3-7

These calculations assume that all the expected sulphide produced that is not accounted for in the sulphide exiting the reactor in the effluent is steered to elemental sulphur within the biofilm. It does not consider other sulphur species or colloidal sulphur suspended in the bulk volume. Thus, any difference between the expected and observed elemental sulphur in the FSB represents the gap in the sulphur balance, potentially occupied by these colloidal and alternate sulphur species.

Chapter 4 Propionate as a potential electron donor for biological sulphate reduction

4.1 Introduction

As highlighted in Section 2.3.2, one of the key issues holding biological sulphate reduction (BSR) back as a popular treatment option for sulphate laden waters, is the cost involved in feeding systems with an effective electron donor for the sulphate reducing bacteria (SRB). Volatile fatty acids (VFAs) such as lactate and acetate are commonly used. VFAs are typically chemically synthesised from fossil fuels but in the past two decades there has been an increase in focus on VFAs produced sustainably from the fermentation of certain organic matter (Bhatia and Yang, 2017). Digestate from various sources of anaerobic digestion (AD) including that of microalgae, food waste and plant waste, is comprised of different ratios of VFAs (Chang *et al.,* 2010; Harrison *et al.,* 2014; Wang *et al.,* 2014; Inglesby *et al.,* 2015). In particular, acetate, propionate and butyrate are the dominant VFAs generated. Depending on the conditions and extent of the AD process, the relative proportions of the VFAs propionate, acetate and butyrate can vary (Harrison *et al.,* 2014). Propionate has not been studied a great deal compared to its VFA counterparts acetate and lactate; however, it has been reported that propionate utilising SRB have relatively fast growth rates and would outcompete syntrophic propionate degraders (Muyzer and Stams, 2008). In some cases, propionate was preferred over acetate for sulphate reduction (Oude Elferink, Vorstman, *et al.,* 1998).

BSR has been carried out using microalgal digestate, containing propionate in the range of 500 – 2000 mg/L, along with acetate and butyrate in varying proportions (Harrison *et al.,* 2014). Preliminary success was shown, particularly at low dilution rates. Motleleng (2020) continued to study BSR using algal digestate by investigating CSTRs fed with this mix of VFAs, and consistently achieved 90% sulphate conversion from a five-day to a two-day hydraulic residence time (HRT). The aim of this study is to expand on understanding of the potential of propionate as an electron donor to improve approaches to effective resource utilisation in BSR. This component of the study focuses solely on propionate to determine the kinetics and characteristics of propionate utilisers to gain better insight into propionate use by a mixed SRB community and thereby inform effective use of this electron donor. The chapter initially describes the colonisation and start-up period of a propionate-fed linear flow channel reactor and the strategies followed to establish a strong SRB community. The resulting microbial community was then investigated. Lastly, the performance of the reactor was ultimately studied via a hydraulic residence time (HRT) study to give insight into the potential and effectiveness of propionate over a range of dilution rates.

4.2 Reactor start-up

The rational for this study was to colonise the LFCR with a culture rich in propionate-using SRBs. Based on the assumption that once the reactor was filled with culture, the communities of microbes present could begin colonising the carbon microfibre supports immediately, it was deemed necessary to start the reactor using a culture with an active propionate utilising SRB community to decrease non-SRB colonisation of the microfibres. In order to culture a community of propionate utilisers, a diverse inoculum as described in Section 3.5.1 was gathered to set up propionate-fed batch culture bottles. These were sub-cultured four times on a propionate supplemented feed over a three-month period before being used to start a propionate-fed LFCR. Propionate utilisation was observed alongside decreasing sulphate levels and accumulation of sulphide and acetate (data not shown). Once started, the LFCR was operated as a “closed” system, with an airtight lid to maintain anaerobic conditions until stable sulphate reduction was observed. The reasons for this were two-fold. Firstly, it minimised any oxygen entering the system which could negatively impact the SRB and lead to more oxygen tolerant microbes colonising the microfibres in their place. Secondly, it allowed higher sulphide levels to be maintained by preventing any sulphide oxidation and the formation of a floating sulphur biofilm (FSB). This created a more selective environment for SRB which have a higher tolerance to sulphide as compared to other anaerobic bacteria (Celis-García *et al.,* 2004; Greben *et al.,* 2005). Although not run as a comparative study, the changes observed in reactor performance from a closed to an “open” system are discussed.

The LFCR was maintained with a sulphate feed of 1000 mg/L for approximately 180 days of continuous operation allowing a period of acclimatisation and/or adaptation (Figure. B-2). By day 180 of reactor operation in a closed system, residual sulphate levels had stabilised to between 400 and 500 mg/L (Figure 4-1). The expected sulphide (based on sulphate reduced) and observed sulphide converged representing a closing of the sulphur balance. This shows that there was little unaccounted for sulphur being channelled to other sulphur species. The significant discrepancy between expected sulphide (based on sulphate reduced) and observed sulphide between days 170 to 180 was attributed to an air leak which lead to some formation of an FSB. The reactor was kept in this state of operation in order to study its performance under anaerobic conditions. On day 246, the reactor lid was removed, allowing air into the headspace of the reactor and thus stimulating full growth of the FSB. Figure 4-1 shows the change from the closed to open reactor operation.

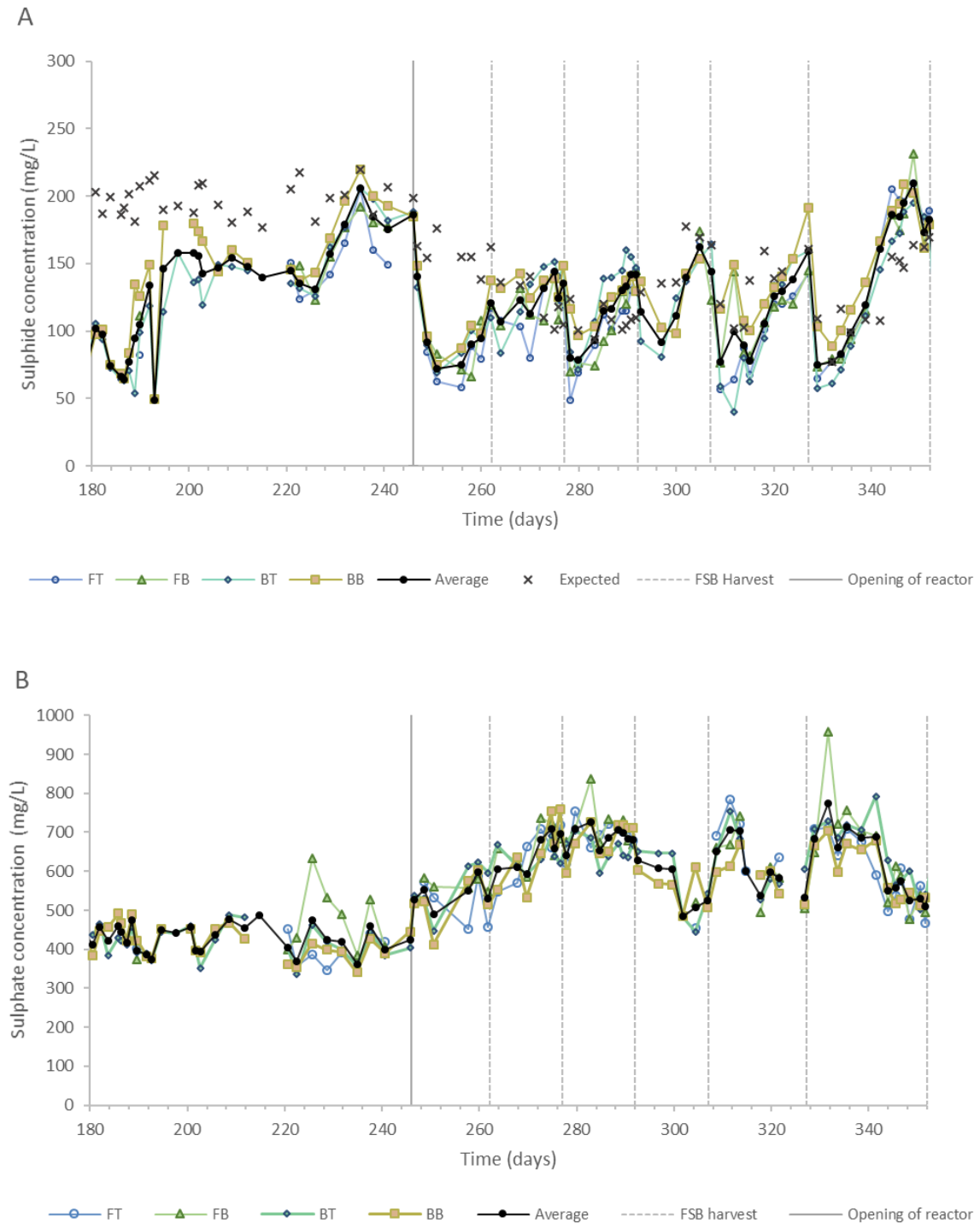


Figure 4-1 Sulphide (A) and sulphate (B) levels within the propionate LFCR for bulk reactor samples for the period before and after opening the reactor headspace (indicated by the solid vertical line), at a 5-day HRT. Front top (FT), front bottom (FB), back top (BT) and back bottom (BB) sample ports are shown. Dotted lines are indicative of harvesting of FSB and thus the end of a cycle run.

Following the period of anaerobic operation shown in Figure 4-1, the lid was removed and the reactor operated as an open system to enable it to be evaluated as a hybrid reactor as described by Marais *et al.* (2020). As mentioned in Section 1.2 however, for the purposes of this chapter, only the BSR component of the reactor will be evaluated. Sulphate reduction within the bulk volume was negatively impacted by the change in operating conditions, resulting from the ingress of oxygen. Measured residual

sulphate concentrations increased for approximately 30 days from 425 to 730 mg/L. Thereafter bulk sulphate concentrations stabilised, during the third cycle of biofilm formation, before beginning to decline over the duration of the fourth cycle. However, the sulphate concentration did not decrease to the same extent as in the anaerobic setup, reaching a low of 485 mg/L residual sulphate (Figure 4-1).

Due to the nature of operation of the LFCR, with cycles of FSB formation following each FSB harvest, typical trends are expected as described in Section 2.6.3. Within each experimental run these trends include a gradual increase in sulphide concentration within the bulk volume from its decrease following FSB harvest up until FSB harvest. Following this, a sharp decline in sulphide concentration is seen as an oxygen influx following FSB harvest promotes high levels of partial sulphide oxidation to sulphur and the FSB is reformed. However, for a period of time following the switch to the open system, several cycles - shown in Table 4-1 (time course data in Figure. B-4 and Figure. B-5) – were marked by significant increases in sulphate alongside these steep drops in sulphide concentration. This simultaneous increase in sulphate demands the question of whether this is due to an inhibition of SRB activity with the oxygen ingress into the system during the early stages of biofilm reformation, or some complete oxidation of sulphide back to sulphate (Equation 2-13), or both. The stable redox potential of the system during this period (-250 to -350 mV, Figure. B-3) indicates that the system maintained a highly anaerobic environment, suitable for SRB growth and activity (Pepper and Gentry, 2015). However, it is possible that the active SRB in the community were particularly sensitive to oxygen. Table 4-1 explores in more detail the predicted outcome of both extremes: firstly, the predicted rise in sulphate if complete oxidation back to sulphate were to account for all the sulphide lost, or secondly, the predicted sulphate due to the sulphate feed if SRB were completely inhibited. The data shows that either complete sulphide oxidation or SRB inhibition could solely account for the sulphate increase observed. It also shows that in most cases at least some of the sulphide is partially oxidised as sulphate levels did not increase to the level predicted if all sulphide was fully oxidised. This rise in sulphate upon harvesting differs from previous work done in a similar, lactate-fed LFCR (Marais *et al.,* 2020). Here, sulphate and sulphide levels were monitored for a 24-hour period post harvesting the FSB; while sulphide levels dropped, sulphate levels were seen to remain stable. Sulphide levels in Marais *et al.*'s study were similar to those observed here, suggesting that the effect lies within the difference in the microbial community that lactate or propionate support, with propionate utilisers being more sensitive to oxygen.

Table 4-1 Correlation of sulphide and sulphate levels post FSB harvesting in propionate-fed reactor. Data quoted for pre-FSB harvest was taken the day of harvesting, while data quoted for post-FSB harvest was taken within the next 48 hours. Time points indicated can be related to time course performance data shown in Figure. B-4 and Figure. B-5. Data represents the mean ± S.D, n=4.

Reactor run time (days)		Period between data points (h)	Sulphate loading (mg/L/h)	Sulphate increase observed (mg/L)	Sulphate increase predicted (mg/L)	
Pre-FSB harvest	Post-FSB harvest				via complete oxidation of all sulphide lost	via complete SRB inhibition
307	309	42	21	101	221	171
327	329	46	21	150	245	184
352	354	46	24	104	227	225
369	370	22	24	139	119	59

The change to the hybrid reactor configuration also affected VFA profiles which were monitored for the period before opening of the reactor headspace and for three cycles afterwards, albeit once sulphate levels had begun to recover (Figure 4-2).

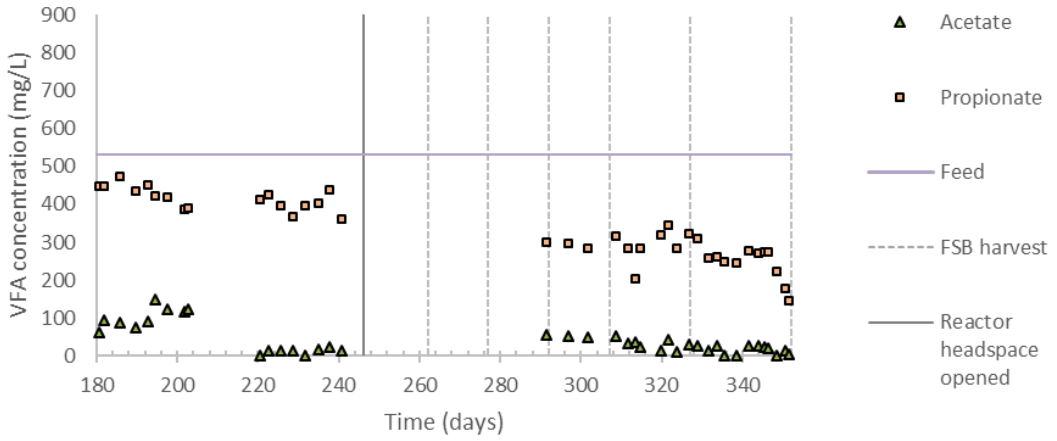


Figure 4-2 Volatile fatty acid (VFA) concentration profiles for periods before and after opening of the reactor headspace.

Surprisingly, despite a significant level of sulphate reduction, there appeared to be little propionate utilisation under the closed system, and the amount utilised could not account for the sulphate reduced (Figure 4-1, Figure 4-2). The explanation for this could be two-fold, involving various pathways that could be taking place (Figure 4-3) and the utilisation of acetate for BSR.

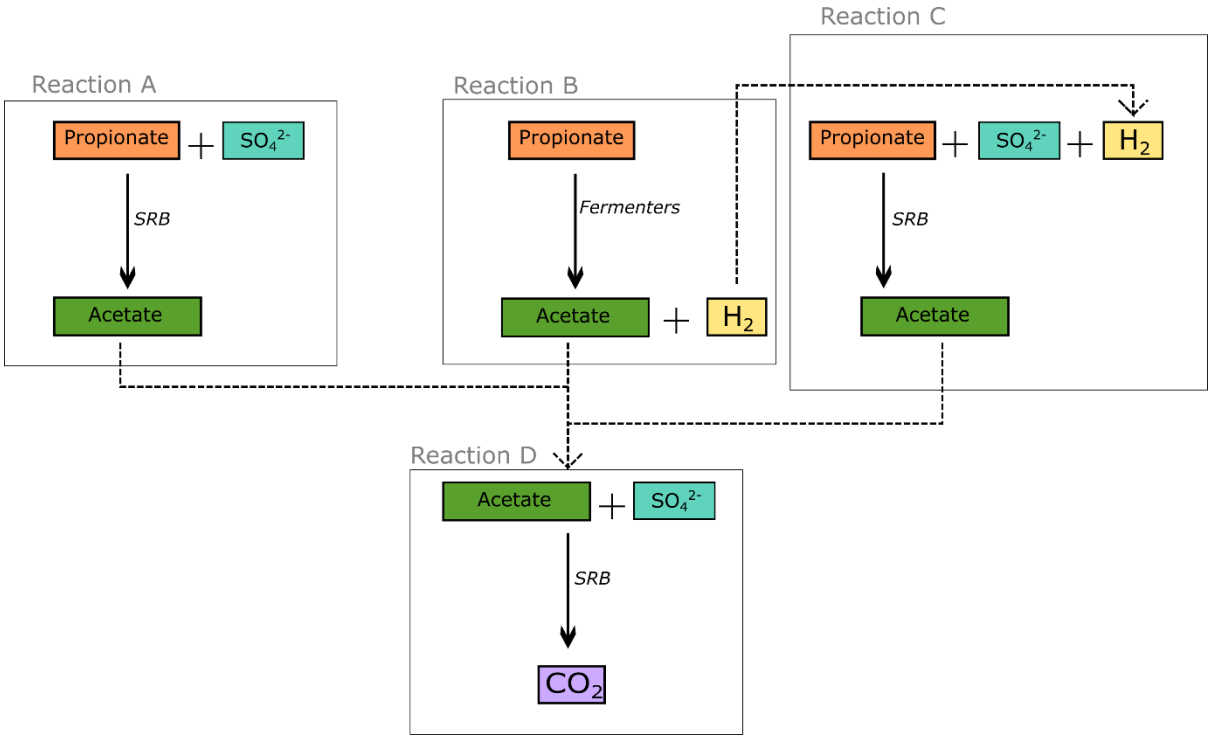


Figure 4-3 Schematic of possible routes of VFA utilisation in the propionate reactor.

Firstly, the propionate utilised - albeit a small amount – would have resulted in the production of acetate (Figure 4-3). As acetate levels remained consistently low, and from approximately day 220 there was near complete utilisation of acetate upon production. Secondly, a further explanation to this could lie with the 0.4 g/L yeast extract (YE) incorporated in the media as a source of amino acids and micronutrients. Hessler (2020) found that this amount of YE appeared to produce 270 mg/L of acetate in similar systems and with the same feed as used in this study. This was deduced from the differences in acetate in the reactor systems across a change in YE concentration from 1g/L to 0.4 g/L (Hessler, 2020). This deduction was supported in the mentioned study by multi-genome sequencing of the reactor communities, which showed the presence of amino acid metabolising pathways. The propionate reactor was in fact colonised in part using inoculum from the reactors used in this study by Hessler (2020) and it has therefore been assumed, in calculations going forward, that the same amount of acetate (270 mg/L) was generated via microbes breaking down the amino acids within the YE. Table 4-2 shows that a significant amount of acetate was used in the reactor as compared to propionate. Taking this, together with the consistently low levels of residual acetate observed, the data suggests that acetate, rather than propionate, was preferentially utilised for BSR, alongside some degree of propionate usage. The significant level of residual propionate remaining suggests a high K_s of the various microbes responsible for propionate utilisation in the system.

Table 4-2 VFA concentration profiles under closed and open reactor systems. The closed system data was taken from days 221 – 241 of operation and the open system data was taken from pseudo-steady-state from the last cycle of a five-day HRT (days 346 to 352). Acetate produced is calculated from propionate and YE metabolism. Data is given ± one SD from the mean, n ≥ 5.

	VFA concentration (mg/L)						
			Acetate produced				
Reactor operation	Residual propionate	Propionate utilised	Propionate metabolism	YE	Total	Residual acetate	Acetate utilised
Closed	394 ± 25	136 ± 25	110 ± 20	270	380 ± 20	18 ± 8	362 ± 21
Open	235 ± 52	295 ± 52	238 ± 42	270	508 ± 42	19 ± 9	489 ± 43

In the open system, a change in VFA utilisation is seen, with an increase in both propionate and acetate utilisation (Table 4-2). Since there was no increase in sulphate reduced this speaks to different pathways occurring in the reactor. To investigate this further, the sulphate reduced, and the most likely pathways used are looked at more closely in Table 4-3. Propionate can be either oxidised by SRB or fermented as shown Figure 4-3, producing an equimolar amount of acetate (Equation 4-1 and Equation 4-2). Fermentation would offer an explanation to the increased propionate usage with decreased sulphate reduction.

$$\text{Propionate} + 0.75\ SO_4^{2-} \rightarrow \text{Acetate} + 0.75\ HS^- + HCO_3^- + 0.25\ H^+ \quad \text{Equation 4-1}$$

$$\text{Propionate} + 3H_2O + H_2 \rightarrow \text{Acetate} + HCO_3^- + H^+ + 3H_2 \quad \text{Equation 4-2}$$

The likelihood of fermentative bacteria becoming active in the open system is considered in Table 4-3. In this table two possible scenarios are given and the resulting distribution of VFAs for sulphate reduction during pseudo steady state before and after opening of the reactor headspace. In the first case, and on one extreme, the utilisation of propionate and acetate for BSR are shown if we assume that all propionate utilised was channelled to sulphate reduction. In the second case, the VFA utilisations are shown if we assume that the propionate-utilising SRB could at maximum only reduce the same amount of sulphate as in the closed system since the HRT remained the same. In this scenario, the remaining propionate utilised is therefore fermented. In considering the resulting data from each of these situations, no fermentation of propionate would suggest an unlikely situation of a dramatic increase in activity of propionate utilisers following the change in operation from closed to open, whilst the activity of acetate utilisers would be impacted heavily. Additionally, with all the propionate going to sulphate reduction a significant proportion of utilised acetate would be unaccounted for (Table 4-2 and Table 4-3). Under the scenario where propionate fermentation is assumed to take place in the open system,

In this instance, the activity of acetate utilisers remains similar from the closed to open system. The proportions of sulphate reduced via each electron donor and under each environment are similar if propionate fermentation was taking place in this manner. As will be further supported in Section 4.4.2, it seems more likely that largely acetate is being utilised for BSR alongside some use of propionate,

thereby supporting the scenario involving propionate fermentation. An increase in activity of fermentative bacteria in the open reactor system would be consistent with literature supporting that fermentative bacteria are more tolerant to oxygen than are SRB (Xu *et al.,* 2012a). Further, propionate fermenters have been shown to be less tolerant to sulphide as compared to SRB (Maillacheruvu *et al.,* 1996). While higher sulphide levels may have inhibited much fermentative action under the closed system, lower sulphide levels in the open system could have encouraged fermentative activity.

It is worth noting that if fermentation was indeed occurring (Figure 4-3 Reaction B) then this avails hydrogen as produced in this pathway for use in the system via Reaction C (Equation 4-3). BSR was otherwise assumed to have occurred via Reaction A (Figure 4-3, Equation 4-1).

$$\text{Propionate} + SO_4^{2-} + H_2 \rightarrow \text{Acetate} + HS^- + HCO_3^- + H_2O \qquad \text{Equation 4-3}$$

Hessler (2020) comments on this in detail and shows hydrogenases found in both non-SRB and SRB, supporting the exchange of hydrogen between these communities in the systems used. Interestingly, this pathway of BSR is more efficient in terms of the ratio of VFA utilised to sulphate reduced as compared to the pathway without hydrogen (one mole of propionate used per mole of sulphate in Equation 4-3 versus one mole of propionate per 0.75 mole of sulphate in Equation 4-1).

Table 4-3 Possible VFA utilisation pathways with respect to sulphate reduction. The proportion of sulphate reduced via each electron donor is given as a percentage of the total sulphate reduced. Closed system data was taken from days 221 – 241 of operation and open system data was taken from pseudo-steady-state from the last cycle of a five-day HRT (days 346 to 352). Data is given ± one SD from the mean where possible, n ≥ 5.

Reactor operation	Total SO42- reduced	SO42- reduced assuming no propionate fermentation				SO42- reduced assuming propionate fermentation in open system			
		via propionate		via acetate		via propionate		via acetate	
	mg/L	mg/L	%	mg/L	%	mg/L	%	mg/L	%
Closed	586 ± 37	135 ± 24	23	451	77	135 ± 24	23	451	77
Open	461 ± 27	291 ± 51	63	170	37	135 ± 24	29	326	71

4.3 Microbial analysis

The 16S rRNA gene is made up of sections of conserved and variable sequences. This gene can be amplified via polymerase chain reaction (PCR) using primers that are designed to bind to conserved regions of the gene with variable regions within the amplified sequence. Sequencing of the variable region is carried out for phylogenetic analysis.

Soon after reactor start up, samples were taken for microbial analysis of the planktonic community via 16S rRNA gene sequencing. Microbial analysis thereby allowed determination of the microbial diversity achieved after the period of draw and fill sub-culturing and at the beginning of community establishment in the reactor. The planktonic community present at the time of reactor start up gives an indication of SRB available for colonisation of the carbon fibres within the community. The microbes which colonise the carbon fibres are those expected to remain throughout the residence time study as they are decoupled from the HRT.

The reactor was found to be dominated by Proteobacteria with a significant proportion of Synergistetes and Bacteroidetes (Figure 4-4). Proteobacteria contains the class Deltaproteobacteria, which in turn includes the SRB that were observed in the reactor (Figure 4-5). Total SRB abundance averaged 17%; however, the diversity was low with only two SRBs above 1 % relative abundance being identified. *Desulfomicrobium* (15.1%) made up the large majority, with small proportion of *Desulfobacter* (1.2%) present. Results shown in Section 4.2 suggest that at least one of these species was able to utilise propionate for BSR, however no SRB that have previously been shown to be strong propionate utilisers - *Desulfobulbus* species in particular, as described Section 2.4.3 - were observed above 1 % relative abundance. It has been previously observed that on the whole, a low number of SRB have been identified as propionate utilisers (Hao *et al.,* 2014). Both genera that were present have been associated with, among other electron donors, acetate oxidation (Laanbroek and Pfennig, 1981; Rotaru *et al.,* 2015; Hessler, 2020). *Desulfobacter* has also been associated with ethanol utilisation (Laanbroek and Pfennig, 1981; Icgen and Harrison, 2006). The *Desulfobacter* OTU was classified as *Desulfobacter vibrioformis,* which has been shown to grow on acetate and not on several other electron donors tested (Lien and Beeder, 1997). *Desulfomicrobium* also associated with several other electron donors including lactate, formate, hydrogen and ethanol (Sharak Genthner *et al.,* 1994; Krumholz *et al.,* 1999; Dias *et al.,* 2008).

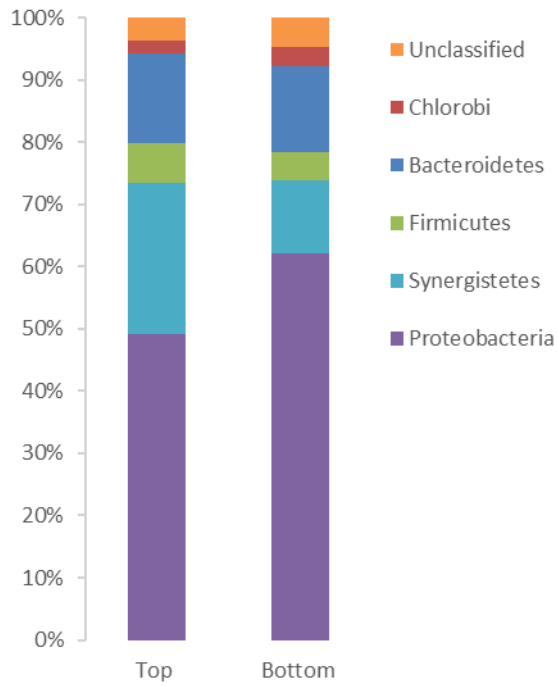


Figure 4-4 Microbial diversity within the propionate reactor at a phylum level. Relative abundance of phyla present at > 2% abundance are shown. Planktonic samples were taken from the top and bottom of the bulk volume at the beginning of reactor operation (Day 5).

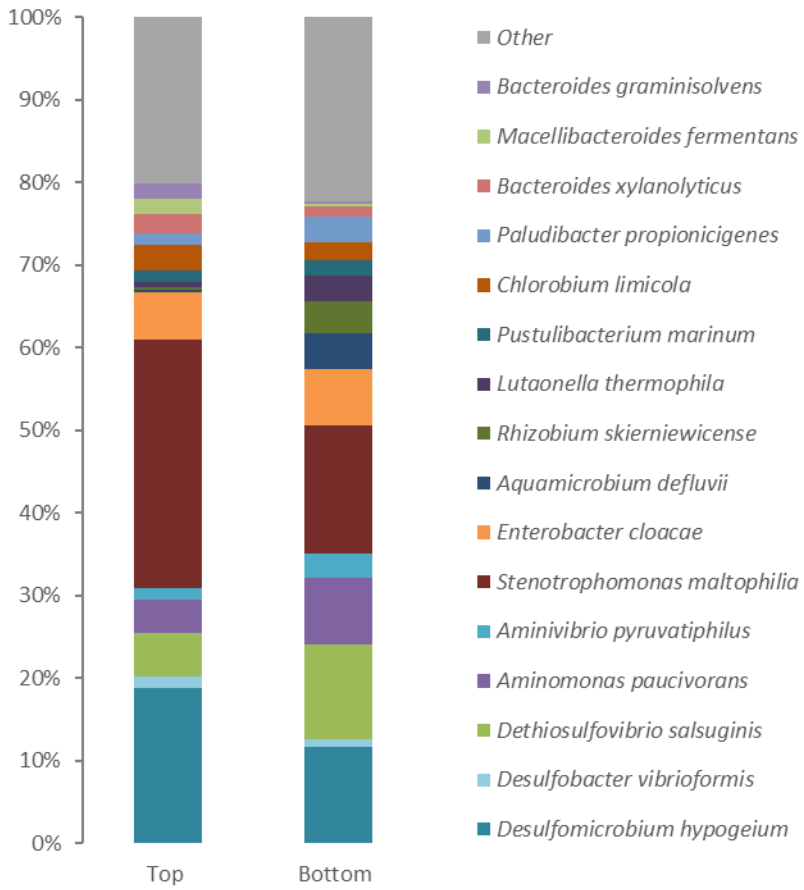


Figure 4-5 Relative abundance of operational taxonomic units (OTUs) within the propionate reactor at >1% abundance in one or both of the samples. SRB are shown at the bottom in blue. Samples were taken from the top and bottom of the reactor volume at the beginning of reactor operation (Day 5).

The data here can be compared with a study where algal anaerobic digestate comprising of acetate, propionate and butyrate was found to sustain a far more diverse SRB community of up to 13 species (Motleleng, 2020). Propionate utilisation was attributed to largely to *Desulfobulbus oligotrophicus* and to some extent with *Desulfarculus baarsii*. Further, Motleleng (2020) showed that the algal digestate, which contributed a mixed VFA feed, sustained a more diverse SRB community as compared to a single electron donor of lactate. The microbial analysis performed in here, together with performance data of the batch culture inoculum (Figure. B-1) and the reactor start-up period (Figure 4-2) suggest that retention of propionate utilisers from the batch culture inoculum to the reactor may have been limited. It is possible that the propionate utilising SRB present in the batch cultures had low growth rates and did not form robust biofilms on the carbon microfibres at the 5-day HRT. Periods between sub-culturing of the batch culture inoculum averaged 17 days and therefore slower growing SRB could have been responsible for propionate degradation seen in the batch culture inoculum (Figure. B-1).

4.4 Hydraulic residence time study

Following successful establishment of a propionate-fed LFCR with stable sulphate reduction, an HRT study was then commenced to more rigorously investigate the propionate utilising SRB. HRT studies are necessary to determine how microbial communities or reactor performance can change with

changing flow rates, and how robust a BSR system is with respect to these changes. Changing flow rate impacts both the volumetric sulphate loading rate and the retention time in the reactor. It also gives a spectrum of data which allows an HRT to be chosen to give the greatest volumetric sulphate reduction rate (VSRR) whilst not compromising sulphate conversion beyond what is desired.

4.4.1 Experimental approach

The system was subjected to increasing dilution rates, beginning with a 5-day HRT, followed by HRTs of four, three and two days. An experimental run of at least four residence times, from one FSB harvest to the next – was carried out for each HRT. Apart from the 2-day HRT run, the reactor was kept at each HRT for several cycle runs and in each case data from the last cycle was used for the HRT study. However, HRT runs differed slightly in duration. This was due to a combination of the impact of harvesting of the biofilm on sulphate levels and the stability of the FSB over longer periods without harvesting. It was found in the initial cycles of biofilm formation and collapse that studies had to be run for 10 - 15 days in order for sulphate levels to have dropped and stabilised back to what they were before biofilm harvest. However, it was also found that the biofilm was not consistently stable for periods of 20 days and longer and risked self-collapsing. Therefore, studies were run for a period of between 15 – 25 days with the data from the last 1.5 residence times in each study being used for the pseudo-steady state data. An extended period of six residence times was used for the 2-day HRT pseudo steady state since this study was prolonged to a total of 10 residence times. Following this pseudo-steady state, the FSB was harvested.

4.4.2 Results and discussion

At an HRT of five days (D = 0.21 $days^{-1}$), residual sulphate levels in the bulk of the reactor averaged 549 mg/L at pseudo-steady state, giving a sulphate conversion of 46% (Figure 4-6, Figure 4-7 C). Sulphate conversion then increased to 53% at a 4-day HRT (D = 0.24), peaking at 58% by a 3-day HRT (D = 0.33 $days^{-1}$). Following this it dropped to 38% by a 2-day HRT (D = 0.50 $days^{-1}$). The VSRR increased linearly from 0.99 mM/day at a 5-day HRT to 1.98 mM/day a 3-day HRT, and remained similar at a 2-day HRT (Figure 4-8). The maximum sulphate conversion and maximum VSRR, therefore, both occurred at a 3-day HRT at a corresponding volumetric sulphate loading rate (VSLR) of 3.42 mM/day. Sulphate levels were generally stable for at least the last 1.5 residence times of each study – the period designated as the pseudo-steady state barring the 2-day HRT study for which the period of pseudo-steady state was increased as previously explained (Figure 4-6). This highest dilution rate showed the least stable sulphate levels, and, as would be expected, the highest levels of residual sulphate (Figure 4-6, Figure 4-7 C). Redox and pH levels remained relatively stable across the HRT study (Figure 4-7 B). Aqueous sulphide concentrations remained steady at approximately 185 mg/L for the first three HRTs, dropping to 130 mg/L alongside the decrease in sulphate conversion at a 2-day HRT (D = 0.50 $days^{-1}$, Figure 4-7 C). Little difference is seen between the expected and observed sulphide indicating that by the time pseudo-steady state was achieved, the FSB was limiting oxygen transfer at the surface of the bulk liquid and little sulphide was channelled to elemental sulphur at this stage of the run. An

increasing trend in sulphide typical of the hybrid LFCR was seen across each HRT study, although this became less pronounced at lower HRTs (Figure 4-6).

The increase in VSRR followed by a levelling off is expected with increasing flow rate, as higher flow rates mean higher loading rates of sulphate and electron donor, therefore activity of SRB will increase until the community reaches its capacity. However, the increase in sulphate conversion with decreasing HRT is counter-intuitive as one would expect a decrease in the fraction of sulphate reduced with an increased flow rate of sulphate into the system as has been seen previously (Oyekola *et al.,* 2010; van Hille *et al.,* 2016). This suggests that the change in flow rate affected either a change in the functioning of the SRB making them more efficient, or a change in the community composition which aided, or removed competition for the SRB. Microbial community analysis throughout the HRT study would be recommended for future studies to determine such changes. Similar trends of increasing sulphate conversion were also observed by Hessler (2020) in two up-flow anaerobic packed bed reactors fed lactate or acetate, as sulphate conversion increased from approximately 40 to 60% in each case over the change from a 4-day to a 2.6-day HRT in the lactate-fed reactor and a 3-day to 2.6-day HRT in the acetate reactor. No microbial changes were observed that could explain this, and these data points were considered by the author as outliers (Hessler, 2020). However, a possible explanation for these observations in their study could have been linked to increased biomass retention.

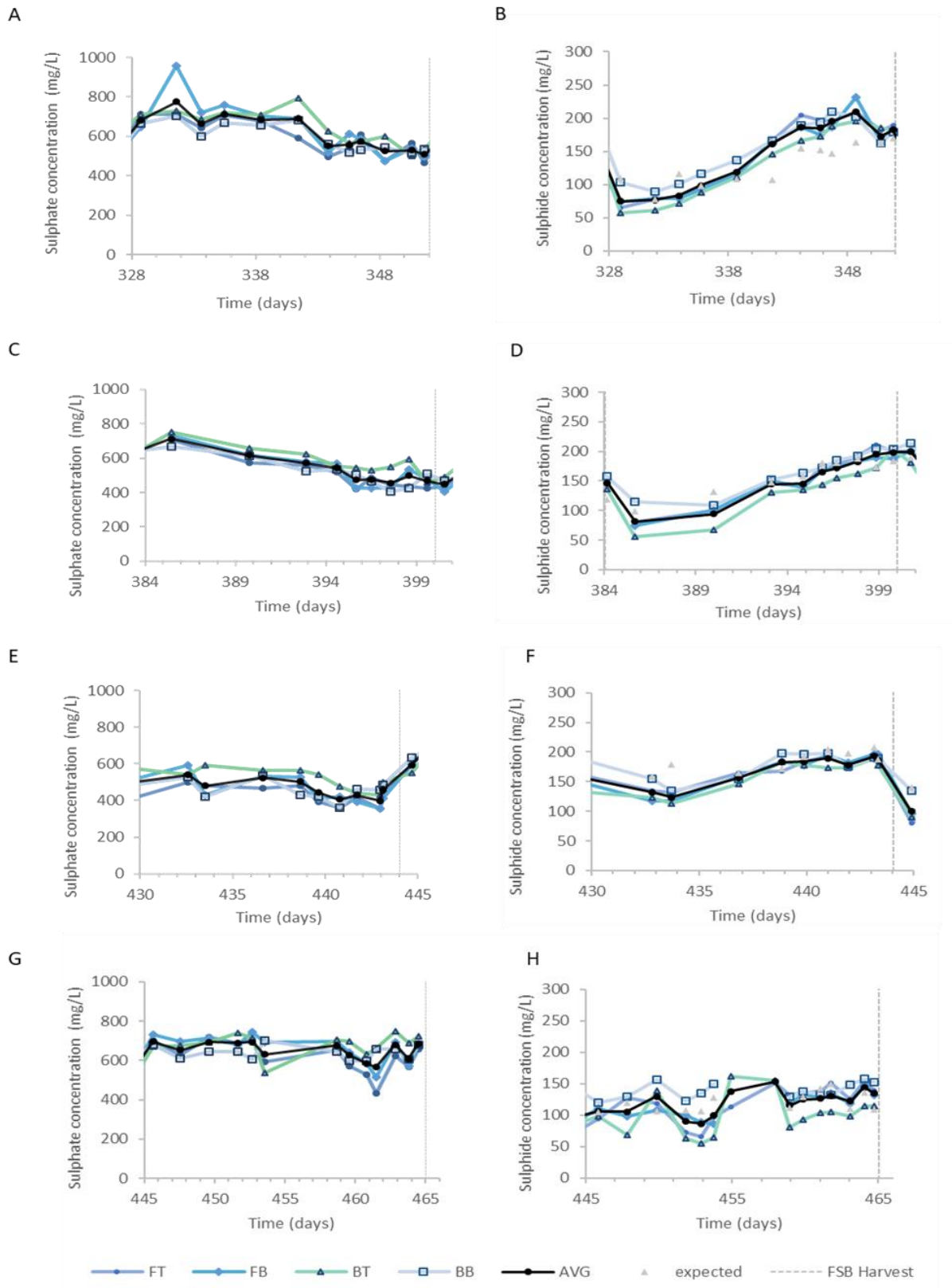


Figure 4-6 Sulphate and sulphide levels across each cycle run for the various HRTs studied: five-day HRT (A and B), four-day HRT (C and D), three-day HRT (E and F) and five-day HRT (G and H). For each HRT one cycle run was used, as shown. Complete time course data across the whole HRT time period can be seen in Appendix B.2.2. Pseudo-steady state was designated by the last 1.5 residence times. For the 2-day HRT pseudo-steady state was taken as the last 4 residence times.

VSRR and conversions achieved can be compared to literature. Similar LFCR systems were run in studies performed by Marias (2020) and by Hessler (2020) on feeds supplemented with lactate or acetate as the electron donor. The reactors were all colonised from the same initial inoculum (Section 3.1) and therefore these studies provide a good basis for comparison to the current study. At a 3-day HRT – where VSRR peaked in this study (1.98 mM/day) – and at the same VSLR, Hessler (2020) reported VSRRs of 1.7 mM/day for both a lactate-fed and an acetate-fed, closed, fully anaerobic LFCR. Marais (2020), using the same reactor configuration as reported here, observed VSRRs at the same HRT and VSLR of 3.4 mM/day in a lactate-fed LFCR and 1.4 mM/day in an acetate-fed LFCR. Maximum VSRRs for an acetate-fed, closed LFCR however, of up to 4.3 mM/day were achieved at HRTs as low as one day (Hessler, 2020). A good comparison can also be made with CSTR reactors studied by Motleleng (2020) which were operated with biomass retention by filtration and recycle and fed on an algal digestate mix of propionate, acetate and butyrate; this system reached VSRRs of 3.2 mM/day at a three-day HRT, with maximum VSRRs of approximately 10.4 mM/day at a 0.5-day HRT. In the study presented here, VSRR remained stable from a three-day to a two-day HRT; the data suggests that the true optimal HRT for highest VSRR without heavily impacted sulphate reduction percentages may occur somewhere between a 2- and 3-day HRT and further fine-tuning should be investigated. Data also indicates that further decrease in HRT below two days would be at the detriment of sulphate conversion (Figure 4-8).

As was seen in Section 4.2, Figure 4-7 also shows near complete utilisation of acetate, evident across the HRTs, with residual concentrations ranging between 2 mg/L at a 3-day HRT (0.33 $days^{-1}$) and 50 mg/L at a 2-day HRT (0.50 $days^{-1}$). Propionate on the other hand was seen to accumulate with decreasing HRT. Unpacking the fate of propionate and the acetate produced in the system again proves to be tricky and is attributed to the complexity of the microbial community and competing reactions taking place between fermentative microorganisms and SRB (Figure 4-3). However, to further explore both the sulphate reduction performance and the VFA utilisations, Figure 4-8 compares the rates of sulphate reduction to the VFA utilisation rates across the residence time study. From a 5-day HRT (0.21 $days^{-1}$) to a 3-day HRT (0.33 $days^{-1}$), a sharper increase in VSRR compared to volumetric acetate utilisation rate (VAUR) is observed, while volumetric propionate utilisation rate (VPUR) decreases slightly over the same period. Therefore, the increase in VSRR was attributed to increased acetate utilisation. However, since an equimolar amount of acetate and sulphate are consumed in BSR via acetate utilisation (Equation 2-8), the sharp increase in VSRR with less pronounced increase in acetate consumption is interesting. It could suggest an increase in the efficiency of BSR with the respective VFA being utilised. As described in Section 4.2, this could be possible if hydrogen generated from the fermentation of propionate allowed BSR via both hydrogen and propionate (Equation 4-3) where a one to one molar ratio of propionate to sulphate is employed, as opposed to Equation 4-1 where the same ratio is one to 0.75 (also see Figure 4-3, reactions B and C). Alternatively, the generated hydrogen could itself be an electron donor via the equation below:

$$4\,H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$$ Equation 4-4

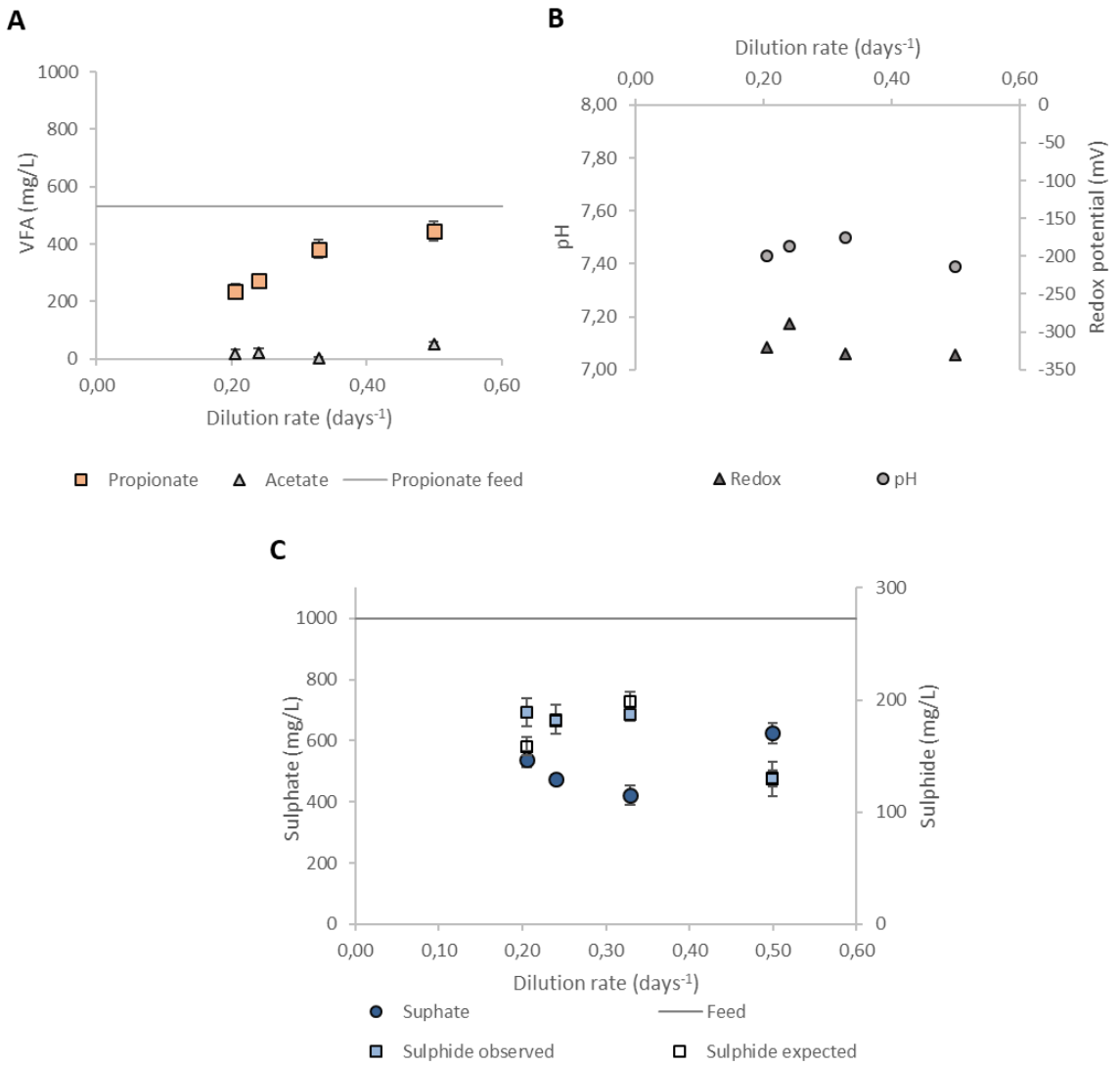


Figure 4-7 Pseudo-steady state solution chemistry data showing A: residual VFA levels, B: pH and redox and C: sulphide and residual sulphate levels for the increases dilution rates tested which correspond to HRTs of five, four, three and two days in the propionate LFCR. Error bars represent one standard deviation ± the mean, n ≥ 4.

Further, the SRB shown in Section 4.3 to be most abundant – *Desulfomicrobium hypogeium* – has been previously reported to utilise hydrogen for BSR (Krumholz *et al.,* 1999). The likelihood of increased fermentation, and therefore hydrogen, would depend on the μ_{max} of the fermenters compared to the SRB. Under a lactate environment, SRB have been shown to outcompete fermentative bacteria under lower dilutions rates, but not at higher dilution rates, due to a lower μ_{max} and lower K_s (Oyekola *et al.,* 2010, 2012b). Maillacheruvu *et al.* (1996) supported this finding in a propionate environment as well, showing propionate fermenters to have higher growth rates than propionate-utilising SRB. This suggests that despite a slight decrease in overall propionate utilisation, more propionate is being fermented at higher dilutions rates. Oude Elferink *et al.* (1998) observed competition between fermenters and SRB for propionate, with just under half the propionate utilised going to fermentation (study conditions of 200 mg/L sulphate, 1700 mg/L COD, of which 300 was propionate, HRT of 4.6 h). However, their study reported at least one known propionate utilising SRB – *Desulfobulbus propionicus* – not observed in the community investigated here at an abundance above 1 % (Figure 4-5), and

propionate was preferred over acetate for BSR (Oude Elferink, Vorstman, *et al.,* 1998). Batch culture work done by Laanbroek and Pfennig (1981) suggested simultaneous usage of propionate and acetate for sulphate reduction with complete depletion of propionate followed by a decrease in sulphate reduction until acetate was also completely depleted. Their study showed that propionate utilisers are able to scavenge propionate well. Again, the presence of a *Desulfobulbus* species was reported (Laanbroek and Pfennig, 1981). Few maximum specific growth rates are reported for either propionate oxidising SRB or propionate fermenters; O'Flaherty *et al.* (1998) reported a μ_{max} of 3.65 days^{-1} for a propionate utilising SRB and Heyes and Hall (1983) reported growth rates of 0.13 days^{-1} and 1.2 days^{-1} for two propionate utilising SRB, however microbial analyses were not performed in these studies. El Houari *et al.* (2017) reported growth rates for *Desulfobulbus oligotrophicus* of between 0.29 and 0.58 days^{-1}. Some of these lower growth rates may support wash out of propionate-utilising SRB in our system, leading to the steady to slight decrease in VPUR observed in Figure 4-8. If washout does occur, this implies that the propionate utilisers were mostly active in the planktonic phase and that the propionate utilisers had slower growth rates than the acetate utilisers in this system.

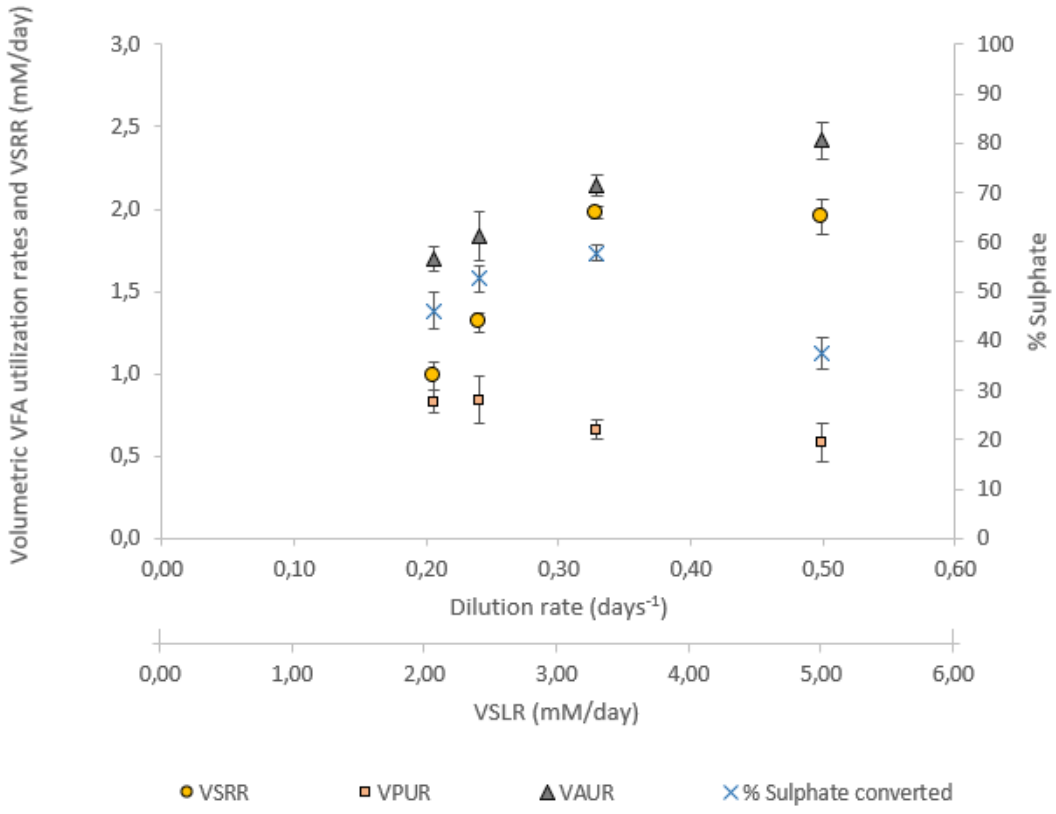


Figure 4-8 Volumetric sulphate reduction rates (VSRR), volumetric propionate utilisation rates (VPUR), volumetric acetate utilisation rates (VAUR) and percentage sulphate conversion with increasing dilution rate. Error bars represent one standard deviation ± the mean, n ≥ 4.

It is also possible that the steep increase in VSRR is due to an increase in the proportion of either propionate or acetate being used for BSR. It is unlikely that more propionate was channelled to sulphate

reduction as residual propionate remained high, indicating that fermentative bacteria and SRB were not competing for propionate. There may have been competition for acetate; non-SRB utilisation of acetate under an anaerobic environment could be due to the activity of methanogens or potentially the less common syntrophic acetate oxidisers (Schwarz *et al.,* 2007). Methanogenic activity is not expected as the batch cultures used to establish the reactor were treated with a methanogen inhibitor as described in Section 3.5.1. Additionally, SRB have been reported to outcompete methanogens at low acetate loading rates (Yoda *et al.,* 1987; Muyzer and Stams, 2008). SRB have been described as efficient scavengers of electron donors, including acetate and lactate, implying that the decrease in residual acetate to negligible levels observed by a three-day residence time is likely due to SRB action (Oyekola *et al.,* 2012; Hessler, 2020).

Figure 4-9 investigates from a different angle which sulphate reduction pathways are more likely or dominant. The figure shows the amount of propionate or acetate utilisation required if all sulphate is reduced by solely propionate (A) or acetate (B), alongside the actual propionate or acetate utilisation observed. It is clear that there is a stark gap between the amount of propionate needed to account for the sulphate reduced, and the actual amount that was observed to have been utilised. On the other hand, the amount of acetate predicted to have been utilised shows that acetate utilisation alone could account for all of the sulphate reduced (Figure 4-9 B). Also evident is the significant degree of unaccounted for acetate utilisation as implied previously, particularly at the higher HRTs.

Essentially, a syntrophic relationship is suggested as has previously been observed between fermenters and SRB developed (Greben *et al.,* 2005) whereby propionate fermentation produces acetate for utilisation by SRB. Whilst not being able to conclusively tell what proportion of propionate was oxidised for sulphate reduction versus being fermented, the data strongly suggests that acetate was oxidised for sulphate reduction preferentially over propionate with potential utilisation of hydrogen as the HRT decreased.

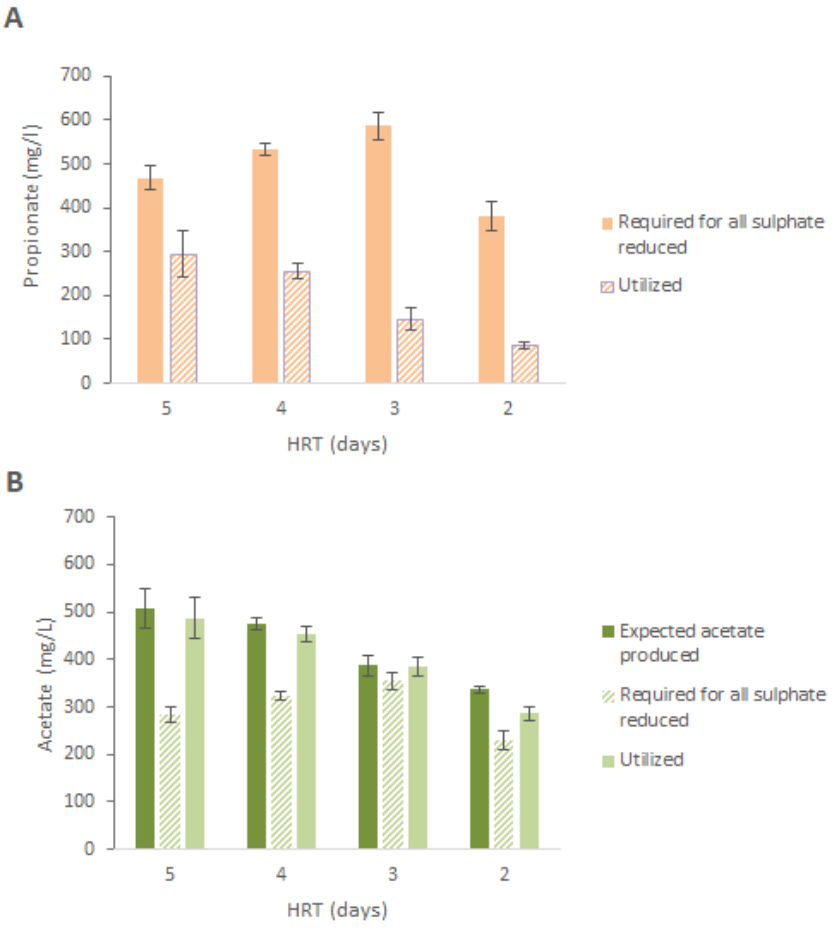


Figure 4-9 Propionate and acetate utilisation comparisons. A: The amount of propionate that would theoretically have been needed if all the sulphate reduced at each HRT was reduced via propionate oxidation alone, is shown alongside the observed amount of propionate utilised. Propionate feed concentration was 530 mg/L. B: The total predicted amount of acetate produced (from propionate and YE metabolism), the amount of acetate that would theoretically have been utilised for BSR if all sulphate reduction occurred via acetate oxidation alone, and the amount utilised acetate. Error bars indicate one SD ± the mean, n ≥ 4.

4.5 Conclusions

In conclusion, acetate produced from propionate metabolism appeared to be used preferentially to propionate itself for BSR in this study. Some propionate was channelled to sulphate reduction but most was utilised by fermentative bacteria. Viewing this in light of the of the microbial community analysis of the inoculum, it was clear that SRB abundance and diversity was limited and the dominant SRBs did not include the genus most associated with propionate utilisation in the literature. Kinetic data for the use of propionate by the SRB observed here are not available; growth rates of propionate utilisers in literature range widely from 0.13 to 3.65 $days^{-1}$. The observed propionate utilisation led to the production of acetate, providing the feedstock for BSR with further utilisation of COD, as well as demonstrating a syntrophic relationship between the propionate fermenters and the acetate utilising SRB. The system however showed some inefficiency in this regard with not all the propionate being utilised, potentially due to the fermentative bacteria having a high K_s, and resulting in high inorganics remaining in the effluent. For propionate to be used successfully it is important to ensure the colonisation and immobilisation of the correct propionate utilising SRB, as these seem low in diversity. It is recommended

that a study involving different support matrices, with low dilution rates at the time of inoculation, could be of benefit.

Chapter 5 Development of a sequential linear flow channel reactor system

5.1 Introduction

This chapter explores the potential of reactors-in-series to address the challenge of acetate accumulation in biological sulphate reducing systems, which use longer chain or more complex electron donors such as butyrate, lactate, ethanol and propionate. These are typically incompletely oxidised and give rise to acetate during BSR (Colleran *et al.,* 1995; Liamleam and Annachhatre, 2007; Muyzer and Stams, 2008). Lactate has been extensively studied as an electron donor for sulphate reduction, with good sulphate conversions observed due to the action of incomplete oxidisers (Oyekola *et al.,* 2010; Bertolino *et al.,* 2012; Hessler *et al.,* 2018; Marais *et al.,* 2020). This is typically associated with the accumulation of acetate, indicating the absence of acetate-utilising sulphate reducing bacteria (SRB) in these communities (Lens *et al.,* 2002; Liamleam and Annachhatre, 2007; Celis *et al.,* 2013; Hao *et al.,* 2014). This contributes to undesirably high residual chemical oxygen demand (COD) in effluents with associated negative environmental impacts.

SRB which utilise acetate for BSR and produce carbon dioxide are complete oxidisers. SRB are often divided into incomplete oxidisers and complete oxidisers, and clearly complete oxidisers do not typically function alongside incomplete oxidisers. This may be explained by their growth rates. Incomplete oxidisers are often faster growing compared to complete oxidisers (Tang *et al.,* 2009) and the oxidation of acetate has been reported as the rate-limiting step in sulphate reducing systems run on acetate (Kaksonen *et al.,* 2006). This sheds light onto why acetate utilisers are not seen to develop in SRB communities fed with higher chain VFAs in a well-mixed reactor.

To address this challenge, this study employs a sequential reactor system, with two separately enriched communities for lactate or acetate. Two linear flow channel reactors (LFCRs), which had each been run previously for several years on either lactate or acetate, were connected so that the acetate-rich effluent from the lactate reactor is fed into the acetate reactor to form one compartmentalised system. This was hypothesised to result in both a lower COD in the effluent and, more importantly, more sulphate reduced per lactate supplied, making the process more cost-effective. This is particularly important as the cost of the electron donor is one of the key factors in techno-economic feasibility of BSR systems (Gopal 2005; Harrison *et al.,* 2014). This chapter explores the efficiency of carbon utilisation alongside process performance in terms of BSR.

Initially, the two LFCRs fed with lactate or acetate as the main SRB electron donor were studied as independent reactors. This is referred to as Study I, the independent reactor study. Following this, the acetate-fed reactor was coupled to the lactate-fed reactor for Study II. Finally, in Study III the lactate concentration in the feed was increased from 1.0 g lactate / g sulphate (1.0 g/L lactate) in Study II to 2.2 g lactate / g sulphate (2.2 g/L lactate) in Study III to determine the effect of an increased

COD/sulphate ratio on this system. (Relevant molar COD/sulphate ratios are: 1.07 for Study II and 2.38 for Study III). Several systems have shown improved sulphate reduction performance when the COD/sulphate ratios were increased in the range of 0.7 to 4.0 g/g (De Smul *et al.,* 1999; Papirio *et al.,* 2013; Reyes-Alvarado *et al.,* 2017). Based on high sulphate reduction observed in studies done on a 2.2 g/g ratio by Oyekola *et al.* (2010) using lactate and similar other parameters to this study, it was anticipated that a higher lactate feed would result in increased sulphate conversion..

The chapter beings with an introductory section of "typical" LFCR operation where the LFCR systems used are described. Results from Study I and II are then compared and discussed, following which Study II is used as a base case of comparison for Study III.

5.2 LFCR normal operation for BSR and typical cycles observed

In contrast to the reactor started up in the previous chapter, those used in this study were established five years prior to its commencement. The reactors had been maintained on the relevant electron donor over this time and operated at HRTs in the range of 2 to 5 days, at temperatures between 10 and 30 °C and influent sulphate concentrations of 1 g/L to 10 g/L. Throughout the Studies I - III a feed of 1 g/L sulphate was used at a temperature of 25 °C. As described in Section 3.4, each reactor unit was kept at a 2-day HRT, meaning that the sequential system as a whole was at a 4-day HRT. A period of operation of the lactate reactor before commencing Study I is shown in Figure 5-1. Typical trends expected of a sulphate reducing, sulphide oxidising LFCR as described in Section 2.6.3 were demonstrated. Fairly stable sulphate levels were seen with increasing trends of sulphide concentration between each harvest of the FSB tending towards the expected sulphide by the end of each cycle. This indicated that the maturation of the FSB over time began to limit oxygen mass transfer into the system, leading to a decrease in partial sulphide oxidation in the bulk volume (Figure 5-1). Sulphate levels fluctuated somewhat, with some disturbances linked to periods of 'desludging'. This will be explained further in Section 5.3.1; however, a robust hybrid system was demonstrated where sulphate levels were not impacted on harvesting of the floating sulphur biofilm (FSB) indicating that the bulk of the reactor remained highly anaerobic and SRB function was not hindered. Samples from all four ports showed similarity in physicochemical conditions indicating reproducibility and a well-mixed system. Further, it showed that FSB harvesting was carried out just as the system became limiting to sulphide removal into the biofilm. However, as mentioned in the thesis overview in Section 1.2, the FSB of this system is studied in detail in Chapter 6, while this chapter focuses on BSR.

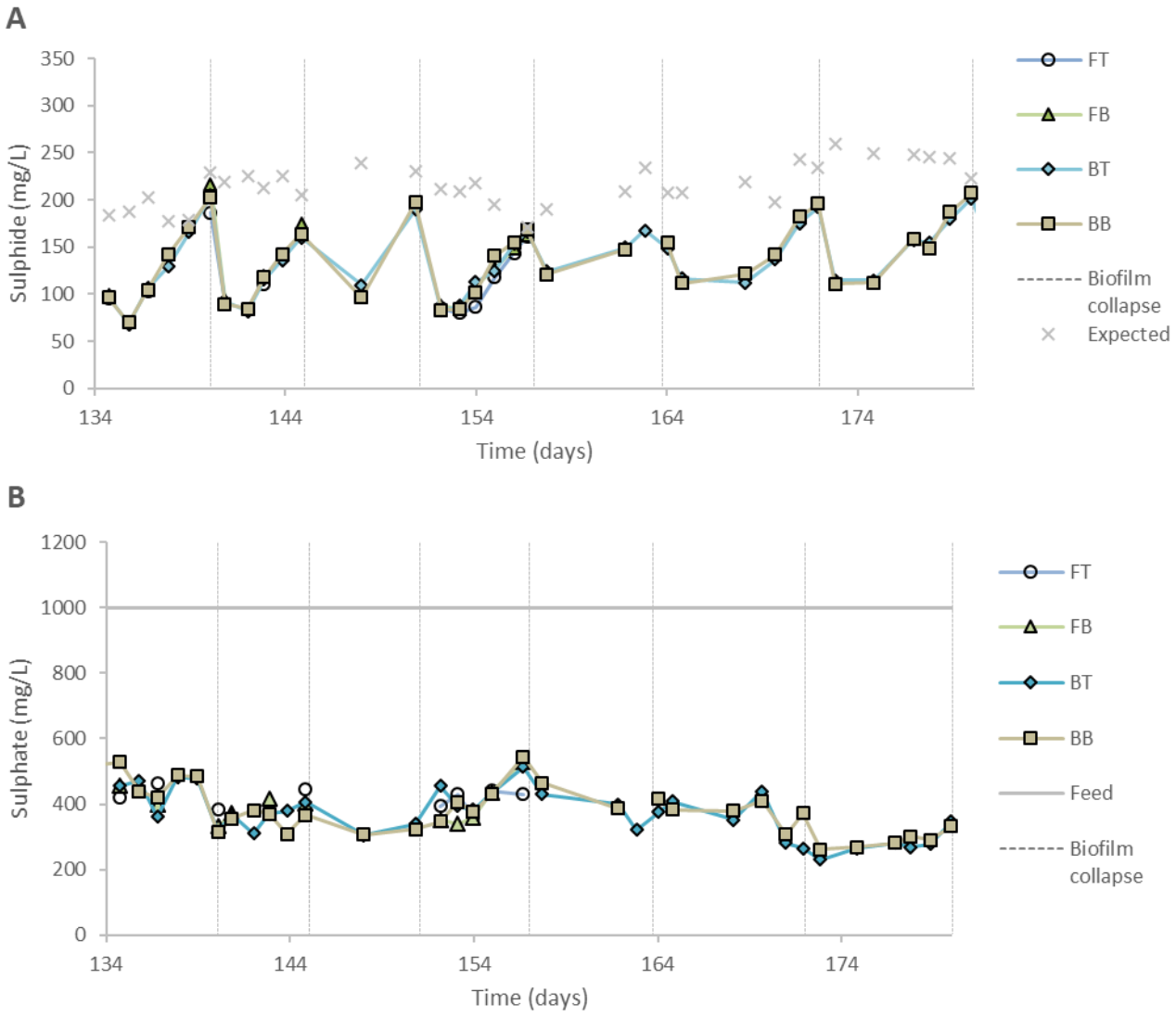


Figure 5-1 Sulphide (A) and sulphate (B) levels across several cycles of operation in the lactate LFCR. Expected sulphide is calculated based on sulphate reduction observed (not considering sulphide oxidation). Vertical lines indicate FSB harvesting points. Operating conditions: 1 g/L sulphate; 2-day HRT, 25 °C.

5.3 Independent and sequential lactate-acetate reactor system: results and discussion

5.3.1 Effect of linking reactors: comparison of Study I and Study II

As independent reactors in Study I, the lactate and acetate LFCRs achieved sulphate conversion efficiencies of 44 and 63% respectively at pseudo-steady state (Figure 5-2, Figure 5-4). The unexpected trend in increasing sulphate concentration across the last harvest cycle of the lactate reactor in Study I was due to a pump failure at the end of the previous cycle which resulted in decreased influent flow rates. In Study II, the system achieved much higher overall sulphate conversions as a sequential reactor system compared to either individual reactor (Figure 5-3; Figure 5-4). The acetate reactor unit was able to further reduce incoming sulphate from the lactate unit. Residual sulphate concentrations at pseudo-

steady state dropped from 533 mg/L exiting the lactate unit to 150 mg/L leaving the acetate unit (Figure 5-4A). This corresponds to an overall sulphate conversion of 85% across the system (Figure 5-4C).

The sulphate conversion efficiency in the lactate unit of Study II was consistent with Study I, as would be expected. In the acetate reactor, a much lower concentration of sulphate was fed into the reactor unit in Study II compared to Study I (Table 5-1) and the proportion of sulphate reduced increased slightly from 63% in Study I to 73% in Study II. This equated to reduction of 627 mg/L of sulphate in Study I, but only 404 mg/L in Study II, leaving residual sulphate of 150 mg/L. The acetate reactor did not remove this residual sulphate even though approximately 270 mg/L acetate was available as the electron donor (Figure 5-6). This implies that either an essential nutrient in the feed became limiting, or that complete oxidising SRB that are capable of scavenging low concentrations of sulphate or acetate at an appropriate rate were absent in the community. The latter will be further investigated through microbial community analysis presented further down in this section. However, at a residual sulphate concentration of 150 mg/L, the coupled reactor system was able to reduce a 1000 mg/L sulphate feed to below 200 mg/L, the limit recommended by South African water quality guidelines (Department of Water Affairs and Forestry, 1996). The sequential reactor can be compared with a similar linked series reactor system studied by Marais (2019). In their study the overflow from a lactate-fed LFCR was fed into an empty secondary LFCR for colonisation from the lactate effluent. It was found that while the secondary reactor was able to further reduce the COD content of the lactate effluent, no further sulphate reduction was observed presumably due to the absence of an acetate-utilising SRB community. This demonstrates the importance of prior acetate-SRB selection and colonisation of biomass support structures.

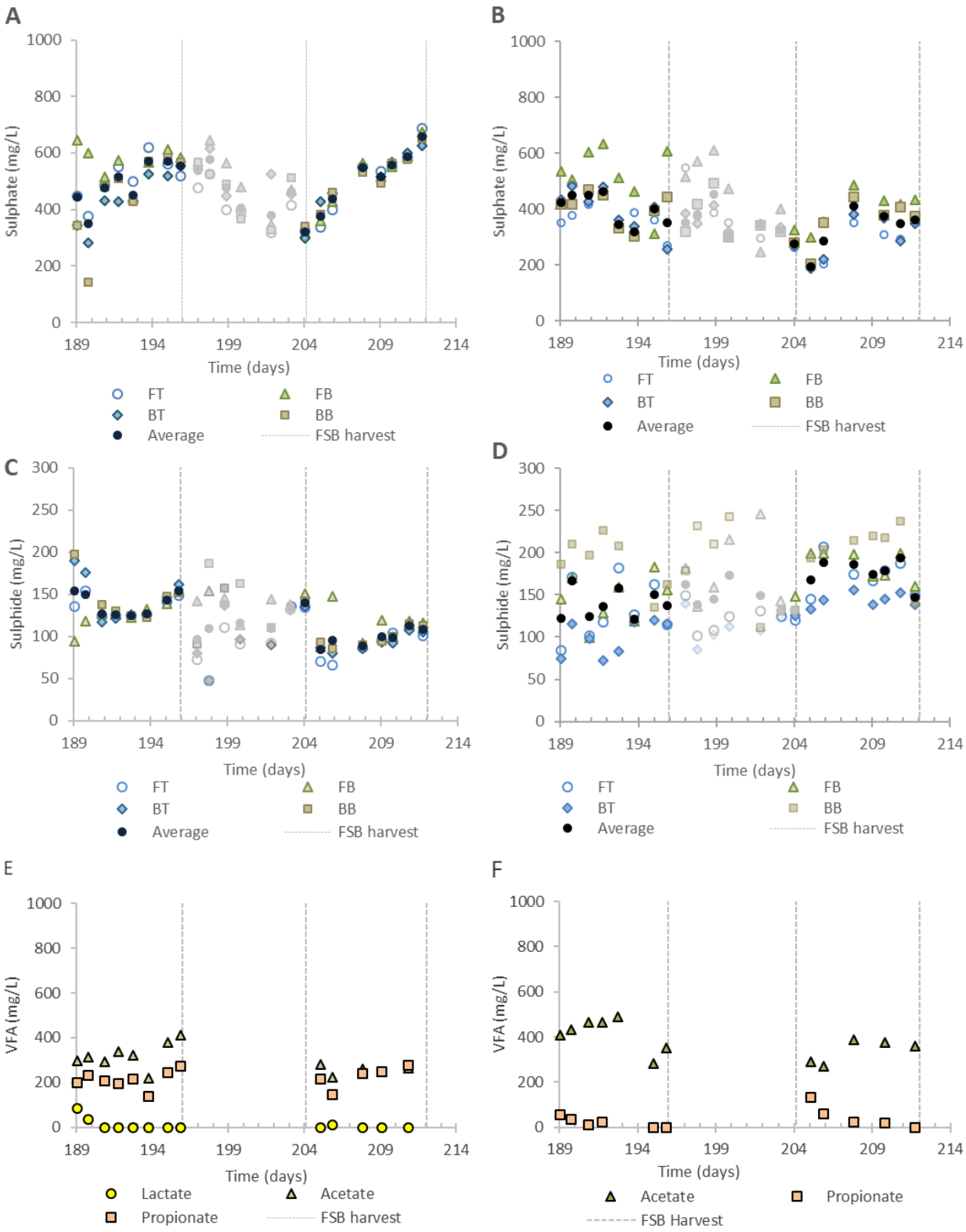


Figure 5-2 Performance data for the lactate-fed (left) and acetate-fed (right) reactors during Study I. A and B: sulphate concentration, C and D: sulphide concentration, E and F: average residual VFA concentrations (n=4). Data is greyed out for the second cycle shown and was not used due to a pump failure disturbing steady state operation. Vertical lines indicate an FSB harvest event.

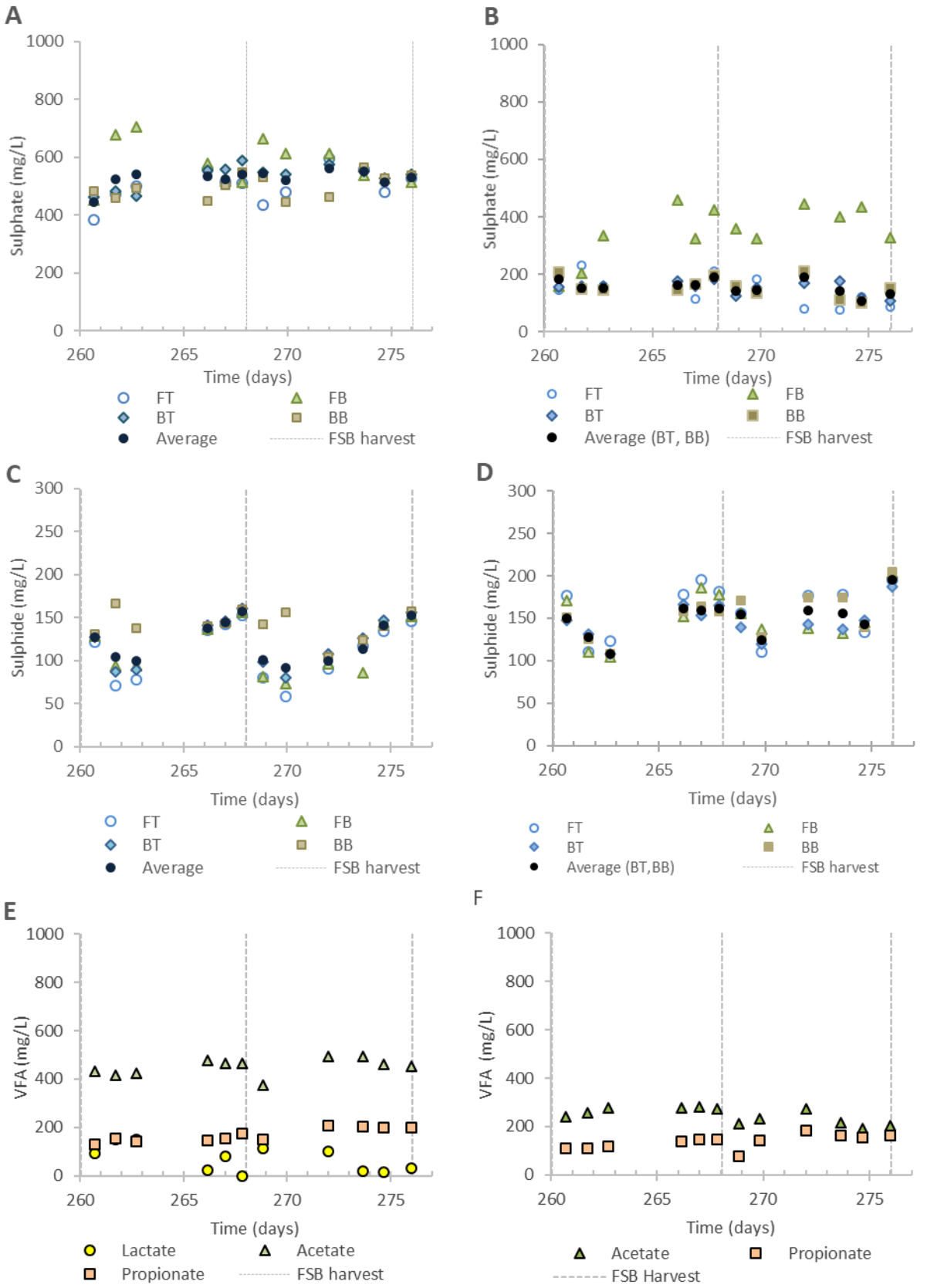


Figure 5-3 Performance data for the lactate-fed (left) and acetate-fed (right) reactors during Study II. A and B: sulphate concentration, C and D: sulphide concentration, E and F: average VFA concentrations (n≥2). FB port data for the acetate reactor was considered as an outlier as detailed in text. VFA levels given are thus averages of at least two ports. Vertical lines indicate an FSB harvest event.

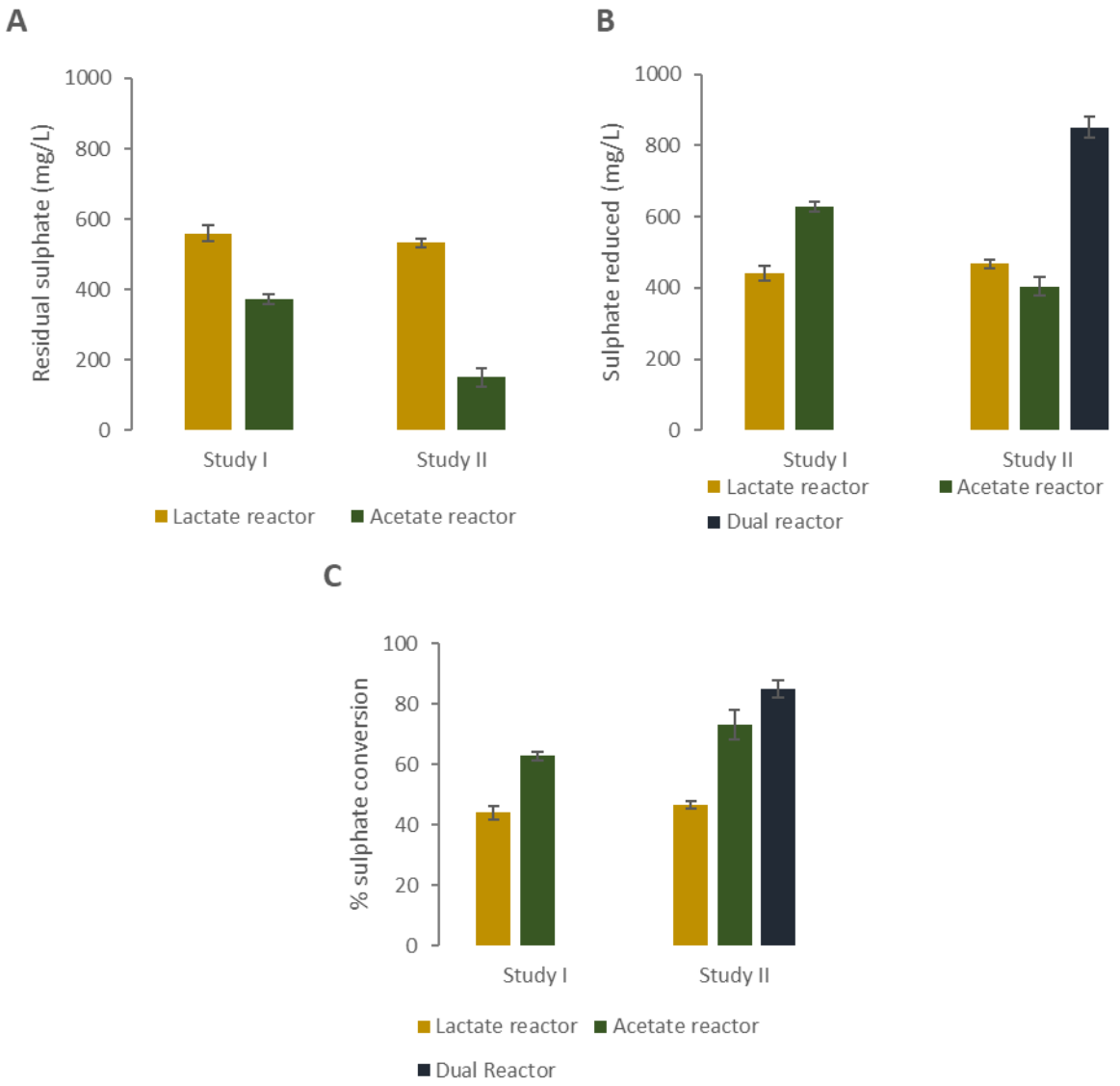


Figure 5-4 Sulphate reduction performance across pseudo-steady state in Study I (lactate and acetate independent reactors) and Study II (dual lactate-acetate reactor system). A:Residual sulphate levels from the bulk of the reactor, B: sulphate reduced, C: percentage sulphate conversion. Error bars show the mean ± one standard deviation, n=6.

Data shown in Figure 5-2 and Figure 5-3 revealed that some degree of stratification occurred in both reactors compared to the data shown in Figure 5-1. An onset of stratification in this reactor system had been observed by Marais (2020) upon a decrease in temperature from 30 to 25 °C and it was concluded in their study that the lower temperature had caused increased biomass at the base of the reactor. The lactate and acetate reactors used here had both been in operation for over five years and a significant accumulation of sludge in the lower half of the reactor was apparent at the start of operation. At this stage shown in Figure 5-1 high similarity between sample ports was observed. However, due to the sludge taking up significant reactor volume, the bulk volume and sludge in each reactor was removed and the reactor was filled with fresh feed in order to 'desludge' the reactors on day 142 of operation, before Study I began. The heavily colonised carbon microfibres allowed for recolonization of the planktonic phase. By the start of Study I, approximately seven weeks later, a build-up of biomass at the base of the reactor was again observed. It was proposed that the stratification observed in Figure 5-2

and Figure 5-3 was due to re-accumulation of sludge with different properties. In the current study, the separate sampling ports tended to converge by the end of each cycle representing a well-mixed system within pseudo-steady state, with the exception of the front bottom (FB) of the acetate reactor in Study II (Figure 5-2, Figure 5-3).

Data from the FB port of the acetate unit in Study II was considered to be an outlier. Here sulphate readings were significantly higher as compared to the rest of the reactor (Figure 5-3). This was attributed to an observed build-up of sludge particularly in the front bottom corner of the reactor near this port (Figure 5-5). LFCR dye tracer studies have previously shown that a small dead zone formed near the inlet front corner of the reactor (Marais *et al.,* 2020; Mooruth, 2013). It is proposed that the sludge was lacking an active SRB population and that it may have also caused a disruption in fluid dynamics, resulting in a longer retention time particularly for sulphate ions in the vicinity of the FB port. Therefore, for Studies II and III it was decided that the back top (BT) and back bottom (BB) ports alone were used for evaluating reactor performance in the acetate reactor.

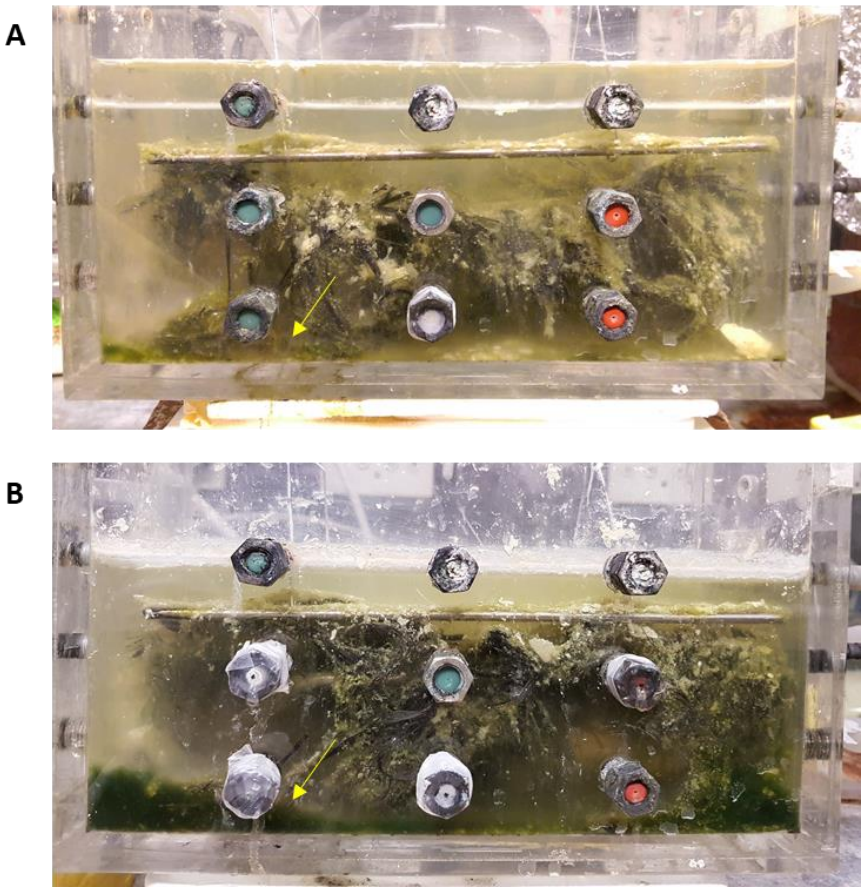


Figure 5-5 Comparison of sludge accumulation at the FB port of the acetate reactor in Study I (A) and Study II (B) as indicated by the yellow arrow. Biomass accumulation was also observed at the back of the reactor in Study II, but this did not extend as far as that of the front corner and was not observed at the BB port.

VFA profiles are shown in Figure 5-6. In considering the pathways by which each VFA was utilised, the following assumptions were made:

i) no propionate was utilised for sulphate reduction, and therefore residual propionate levels were used to calculate the amount of lactate fermented,

ii) lactate was used preferentially for BSR followed by acetate, and

iii) the degradation of the carbohydrate component of yeast extract (YE; 400 mg/L) by microbial action in the reactor resulted in the production 270 mg/L acetate as reported by Hessler (2020).

Lactate has been previously shown to be used preferentially over acetate and propionate (Cao *et al.,* 2012). The assumptions with regards to fermentation follow observations seen in lactate-fed sulphate reducing reactors where increases in propionate were associated with decreases in both sulphate reduction and acetate production indicating fermentative action (Oyekola, 2008). Chapter 4 of this study also supports this as acetate was determined to be used preferentially over propionate for sulphate reduction. As shown further below (Figure 5-8), the SRB microbial component in these reactors were similar to those observed in the propionate reactor of Chapter 4.

VFA profiles showed that lactate was completely utilised in the single-stage lactate reactor unit in Study I; however, significant levels of acetate and propionate remained (Figure 5-6). Similarly, the single-stage acetate reactor in Study I showed high levels of acetate remaining in the bulk. The data from Study I suggested firstly that the acetate reactor was receiving more acetate than it could utilise under the current conditions. This agrees with findings by (Marais, 2019) where residual acetate and sulphate concentrations were lower at a 5-day HRT compared to a 2-day HRT with similar feed sulphate and acetate concentrations to this study. Secondly, the data shows that the lactate reactor favoured incomplete oxidation, resulting in sufficient residual VFAs in the overflow, especially acetate, giving potential to sustain sulphate reduction in a secondary reactor receiving this acetate. Table 5-1 compares substrates fed into the acetate reactor in Study I and from the lactate unit effluent in Study II. The level of propionate in Study II remained stable from the lactate reactor to the acetate reactor showing that no lactate was fermented in the acetate reactor (Figure 5-6).

Table 5-1 Influent concentrations into the acetate reactor in Study I versus the acetate reactor unit as part of the sequential reactor system. Values show the average ± one SD where applicable (n=6).

	Influent concentrations (mg/L)			
	Sulphate	Lactate	Propionate	Acetate
Study I	1000	-	-	930
Study II	553 ± 41	10 ± 15	191 ± 16	478 ± 4

Figure 5-6 also shows that a significant amount of acetate could not be accounted for across the two studies. This may be linked to the amount of acetate produced from YE which was assumed to be similar to that seen by Hessler (2020). As this was the only significant source of acetate production that could not be directly determined, it was concluded that the amount of acetate generated from degradation of YE must vary due to differing microbial communities. If the unaccounted-for acetate utilisation indeed lies with YE, it appears that YE was fully metabolised in the lactate reactor leaving no unaccounted-for acetate in the acetate reactor in Study II. Alternative explanations for this acetate usage could lie with methanogenic activity as methanogens are well known to compete with SRB for

acetate utilisation under anaerobic environments (Parkin *et al.,* 1990; O 'Flaherty *et al.,* 1998; Oude Elferink *et al.,* 1998a; Oude Elferink *et al.,* 1998b; Scholten *et al.,* 2000; Rotaru *et al.,* 2015).

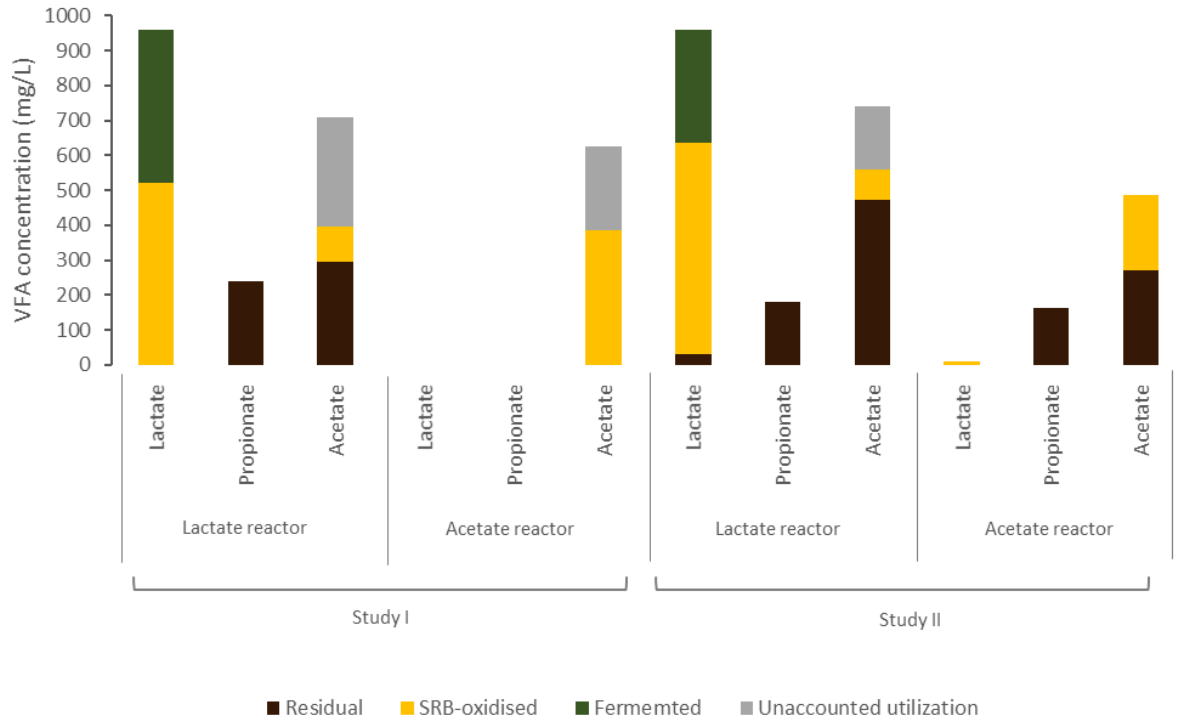


Figure 5-6 Fate of VFAs within each reactor unit across Studies I and II.

Microbial diversity (>2%) was determined by 16S rRNA gene sequencing and is shown in Figure 5-7 with SRB relative abundances (>1.5%) shown in Figure 5-8. In Study I, the community supported by the carbon microfibres (the "attached" samples) can be compared to the planktonic communities in each reactor. Attached samples were only sequenced for one time point due to time constraints of the study. It can be seen that the carbon microfibres allowed a higher proportion of SRB to be supported as compared to the planktonic samples. However, only five SRB species (at abundance > 1.5%) were detected across the studies and were present to some degree in all the samples. Three of these were *Desulfovibrio* (*Desulfovibrio intestinalis, Desulfovibrio oximicus* and *Desulfovibrio alcoholivorans*) with the other two belonging to the genera *Desulfomicrobium* (*Desulfomicrobium hypogeium*) and *Desulfobacter* (*Desulfobacter vibrioformis*). Significant proportions of *Veillonella*, a lactate fermenter, were also observed in the lactate reactor. Marias (2020) observed an increase in this genus on decreasing HRT to two days which was linked to an increase in lactate fermentation. In Study II a dramatic increase in a *Stenotrophomonas* species was evident. The role of this organism in the community was not clear. The only observed change that could be attributed to this, was a change in VFA utilisation in the lactate reactor from Study I to Study II which resulted in less residual propionate and more residual acetate (Figure 5-2E and Figure 5-3E).

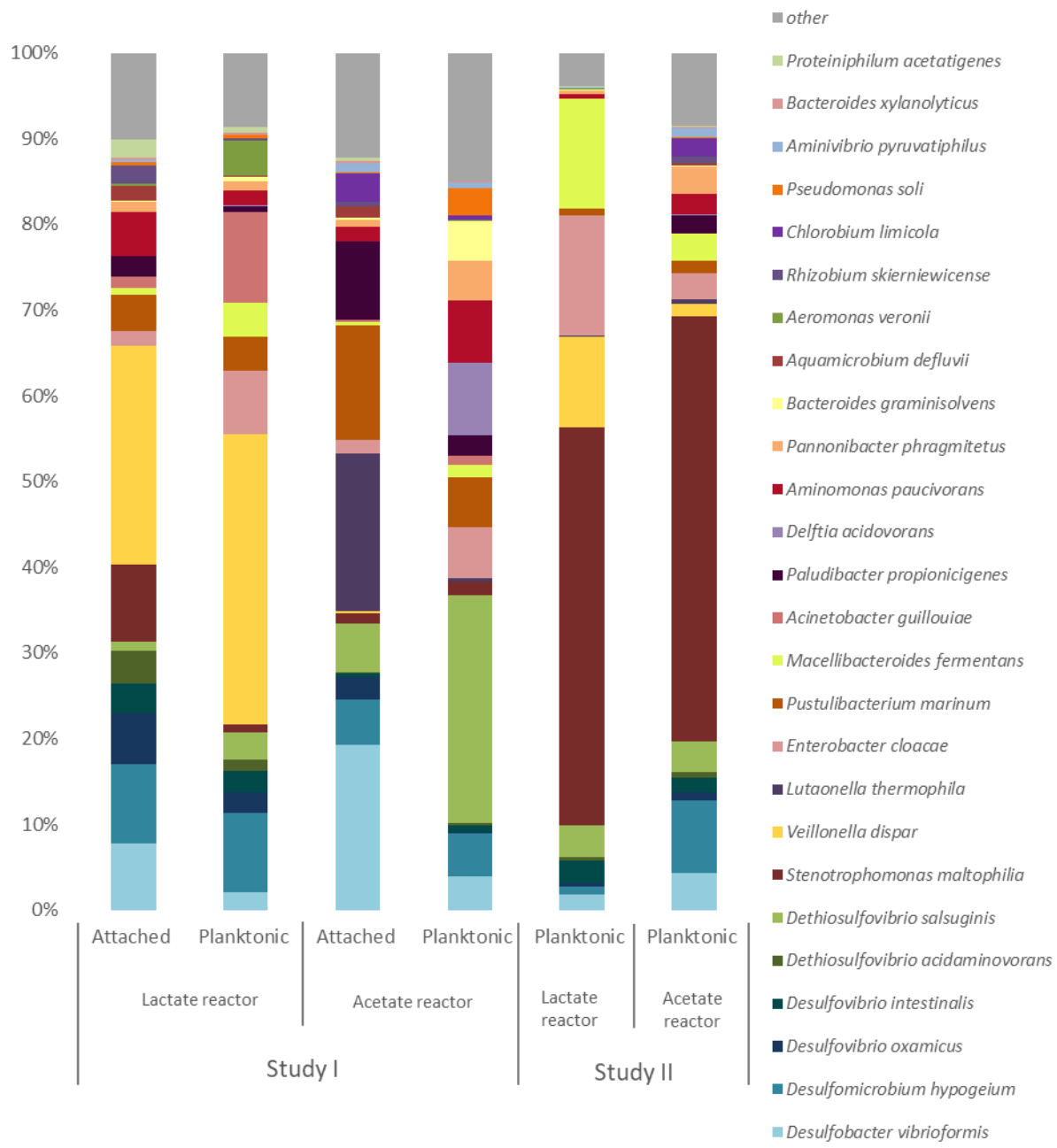


Figure 5-7 Microbial diversity in the lactate and acetate reactor units across Studies I and II. Relative abundances of all OTUs with ≥2% abundance in at least one sample are shown.

Since no observed change in sulphate reduction occurred from Study I to Study II in the lactate reactor, it was assumed that the relative proportion of the *Stenotrophomonas* species caused a skew in the planktonic data from Study I to Study II with respect to the relative proportions of SRB present. To overcome this, Figure 5-8 looks exclusively at the relative proportions of the SRB. *Desulfovibrio* species are often linked to lactate utilisation (Postgate and Campbell, 1966; Bryant *et al.,* 1977; Traore *et al.,* 1982). Hessler (2020) observed lactate utilisation pathways in both *Desulfovibrio* and *Desulfomicrobium.* Higher proportions of *D. oxamicus* and *D. intestinalis* can be seen in lactate reactor as compared to the acetate reactor in Study I. In the acetate planktonic samples, higher proportions of each of these can be seen in Study II as compared to Study I. This indicates that these species were

carried through from the lactate unit of the coupled system and thereby could have contributed to lactate oxidation in the acetate unit, relevant to the data shown in the next Section 5.3.2 below. Lactate oxidation in the acetate unit will be seen again in Section 5.3.2 where it was more pronounced due to higher lactate concentrations. From the species present, *Desulfobacter vibrioformis* was likely the dominant acetate utilising SRB. *Desulfobacter* have been shown to contain acetate utilising species (Laanbroek and Pfennig, 1981) and *D. vibrioformis* has been found to use only acetate out of a range of other organic carbon sources (Lien and Beeder, 1997). This species was the most dominant SRB in the acetate attached phase and maintained planktonic levels from Study I to Study II. Interestingly, Hessler *et al.* (2018) reported this same *Desulfobacter* OTU to be particularly present at higher concentrations of acetate at the inlet of an acetate-fed up-flow anaerobic packed bed reactor (UAPBR) and found other SRBs – a *Desulfatitalea* and a *Desulfatiglans* – to be present in the effluent zones of these reactors in acetate environments, and more able to scavenge sulphate as compared to *Desulfobacter*. The inability of the acetate unit to reduce as much sulphate in Study II as compared to Study I (403 versus 627 mg, respectively; Figure 5-4) could speak to the absence of key acetate utilising, sulphate-scavenging SRB.

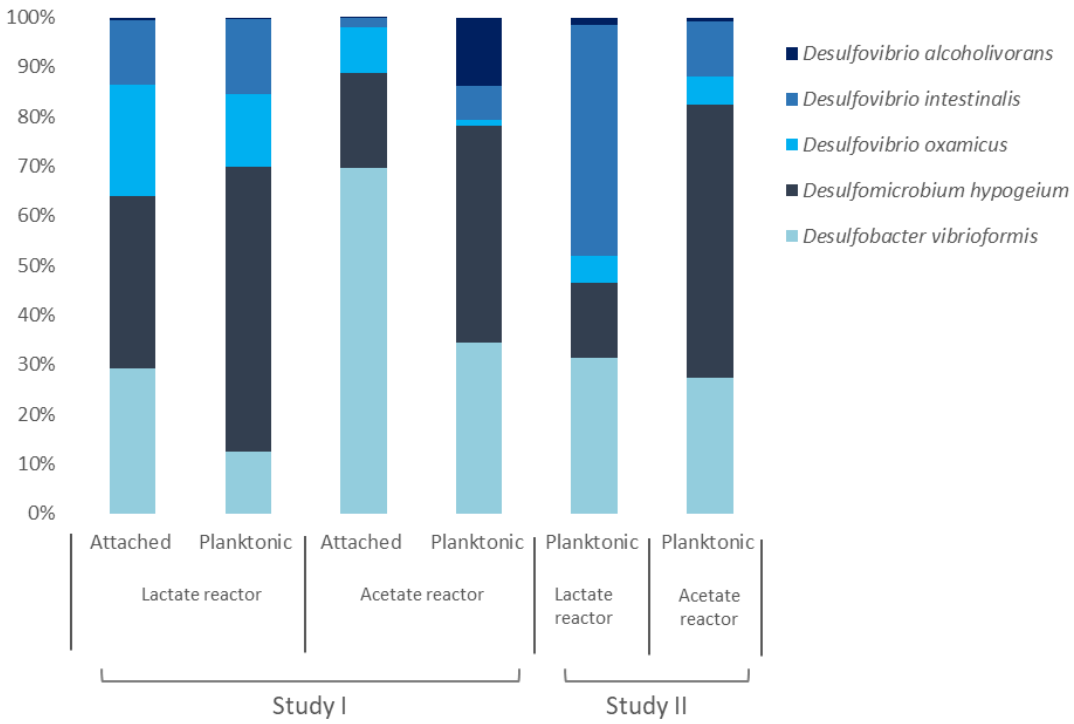


Figure 5-8 SRB diversity in the lactate and acetate reactor units across Studies I and II. Relative abundances of all OTUs with ≥1.5% abundance in at least one sample are shown.

5.3.2 Effect of increase in lactate concentration in the sequential LFCR system: comparison of Studies II and III

The increase in lactate concentration allowed determination of the effect of the COD/sulphate ratio on the sequential reactor system. In this section Study II was used as a reference for comparison to Study

III. Considering that lactate was completely utilised via partial oxidation in the lactate reactor with only just under 50% sulphate conversion in this unit in Studies I and II, it was hypothesised that more available lactate would lead to increased sulphate reduction in the lactate reactor. However, Figure 5-9 shows only a minimal increase in performance with increased lactate supplied; sulphate conversion in the lactate unit increased from 47 % in Study II to just 54 % in Study III, and across the sequential reactor from 85 to 90% (see Figure. B-7 for time course performance data).

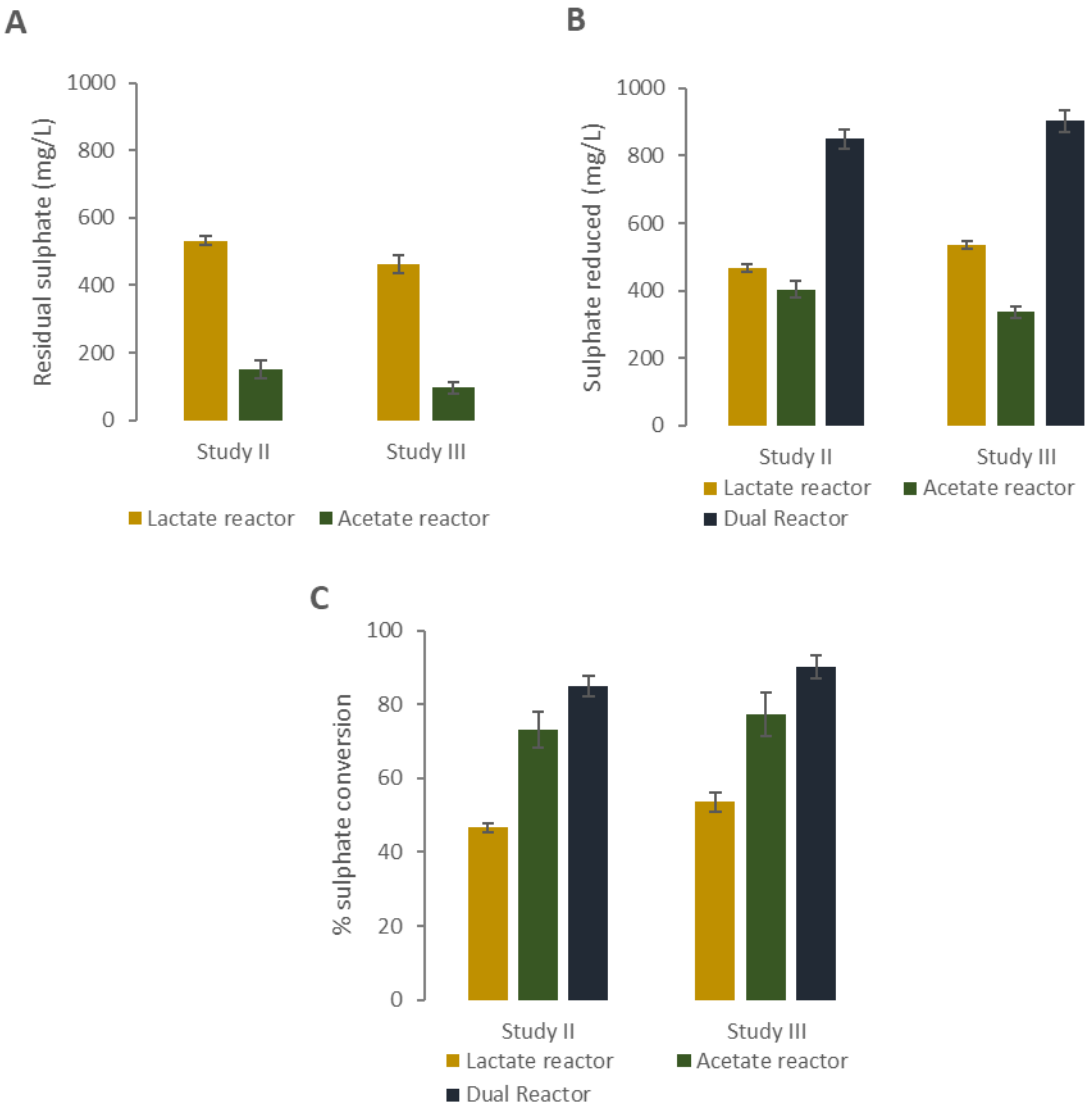


Figure 5-9 Pseudo-steady state sulphate reduction performance over Study III (COD/sulphate ratio of 2.2) with Study II (COD/sulphate ratio of 1.0) shown for reference. A: bulk residual sulphate, B: sulphate reduced, C: sulphate conversion for each reactor unit as well as the sequential reactor as a whole. Error bars show the mean ± one standard deviation, n=6.

Figure 5-10 shows that increased lactate concentration increased the activity of fermentative bacteria dramatically. More sulphate was reduced via lactate metabolism in Study III as compared to Study II. However, some residual lactate remained unused by either SRB or fermenters in the lactate reactor. This suggests that there was no competition for the remaining lactate. This was unexpected especially as the community present was able to fully scavenge the lactate to levels of 30 mg/L and below in Studies I and II (Figure 5-6). Considering that a significant amount of sulphate remained in the lactate

reactor (463 mg/L; Figure 5-9A), it was expected that the SRB should have been able to carry out more sulphate reduction via lactate oxidation. Oyekola *et al.* (2012) showed lactate-utilising SRB to have a much lower K_s (0.12 g/L) as compared to that of lactate fermenters (3.3 g/L). It is possible that at a 2-day HRT of the reactor unit, lactate was entering the system faster than the microbial consortia could metabolism. It is also possible, due to the slow growth of SRB, that the populations had not grown to their maximum size possible under the increased lactate load. However, similar performance data from subsequent studies does not show any increasing trend of utilisation (Figure. B-7 - Figure. B-9). Lastly, the data could also suggest that an essential macro- or micro-nutrient became limiting thus inhibiting an increase in biomass with more substrate. Oyekola *et al.* (2010) observed complete lactate utilisation and 85% sulphate conversion in a CSTR fed with a similar feed make-up and with the same lactate and sulphate concentrations at the same dilution rate, but with a higher yeast extract concentration than was used in this study.

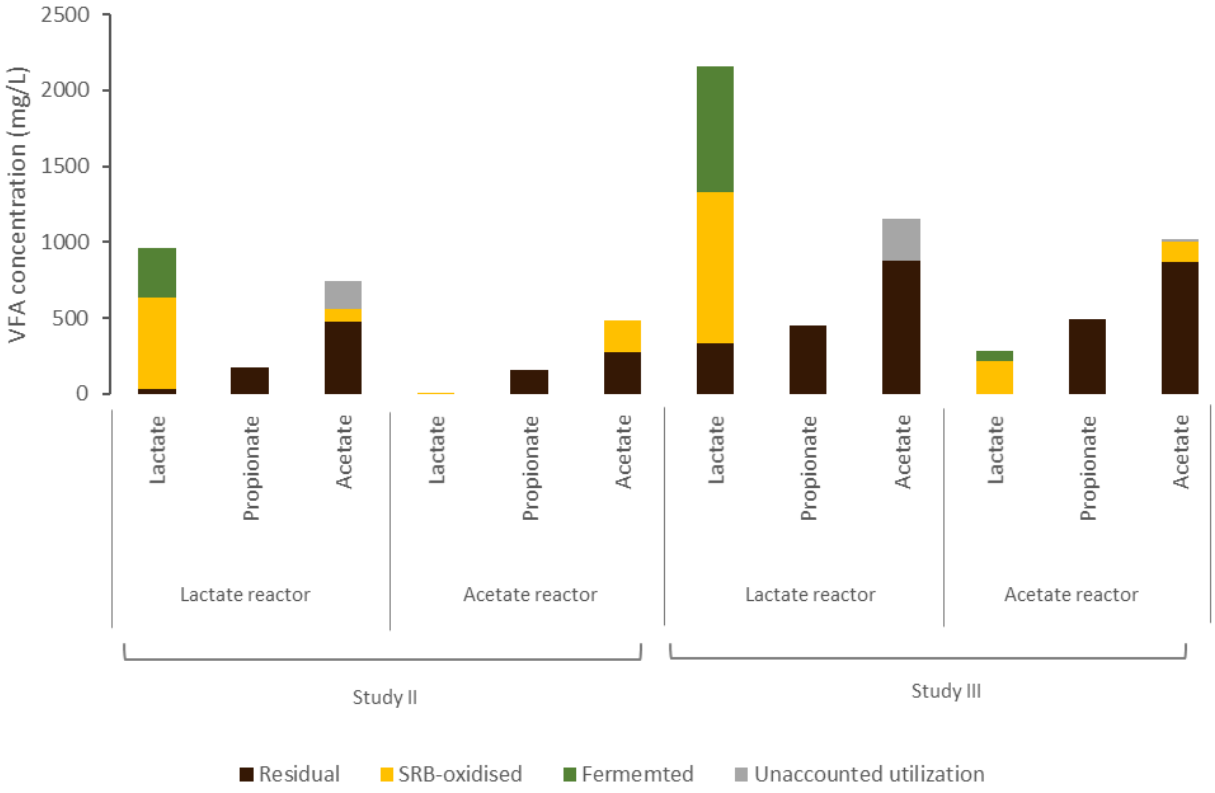


Figure 5-10 VFA profiles showing the fate of each VFA in Study III across each reactor unit, with Study II included for reference.

5.3.3 Summary analysis for Studies I – III

Table 5-2 gives a summary of analysis for the work shown in this chapter. Most notably the yield coefficient of sulphate reduced / lactate consumed can be compared. The table shows that this increased almost two-fold from 0.44 in the single lactate reactor in Study I to 0.85 across the sequential reactor in Study II. Comparing the lactate reactor of Study I to the overall system in Study II it can also be seen that VSRR was maintained. Additionally, the compromise of raising lactate concentration is evident as the sulphate reduced / lactate consumed ratio dropped to 0.41 across the sequential reactor.

Table 5-2 Summary of performance and process efficiency through Studies I-III at pseudo-steady state.

	Study I		Study II			Study III		
	Lactate reactor	Acetate reactor	Lactate reactor	Acetate reactor	Overall	Lactate reactor	Acetate reactor	Overall
Feed sulphate (mg/L)	1000	1000	1000	553	1000	1000	433	1000
Feed lactate (mg/L)	1000	0	1000	10	1000	2200	279	2200
Feed acetate (mg/L)	0	660	0	478	478	0	872	872
Predicted acetate produced (mg/L)	710	270	741	7	748	1154	148	1302
Total acetate available (mg/L)	710	930	741	485	748	1154	1020	1302
Residual sulphate (mg/L)	560	373	533	150	150	463	97	97
Residual lactate (mg/L)	0	0	10	0	0	338	0	0
Residual acetate (mg/L)	296	341	474	272	272	874	872	872
Residual sulphide (mg/L)	124	155	141	167	167	140	184	184
Sulphate reduced (mg/L)	440	627	447	404	851	540	360	900
Sulphate conversion (%)	44	63	47	73	85	54	83	90
Lactate consumed (mg/L)	1000		990	10	1000	1862	279	2200
Acetate consumed (mg/L)		589	267	213	480	280	148	430
Sulphate reduced / lactate consumed	0.44		0.45		0.85	0.29		0.41
Sulphate reduced /acetate consumed		1.97		1.96			*	
VSRR (mg/L/day)	220	313.5	223.5	202	212.75	270	180	225

* Value omitted due to sulphate reduction occurring via both lactate and acetate in this instance.

5.4 Conclusions

This study has shown the successful development of a sequential lactate-acetate LFCR allowing more complete utilisation of lactate and improved sulphate reduction performance. The separately colonised acetate reactor allowed for a strong acetate utilising community to develop, which, when coupled to the lactate reactor, could utilise much of the accumulated acetate and remaining sulphate to acceptable levels. The community in the acetate reactor was able to function despite being fed a feed that would have been somewhat depleted in nutrients used by the lactate reactor. This acetate community does not develop within a lactate fed environment where lactate utilisers outcompete acetate-utilisers. The compartmentalised reactor therefore allows both communities to be maintained in one system. This results in more complete utilisation of the lactate feed making the use of this electron donor more economically viable. The acetate reactor unit of the coupled system also showed capacity to utilise residual lactate for BSR. The sequential nature means that planktonic species in the lactate unit were carried into the acetate unit allowing for complete utilisation of any residual lactate. Despite

expectations, an increase in lactate concentration, giving a COD/sulphate ratio of 2.2 did not result in significantly better performance in either reactor. The reasons for this were not clear, particularly within the lactate reactor.

Relevant further investigation for this system may revolve around loading the sequential reactor system with higher sulphate. The acetate reactor showed potential to reduce more sulphate but was limited by its inability to scavenge sulphate. This could be overcome by seeding the reactor with the acetate scavengers selected for in the zoned acetate reactor presented by (Hessler *et al.,* 2018).

Chapter 6 Optimisation of biological sulphide oxidation and sulphur recovery

6.1 Introduction

Chapters 4 and 5 have focused on biological sulphate reduction within the linear flow channel reactor (LFCR) as the first process of this hybrid reactor system. This chapter investigates the second process, namely partial sulphide oxidation of the generated sulphide to elemental sulphur. The passive generation of a floating sulphur biofilm (FSB) within the same reactor unit as the sulphate reduction has proved a promising sulphide management option (Marais *et al.,* 2020). FSBs have been studied extensively by Molwantwa (2008) and Mooruth (2013) in solely sulphide oxidation reactors. Marais *et al.* (2020) extended their use into a single-stage hybrid reactor encompassing both sulphate reduction and partial sulphide oxidation. Here this is extended to the sequential hybrid reactor. The studies described in this chapter look at the formation of the FSB in the sequential LFCR system described in Chapter 5. From these initial observations of the FSB, changes in the feed composition with regard to the magnesium and phosphate concentrations were recommended. These changes were tested to determine their effect on biological sulphate reduction (BSR), biological sulphide oxidation (BSO) and sulphur recovery.

6.2 Floating sulphur biofilm formation and initial observations

Stages of growth and maturation of this biofilm in the hybrid reactor used in this thesis, from one biofilm harvest to the next, are shown in Figure 6-1. In the reactors studied, a thin, translucent layer formed over the initial 24 hours, which can be related to the ‘sticky’ stage described by Molwantwa (2008). The biofilm became uniformly opaque by day three and by the end of the study had taken on a ‘brittle’ nature which was easily scraped off the surface of the reactor for harvesting. SEM images of mature FSB from the lactate and acetate LFCRs show biomass made up of rod-shaped bacteria (Figure 6-2, B) with strands of extracellular polymeric substances (EPS) visible (Figure 6-2, A). However, numerous crystal structures were also observed and were pervasive throughout the biofilm (Figure 6-2). Similar structures have been previously reported by Marias (2020) where they were shown to be magnesium phosphate crystals. These were determined to be the result of using a modified Postgate media - as was used in the current study, a nutrient-rich media that has historically been used for SRB cultivation (Postgate, 1984). Magnesium phosphate is soluble in dilute mineral solutions, particularly in the presence of ammonia, allowing it to dissolve in Postgate media; however, in water it is sparingly soluble (Weast, 1979; O’Neil *et al.,* 2001). Therefore, as ammonia is utilised by the microbial community in the reactor, excess magnesium phosphate could be expected to precipitate. Further, charged polysaccharides and proteins within the EPS of biofilms can promote the sorption of inorganic ions (Flemming and Wingender, 2010). Marais (2020) proposed that sulphur recovery in the FSB could be hindered by the

presence of these ions in excess in the feed and their resulting accumulation in the biofilm. This hypothesis formed the basis of the studies carried out in this chapter.

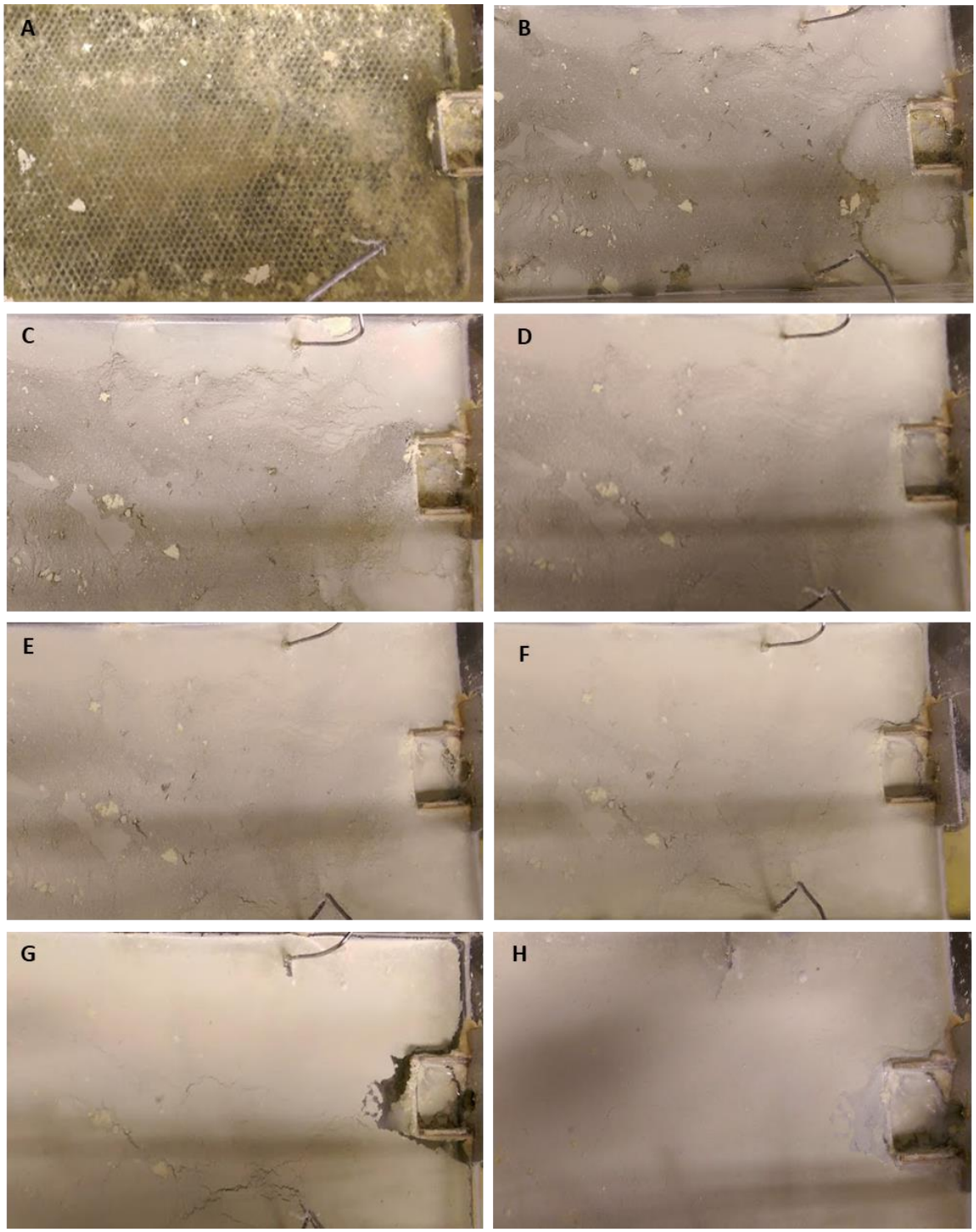


Figure 6-1 Typical formation of the FSB showing its maturation over a cycle run of the LFCR (acetate reactor, Study I). A: Day 0, B: Day 1, C: Day 2, D: Day 3, E: Day 4, F: Day 5, G: Day 6, H: Day 8.

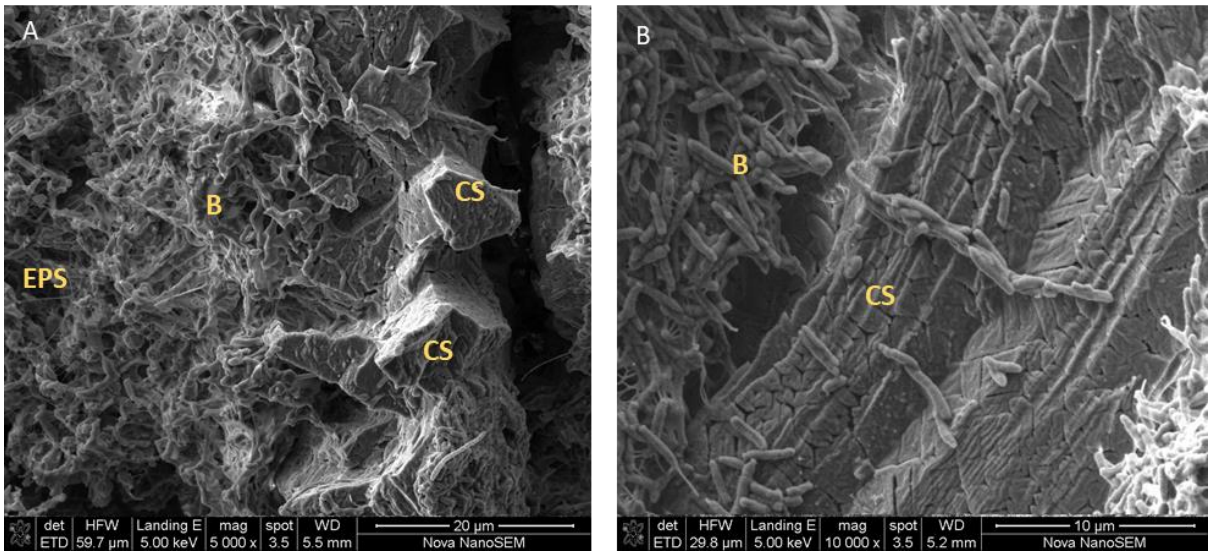


Figure 6-2 SEM images of the FSB showing the microbes making up the biofilm as well as crystalline structures ubiquitous throughout the biofilm. Samples were mature, eight-day old FSB. A: FSB taken from Study III of the lactate-fed LFCR, crystal structures protruding out of the biofilm. B: FSB from Study III of the acetate-fed LFCR, crystal lattice structure and rod-shaped microorganisms. Observed features: biomass (B), strands of extracellular polymeric substances (EPS) and crystalline structures (CS). Scale bars shown.

6.3 Effect of inorganics in feed on sulphur recovery

6.3.1 Introduction

The studies described here were carried out to determine if the build-up of inorganics in the FSB as observed by Marais (2020) contributed to low sulphur incorporation into the biofilm. Studies performed by Marias (2020), with similar parameters to those carried out here, highlighted two problems with sulphur recovery in the FSB of a hybrid sulphate reducing and sulphur oxidising LFCR. Firstly, up to 21% of the total sulphur load could not be accounted for in attempting to close the sulphur balance. This 'sulphur gap' was attributed to the loss of FSB fragments, as well as the production of colloidal sulphur in the bulk of the reactor. Secondly, low conversion levels of sulphide to elemental sulphur in the range of 20% were achieved. This contrasts with studies by Mooruth (2013) where sulphur recoveries averaged 48% of the sulphide generated in a sulphide oxidation LFCR. In the study by Mooruth (2013), the outflow from a degrading packed bed reactor (DPBR) supplied the feed for the sulphide oxidising reactor and this was only supplemented at times with acetate. This further supports an investigation into lowering the inorganic content in the modified Postgate medium. These two problems, namely the sulphur gap and the poor conversion of sulphide to sulphur, are considered with regards to improvement in sulphur recovery. Therefore, key questions for the studies presented here were as follows:

- Does a decrease in magnesium or phosphate in the feed impact sulphate reduction performance?
- Will a reduction of magnesium and phosphate in the FSB result from the changes in feed concentrations?
- Will this in turn lead to better sulphur recovery in the FSB?

- If increased sulphur recovery is achieved is this due to increased sulphide conversion and/or a closing of the sulphur gap previously observed?

6.3.2 Experimental approach

The studies examined here are Studies III – V of the sequential lactate-acetate reactor. Study III was discussed in the previous chapter with regards to sulphate reduction. Here this study shall be used as a control for the following two studies, namely Studies IV and V. Two sequential changes were made to the feed composition, described in Table 3-1 to alter the amount of magnesium and phosphate fed into the reactor system. In Study IV, the magnesium concentration was decreased by 50% from 100 mg/L to 50 mg/L, and in Study V the phosphate concentration was decreased by 30% from 250 mg/L to 175 mg/L in the presence of the reduced magnesium concentration of 50 mg/L. All other parameters were kept constant. As described in Section 3.4.2, each study was comprised of two experimental runs of 4 residence times each. The FSB from each run was harvested, observed with SEM imaging and analysed for sulphur, magnesium and phosphate. Levels of elemental sulphur were determined via energy dispersive X-ray spectroscopy (EDS) analysis in conjunction with SEM imaging (Section 3.7.7) as well as by CHNS analysis (Section 3.7.6). Similarly, levels of magnesium and phosphate in the biofilm were determined in two ways: firstly, via EDS analysis in conjunction with SEM imaging, and secondly via solution ICP-MS of acid digested powdered biofilm (Section 3.7.5).

6.3.3 Results

Firstly, the effect of the changes in magnesium and phosphate concentration on sulphate reduction is reported. Table 6-1 shows that sulphate reduction remained relatively stable across all three studies. This implies that the magnesium and phosphate supplied were in excess for the needs of the SRB and any other microbes working synergistically alongside them.

Table 6-1 Effect of changes in inorganic feed components on sulphate reduction in pseudo-steady state. Feed sulphate concentration was 1 g/L. Error bars represent one SD ± the mean (n=6).

Study	Sulphate reduced (mg/L)	
	Lactate reactor unit	Acetate reactor unit
III	537 ± 37	377 ± 27
IV	563 ± 56	333 ± 52
V	523 ± 26	338 ± 45

The elemental sulphur recovery is first considered over the whole system (Figure 1-3) and thereafter in the lactate unit (Figure 6-4) and acetate unit (Figure 6-10). In each case the expected mass of elemental sulphur in the biofilm is compared to the recovered mass of elemental sulphur recovered. The expected and recovered amounts of elemental sulphur were calculated as detailed in Section 3.8 (time course performance data is shown in Figure. B-7 to Figure. B-9). Briefly, the recovered sulphur was determined using the mass of FSB harvested and the measured percentage of sulphur within the biofilm. The expected sulphur in the biofilm is determined using the expected sulphide in the bulk of the reactor based on sulphate reduction, and the measured amount of sulphide exiting the reactor at the effluent

port. Looking at the sequential reactor system as a whole, an increase in the amount of elemental sulphur recovered across a run cycle can be seen from 0.9 g in Study III to 1.3 g in Study IV (Figure 6-3 A), suggesting that excess Mg and its crystallisation negatively impacted sulphur recovery. The decrease in phosphate in Study V, in addition to magnesium, appears to have had no effect on sulphur yield across the system as a while. Percentage sulphur recovery from the total feed sulphur load for the sequential system therefore reached a maximum of 48% in Study IV and remained stable with an added decrease in phosphate. Examining FSB formation and sulphur recovery in the two reactor units of the coupled system independently gives more insight into the effect of the inorganic changes in each reactor unit. Therefore, the lactate reactor unit and acetate reactor unit will be discussed separately over the following two sections.

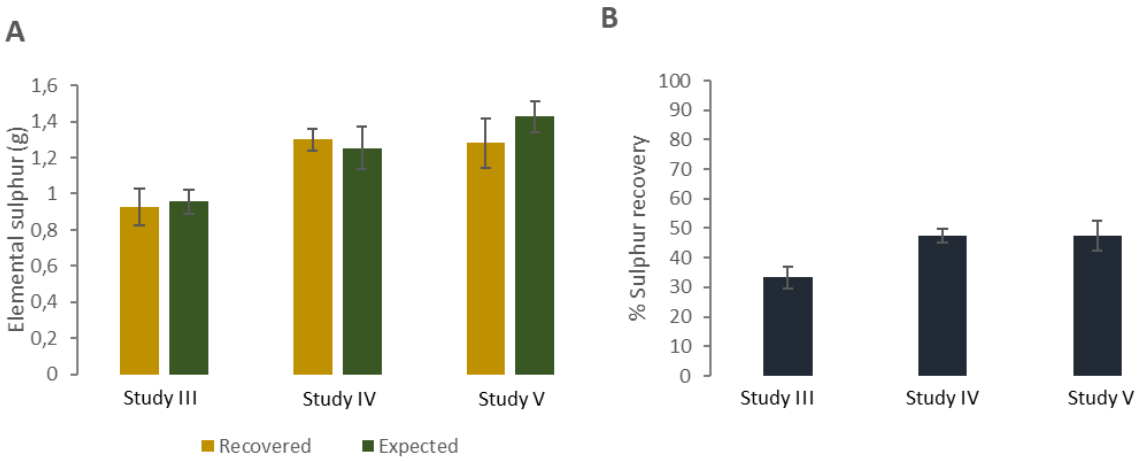


Figure 6-3 Effect of magnesium and phosphate feed concentrations on sulphur recovery in the lactate-acetate coupled reactor system. Concentrations in each study are as follows: Study III: 100 mg/L magnesium, 250 mg/L phosphate; Study IV: 50 mg/L magnesium, 250 mg/L phosphate; Study V: 50 mg/L magnesium, 175 mg/L phosphate. A: The amount of elemental sulphur recovered in the FSB and the amount of sulphur expected based on sulphate reduction observed. B: The percentage of elemental sulphur recovered as a proportion of the total sulphur loaded into the system as sulphate. Error bars represent one SD ± the mean, n=2.

Lactate reactor unit

In the primary lactate reactor unit, both a decrease in magnesium and a decrease in phosphate improved both the sulphur recovery and the percentage of sulphide converted to sulphur in the biofilm while reducing the residual aqueous sulphide (Figure 6-4). When considering sulphur recovery in the system, it is useful to look at both the amount of sulphur recovered as a proportion of the total sulphur load fed to the system, as well as of the total sulphide produced by BSR. The percentage sulphur recovered from the total sulphur load increased from 11% in Study III (the control) to 21% in Study IV (reduced magnesium) and further increased to 26% in Study V (reduced magnesium and phosphate). These percentages respectively correlate to 20, 37 and 50% sulphur recovery from the total sulphide produced in this reactor unit (Figure 6-4, B). An increase in sulphur yield could be attributed to an increase in the amount of FSB formed and harvested (FSB mass recovered in Figure 6-4 A), and/or an increase in the concentration of sulphur within the FSB. In this instance, some increase in the weight of biofilm harvested, from 0.9 g in Study III to 0.12 in Study IV, was observed however this did not change significantly in Study V. However, a steady increase in the concentration of sulphur incorporated in the biofilm is seen from the percentage sulphur composition in the FSB shown in Figure 6-4 B. This

increased from 34% in Study III to 52% in Study IV and 69% by Study V. It can be noted that the error associated with the percentage sulphur composition in the FSB is small compared to the error associated with the total amount of FSB harvested. This observation was consistent across the other experimental runs carried out during the course of this study (data not shown). This implies that the increase in sulphur yield seen across these two studies in the lactate reactor unit was largely due to a change in the proportion of sulphur in the FSB rather than an increase in the actual amount of FSB produced.

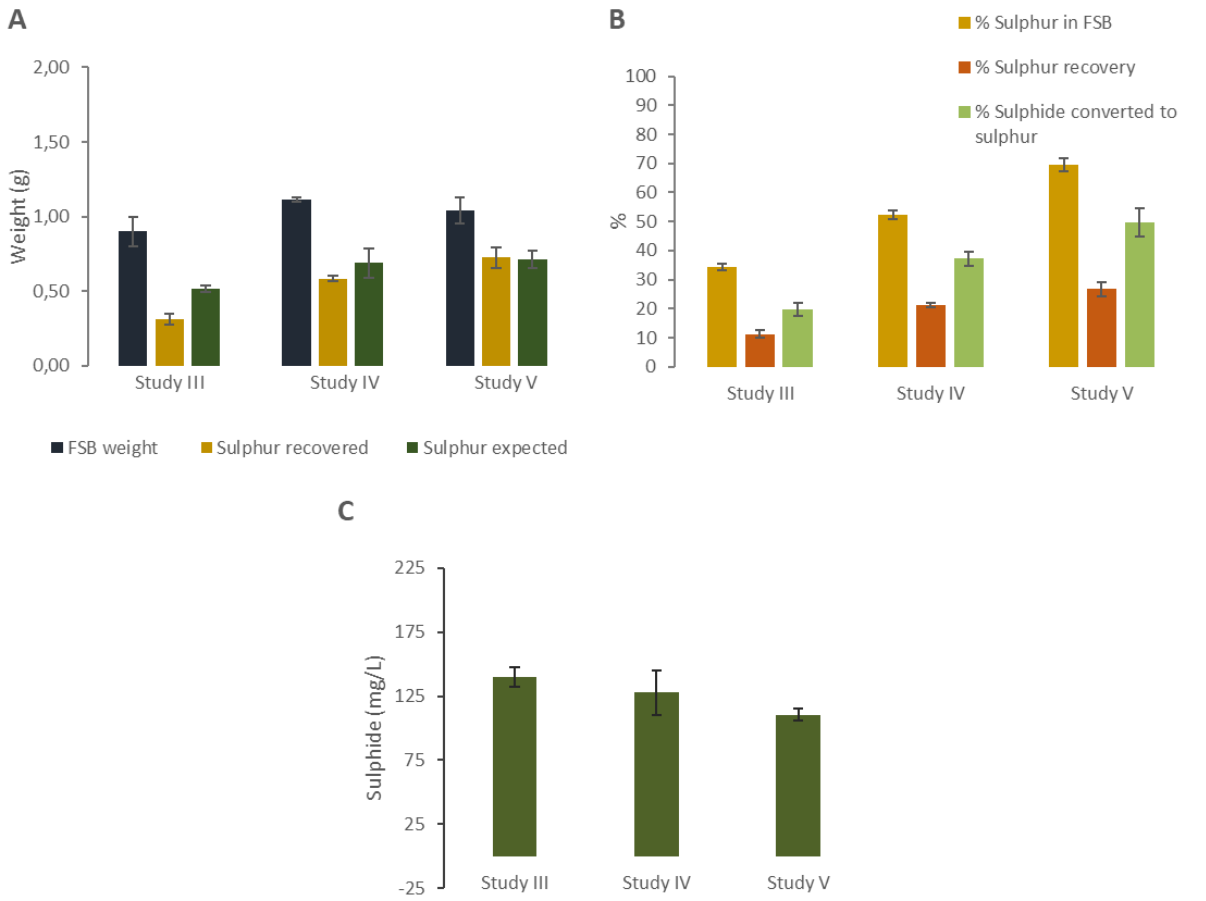


Figure 6-4 Sulphide oxidation and sulphur recovery in the lactate reactor unit of the lactate-acetate sequential reactor with varying inorganic ion contents in the feed of Studies III - V. A: Total FSB weight is compared to the amount of sulphur contained in the FSB as well as the amount of sulphur expected. Sulphur expected is calculated using the expected sulphide available based on the sulphate reduction achieved and observed sulphide levels in the effluent. B: Percentages of sulphur recovery. The percentage sulphur recovery denotes the amount of sulphur recovered as a proportion of the total sulphur load, while the percentage sulphide converted to sulphur compares the sulphide produced within the single reactor unit to that reporting to the biofilm. C: Residual sulphide levels at pseudo steady state. Error bars represent one SD ± the mean (n≥2).

By Study V, FSB formation was somewhat impacted with visible fractures forming in the biofilm by day 5 of the run (Figure 6-5 A); however, despite these tears the biofilm matured to a level comparable to typical formation shown in Figure 6-1, albeit that it was less brittle upon harvesting. The change of general topology of the surface of the biofilm with decreasing crystal formations was also observed (Figure 6-8 B), however, some crystal structures were still observed in Studies IV and V.

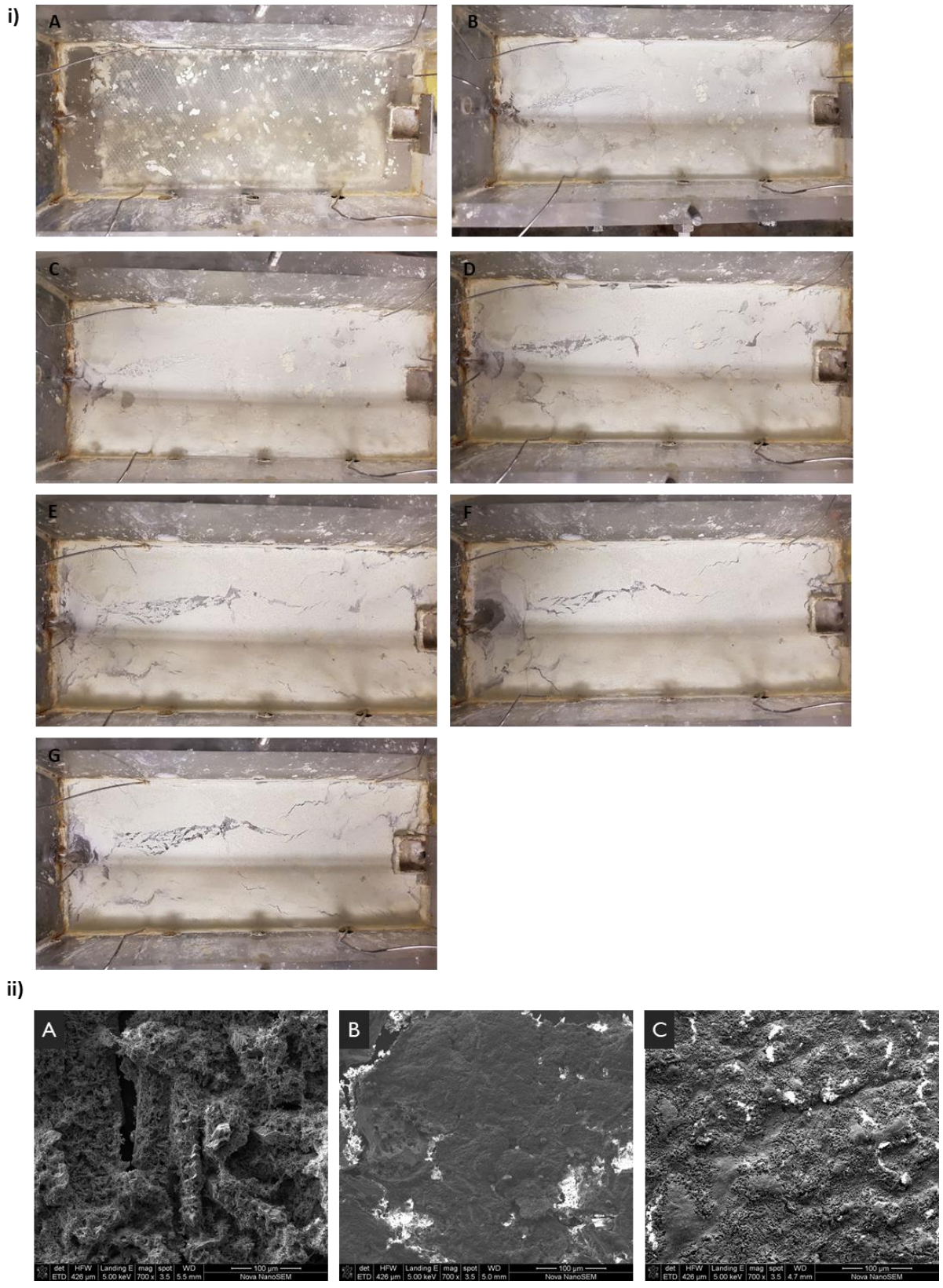
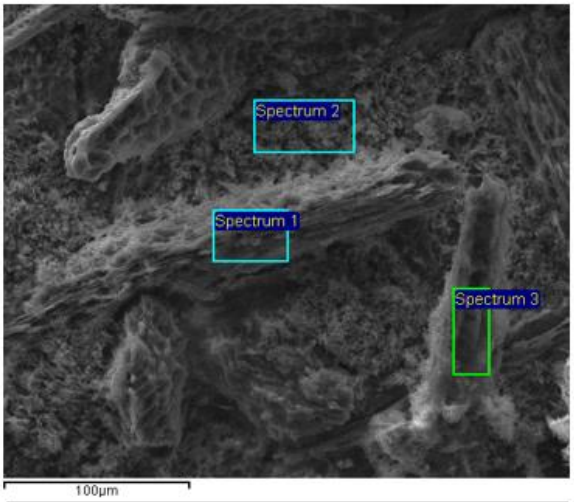


Figure 6-5 FSB formation and structure in the lactate reactor unit of the sequential lactate-acetate LFCR system. i): Formation of the FSB over the course of a run of Study V. A: Day 0, B: Day 3, C: Day 4, D: Day 5, E: Day 6, F: Day 7, G: Day 8. ii): SEM images of the FSB over decreasing magnesium and phosphate concentrations. A: Study III, B: Study IV, C: Study V. Magnification as shown by scale bars.

Surface structure and composition was further investigated immediately before biofilm harvest on Day 8 with EDS analysis of SEM images at higher and lower magnifications (Figure 6-6 and Figure 6-7).

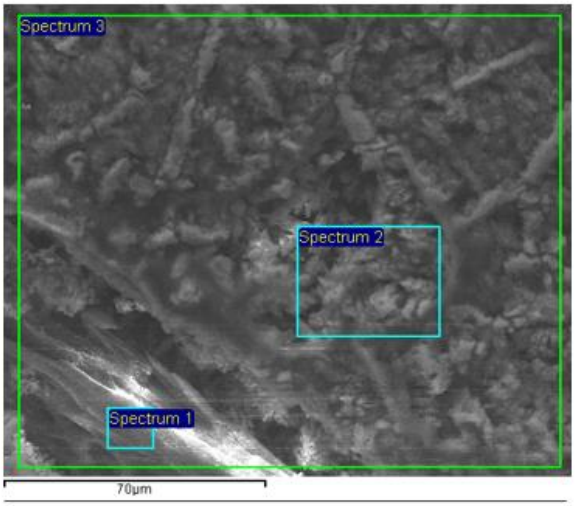
Spectra of crystal structures, given as spectra 1 and 3 in Figure 6-6 A (Study III) and spectrum 1 in Figure 6-6 B (Study IV) confirm the presence of high levels of magnesium and phosphorus compared to surrounding areas of biofilm. Spectrum 2 in both Figure 6-6 A and B present higher relative abundances of sulphur compared to the other spectra. This shows that sulphur is deposited less at areas of magnesium phosphate crystals. These areas of higher sulphur deposition can be seen to be rich in biomass. Furthermore, the biomass-rich area showed a higher relative sulphur (41%) concentration in Study IV as compared to Study III (19%). Percentage relative elemental abundances from lower magnification analysis showed magnesium and phosphate concentrations to decrease from Study III to Study IV while, for the same period, sulphur increased markedly from 9 to 32% (Figure 6-8). By Study V the reduction in observable crystal structures was markedly noticeable with consistently high sulphur averaging 29%, and consistently low magnesium and phosphate both averaging <0.5 % (Figure 6-6). It should, however, be noted that EDS data penetrates approximates 1 µm into the sample whereas FSBs have been shown to be in the range of 60 – 400 µm thickness based on previous studies (Molwantwa, 2008; Mooruth, 2013). This data therefore gives an indication of the composition on the very surface of the biofilm.

A



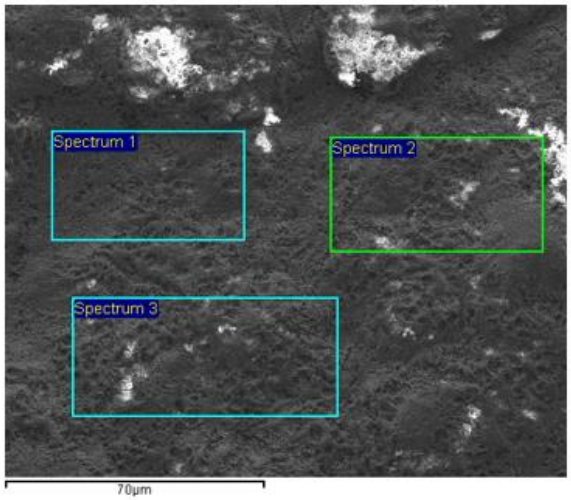
Spectrum	Element (%)					
	C	N	O	Mg	P	S
Spectrum 1	46.68	5.75	36.69	4.15	4.61	2.13
Spectrum 2	72.82	0	7.66	0.23	0.48	18.81
Spectrum 3	60.04	4.94	26.97	3.19	3.86	1.00

B



Spectrum	Element (%)					
	C	N	O	Mg	P	S
Spectrum 1	24.28	5.82	32.35	7.51	18.23	11.82
Spectrum 2	54.36	0	4.1	0.08	0	41.47
Spectrum 3	60.19	0	6.53	0.17	0.52	32.58

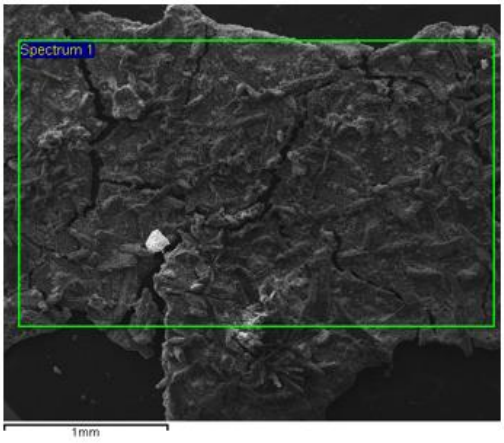
C



Spectrum	Element (%)					
	C	N	O	Mg	P	S
Spectrum 1	53,92	5,35	13,59	0	0,71	26,42
Spectrum 2	51,4	4,33	11,01	0,15	0,49	32,63
Spectrum 3	53,61	4,36	12,62	0	0,44	28,98

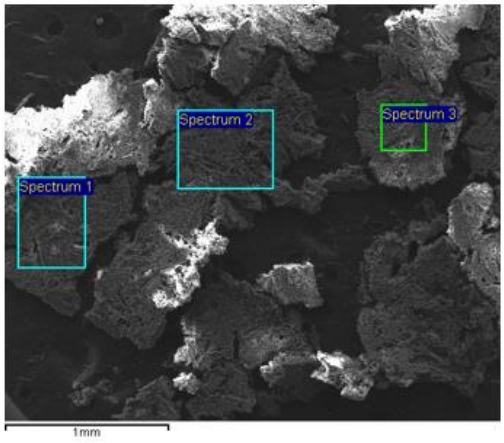
Figure 6-6 High magnification EDS analysis of various structures and areas of the FSB of the lactate reactor unit in Study III (A), Study IV (B) and Study V (C). Relative percentage abundances for each element shown are given below each SEM image for the spectra indicated. Magnifications as shown by scale bars.

A



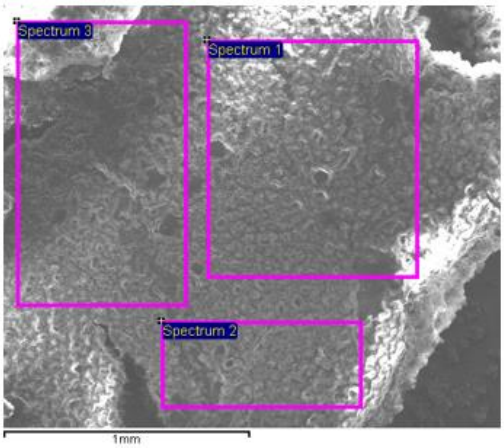
Spectrum	Element %					
	C	N	O	Mg	P	S
Spectrum 1	59.09	4.54	23.98	1.79	2.07	8.53

B



Spectrum	Element %					
	C	N	O	Mg	P	S
Spectrum 1	59.67	0	11.36	0.2	0.42	28.36
Spectrum 2	59.83	0	9.11	0.22	0.39	30.45
Spectrum 3	55.28	0	8.13	0.17	0.29	36.13
Average	58.26	0.00	9.53	0.20	0.37	31.65

C



Spectrum	Element %					
	C	N	O	Mg	P	S
Spectrum 1	52.85	3.77	15.92	0	0.53	26.92
Spectrum 2	55.75	4.12	16.74	0	0.61	22.78
Spectrum 3	53.04	2.98	14.93	0	0.44	28.61
Average	53.88	3.62	15.86	0.00	0.53	26.10

Figure 6-7 Low magnification (scale bars shown) EDS analysis of the FSB of the lactate reactor unit in Study III (A), Study IV (B) and Study V (C). Relative percentage abundances for each element shown are given for the spectra indicated. It should be noted that the biofilm in (B) fragmented whilst mounting the sample.

In order to determine the amount of magnesium and phosphorus in the biofilm more robustly, samples of acid digested powdered biofilm were analysed by ICP-MS (Figure 6-8). Decreasing trends of both the concentration and overall amount of magnesium and phosphorus were seen from Studies III to V as expected. Both magnesium and phosphorus decreased from Study III by approximately one third in Study IV, and one half in Study V. This confirms that a decrease in magnesium or phosphate in the feed supplied, and therefore the bulk reactor volume, caused a concomitant decrease of these elements in the biofilm. Additionally, a decrease in either element in the media caused both elements to decrease in the biofilm since phosphate levels were impacted by magnesium reduction in Study IV and

magnesium further decreased alongside a reduction of phosphate in Study V. This further supports that these ions react together to form magnesium phosphate crystals in the FSB.

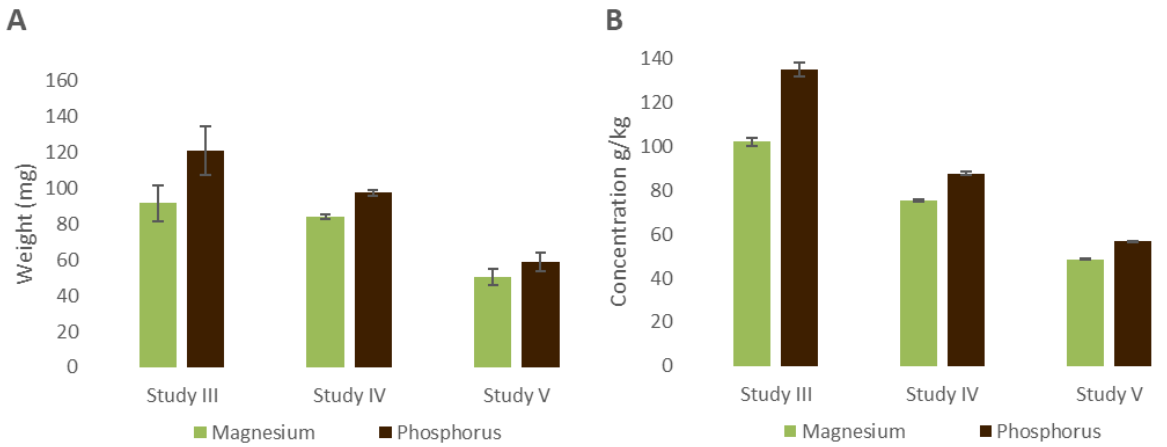


Figure 6-8 ICP-MS elemental analysis of the amount (A) and concentration (B) of magnesium and phosphorus in the FSB of the lactate reactor unit.

Acetate reactor unit

The acetate reactor unit of the coupled system demonstrated a different story to the lactate unit with regards to the FSB, both in terms of structure and composition. Firstly, macroscopic observable changes were evident in the biofilm structure by Study IV; though the biofilm developed to maturity in Study IV, it had lost all brittleness. By Study V, it formed very poorly, having generated only a thin and sparse layer of biofilm by day 5, which remained patchy with large translucent areas till the end of the run (Figure 6-9, (i)). This can be compared to typical biofilm formation in the acetate reactor unit in Study I shown in Figure 6-1. Visible tears in the biofilm from Study IV which can be seen at the microscopic level may be an indication of deterioration of the FSB (Figure 6-9). These were identified as unnatural tears due to the jagged edges, different in nature to pores commonly present in biofilms and observed by Mooruth (2013) in FSBs. The images shown in Figure 6-9 (ii) can be compared to those of the lactate unit in Figure 6-5 B which remained structurally intact.

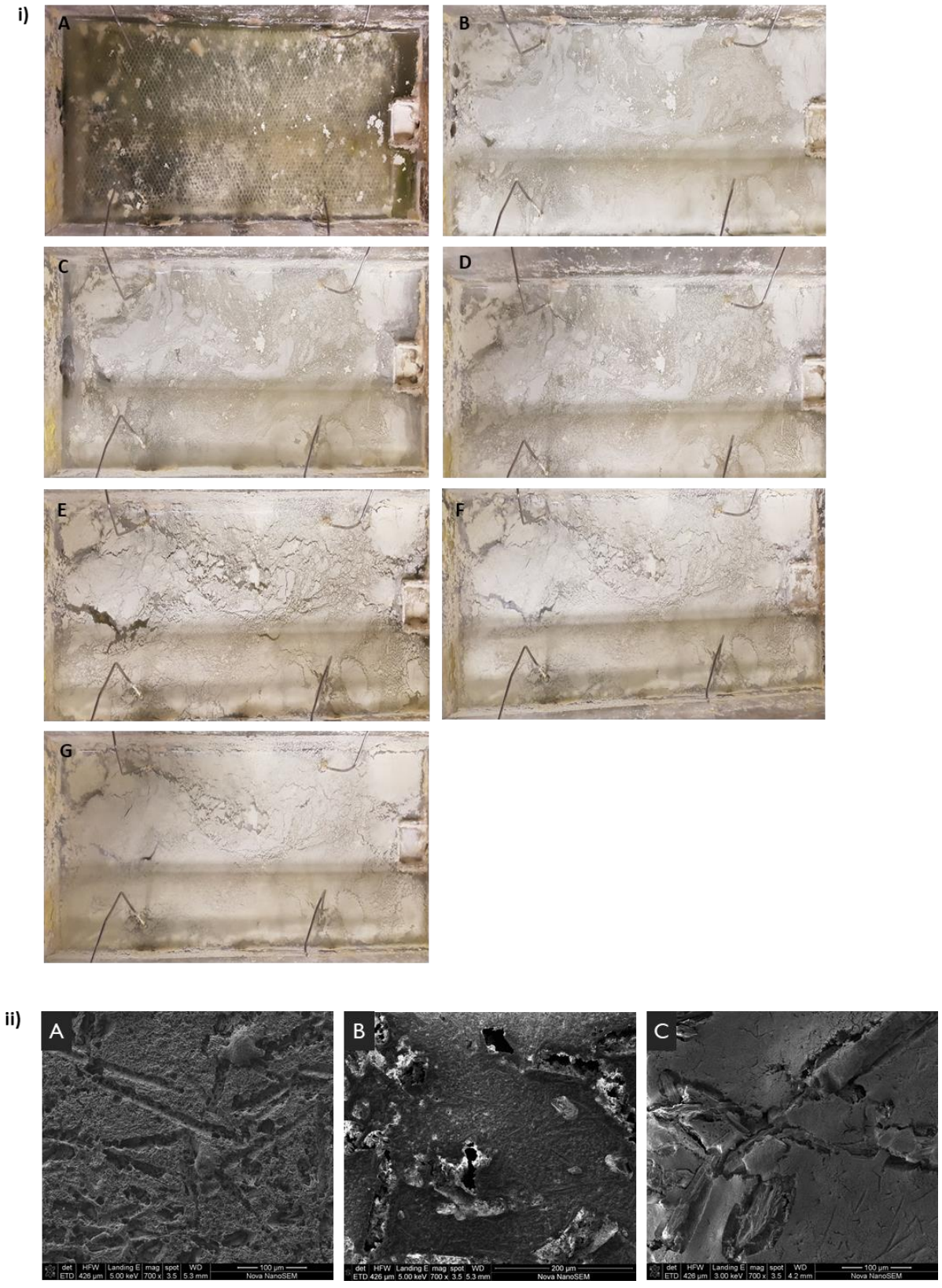


Figure 6-9 FSB formation and structure in the acetate reactor unit of the sequential lactate-acetate LFCR system. i): Formation of the FSB over the course of a run of Study V. A: Day 0, B: Day 3, C: Day 4, D: Day 5, E: Day 6, F:Day 7, G: Day 8. B: SEM images of the FSB over Studies III – V. A: Study III, B: Study IV, C: Study V. Magnification as indicated by scale bars.

Interestingly however, despite the poor biofilm development and the decreasing amount of biofilm harvested, sulphur recovery remained relatively consistent throughout the studies (Figure 6-10).

Though there was no increase in overall sulphur yield, an increase in the percentage of sulphur in the biofilm was observed, as in the lactate unit, from Study III (35%) to Study IV (47%). A slight decrease in sulphur yield by Study V was solely due to decreased biofilm produced. Some increase in percentage sulphide converted is seen from Study III to Study IV, however residual sulphide is relatively stable across the studies (Figure 6-10, B and C). Interestingly, ICP-MS analysis revealed that magnesium concentrations remained similar throughout the three studies with some minor decrease in phosphorus seen (Figure 6-11). This contrasts with the lactate reactor where clear reductions of the inorganics in the biofilm resulted from the media concentration changes.

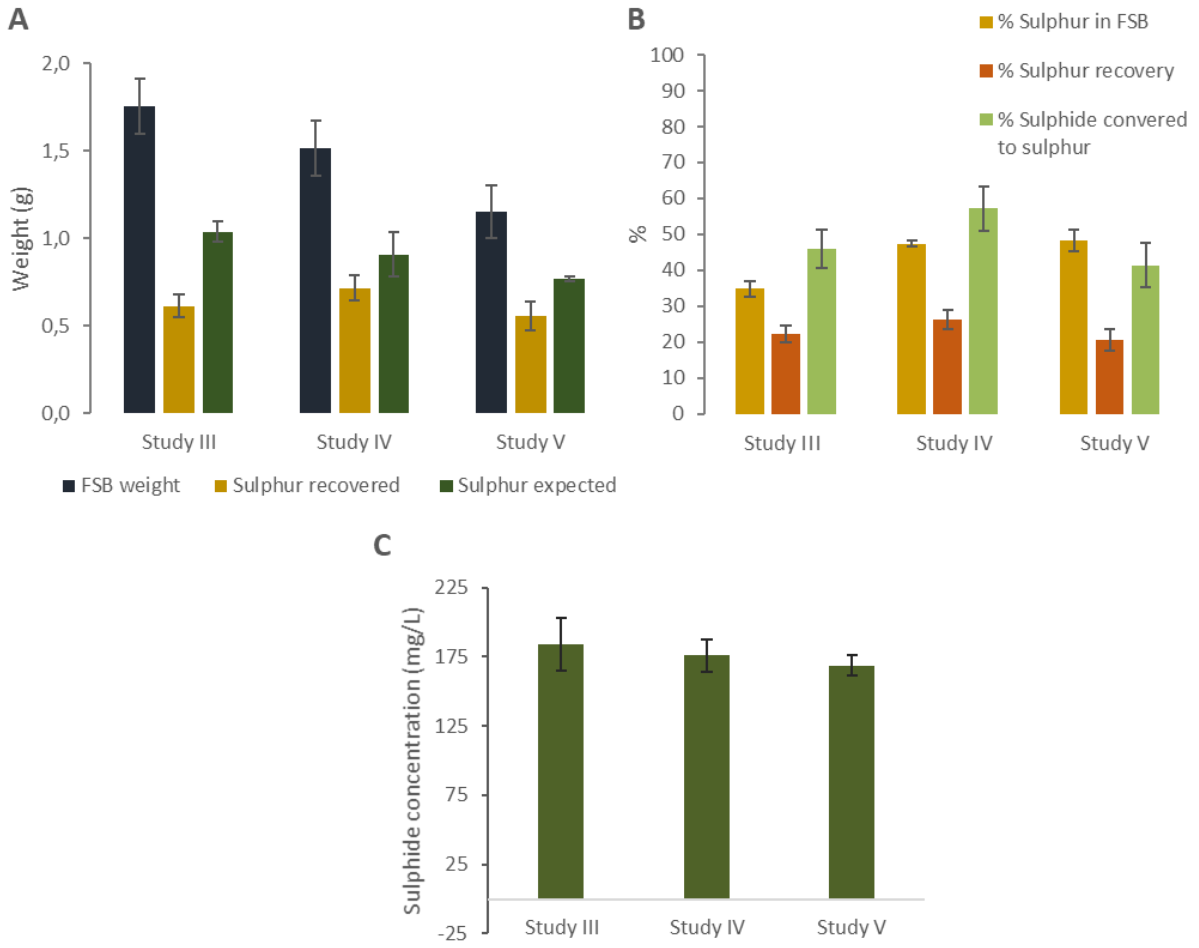


Figure 6-10 Sulphide oxidation and sulphur recovery in the acetate reactor unit of the lactate-acetate coupled LFCR across the different the varying inorganic ion contents in the feed of Studies III - V. A: Total FSB weight is compared to the amount of sulphur contained in the FSB as well as the amount of sulphur expected. Sulphur expected is calculated using the expected and observed sulphide levels in the effluent. B: Percentages of sulphur recovery and sulphide oxidation. The percentage sulphur recovery denotes the amount of sulphur recovered as a proportion of the total sulphur load, while the percentage sulphide converted to sulphur is with regards to the sulphide produced within the single reactor unit. C: Residual sulphide levels at pseudo-steady state. Error bars represent one SD ± the mean (n≥2).

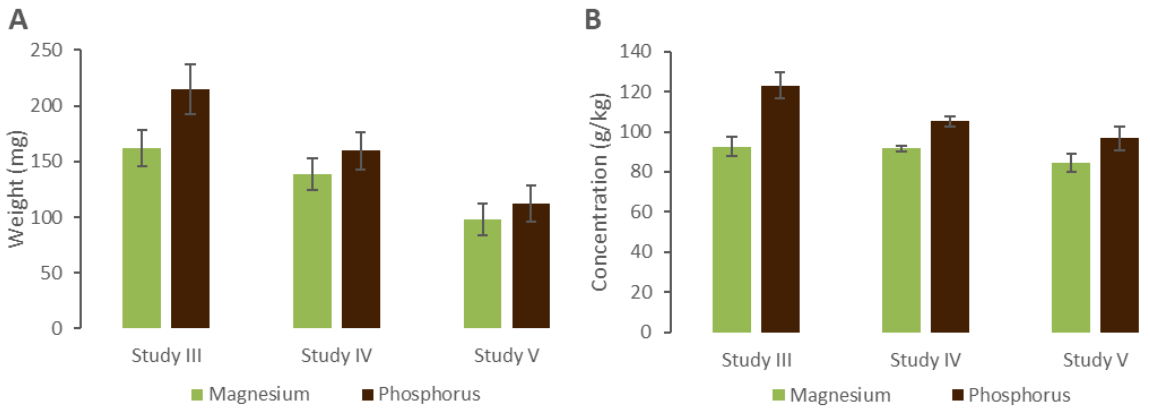


Figure 6-11 ..ICP-MS elemental analysis of the amount (A) and concentration (B) of magnesium and phosphorus in the FSB of the acetate reactor unit. Error bars represent ± one SD from the mean (n=2).

6.3.4 Discussion

The results presented above have shown that reducing the amounts of magnesium and phosphate in the media supplied to a system such as that described here has a positive effect of increasing the amount of sulphur recovered as elemental sulphur. This was clearly the case in the lactate unit of the coupled reactor. Here, it can be postulated that a lower number of crystallised inorganics in the FSB allowed for a greater mass transfer of oxygen to the surface of the bulk volume to allow for partial oxidation of the sulphide to sulphur. In the typical functioning of the FSB, the biofilm itself creates optimal conditions for partial oxidation to occur as it limits oxygen transfer preventing full oxidation of sulphide to sulphate. However, as it matures and becomes thicker it allows less mass transfer of oxygen, thus reaching a point where it causes oxygen availability to become rate-limiting and partial oxidation rates decrease (Mooruth, 2013). In Study III, the build-up of inorganic crystals as well as sulphur and biomass resulted in the biofilm becoming limiting at a lower sulphur recovery. A reduction in the inorganics in Study IV and V meant that the period of optimal partial sulphide oxidation conditions was prolonged, allowing additional sulphur recovery. Additionally, in many cases less biofilm growth was observed immediately surrounding crystal structures. Essentially, the space taken up in the biofilm by unnecessary crystal structures was replaced by sulphur oxidising bacteria.

The acetate reactor unit behaved differently from the lactate reactor unit. Being the secondary reactor unit, concentrations of solutes into the reactor are expected to be much lower than those feeding into the lactate unit. Study III shows that despite this, as was investigated in Chapter 5, the acetate reactor still received enough of the necessary feed components for both its sulphate reducing microbial component, as well as its sulphide oxidisers and the heterotrophs needed to form the initial EPS. It is not clear whether the decrease in magnesium in Study IV adversely affected the biofilm in the acetate unit. Sulphur yield remained consistent with Study III because although there was a decrease in the biofilm harvested, the concentration of sulphur increased and negated the effect from the smaller harvest (Figure 6-10). In Study V, the FSB appeared to be negatively impacted but this did not significantly impact the sulphur recovery or affect the sulphate reduction despite only a very thin biofilm for much of the study (Table 6-1, Figure 6-10). The data from Study V suggests that phosphorus decreased to a level too low to support formation of a biofilm as structurally robust as previously and

that this was perhaps due to a negative impact on some of the microbes within the biofilm – perhaps those associated with EPS formation. The change did not come from changes of the inorganics within the biofilm as these remained stable. Importantly however, the SOB community did not appear to have been jeopardised since sulphur recovery remained stable.

As mentioned in Section 6.3.1, a previous study by Marias (2020) in the hybrid LFCR achieved sulphide conversion to sulphur in the range of 20%. However, in a sulphide oxidising LFCR, Mooruth (2013) achieved much higher maximum sulphide conversion to sulphur (78%). This study confirms that this discrepancy can in part be attributed to the rich Modified Postgate B media required for SRB functioning. Figure 6-4 shows that sulphide conversion to sulphur in Study III was 20%, similar to that achieved by Marais (2020). The percentage of sulphide conversion in this study reached maximums of 50% in the lactate reactor in Study V (Figure 6-4) and 57% in the acetate reactor in Study IV. With regards to improving sulphur yield further, Study V suggests that additional reduction of the phosphate component in the feed would not be recommended, but further lowering of the magnesium content could be investigated. Previously, evidence for increased mechanical stability in biofilms from the crosslinking of multivalent ions such as calcium with components of the EPS has been shown (Körstgens *et al.,* 2001). This suggests that too great a decrease in these ions may compromise the structural integrity of the biofilm, in addition to potentially impacting some of microbial community as appeared to be the case in the acetate reactor unit in Study V.

Further to what has been discussed so far, it should be noted that certain differences in the harvesting of the biofilm could have also led to increases in sulphur recovery in this study compared to that of Marais (2020). Firstly, in the work described here, the biofilm was harvested every four residence times. This contrasts with Marais (2020) where the biofilm was collapsed onto a wire gauze just below the surface of the bulk volume after several residence times, where it remained and was harvested with the next biofilm collapse. The first biofilm may have broken down somewhat during the second cycle run as was noted in the mentioned study. Secondly, the use of a wire gauze to harvest an FSB results in some loss as residues from the biofilm are caught in the mesh. In the studies performed here, a wide spatula was used to scrape the biofilm off the surface of the reactor and the wire gauze was simply used as a back-up harvesting method to catch any pieces that broke away so that they were not lost to the base of the reactor. This may have been a more efficient method of harvesting.

Indeed, these changes may have also affected the ‘sulphur gap’ observed in this study. Previously, a significant sulphur gap has been observed in attempting to close the sulphur balance in the similar system mentioned (Marais, 2020). The difference between the sulphur expected and sulphur recovered represents the gap in the sulphur balance. Figure 6-4 A shows that the sulphur gap progressively closed from Study III to Study V. This is explored in more detail in Table 6-2 where it given as a percentage of the total sulphur load.

Across the sequential system, a closing of the sulphur gap can be seen in the lactate unit with decreasing inorganics in the feed. However, because the percentages are relatively small the error associated is proportionally large in some cases and this data should be regarded under this light. In particular, introduced error in this data is associated with fluctuating sulphate and sulphide

concentration levels in the effluent. In order to calculate the sulphur balance, the amount of elemental sulphur recovered is added to the cumulative amount of sulphate and sulphide released into the effluent stream over the course of the run. While the elemental sulphur recovered is an absolute measurement of the continuous process of sulphur deposition into the biofilm, the cumulative sulphate and sulphide are estimates from distinct data points taken across the run and their reliability is limited by the number of data points. Overall, the increase in sulphur recovery across the studies was attributed both to an increase in partial sulphide oxidation and sulphur recovery, as well as to an apparent closing of the sulphur balance.

Table 6-2 The percentage sulphur gap across the sequential system as a whole as well as each reactor unit. The sulphur gap is the amount of unaccounted-for sulphur after balancing the amount of sulphate in the with the sulphate remaining, sulphide measured, and sulphur recovered. Error bars represent one SD ± the mean (n≥2).

	% Gap in sulphur balance		
	Dual system	Lactate reactor unit	Acetate reactor unit
Study III	23 ± 4.4	7.3 ± 1.5	15 ± 3.2
Study IV	11 ± 6.3	3.8 ± 3.8	7.0 ± 5.3
Study V	7.3 ± 5.6	-0.5 ± 3.3	7.8 ± 3.0

Further to the work presented in chapter 5, the value of the sequential reactor can be seen with respect to sulphide removal and elemental sulphur recovery. Comparing the lactate and acetate reactor from the base case in Study III, sulphur recoveries were 0.311 g and 0.614 g respectively, with total sulphur recovery across the system being 0.925 g (Figure 6-4 and Figure 6-10). The secondary reactor therefore provided substantially more sulphur recovery to the system. In fact, the relative increase in sulphur yield that the secondary reactor contributes, is even greater than the relative increase in sulphate reduction contribution. The secondary reactor receives sulphide not channelled to the lactate reactor FSB in the overflow from the lactate reactor, as well as producing more sulphide from BSR, and can channel both these streams to the FSB.

Previous work has shown simultaneous sulphate reduction and sulphide oxidation within the same reactor unit via tightly controlled micro-aeration of a sulphate reducing bioreactor (Xu *et al.,* 2012b). Sulphide conversion to elemental sulphide was estimated in this study via the gap in the sulphur balance and peaked at 72% on a sulphate load of 1.8 g/L/day. This compares with the sulphate load utilised in the present study of 1.1 g/L/day. The results obtained in the current work could be further improved by adjusting the surface area to volume ratio, as has been previously demonstrated, which would allow for a great area for FSB formation (Marais, 2019). Optimal FSB formation and harvest has also been studied extensively with respect to other factors such as hydraulic residence time (HRT) and the frequency of harvesting (Marais, 2020; Mooruth, 2013) and therefore, the results presented here should be taken in conjunction with these studies to achieve good sulphur recovery.

6.4 Conclusion

This chapter has successfully demonstrated improved sulphur recovery by reducing magnesium and phosphate inorganic ions in the feed, without jeopardizing sulphate reduction in the hybrid LFCR. In the

lactate reactor unit, both the elemental sulphur yield and the concentration of sulphur in the FSB increased significantly with reductions in inorganics. It is proposed that the reduced extent of magnesium phosphate crystallisation in the FSB allows increased oxygen mass transfer across the biofilm. Reduced phosphate concentrations led to visible deterioration of biofilm formation in the secondary, acetate reactor of the sequential system. However, the yield in the secondary reactor remained stable, with an increase in the concentration of sulphur in the FSB of this reactor as well. Additionally, throughout the studies the secondary acetate-metabolising reactor added significantly to the overall sulphur yield, recovering substantially more sulphur than the lactate reactor in Studies III and IV, adding further value to the sequential reactor system demonstrated in this thesis.

Chapter 7 Conclusions and recommendations

BSR is proposed as a potential treatment option for low-flow, sulphate-rich wastewaters, such as acidic or neutral rock drainage. Operation of the BSR process as a semi-passive process using the hybrid LFCR for concomitant sulphate reduction and partial sulphide oxidation, facilitating an elemental sulphur-rich product with potential as a fertiliser is receiving interest as a route to treat distributed rock drainage streams associated with South Africa's mining legacy. For such processes, achieving remediated fit-for-purpose water as a product of the process is a requirement. In this thesis, the research conducted has sought to improve the hybrid LFCR BSR / BSO process through addressing key issues of a BSR system with elemental sulphur recovery, surrounding electron donor usage efficiency and cost, optimisation of sulphide removal through elemental sulphur recovery, and final water quality. These are all noted as key requirements for a techno-economically feasible BSR process.

A more cost-effective electron donor than commonly used lactate or ethanol would be of benefit for wide-scale and geographically distributed treatment of low-flow mine drainage associated with mining operations. Propionate was chosen as a potential low-cost electron donor for investigation as it can be generated from fermentation or digestion of organic waste streams. A propionate-fed hybrid LFCR reactor with BSR and concomitant partial BSO was set up and studied in this thesis to explore this potential. Despite enrichment of the inoculum on propionate, the microbial consortium colonising the LFCR appeared low in propionate-utilising SRB. Acetate formed as the product of propionate fermentation and was used preferentially for BSR, giving an intermediate VSRR of 190 mg/L/day. It has been noted previously that propionate utilising SRB are less common in the literature. Further, the small number of propionate-utilising SRB reported in literature were not evident at >1.5% abundance in the microbial ecology analysis undertaken.

To further address the challenge of a cost-effective electron donor; improvement of the efficiency of electron donor use over the typically reported incomplete utilisation of more complex VFAs than acetate was explored using lactate as the model VFA, owing to the copious research reported on it. Lactate is one of several more complex electron donors that provide a more robust SRB community which can handle varying sulphate loads more easily. However, these VFAs leave high COD in the effluent in the form of acetate due to incomplete oxidation. Therefore, while the robustness offered by these carbon sources is desired, a mechanism of handling the remaining COD is required. This led to the development of the sequential lactate-acetate reactor system which allowed increased electron donor efficiency for BSR via the utilisation of acetate in the secondary reactor, which was pre-colonised with acetate-oxidising SRB. Overall increased biological sulphate conversion from some 50% in the single lactate-fed LFCR to some 85% in the sequential reactor system was demonstrated with decreased residual sulphate and COD concentrations, from 480 to 150 mg sulphate/L and from 400 to 270 mg acetate/L. This was accompanied by a 1.9-fold increase in the sulphate reduced per unit lactate used in the sequential reactor, with no compromise on the VSRR of some 220 mg/L/day. The achieved residual sulphate levels lay below the recommended 200 mg/L national limit in South Africa. The concept of this reactor setup could be extended to many reactor types that are fed with more complex

organic electron donors that lead to the accumulation of acetate, providing that a system of biomass retention is used to facilitate the appropriate microbial consortia in each reactor. Prior separate colonisation of the biomass retention structures – in this case the carbon microfibers – of the acetate LFCR allowed for a strong community of acetate utilising SRB to be incorporated into a lactate-fed reactor system upon linking this reactor in series to the lactate LFCR. A key aspect of the sequential lactate-acetate LFCR system was the ability to create compartmentalised, community specific units in an otherwise well-mixed system.

The sequential reactor system not only improved BSR and water quality but substantially increased sulphur recovery. The overall sulphur yield increased in the sequential system compared to the independent lactate reactor by three-fold in the base case study; a proportionally higher rise than the increase in BSR from the independent to the overall sequential system.

Additionally, sulphide oxidation and sulphur recovery were optimised in the sequential reactor system by the reduction of the feed inorganic ions, magnesium and phosphate. These ions are often present in high concentrations in BSR systems due to their presence in Postgate media, which is commonly used for SRB selection. This work has demonstrated that the inorganics are not necessary in such high concentrations; sulphate reduction was not jeopardized upon reducing both inorganic ions. Further, sulphide removal and sulphur recovery to biofilm were increased. The presence of these ions in high concentrations was therefore detrimental to sulphur incorporation into the biofilm, presumably due to decreased oxygen mass transfer across the biofilm. Apart from the increased value of higher elemental sulphur recovery, the work demonstrated may allow cost savings with respect to the amount required of these inorganics for supplementation into a BSR system.

Recommendations to further this work most notably include challenging the LFCR system described with a ‘real world’ ARD stream which would have a much lower nutrient content. Fine-tuning the inorganic concentrations needed, particularly with regards to magnesium, in order to determine the minimum concentration that would fulfil the needs of the SRB and SOB communities would be of benefit and may lead to greater sulphur yields. Increased sulphate loading in the sequential LFCR would be of interest to determine how a range of sulphate loading is managed by the system and to establish the maximum load that can be tolerated.

To conclude, South Africa, as with 40% of the global population, is water stressed, hence water quality and resource efficiency is of utmost importance. Where mining operations or, more particularly, mining legacies are present, economically feasible and sustainable strategies are required to mitigate the effects of ARD. This work has provided further research into and understanding of BSR in semi-passive systems particularly relevant to diffuse, low-flow, sulphate-rich ARD sources.

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Appendix A **Analytical methods**

A.1 Sulphate assay standard curve

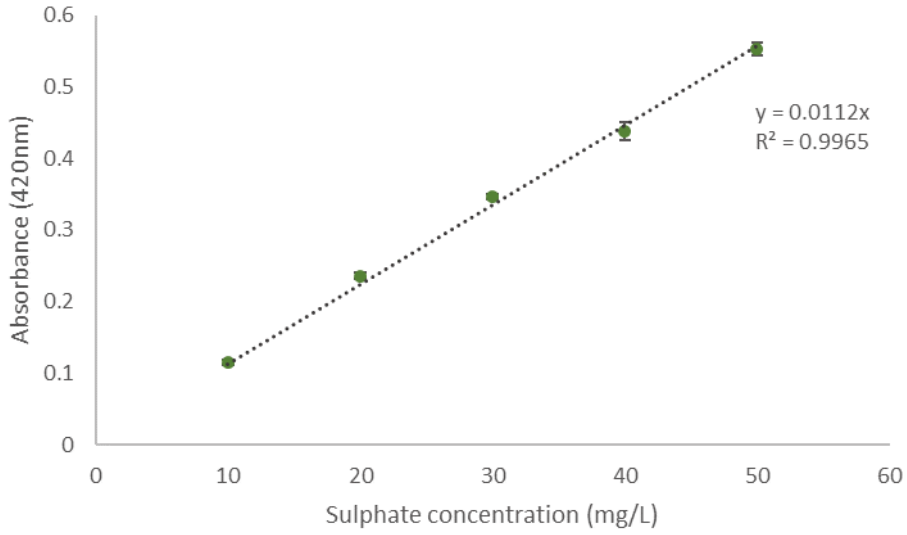

Figure. A-1: Sulphate standard curve generated using a 1 g/L sodium sulphate solution. Equation of the line and R^2 value is indicated. Error bars show the mean ± one SD (n=3).

A.2 Volatile fatty acid standard curves

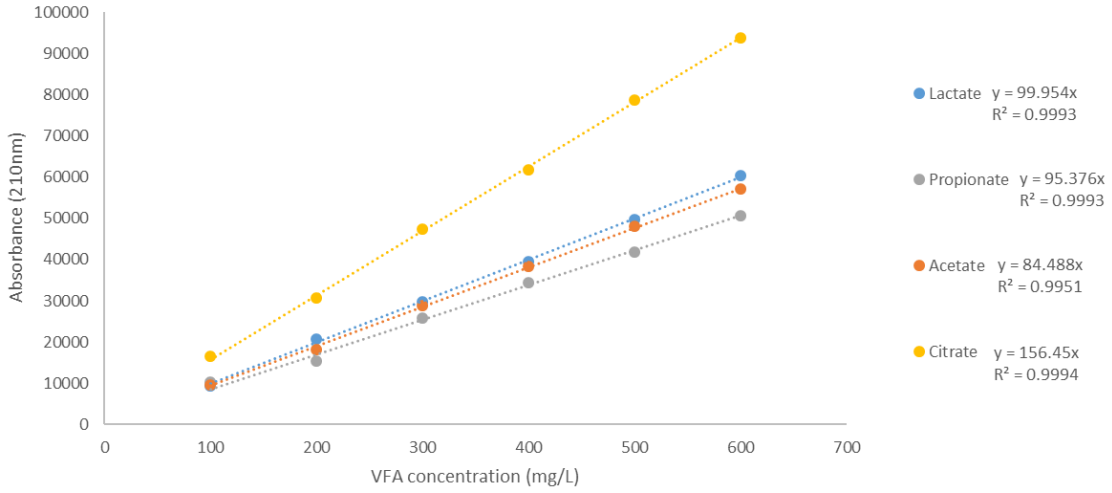

Figure. A-2: VFA standard curve; the gradients and associated R^2 values are indicated next to the legend entry for each VFA.

A.3 Sulphide standard curve

A sulphide standard curve was generated using a 100 mg/L sulphide standard solution made up using sodium sulphide nonahydrate.

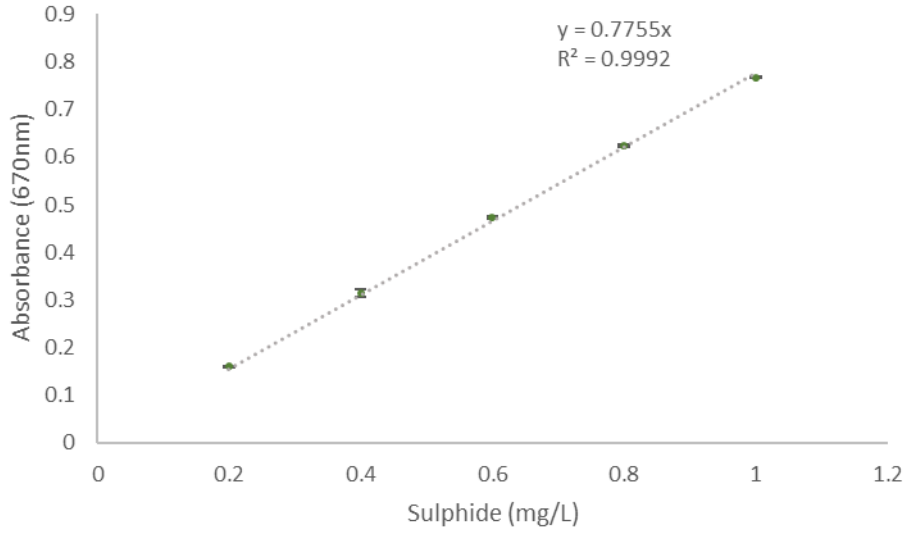

Figure. A-3: Sulphide standard curve generated using a 100 mg/L sulphide solution. Equation of the line and R^2 value are indicated. Error bars represent the mean ± one SD (n=3).

A.4 Miscellaneous regents

A.4.1 Phosphate buffered saline

A 10x phosphate buffered saline (PBS) stock solution was prepared using 80 g/L sodium chloride, 2.0 g/L potassium chloride, 14.4 g/L di-sodium hydrogen phosphate, 2.4g of potassium dihydrogen phosphate. The buffer was adjusted to pH 7.4.

Appendix B **Supplementary results**

B.1 Propionate batch cultures

Several batch cultures were started to select for and propionate-utilising SRB and to the start the propionate-fed LFCR with an active SRB community for effective colonization of the carbon microfibres. Figure. B-1 shows time course data for the culture selected to start up the propionate reactor.

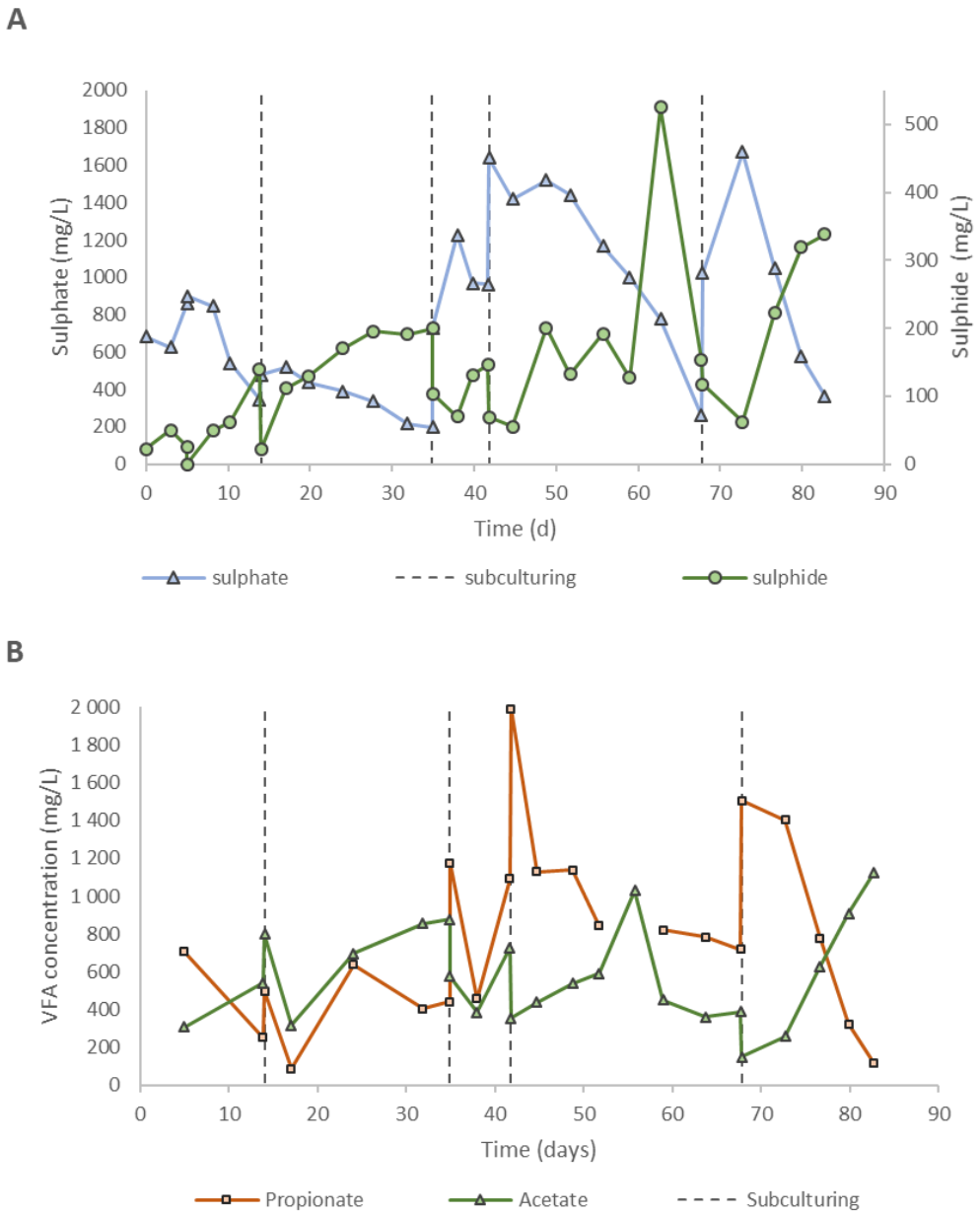

Figure. B-1: Batch culture raised for inoculation of the propionate-fed LFCR. The culture was started and maintained for the time period shown until start of the reactor. Dotted lines indicate draw and fill sub-culturing events. A: Sulphate and sulphide levels, B: propionate and acetate levels.

B.2 Propionate LFCR

B.2.1 Reactor start-up period

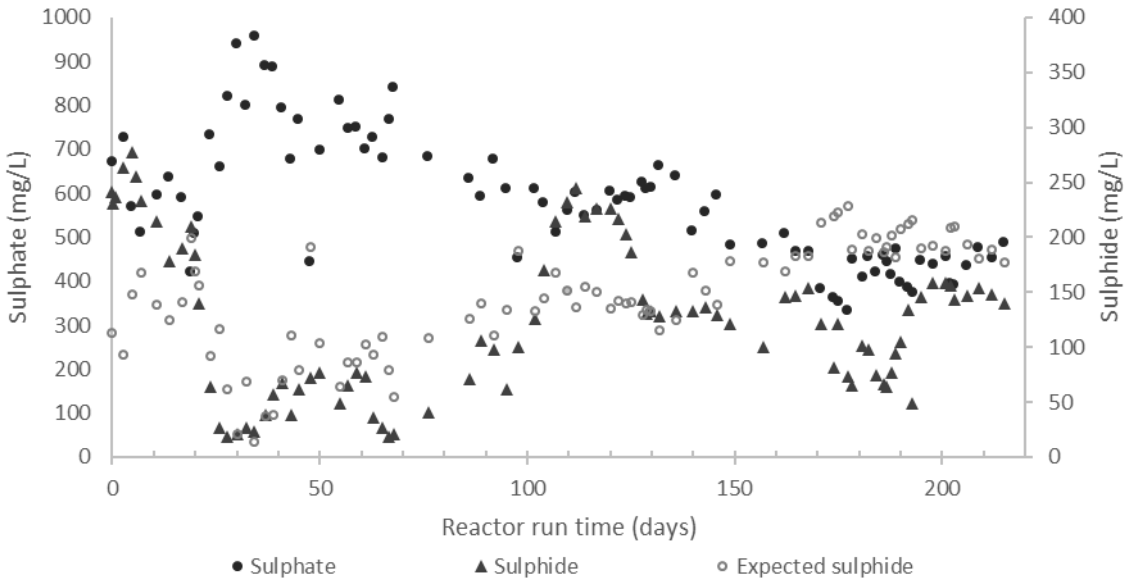

Figure. B-2: Start up period of the propionate LFCR showing average bulk sulphate and sulphide levels.

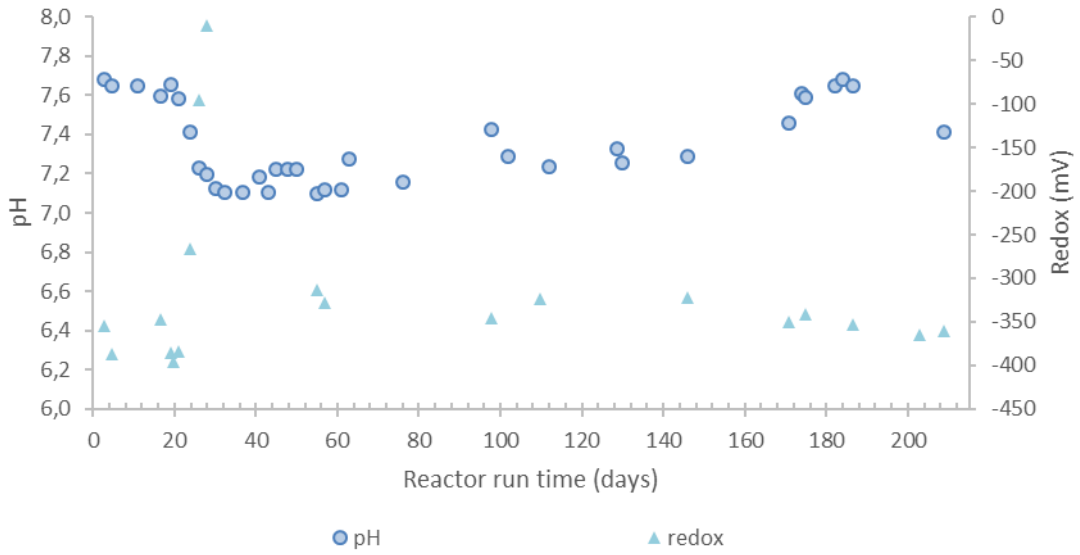

Figure. B-3: Redox and pH readings across the start-up period of the propionate LFCR.

B.2.2 HRT study

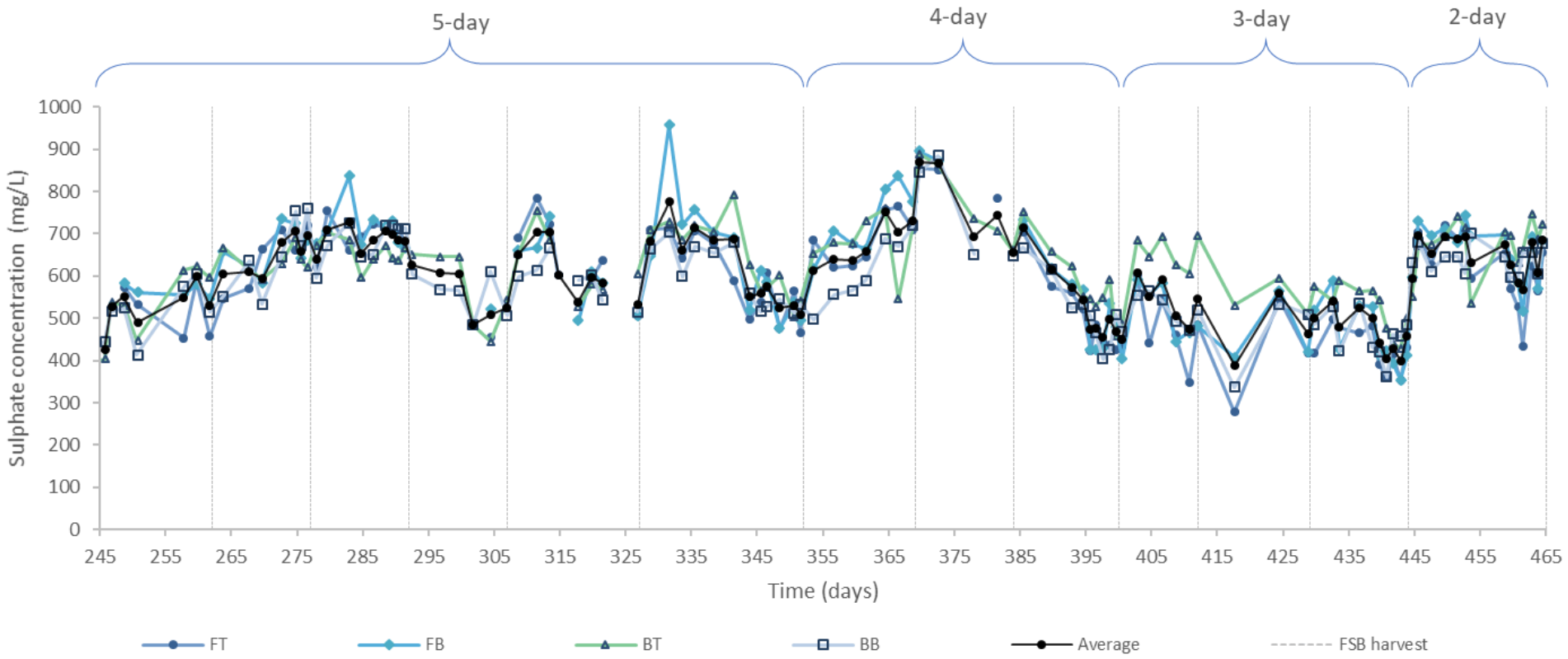

Figure. B-4: Bulk sulphate levels time course data across the HRT study.

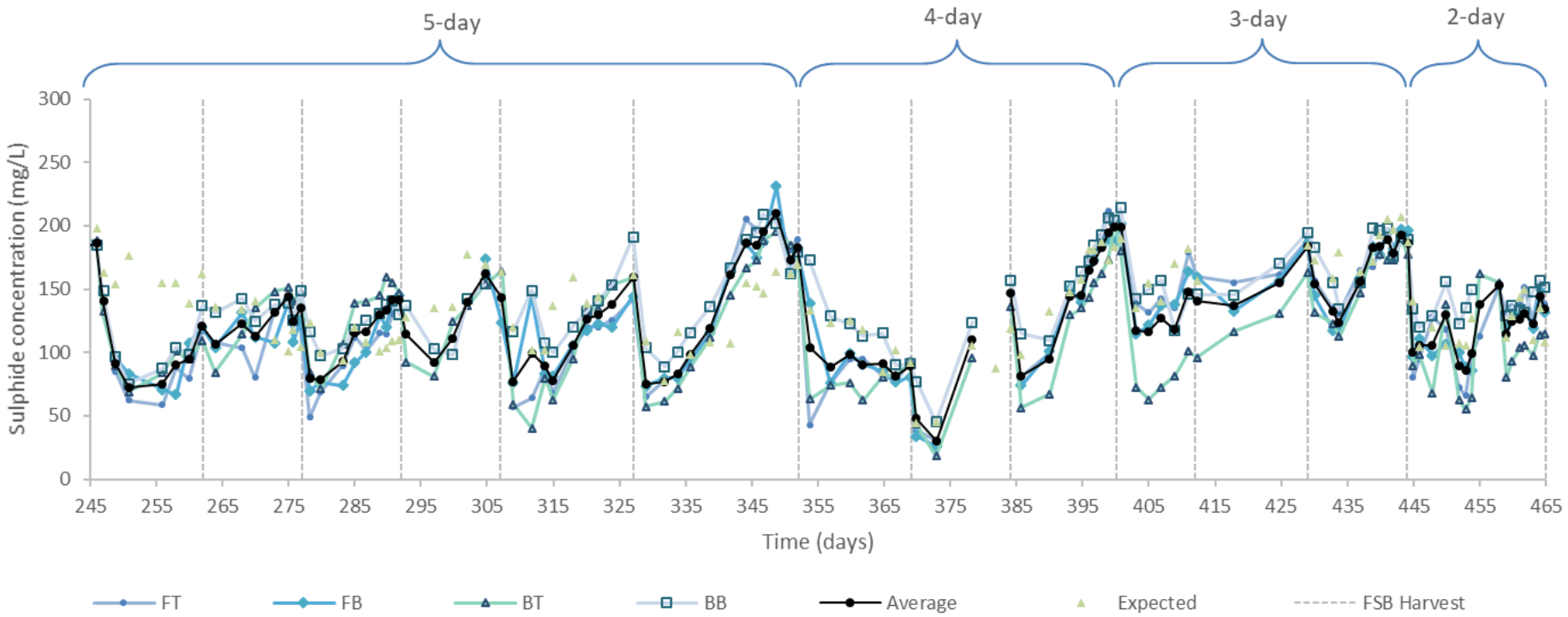

Figure. B-5: Bulk sulphide levels time course data across the HRT study.

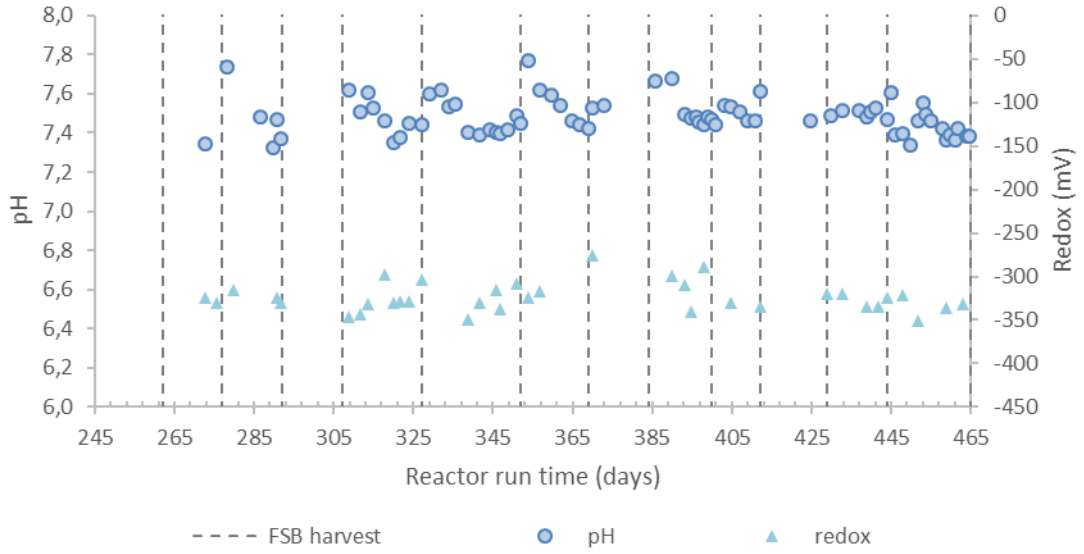

Figure. B-6: Redox and pH readings across the HRT study.

B.3 Dual lactate-acetate reactor

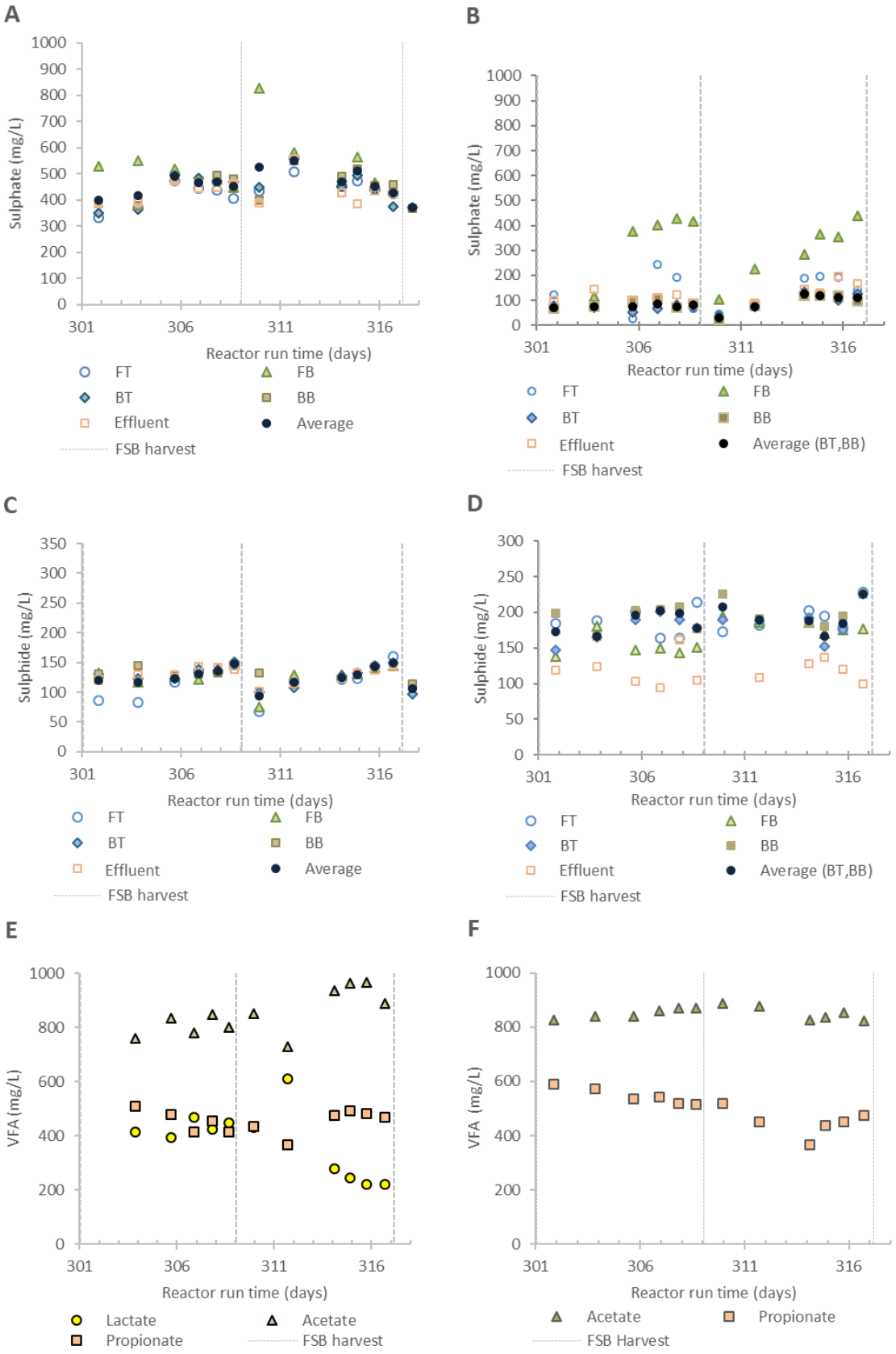

Figure. B-7: Performance time course data for the sequential reactor, Study III. Dotted lines indicate FSB harvest events.

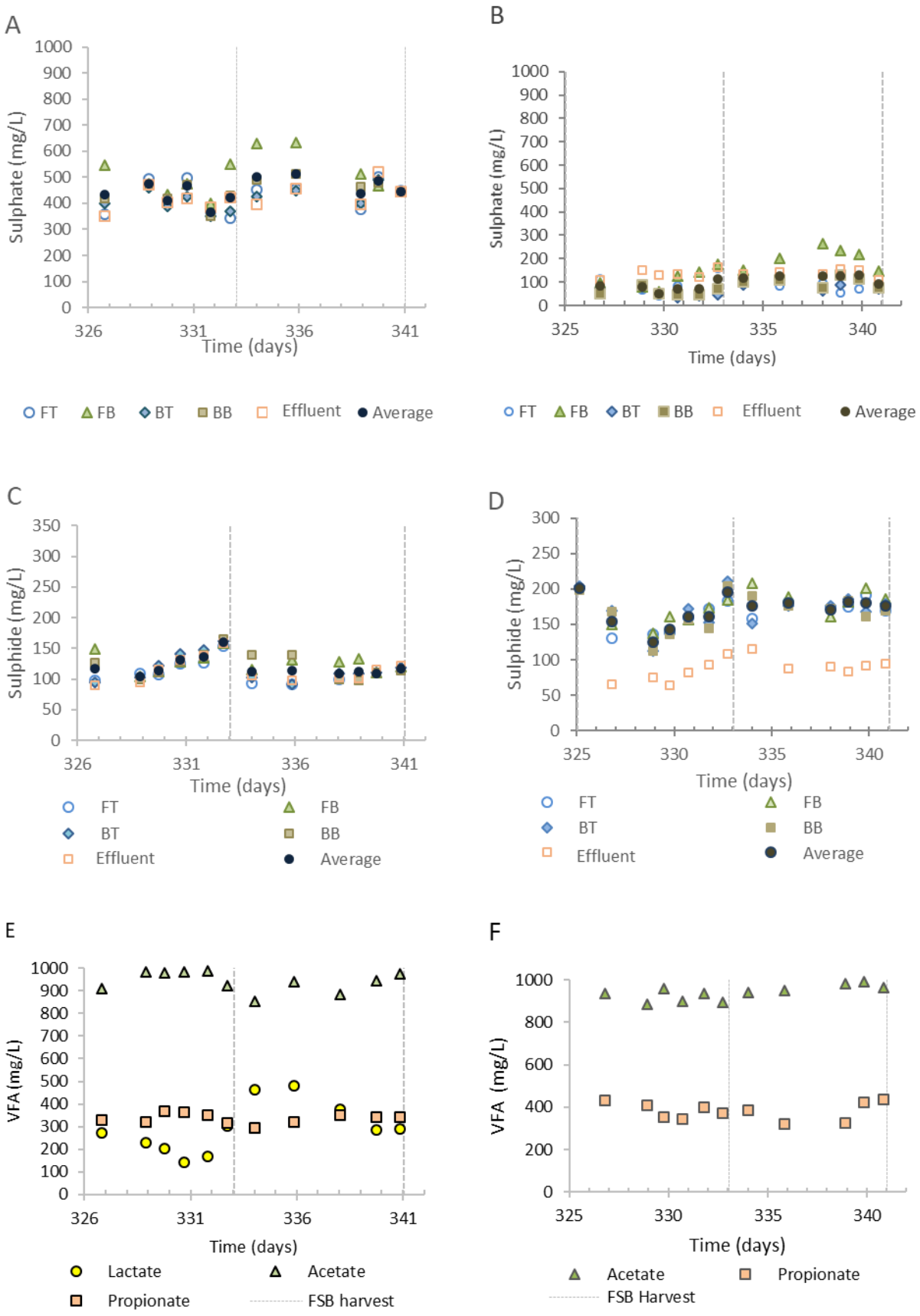

Figure. B-8: Performance time course data for the sequential reactor, Study IV. Dotted lines indicate FSB harvest events.

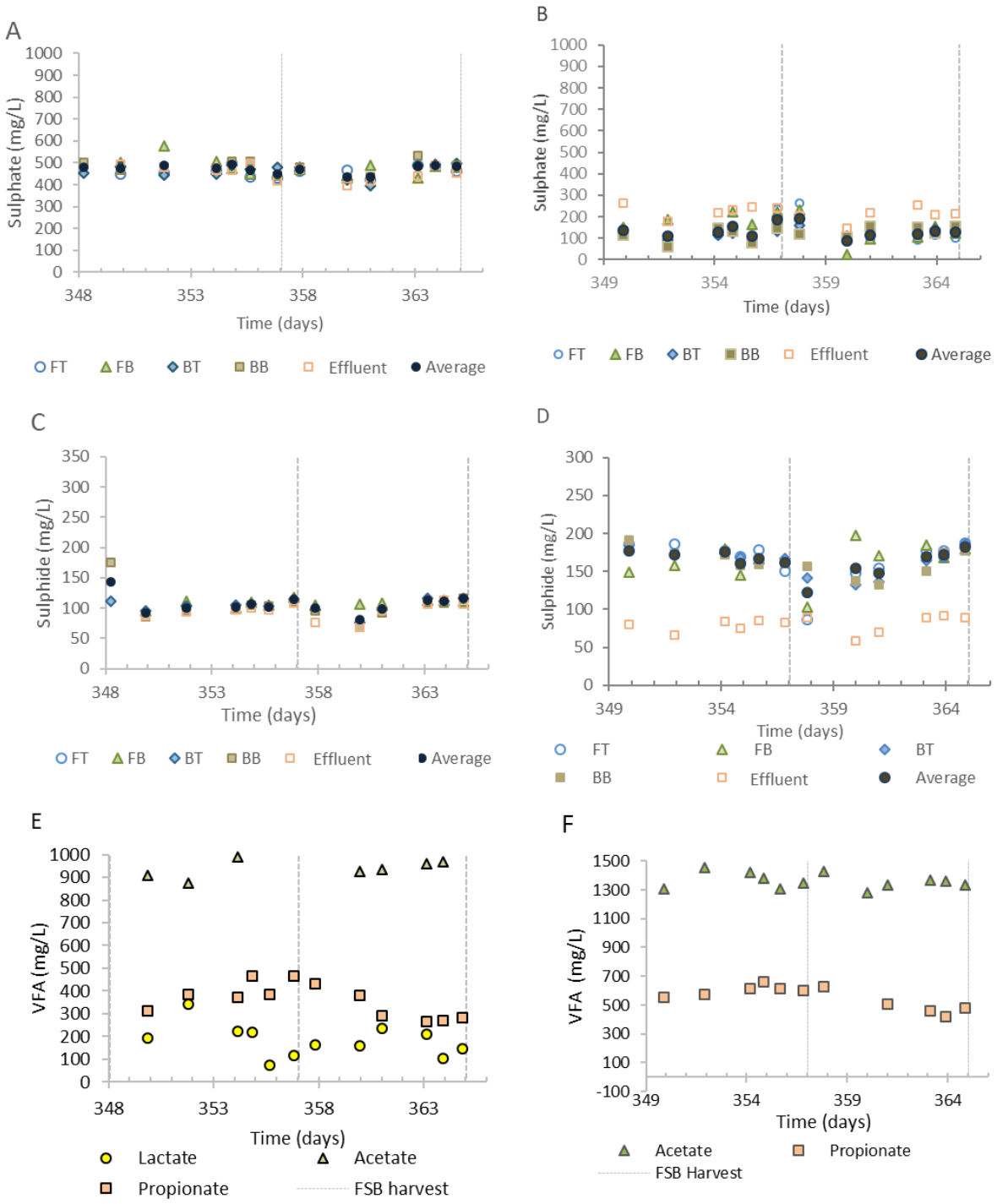

Figure. B-9: Performance time course data for the sequential reactor, Study V. Dotted lines indicate FSB harvest events.