Linking microbial community dynamics and performance of a biological sulphate reducing system using a mixed volatile fatty acid stream as electron donor

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This work is dedicated to my mother ‘M’e ‘Manyatso Motleleng and my grandmother Nkhono Halitšoane ‘Makherehloa Molapo Matela for being mighty and masterpieces in my life.

ABSTRACT

Mining for the recovery of minerals and coal can result in acid mine drainage (AMD) which presents an environmental risk. Acid mine drainage, as the name suggests, is acidic run-off water from mostly mine waste dumps. It affects water quality by lowering its pH and increasing its metal and sulphate loading, thus making it unsuitable for use by many forms of life. AMD must therefore be treated before entering nearby water systems and soils. An effective treatment technology is considered as the one that can result in water neutralisation and removal of metals and sulphate. Biological sulphate reduction (BSR) technologies, mediated by sulphate reducing bacteria (SRB), have attracted attention as a sulphate remediation strategy as they offer a cheap alternative to other sulphate removal technologies such as chemical approaches. In addition, the concomitant generation of alkalinity and soluble sulphide assist in neutralisation and heavy metal removal. One of the challenges associated with BSR is the supply of a cost-effective carbon source which also acts as an electron donor for the anaerobic reduction of sulphate.

Studies have reported that both the choice of carbon source and electron donor and the microbial communities present influence the sulphate reduction process, the former frequently defining technoeconomic feasibility. The feed sulphate concentration and residence time, together defining the volumetric sulphate loading rate, have also been reported to influence the efficacy of the sulphate reduction process and needs to be optimised for the microbial community present and the chosen electron donor. The identification and characterisation of the microbial communities involved and investigating how these change with changes in operating conditions is crucial in the optimisation of BSR processes. Currently, there are no commonly used molecular tools which can be used for routine analysis of SRB communities in real time and on a regular basis and cost effectively. This makes it difficult to understand the link between changes in the mixed BSR microbial community structure and process performance. The study presented in this thesis had three main objectives. Firstly, to evaluate the use of an anaerobic digestate, obtained from a partially anaerobically digested Cyanobacteria species (*Arthrospira platensis*, commonly known as Spirulina), as a carbon source and electron donor for BSR. Secondly, to validate, optimise and apply the molecular tools for analysis of the relative abundances of species within the mixed BSR microbial community in this study. Thirdly, to compare the microbial community dynamics and performance of BSR using the complex anaerobic digestate as carbon source and electron donor to BSR using a single electron donor source, lactate.

Chemostat studies using a mixed SRB consortium were carried out using anaerobic digestate, characterised as containing a mixture of acetate, propionate and butyrate, as a carbon source and electron donor for BSR. Upon reaching steady-state, the concentrations of sulphate, bicarbonate, acetate, propionate and butyrate were measured and used to estimate the BSR kinetics and reaction

stoichiometry. A 16S rRNA gene survey of the BSR inoculum used for this thesis was performed by constructing a 16S rRNA gene clone library and analysis of the diversity of clones was performed using amplified ribosomal DNA restriction analysis (ARDRA). These 16S rRNA sequences were used to provide insight into the diversity and phylogenetic relatedness of the bacterial community and key species within the mixed BSR inoculum. *In silico* analysis of the 16S rRNA sequences captured from the clone library was performed to design novel genus specific quantitative real-time PCR (qPCR) primers and to validate the specificity of previously published primers. Fluorescence *in situ* hybridisation (FISH) techniques were optimised for the visual characterisation of this microbial community. FISH and qPCR were then applied to assess how the mixed microbial community structure was affected by the changes in the volumetric sulphate loading rate (VSLR), mediated through dilution rate and feed sulphate concentration, when anaerobic digestate (mixed carbon source) and lactate (simple carbon source) were used as an electron donor for BSR. The results obtained were used to examine and compare the link between microbial community dynamics and performance of sulphate reducers between the mixed and the simple carbon source.

The results obtained from this thesis suggested the simultaneous utilisation of all the three volatile fatty acids (acetate, propionate and butyrate) present in anaerobic digestate which contributed to the robustness of the chemostat reactors as indicated by higher sulphate, propionate and butyrate conversion efficiencies. The kinetic profiles of the volumetric sulphate reduction rate (VSRR) obtained with anaerobic digestate were well matched with the kinetics observed in previous studies when single carbon sources and electron donors were used for BSR. At a feed sulphate concentration of 1.0 g l^{-1}, the oxidation of acetate, propionate and butyrate and concomitant sulphate reduction were observed across the dilution rates of 0.0083 to 0.083 h^{-1}. The stoichiometry of BSR utilising propionate and butyrate as carbon and electron donor suggested that by increasing feed sulphate concentrations from 1.0 to 2.5 and 5.0 g l^{-1} acetogenic reactions were favoured at the higher dilution rates of 0.042 and 0.083 h^{-1}. However, increasing the feed sulphate concentration at the lower dilution rates of 0.0083 to 0.021 h^{-1} did not alter the oxidation of volatile fatty acids (VFAs) and concomitant sulphate reduction, suggesting that the sensitivity of the propionate and butyrate oxidisers was related to specific growth rate rather than the sulphate loading. A previous mathematical model developed by Moosa *et al*. (2002) was used to determine microbial growth constants (μ_{max} and K_s) and energetic coefficients ($Y_{x/s}$) for SRB at each feed sulphate concentration to describe the microbial growth kinetics obtained with anaerobic digestate.

A 16S rRNA gene survey, performed by 16S rRNA library construction and 16S rRNA gene amplicon sequencing, revealed a more diverse microbial community in the inoculum obtained from a lactate operated BSR reactor than previously reported. qPCR was used to confirm the presence and relative abundance of these species within the reactors receiving anaerobic digestate or lactate as carbon source and electron donor. The 16S rRNA sequences captured were found to have high similarity to well-

known SRB species belonging to the *Desulfomicrobium, Desulfovibrio, Desulfuromonas, Desulfobulbus* and *Desulfocurvus* genera. Other "non-traditional SRB" species belonging to the *Firmicutes* and *Citrobacter* genera containing a specific molecular target for the detection of SRBs, the *dissimilatory sulphite reductase* gene (*dsrAB*), within their genomes were also detected. DsrAB is the key enzyme catalysing the last and main energy-generating step during sulphate reduction. Non-SRB species present were identified as members of the *Sphaerochaeta, Synergistetes, Chloroflexi, Mesotoga, Acholeplasma, Bacteriodetes, Petrimonas* and *Bacteriodes* genera. A 16S rRNA gene survey by 16S rRNA variable region amplification from metagenomic DNA extracted from microbial biomass associated with continuous stirred tank reactors (CSTRs) operated on anaerobic digestate or lactate was performed to validate the qPCR results and assist with the identification of the "other SRB" and non-SRB species. The 16S rRNA gene survey suggested the presence of 13 known SRB species *Desulfomicrobium* groups (*Desulfomicrobium hypogeium* and *Desulfomicrobium aestuarii*), *Desulfovibrio* species (*D. aminophilus, D. vulgaris, D. desulfuricans, D. intestinalis, D. oxamicus*, and *D. sulfodismutans*), *Desulfobulbus oligotrophicus, Desulfocurvus vexinensis, Desulfococcus biacutus, Desulfarculus baarsii, Desulfomonile tiedjei* and *Desulfobacca acetoxidans* in CSTRs operated on anaerobic digestate. Only up to 10 SRB species, *Desulfomicrobium hypogeium, Desulfomicrobium aestuarii, Desulfovibrio* groups (*Desulfovibrio aminophilus, Desulfovibrio vulgaris, Desulfovibrio desulfuricans, Desulfovibrio intestinalis, Desulfovibrio sulfodismutans, Desulfovibrio mexicanus, Desulfobulbus oligotrophicus* and *Desulfocurvus vexinensis* were observed in reactors with lactate, suggesting that the multiple VFAs present in the anaerobic digestate (acetate, propionate and butyrate) were able to support a more diverse SRB community than a single electron donor (lactate). Various non-SRB bacterial genera as well as known elemental sulphur reducing bacteria *Desulfuromonas acetexigens* and *Dethiosulfovibrio acidaminovorans* were also found to be present, with the latter being associated only with the lactate operated reactor.

qPCR results indicated that despite being present in high proportions at the lowest VSLRs, the *Desulfomicrobium* species were washed out of the reactors at higher VSLRs regardless of carbon source and electron donor was provided. Species from the *Desulfovibrio* genera, which were present at lower abundances than the *Desulfomicrobium* species, were more resistant to changes in dilution rates and remained present within the reactors at the higher VSLRs, 0.104 and 0.208 g l^{-1} h^{-1}. In the reactors operated on anaerobic digestate, the decline in the abundance of *Desulfovibrio* species at VSLRs of 0.052 and 0.104 g l^{-1} h^{-1}, correlated with a noticeable decline in sulphate conversion from 60.4 to 49.4% at feed sulphate of 2.5 g l^{-1}, and from 66.9 to 22.6% at feed sulphate of 5.0 g l^{-1}. These findings suggest that *Desulfovibrio* species may play a critical role in sustained sulphate reduction at lower VSLRs. 16S rRNA gene amplicon data validated the qPCR data showing that increasing the VSLR, resulted in a change in the SRB community structure and a decrease in the proportion of total SRB within the microbial community. In agreement with the FISH and qPCR findings, *Desulfomicrobium hypogeium*

was identified as the most abundant operational taxonomic unit (OTU) belonging to SRB present at the lowest dilution rate (D) tested (0.0083 h^{-1}, retention time (RT = 1/D) of 5 d) when anaerobic digestate was used as an electron donor for BSR. Washout of most SRB species was also observed when the dilution rate was increased from 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d) in these reactors. Species such as *Desulfovibrio sulfodismutans*, *Desulfomonile tiedjei*, the acetate oxidiser *Desulfococcus biacutus* and the elemental sulphur reducing *Desulfuromonas acetexigens* were found to tolerate higher VSLRs of 0.104 and 0.208 g l^{-1} h^{-1} (dilution rate of 0.042 h^{-1}), suggesting fast enough growth rates to remain in these reactors at the higher dilution rate of 0.042 h^{-1}. A decrease in the abundance of the incomplete propionate oxidiser *Desulfobulbus oligotrophicus* correlated to a decrease in propionate oxidation at a VSLR of 0.104 and 0.208 g l^{-1} h^{-1} suggesting that this SRB was responsible for the oxidation of propionate and concomitant sulphate reduction observed in these reactors.

Similar to the reactors receiving anaerobic digestate, increasing the dilution rate from 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d) resulted in washout of most SRB OTUs in CSTRs operated on lactate. At a feed sulphate concentration of 10.0 g l^{-1}, increasing the dilution rate from 0.0083 to 0.042 h^{-1} resulted in an increase in the proportion of the lactate oxidiser *Desulfocurvus vexinensis* from 25 to 98% of the total SRB proportion. At this dilution rate (0.042 h^{-1}), other SRB species observed were the lactate oxidisers *Desulfovibrio sulfodismutans* and *Desulfobulbus oligotrophicus* which can oxidise lactate and the product of its incomplete oxidation, propionate. Although the abundance of these two SRB at the dilution rate of 0.042 h^{-1} was much lower than that of *Desulfocurvus vexinensis*, studies with anaerobic digestate suggested *Desulfobulbus oligotrophicus* which was abundant at only 0.004% and was identified as the only propionate degrader in the CSTR resulted in propionate conversion of 21.7%. This suggested that the less abundant *Desulfovibrio sulfodismutans* and *Desulfobulbus oligotrophicus* may have also played a role in sulphate reduction at the dilution rate of 0.042 h^{-1} in the CSTR with lactate. In addition, *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus* were able to function at a VSLR of 0.42 g l^{-1} h^{-1} which suggests these two SRB species could be used effectively to reduce sulphate to hydrogen sulphide in wastewaters with higher VSLRs of up to 0.42 g l^{-1} h^{-1} when lactate was provided as an electron donor for BSR. The acetate specialist, *Desulfobacca acetoxidans*, the butyrate oxidiser *Desulfarculus baarsii* and the propionate oxidiser *Desulfobulbus oligotrophicus*, were able to function at a VSLR of 0.208 g l^{-1} h^{-1} suggesting that a combination of these three SRB species could be used in BSR treatment processes with VSLRs of up to 0.208 g l^{-1} h^{-1} where anaerobic digestate is provided as an electron donor. The ability for anaerobic digestate to support diverse SRB communities even at higher VSLRs may add to the robustness of the reactors to maintain sulphate reduction even at high VSLRs.

This thesis showed that both the presence and diversity of SRB species are subject to the carbon source and VSLR. To the author's knowledge, this is the first study to indicate the relationship between the

change in SRB community structure and sulphate reduction performance when anaerobic digestate (a complex carbon source) is used as a carbon source and electron donor for BSR. Results from this thesis suggest that the use of a mixed volatile fatty acid stream generated for the partial digestion of a suitably digestible biomass may be used as electron donor and carbon source to support a robust BSR process for the treatment of AMD. Using a mixed volatile fatty acid stream also has potential to result in the development of a more economically viable AMD treatment process.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ALDs	Anoxic limestone drains
AMD	Acid mine drainage
APHA	American Public Health Association
APS	Adenosine phosphosulphate
ARDRA	Amplified rDNA restriction analysis
AS	Assayed sulphide
ATP	Adenosine triphosphate
AUR	Acetate utilisation rate
BC	Butyrate conversion
BSR	Biological sulphate reduction
BOB	Butyrate oxidising bacteria
BOR	Butyrate oxidation rate
bp	Base pair
BUB	Butyrate utilising bacteria
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
Ct	Threshold cycle
D	Dilution rate
CV	Coefficient of variance 4′,
DAPI	4′, 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DFAFBR	Down flow anaerobic packed bed reactor
DNA	Deoxyribonucleic acid
DS	Dissolved sulphide
EDTA	Ethylenediaminetetraacetic acid
EGSB	Expended granular sludge bed
ES	Expected sulphide
FAD	Flavin adenine dinuclotide (reduced)
FBR	Fluidised-bed reactor
FISH	Fluorescence *in situ* hybridisation
gDNA	Genomic DNA
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
l	Litre
kb	Kilobase
Mbp	Megabase pair
MBR	Membrane bioreactor
MPA	Methanogens
MPB	Methane producing bacteria
n	Number of observations
NADH	Nicotinamide adenine dinucleotide (reduced)
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PC	Propionate conversion
PCR	Polymer chain reaction
POB	Propionate oxidising bacteria
POR	Propionate oxidation rate
PUR	Propionate utilisation rate
PP	Pyrophosphate

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
qPCR	Quantitative real time PCR
RE	Restriction enzyme
RFUs	Relative fluorescence units
RT	Residence time
SC	Sulphate conversion
SDS	Sodium dodecyl sulphate
SR	Sulphate reduction
SRA	Sulphate reducing archaea
SRB	Sulphate reducing bacteria
SRM	Sulphate reducing microorganisms
t_d	Bacterial doubling time
T_m	Melting temperature of DNA
T-RFLP	Terminal restriction fragment length polymorphism
TCA cycle	Citric acid cycle
TS	Total sulphide
UASB	Upflow anaerobic sludge blanket reactor
UAPB	Upflow anaerobic packed bed reactor
UPBR	Upflow packed bed reactor
VFA	Volatile fatty acids
VAPR	Volumetric acetate production rate
VAUR	Volumetric acetate utilisation rate
VBUR	Volumetric butyrate utilisation rate
VPUR	Volumetric propionate utilisation rate
VSLR	Volumetric sulphate loading rate
VSRR	Volumetric sulphate reduction rate
w/v	Weight by volume

NOMENCLATURE

		UNITS
C_A	Residual acetate concentration	g l^{-1}
C_B	Residual butyrate concentration	g l^{-1}
C_P	Residual propionate concentration	g l^{-1}
C_S	Residual sulphate concentration	g l^{-1}
C_{S0}	Feed sulphate concentration	g l^{-1}
C_X	Bacterial concentration	g l^{-1}
D	Dilution rate	h^{-1}
e^-	Electron	
F	Feed flowrate	l h^{-1}
I	Concentration of the inhibitory compound	g l^{-1}
K_{e-d}	Half saturation constant for electron donor	g l^{-1}
K_1	Half saturation constant for inhibitory compound	g l^{-1}
$K_{SO_4^{2-}}$	Half saturation constant for sulphate	g l^{-1}
m_s	Maintenance coefficient	g g^{-1} h^{-1}
q_s	Specific substrate utilisation rate based on substrate S	g g^{-1} h^{-1}
r_s	Volumetric acetate production rate	g l^{-1} h^{-1}
r_A	Volumetric acetate utilisation rate	g l^{-1} h^{-1}
r_B	Volumetric butyrate utilisation rate	g l^{-1} h^{-1}
r_P	Volumetric propionate utilisation rate	g l^{-1} h^{-1}
r_S	Volumetric sulphate reduction rate	g l^{-1} h^{-1}
r_X	Rate of biomass formation	g l^{-1} h^{-1}

T	Temperature	°C
t_d	Bacterial doubling time	h
V	Reactor working volume	l
$Y_{x/s}$	Yield coefficient of bacteria X based on substrate S	g g^{-1}
ΔG_{rxn}	Gibbs free energy of reaction	kJ mol^{-1}
ΔG_0	Gibbs free energy of formation	kJ mol^{-1}
ΔR_n	Fluorescence signal	RFU
μ	Specific microbial growth rate	h^{-1}
μ_{max}	Maximum specific microbial growth rate	h^{-1}

GLOSSARY

Abiotic	A reaction condition in the absence of microorganisms
Acetogenesis	A reaction that degrades short chain fatty acids such as butyric acid, propionic acid, or longer chain fatty acids, as well as other intermediates such as ethanol, to acetic acid and hydrogen
Aerotaxis	Movement of an organism, especially a bacterium, toward or away from air or oxygen
Amphipathic	Pertains to a molecule with hydrophilic ("water-loving") or polar end and a hydrophobic ("water-fearing") or nonpolar end
Anaerobic digestion	A process by which organic wastes are biologically transformed in the absence of oxygen
Archaea	A group of microorganisms, genetically and metabolically different from bacteria. They appear to be survivors of an ancient group of organisms
Autotrophic	Microorganism that utilises inorganic carbon as its sole carbon source
Batch culture	Microbial culture produced by inoculating a culture vessel containing a single batch of medium with no nutrient addition with time. Conditions within batch culture change dynamically with time
Bacterial community	A group of organisms inhabiting a particular ecological niche, which could include any number of species
Chemical oxygen demand	A measure of the total amount of oxygen consumed through oxidation of both organic and inorganic material in the waste stream
Chemostat	A continuous bioreactor culture into which medium is fed at the same rate as it is removed by reaction and flow out of the reactor, resulting in a constant chemical concentration as a function of time under steady-state
Citric acid cycle (TCA cycle)	The metabolic cycle that oxidises acetyl coenzyme A to carbon dioxide and generates NADH and $FADH_2$ for oxidation in the electron transport chain with concomitant energy storage as ATP
Commensalism	An interaction of two different species where one species benefits and the other species is not significantly affected
Community resilience	The ability of a heterogeneous community to recover from perturbation
Community stability	Resistance to perturbation or physicochemical changes
Community structure	The numerical composition of different species in a mixed community
Ecophysiology	The interaction of the environment, both physical and biological, with the physiology of an organism
Endogenous metabolism	All chemical activities performed by organisms in the absence of utilizable extracellular materials serving as sources of energy and building stones for assimilation and growth
Eukaryotes	Organisms with cells having chromosomes with nucleosomal structure separated from the cytoplasm by a two membrane nucleus envelope as well as compartmentalisation of function in distinct cytoplasmic organelles
Heterotroph	A microorganism that utilises simple or complex organic molecules as its sole carbon source

Homologous genes	Two or more genes that descend from a common ancestral DNA sequence
Hybridisation	A reaction by which the pairing of complementary strands of nucleic acid occurs
Inhibition	Suppression of bacterial function
Inoculum	Microbial cells added to start a culture
Mesophile	An organism that can grow in the temperature range 20 to 45°C
Metabolism	Physiochemical transformations through which simple substrates are either synthesised into complex elements or complex substances are converted into simple ones and energy is made available for use by the organism
Methanogenesis	The process through which low molecular weight substrates are degraded to form methane. It is a terminal stage of the anaerobic digestion involving methane producing microorganisms called methanogens.
Micelles	Lipid molecules that arrange themselves in a spherical form in aqueous solutions as a response to the amphipathic nature of fatty acids. Micelles contain both hydrophilic regions (polar head groups) as well as hydrophobic regions (the long hydrophobic chain).
Microbial community dynamics	The change in relative dominance of community members in response to physicochemical changes. The ability of a microbial system to react and adapt to environmental perturbations
Microbial diversity	A measure of the abundance and the uniformity of distribution of different microbial members in a microbial consortium
Mixed culture	Microbial population consisting of two or more types of microorganisms
Orthologous genes	Genes in different species that evolved from a common ancestral gene by speciation
Paralogous genes	Genes in the same organism at different chromosomal locations that have structural similarities which is indicative of a common ancestral gene and have since diverged from the parent copy by mutation and selection
Phylogenetic	Related to the evolutionary development and history of microorganisms or higher taxonomic grouping of organisms
Reactor performance	The rate of substrate removal or degradation in a reactor system
Syntrophic	A syntrophic reaction is one in which two (or more) organisms interact metabolically to consume a substrate that neither can consume independently
Thermophile	An organism that can grow at high temperatures ≥45°C

CHAPTER 1
GENERAL INTRODUCTION

1.1 BACKGROUND

The mining of minerals and coal contributes towards world economies and industry, and provides primary energy needs such as electricity and is important in supporting our modern day lifestyle. While efforts are being made to move towards a green economy, we continue to heavily rely on the supply of metals accessed through traditional mining activities and, increasingly a new spectrum of metals essential to deliver the green economy. Mineral and coal mining pose several environmental risks when poor and insufficient planning has been made, particularly with respect to waste handling and mine closure. One of these risks is the generation of acid mine drainage (AMD) from the oxidation of sulphide-bearing minerals, primarily pyrite (FeS_2), contained in waste rock, mine tailings and coal tailings on its exposure to atmospheric oxygen and water (Reaction 2.1 to 2.5) (Akcil and Koldas, 2006). This process occurs naturally, although mining accelerates the generation of AMD by increasing the quantity of sulphide-rich rocks exposed to oxygen (Baker and Banfield, 2003; Akcil and Koldas, 2006; Egiebor and Oni, 2007; Lottermoser, 2010; Parbhakar-Fox and Lottermoser, 2015). The rate of supply of oxidant (typically oxygen or ferric iron) to the mineral surface influences the rate at which pyrite dissolves (Baker and Banfield, 2003; Parbhakar-Fox and Lottermoser, 2015). Mining and industrial activities in which sulphides are exposed have resulted in the increase of the generation of AMD which, in turn, has affected run-off water quality through increased acidity, metal and sulphate content. AMD is of major concern in especially semi-arid regions like South Africa where water is a limited natural resource and rural communities are often dependent on natural rivers and streams for drinking, cooking, washing and agriculture (Bell *et al.*, 2001; Hesketh et al., 2010; Naicker *et al*., 2003; Tutu *et al*., 2008; McCarthy, 2011).

Sulphate is a major pollutant contained in AMD, and also neutral mine drainage, highlighting the need to develop technologies that reduce sulphate to acceptable levels. Biological sulphate reduction (BSR) methods have attracted much attention as they offer potential of a cheap alternative compared to conventional remediation strategies which include reverse osmosis, ion exchange and chemical treatment. In addition, the concomitant generation of alkalinity and soluble sulphide assist in neutralisation and heavy metal removal. Valuable metal sulphides can be recovered selectively as compact metal sulphide precipitates (Reaction 1.1 to Reaction 1.3) (Drury, 1999; Liamleam and Annachhatre, 2007; Ndlovu 2014; Akinwekomi *et al.*, 2016). Alternatively, sulphides can be oxidised to sulphur and recovered in its elemental form for use in the fertiliser industry according to Reaction 1.4 (Marais *et al*., 2018).

$$Electron\ donor\ (carbon\ source) + SO_4^{2-} \rightarrow HS^- + HC0_3^- \qquad \text{Reaction 1.1}$$

$$Me^{2+} + HS^- \rightarrow MeS\downarrow + H^+$$ Reaction 1.2

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2$$ Reaction 1.3

$$HS^- + \tfrac{1}{2}O_2 \rightarrow S^0 + OH^-$$ Reaction 1.4

Some of the challenges associated with BSR include maintaining adequate sulphate conversion rates and the enhancement of reaction rate (Gopal 2005, Marais *et al.*, 2017; Hessler *et al*., 2018), the supply of cost-effective carbon sources and electron donors (van Hille *et al.*, 1999; Boshoff *et al*., 2004, Gopal 2005), and the management of sulphur species for permanent removal (Gopal 2005, Molwantwa, 2008; Marais *et al.*, 2018). Various carbon sources have been studied as potential carbon sources for BSR for the treatment of AMD. A key consideration when choosing a suitable carbon source is the availability of the carbon source at the site of AMD treatment (Gopal 2005; Boshoff *et al*., 2004; Liamleam and Annachhatre, 2007, Hiibel *et al.*, 2011). An ideal carbon source is one that can sustain continuous BSR and is not limited by seasonality (Rose *et al*., 1998).

Suitable carbon source and electron donors for BSR are selected based on their cost per unit of sulphate converted to sulphide, and their ability to facilitate the complete reduction of sulphate while minimising the occurrence of other pollutants in the effluent (van Houten *et al*., 1994). While a variety of natural organic substrates such as leaf mulch, animal manure and wood chips are cost-effective, these often result in reduced sulphate reduction kinetics compared to that achieved with small, soluble molecules as substrates (Waybrant *et al.,* 1998). Small molecular weight organic compounds such as ethanol may result in efficient kinetics (Erasmus, 2000) but can be expensive to acquire, mostly due to transportation costs (Gopal 2005). Carbon rich feedstocks subjected to partial anaerobic digestion, to facilitate the formation of volatile fatty acid process intermediates, may be a suitable carbon source and electron donor for BSR.

Cyanobacterial biomass derived from the cultivation of species from the *Arthrospira* genus, commonly referred to as Spirulina, is a good substrate for anaerobic digestion (AD) due to its high chemical oxygen demand (COD) and easily digestible cell walls, compared to agricultural residues and microalgal biomass. AD process intermediates may build-up if biogas formation is minimised by increased feedstock loading and daily removal of effluent from the AD. This effluent (hereafter referred to anaerobic digestate) was found to contain a high residual COD content primarily in the form of the volatile fatty acids (VFAs): acetate; propionate and butyrate (Inglesby, 2011; Inglesby *et al*., 2015). Spirulina could potentially be cultivated on-site, by using existing water bodies or treated effluent as the basis for the media (Boshoff *et al*., 1996; Rose *et al*. 1998), thus increasing its potential as a cost-effective carbon source and electron donor for BSR.

Together with the acquisition of a cost-effective carbon source and electron donor, an understanding of the achievable BSR rates and operating conditions required to successfully remediate sulphate with the chosen electron donor is needed. The potential of the mixed VFA stream obtained from the partial AD digestate of Spirulina biomass as carbon source and electron donor for BSR is assessed in this thesis. Key to this is a semi-quantitative understanding of the microbial communities involved in BSR with the selected carbon source. A number of studies have been performed with single carbon sources such as acetate (Moosa, 2000; Moosa *et al*., 2002; 2005; Moosa and Harrison, 2006), ethanol (Erasmus, 2000; Hansford *et al*., 2007) and lactate (Oyekola *et al*., 2009; 2010; 2012). These studies have used various techniques such as fluorescence *in situ* hybridisation (FISH) and restriction enzyme (RE) digests to show that changes in the BSR microbial community occur with increased sulphate loading and dilution rates. One limitation of most of these techniques is that samples cannot always be analysed in real-time and regularly, and cost effectively and thus often community shifts are not tracked as operating conditions are changed. Chemostat studies performed using a mixed microbial consortium of sulphate reducing bacteria (SRB) receiving lactate as a sole carbon source and electron donor demonstrated a shift in the microbial community structure as sulphate loadings and residence times were changed. A shift in the relative utilisation of lactate for sulphate reduction versus fermentation with change in sulphate loading was also demonstrated (Oyekola *et al*., 2010; Oyekola *et al*., 2012). Similarly, a shift in the community structure of a mixed SRB culture was also observed when acetate served as the sole carbon source and electron donor for BSR (Icgen *et al*., 2006). These studies and the work of Hiibel *et al*. (2011) and Hessler *et al*. (2018) also demonstrated that different carbon sources and electron donors support different microbial communities. To track microbial community dynamics regularly through changing reactor conditions, a readily accessible molecular-based analysis tool is required, allowing rapid access to the data. One such approach is quantitative real-time qPCR. At the outset of this study, the lack of availability of genus- and species-specific quantitative real-time (qPCR) primer sets made it difficult for researchers to evaluate loss of species or changes in relative proportions of species as a result of changes in operating conditions and carbon source.

In this thesis, the development of genus and species specific quantitative real-time (qPCR) primer sets for the rapid and real-time analysis of changes in BSR operating conditions was undertaken. These primers were applied to study changes in the BSR microbial community dynamics operated with anaerobic digestate as carbon source and electron donor. Further the resultant qPCR capability was used in combination with kinetic studies of biological sulphate reduction in continuous stirred tank reactor systems to explore the potential of the anaerobic digestate as carbon source and electron donor and to map the links between performance of the BSR system, utilisation of volatile fatty acids contained in the anaerobic digestate and microbial community dynamics.

1.2 THESIS STRUCTURE

A literature review of previous relevant and related studies is presented in **Chapter 2**. Sections 2.1 to 2.2.4 examine generation of AMD, its sources and its impact on the environment. Control and treatment methods are discussed in Section 2.3 with emphasis placed on anaerobic biological sulphate reduction. The microorganisms detected in AMD treatment processes and sulphate reducing environments, known as sulphate reducing bacteria (SRB), are discussed in Section 2.4. Section 2.5 focuses on anaerobic digestion and the volatile fatty acid metabolism of SRB species. Section 2.6 describes biological sulphate reduction kinetics. Thereafter, molecular methods used to characterise microbial communities are examined in Section 2.7. Chapter 2 is concluded with a research motivation, hypotheses and research objective.

Experimental procedures and apparatus used in this study are described in detail in **Chapter 3**, with a brief account of general methodology provided within each of the experimental Chapters 4, 5 and 6.

In **Chapter 4**, molecular tools to characterise mixed microbial SRB communities, using the inoculum for this study as the case study, were investigated. Fluorescence *in situ* hybridisation (FISH) was performed and a 16S rRNA gene clone library was constructed and de-replicated by amplified ribosomal DNA restriction analysis (ARDRA). The 16S rRNA gene sequences captured from the clone library were used to design novel SRB genus specific qPCR primers. The 16S rRNA plasmids from the clone library were used to optimise and validate the qPCR conditions for the newly designed and existing BSR primers modified from literature.

A chemostat study of BSR was carried out using anaerobic digestate as a carbon source and electron donor. These data and their kinetic analysis are presented in **Chapter 5**. Here the effect of the volumetric sulphate loading rate (VSLR), which was mediated through dilution rate and feed sulphate concentration on volumetric sulphate reduction rate (VSRR), is reported and discussed. Mathematical models previously used for acetate, ethanol and lactate were fitted to describe the effect of feed sulphate concentration on the BSR kinetics when anaerobic digestate was used as a carbon source and electron donor.

In **Chapter 6**, the optimised FISH conditions and the qPCR primers and conditions, developed and validated in **Chapter 4**, were used to assess the microbial community dynamics when anaerobic digestate was used as a complex carbon source for biological sulphate reduction. The effect of the VSLR mediated through dilution rate and feed sulphate concentration on the mixed microbial community composition was investigated. This information was used to highlight the link between the BSR kinetics, utilisation of anaerobic digestate and microbial community dynamics. qPCR analysis was also carried out BSR reactors receiving a simple carbon source (lactate) under steady state conditions. The

BSR community dynamics between anaerobic digestate and lactate was compared. A 16S rRNA gene survey of the metagenomic DNA obtained from key samples was carried out to validate the qPCR results and characterise the 'other' SRB and non-SRB species obtained.

Conclusions and recommendations for further studies are presented in **Chapter 7**.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

The United Nations estimates the current world population to be 7.7 billion and expects it to increase to 8.5 billion by 2030 and to 9.7 billion by 2050 (Department of Economic and Social Affairs UN, 2019). Africa is expected to account for more than half of the world's population growth between 2015 and 2050 (Melorose *et al*., 2015; Department of Economic and Social Affairs UN, 2019). In addition to growth, population in the urban areas is growing even more rapidly as people are moving from rural areas to find jobs in the urban areas. Statistics South Africa reported that the degree of urbanisation in South Africa has increased from 60.6% to 65.8% between 2007 and 2017 (Statistics South Africa, 2018). This increase in urbanisation has resulted in a high demand for metals and hence minerals and mining activities which are essential for sustaining industrial economies and providing primary energy needs such as electricity, which is important in supporting our modern day lifestyle. These mining and industrial activities increase the generation of wastewaters, including potential for acid mine drainage (AMD). One example that provides the long-term relationship between the increase in the demand for minerals and metals due to urbanisation and AMD is demonstrated by the increase in the rising sulphate concentration and salinity of the water in the Middleburg and Witbank dams due to the mining of coal in order to supply coal to the growing gold and diamond mining in the area and to provide electricity to South Africa. In November 1978, the sulphate concentration in the Middleburg dam was approximately 150 mg/L. By July 2007, the sulphate concentration had elevated to 600 mg/l, and is reported to regularly exceed 200 mg/l in recent years (McCarthy, 2011). While current mining practices implemented for new mining activities strive to limit AMD generation, it is often only delayed not prevented. Further, where AMD generation occurs, it continues for decades to hundreds of years; hence abandoned mines still remain as the legacy of AMD sources (Gazea *et al.*, 1996; Johnson and Hallberg, 2003, Parbhakar-Fox and Lottermoser, 2015).

AMD affects water quality, mine run-off water or downstream water resources, by lowering its pH and increasing its metal and sulphate loading (Bell *et al.*, 2001; Naicker *et al*., 2003; Tutu *et al*., 2008; McCarthy, 2011; Parbhakar-Fox and Lottermoser, 2015). The exact factors influencing AMD generation rates vary from one site to another. Therefore, prediction, prevention, containment and treatment of AMD is site specific and must be considered carefully (Simate and Ndlovu, 2014, Broadhurst *et al*., 2015; Parbhakar-Fox and Lottermoser, 2015). While prevention of AMD formation is preferred (Hesketh *et al*., 2010; Kazadi-Mbamba *et al.*, 2012; Kotsiopoulos and Harrison, 2017), this is often not achieved and legacy sites still remain.

2.2 ACID MINE DRAINAGE

2.2.1 Generation of acid mine drainage

Acid mine drainage, as the name suggests, is acidic run-off water from mostly mine waste dumps. It is generated from the oxidation of sulphide minerals, primarily pyrite (FeS_2) contained in waste rock and tailings, mine workings, coal tailings, on exposure to atmospheric oxygen and water (Reaction 2.1 to 2.5) (Evangelou and Zhang, 1995; Baker and Banfield, 2003; Akcil and Koldas, 2006; Egiebor and Oni, 2007; Lottermoser, 2010; Parbhakar-Fox and Lottermoser, 2015). This process occurs naturally, typically referred to as acid rock drainage, however, mining operations promote the generation of AMD by increasing the quantity of sulphides exposed and liberated. The rate of the dissolution of mineral sulphides is determined by many factors such as: temperature; mineral and rock type; dissolution mechanism; fluid chemistry and fluid flow (Evangelou and Zhang, 1995; Baker and Banfield, 2003; Egiebor and Oni, 2007; Lottermoser, 2010). For pyrite dissolution, the rate of supply of oxidant (typically oxygen or ferric iron) to the mineral surface influences the rate of dissolution (Baker and Banfield, 2003; Akcil and Koldas, 2006), with ferric iron identified as a more effective sulphide oxidant than oxygen (Evangelou and Zhang, 1995; Johnson and Hallberg, 2005). The oxidation of pyrite (FeS_2), the most prevalent sulphide minerals, is commonly used to illustrate the generation of AMD (Banks *et al*., 1997; Akcil and Koldas, 2006). The initial step in the presence of atmospheric oxygen is described in Reaction 2.1. (Evangelou and Zhang, 1995).

$$FeS_2 + \frac{7}{2}O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad \text{Reaction 2.1}$$

Reaction 2.1 results in the increase of total dissolved solids and acidity of the water, and hence a decrease in pH, unless neutralised by minerals with a high acid neutralising capacity (Edwards *et al*., 1998; Akcil and Koldas, 2006). Depending on the oxygen concentration, pH and microbial activity, most of the ferrous iron (Fe^{2+}) oxidises to ferric ion (Fe^{3+}) according to Reaction 2.2.

$$Fe^{2+} + \frac{1}{4}O_2 + H^+ \rightarrow Fe^{3+} + \frac{1}{2}H_2O \qquad \text{Reaction 2.2}$$

Around neutral pH, Fe^{3+} has a low solubility and does not influence dissolution significantly. However, as the concentration of dissolved ferric iron Fe^{3+} decreases with increasing pH, the solubility of Fe^{3+} becomes limited. Therefore, if the pH increases to above pH 3, then reactions demonstrated in Reactions 2.3 and 2.4 occur (Lottermoser, 2010; Parbhakar-Fox Lottermoser, 2015) resulting in the precipitation of Fe^{3+} hydroxides ($Fe(OH)_3$) and oxy-hydroxides (FeOOH) (Evangelou and Zhang, 1995). Ferric hydroxide is observed as an orange precipitate and it is distinctive for the orange-red colour observed for AMD effluents (Ravengai *et al*., 2005).

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \qquad \text{Reaction 2.3}$$
$$Fe^{3+} + 2H_2O \rightarrow FeOOH + 3H^+ \qquad \text{Reaction 2.4}$$

However, if the pH decreases to ≤pH 4.5, Reaction 2.5 is triggered (Dold, 2010; Parbhakar-Fox Lottermoser, 2015).

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \qquad \text{Reaction 2.5}$$

AMD generation can be accelerated by naturally occurring microorganisms, particularly mesoacidiphilic chemolithotrophs such as the iron and sulphur oxidising microorganisms: *Acidithiobacillus thiooxidans* (*A. thiooxidans*); *Acidithiobacillus* caldus *(A. caldus)*, *Leptospirillum ferrooxidans* (*L. ferrooxidans*) and *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*) formerly known as *Thiobacillus ferrooxidans* (*T. ferrooxidans*). The biological ferrous iron oxidation can regenerate the leach agent Fe^{3+} for the dissolution of the sulphide minerals or oxidation of sulphur, by *Acidithiobacillus caldus* for example, thus generating acidity (Edwards *et al*., 2000; Kelly and Wood, 2000; Fowler *et al*., 2001; Blowes *et al.*, 2013). Reports by Singer and Stumm (1970) indicated that *Acidithiobacillus ferrooxidans* can increase by a factor of hundreds. However, these observations were reported to be an overestimation by Morin and Hutt (2010), who gave evidence from Leathen *et al*. (1953a, b) and Morth *et al*. (1972), who reported oxidation to proceed 2–5 times and 10–50 times faster, respectively.

Pyrite is not the only sulphide containing mineral which can generate AMD. Table 2.1 gives an overview of other sulphide minerals such as pyrrhotite (FeS), sphalerite (ZnS) and the copper-containing minerals chalcopyrite ($CuFeS_2$), covellite (CuS) and chalcocite (Cu_2S) which are also oxidised, and generate AMD, if present within exposed rock. These minerals are oxidised with varying stoichiometry which depends on the amount of Fe present, with Fe sulphides generating the most acidity (Plumlee, 1999; Dold, 2010; Thurston, *et al*., 2010; Simate and Ndlovu, 2014; Parbhakar-Fox and Lottermoser, 2015; Dold, 2017). To date, these have not been considered much in the AMD literature, however, their role in AMD generation is increasingly recognised (Lottermoser, 2010; Simate and Ndlovu, 2014; Parbhakar-Fox Lottermoser, 2015). Further mineral dissolution reaction stoichiometry for these metal sulphide types is presented in the mineral bioleaching literature (Mok and Wai, 1994; Thurston, *et al*., 2010 Tupikana, *et al*., 2011; Ghorbani *et al*., 2013; Ngoma *et al*., 2018).

Table 2.1. Some important metal sulphides which can generate AMD

Metal Sulphide	Chemical Formula
Pyrite	FeS_2
Marcasite	FeS_2
Pyrrhotite	$Fe_{1\_x}S$
Chalcocite	Cu_2S
Covellite	CuS
Chalcopyrite	$CuFeS_2$
Molybdenite	MoS_2
Millerite	NiS
Galena	PbS
Sphalerite	ZnS
Arsenopyrite	FeAsS

2.2.2 Sources of acid mine drainage

Mining of coal and metallic sulphide ore deposits has the potential to expose sulphide mineral and generate AMD. Sulphide waste rock, spent heap-leach piles, spoil piles and tailing dams have the potential for sulphide oxidation as described in Section 2.2.1, generating extreme acid levels (Kuyucak, 1999; Kuyucak, 2001; Akcil and Koldas, 2006; Lottermoser, 2010; Broadhurst *et al*., 2015). AMD can form as a result of various processes such as discharge of spent process waters occurring from tailing dams and spoil piles, run-off from rain fall interacting with mining operations and ground water entering pits and surface excavations (Lottermoser, 2010; Broadhurst *et al*., 2015). However, the accurate prediction of the pH and chemical concentrations in these waters is a challenge (Kuyucak, 1999; Johnson and Hallberg, 2005; Akcil and Koldas, 2006; Mccarthy *et al*., 2011). While mitigation methods might be put in place to prevent exposure of sulphide minerals to oxygen and water, these typically delay AMD generation, but do not remove the risk of legacy AMD generation post mine closure.

2.2.3 Acid mine drainage characteristics

AMD is characterised as waters with high metal and sulphate concentrations and a low pH (Banks *et al*., 1997; Foucher *et al*., 2001; Naicker *et al*., 2003; Akcil and Koldas, 2006; Behum *et al*., 2011; Caraballo *et al*., 2011; Broadhurst *et al*., 2015). Chemical compositions of AMD are highly variable. The constituents of AMD streams in Africa and elsewhere are indicated in Table 2.2 through examples from literature. The characteristic heavy metals and anions indicated in Table 2.2 are far above the guideline values of metals in various water categories given by the Initiative for Responsible Mining Assurance (IRMA), (Table 2.3). Therefore, AMD treatment aims to increase the pH to acceptable levels and to remove heavy metals and sulphate.

Table 2.2. Recorded chemical characterisations of various acid mine drainage streams in South Africa (SA) and the world. All concentrations are in mg l^{-1}

AMD Stream	pH	Fe	Zn	Cu	Mg	Na	K	SO_4^{2-}	NO_3^-	Co	Ni	Cr	Mn	Pb
South Africa and Africa														
Highveld, SA[a]	1.2- 2.9	0.25- 6110	9.5- 49	nd	0.03- 2800	0.1- 4100	11-52.6	6.2- 14 900	0.4- 160	1.2- 4.6	2.4- 8.0	nd	0.32- 240	nd
Klipspruit, SA[b]	4- 6.2	0.07- 0.35	0.013- 0.23	nd	nd	nd	nd	180- 400	nd	nd	nd	nd	1.6- 3.4	nd
West Rand, SA[c]	3.3	180	3.2	21	124.6	46.5	4.4	1910	nd	2.05	5.6	0.26	63.7	0.03
Natalspruit, SA[d]	4.0- 7.90	0.62- 453.4	0.1- 8.0	0.1- 6.0	nd	10.2- 49.3	nd	250- 2080	nd	0-14.30	0.10- 17.9	1.7- 10	0.3-86.6	0- 0.7
Witbank Coalfieds, SA[e]	1.8- 6.6	128- 193	nd	nd	31- 90	65- 399	7.3- 9.4	1440- 3250	0.02- 0.18	nd	nd	nd	9.3-18	nd
Witwatersrand Basin, SA[f]	2.3- 9.1	0.08- 1010	0.001- 92	0.015- 5.8	4.1- 99.8	13.1- 198	2.8-52.9	18.3- 5080	0.130- 144.7	0.012- 17.1	0.13- 71.1	nd	0.008- 68.3	nd
Iron Duke mine, Zimbabwe[g]	2.0	246-441	1.4-3.9	0.31- 0.58	nd	nd	nd	2.2- 7167	nd	0.92- 5.5	0.96-4.8	nd	nd	0.15- 0.49
Non-African														
Tinto river, Spain[h]	2.2 -5.0	0.31- 2804	2.2- 152.5	0.2- 84.3	8.9- 363	6.7-97.2	1.0-23.6	150- 5547	nd	52- 3754	16- 742	<2- 86	0.7-39.2	<7-689
Odiel River, Spain[h]	3.0-5.1	0.31- 23.5	1.3- 36.4	0.5- 17.1	10.1- 224.1	7.9-32.7	6.2- 37.3	110- 23790	nd	33- 938	19-500	<2- 16	0.9-32.1	<7-267
Yunfu mine, China[i]	2.6	9700	378	nd	597	17.9	nd	nd	nd	0.20	0.85	nd	447.9	0.34

[a]Gitari *et al*., (2005) [c] De Beer *et al*., (2010) [e] Bell *et al*., (2001) [g] Ravengai *et al*., (2005) [i] Ruihua *et al*., (2011)
[b] (Dabrowski and Klerk, (2013) [d] Naicker *et al*., (2003) [f] Tutu *et al*., (2008) [h] Nieto *et al*., (2007) nd: not determined/ detected

Table 2.3. Water standards for metals, metalloids, non-metals and anions in various water categories (Adapted from IRMA, 2016 and USEPA, 2018).

Determinant	Guideline for each category (mg l^{-1})									
	Surface Fresh Water	**Fresh Groundwater**	**Salt Water**	**Aquatic Organisms Fresh Water**	**Aquatic Organisms Salt Water**	**Human Drinking Water**	**Agriculture Irrigation Water**	**Aquaculture Fresh Water**	**Aquaculture Marine Water**	**Recreational Water**
Metals/ Metaloids										
Co	0.05	-	0.001	-	0.001	0.05	0.05	-	-	-
Cr (Total)	0.05	0.05	-	-	-	0.05	0.1	-	-	0.05
Cr (III)	0.2	-	0.03	0.57*	0.03	-	-	-	-	-
Cr (IV)	-	-	0.004	0.001	0,004	-	-	0.02	-	-
Cu	-	0.02	0.001	-	0.001	1.0	0.2	-	-	-
Fe	0.3	0.03	0.01	0.3	-	0.3	5.0	0.01	0.01	0.3
K	-	-	-	-	-	X	-	-	-	-
Mg	-	-	-	-	-	-	-	-	-	-
Mn	0.01	0.05	0.01	1.7	-	0.005	0.2	0.01	0.01	0.1
Na	-	-	-	-	-	30-60	-	-	-	-
Ni	0.07	0.02	0.07	0.5*	0.07	0.002	0.2	0.1	0.1	0.1
Pb	0.01	0.01	-	0.082*	0.004	0.01	0.1	-	-	0.05
Zn	0.3	2.0	0.05	0.12*	0.015	5.0	2.0	0.05	0.05	-
Non-metals/ Ions										
pH	6.5- 8.4	6.5- 8.4	6.5- 8.7	6.5- 9.0	6.5- 8.7	6.5-8.5	6.5-8.4	6.5-9.0	6.5-9.0	6.5-8.5
NO_3^-	10	10	13	13	13	10	10	50	100	10
SO_4^{2-}	400	500	-	-	-	500	1000	-	-	400

X= Occurs in levels below health concern (WHO, 2011)
* Value from USEPA (United States Environmental Protection Agency)

2.2.4 Impacts of acid mine drainage

The USA Environmental Protection Agency (US-EPA) considers AMD to be second only to global warming and ozone depletion in terms of ecological risk, making AMD one of the most significant forms of pollution (Moodley *et al*., 2018). AMD is toxic to almost all forms of life (Singh, 1987; Costello 2003; Bell *et al*., 2001; Ruihua *et al.*, 2011) and can contaminate rivers, groundwater and soils (Tabak *et al*., 2003; Ruihua *et al.*, 2011). AMD aggravates the deportment of heavy metals (Broadhurst *et al*., 2015), demonstrated as both acute (short-term, high concentration) and long term (long-term, low concentration) toxicity (Singh *et al*., 2011; Ndlovu *et al.*, 2011), affecting human health. In humans and animals, heavy metals can accumulate in vital organs and glands and can interfere with the vital nutritional minerals in the body disrupting metabolic activities and hindering biological functions (Singh *et al*., 2011; Simate and Ndlovu, 2014). Heavy metals can accumulate in various tissues and organs such as the liver, kidney, gills and muscles of aquatic organisms, generating reactive oxygen species (ROS) which can damage DNA, lipid and protein (Fatima *et al*., 2014). High concentrations of heavy metals in plant tissues can result in oxidative stress, causing cellular damage and disturbance of cellular ionic homeostasis (Yadav, 2010; Tangahu *et al*., 2011). Heavy metals persist in natural ecosystems for an extended period (Ndlovu *et al*., 2011; Tangahu *et al*., 2011) and accumulate in successive levels of the biological chain. The presence of heavy metals in AMD can affect aquatic life by either acute exposure or chronic exposure (Lewis and Clark, 1997; Khayatzadeh and Abbasi, 2010; Jiwan and Kalamdhad, 2011). Acute exposure (short-term, high concentration) of these metals can kill organisms directly. Chronic exposure (long-term, low concentration) on the other hand, can result in either death or developmental effects such as reduced reproduction, deformities and stunted growth (Lewis and Clark, 1997). Studies by Ahmed *et al.* (2016) demonstrated that the absorption of metal ion to fish is not only tissue specific but also specific fish species.

AMD can affect benthic organisms (small animals that feed at the bottom of the water) through the precipitation of iron oxides and hydroxides. These precipitates can deposit and embed on rivers, streams or ocean beds cementing substrates (Charkavorki *et al*., 2000). As a result, these organisms can no longer feed and therefore are eventually depleted, in turn affecting fish and other animals that feed on them (Charkavorki *et al*., 2000). The low pH resulting from AMD promotes solubilisation of copper, iron and other metals, allowing them to enter streams and rivers, negatively affecting fish, wildlife and drinking water (CSIR, 2013). Low soil pH may result in the insufficient presence of calcium and magnesium, which are essential nutrients for plant growth (Halcomb and Fare, 2002). Low soil pH also hinders microbial activity in the soil which plays a crucial role in nutrient availability for plant development, while the contamination of soil by heavy metals is also a critical environmental concern (Halcomb and Fare 2002; Bobbins, 2015). The survival of aquatic organisms at pH 2 and lower or pH 10 and above is minimal (Clark *et al*., 2004). For maximum productivity of most aquatic organisms,

the pH should be maintained between 6.5 and 8.5. Therefore, low pH, a characteristic of AMD, affects survival of most organisms (Singh, 1987; Lewis and Clark, 1997; Costello 2003; Bell *et al*., 2001; Ruihua *et al.*, 2011; Moodley *et al*., 2018). Low pH water is also corrosive to plumbing causing deterioration of pipe lines and the leaching of heavy metals such as zinc, lead and iron.

Although the WHO has not proposed a health standard for sulphate, most countries recommend a drinking water standard between 250 and 500 mg/L for sulphate which is often based on odour and threshold, as higher sulphate concentration may result in an undesirable taste in water (Gorchev and Ozolins, 2011). Infants are more sensitive to high sulphate concentrations than healthy adults (Gorchev and Ozolins, 2011). Prolonged exposure to excess levels of sulphate have been reported to cause gastrointestinal tract problems such as diaerrhoea, inflamation of the bowel and nausea in humans (Bashir *et al*., 2012). Sulphate is extremely corrosive and can cause burns and irritation to the skin, irritation to eyes resulting in blindness and pulmonary edema (excess fluid in the lungs). Ingestion through drinking may cause burns to the throat, mouth, oesophagus and stomach (CSIR, 2013). High concentrations of sulphates in drinking water can decreased forage digestion in cattle and their ruminal metabolic activity which negatively affect their health (Coria *et al*., 2007). In soil and sediments, high sulphate concentration can cause release of phosphates bound to sediments which could potentially lead to eutrophication (Geurts *et al*., 2009).

2.3 CONTROL AND TREATMENT TECHNOLOGIES

As indicated by Reaction 2.1 to Reaction 2.5 (Section 2.21), oxygen and water are two of key reactants in the generation of AMD (Akcil and Koldas, 2006; Dold, 2010; Parbhakar-Fox Lottermoser, 2015). Therefore control methods are directed toward the removal of oxygen or water or both from the site of AMD or potential AMD generation (Kuyucak, 1999; Johnson and Hallberg, 2005; Pozo-Antonio *et al*., 2014; Kotsiopoulos and Harrison, 2017; 2018). Examples of this is the flooding and sealing of old mines (Kuyucak, 1999), the saturation of tailing storage facilities and the capping of waste deposits (Kotsiopoulos and Harrison, 2017; 2018) to create anoxic environments. However, control of AMD generation has proven to be practically difficult (Kuyucak, 1999; Johnson and Hallberg, 2005) mostly due to the size of waste rock piles. Therefore, AMD mitigation techniques may be more feasible than prevention strategies (Kuyucak, 1999; Johnson and Hallberg, 2003; Johnson and Hallberg, 2005; Simate and Ndlovu, 2014; Kazadi-Mbamba *et al.*, 2012; 2013). AMD risk removal by removal of the sulphide present may be a practical strategy for AMD mitigation (Kazadi-Mbamba *et al.*, 2012; 2013).

Most existing mine sites do not have AMD mitigation strategies in place and thus treatment of existing AMD is required. Diverse technologies have been developed for the treatment of AMD (Simate and Ndlovu 2014; Akinwekomi *et al.*, 2016). The remediation method suitable for application depends on

the volume of the effluent as well as the type and concentration of contaminants present (Gazea *et al*., 1996; Kuyucak, 1999). An effective treatment seeks to ensure water neutralisation, metal removal and sulphate removal or at least reduction concentrations to within the limits allowed for safe discharge into the environment (Gazea *et al*., 1996; Taylor *et al*., 2005). Maximum permissible limits of heavy metals for different water categories are shown in Table 2.3 (Section 2.2.3). Active and passive treatment technologies combine physical, biological and chemical approaches (Skousen *et al*., 2000; Johnson and Hallberg, 2005; RoyChowdhury *et al*., 2015). A broad range of passive and active treatment approaches have been described for the remediation of AMD (Hedin *et al*., 1994; Gazea *et al*., 1996; Costello, 2003; Johnson and Hallberg, 2005; Skousen and Ziemkiewicz, 2005; Taylor *et al*., 2005; Masindi *et al*., 2015). Some remediation methods may operate as either active or passive systems. One such process is biological sulphate reduction (Rose *et al.*, 1998; Gibert *et al*., 2002; Neculita *et al*., 2007; Sheoran *et al.*, 2010), the treatment on which this study focuses.

2.3.1 Active treatment technologies

To be considered an attractive remediation strategy, the process must be easy to install and maintain, produce limited quantities of solid by-products and be low cost (Gazea *et al.*, 1996). Active treatment technologies include chemical treatment, ion exchange, reverse osmosis and biological sulphate reduction. These are used for operational mines and occasionally for closed mines (Taylor *et al*., 2005). Active treatment technologies are fast and effective for the removal of acidity and metals (Coulton *et al*., 2003; Gopi Kiran *et al*., 2017). The major disadvantages associated with active treatment technologies is the need for active operation, resulting in ongoing operation costs and the regular maintenance which increases the overall cost of these technologies (Skousen et al., 2000; Skousen and Ziemkiewicz, 2005), especially when their operating lifespan needs to exceed that of the active mine.

2.3.1.1 Chemical treatment

The most generally applied chemical approach in the treatment of AMD is the addition of alkaline reagents such as lime [$Ca(OH)_2$] (Reaction 2.6), slaked lime, limestone ($CaCO_3$) (Reaction 2.7), sodium carbonate (Na_2CO_3) or sodium hydroxide (NaOH) (Smit, 1999; Kalin *et al*., 2006; de Beer *et al*., 2010). Lime (Reaction 2.6), the most widely used chemical, increases pH and precipitates dissolved metals as metal hydroxides (Johnson and Hallberg, 2005; de Beer *et al*., 2010; Kusin *et al*., 2013). This results in the removal of both acidity and metal toxicity (de Beer *et al.*, 2010). Liming also precipitates out some sulphate by the formation of gypsum ($CaSO_4.2H_2O$) (Reaction 2.8). To be effective, it is necessary to remove the precipitate formed and re-purpose it. Where not removed, due to the instability of the sludge (gypsum and metal hydroxides) metal re-solubilisation can occur at lowered pH (Johnson and Hallberg, 2005; Kuyucak, 1999). The disadvantages of the chemical treatment of AMD are the high costs

incurred, the limited availability of lime and the challenge associated with the selective recovery of metals (Simate and Ndlovu, 2014).

$$Ca(OH)_2 + Me^{2+} + H_2SO_4 \leftrightarrow Me(OH)_2 \downarrow + CaSO_4 \downarrow + 2H^+ \quad \text{Reaction 2.6}$$

$$CaCO_3 + H_2SO_4 + H_2O \leftrightarrow CaSO_4.2H_2O + CO_2 \quad \text{Reaction 2.7}$$

$$Ca(OH)_2 + H_2SO_4 \leftrightarrow CaSO_4.2H_2O \downarrow \quad \text{Reaction 2.8}$$

A number of technologies have been developed with the aim of recovering valuable metals from AMD (MacIngova and Luptakova, 2012; Akinwekomi *et al*., 2016). A study by Akinwekomi *et al*. (2016) demonstrated that pre-treatment of AMD first with $Mg(HCO_3)_2$ or $Mg(OH)_2$, and then $Ca(OH)_2$, prevents gypsum precipitation along with metal hydroxides. Therefore, metal hydroxides and gypsum are obtained separately (Figure 2.1). The metal hydroxides obtained and gypsum can be used for different industrial applications (Akinwekomi *et al*., 2016).

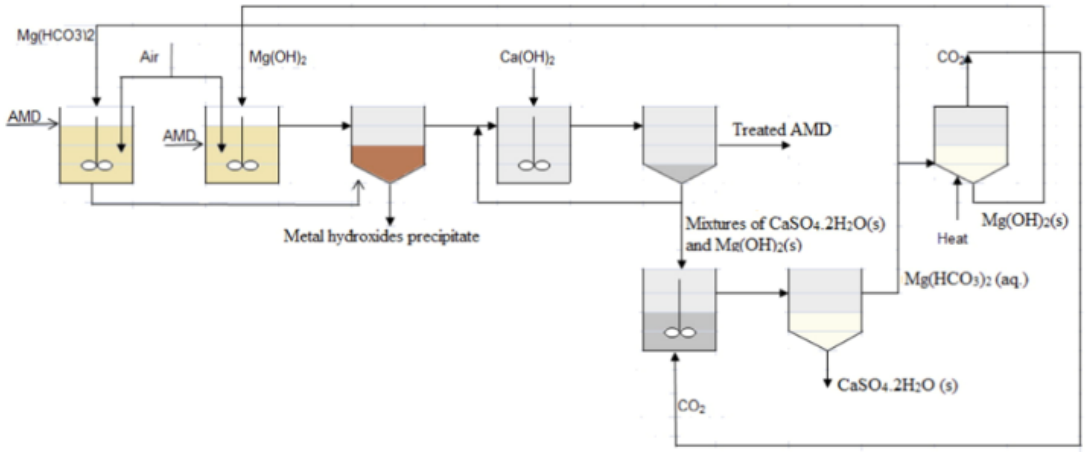


Figure 2.1. Schematic diagram of AMD treatment using $Mg(HCO_3)_2$ or $MgOH_2$ and $Ca(OH)_2$ for separate removal of metals and sulphate (Taken from Akinwekomi *et al*., 2016, with permission).

2.3.1.2 Ion exchange

Ion exchange, defined as the exchange of ions between a solid substrate and surrounding medium (Dabrowski *et al*., 2004; Hubicki and Kołodyńska, 2012), is designed to remove traces of ionic impurities from water and process streams and give a product of desired quality. Therefore, ion exchange is used as a polishing step (Dill *et al*., 1998). Selective cations or anions may also be exchanged by amphoteric exchangers. This will depend on the pH of the solution. These ion exchangers are also called bipolar electrolyte exchange resins or zwitterionic ion exchangers (Nesterenko and Addad, 2000; Hubicki and Kołodyńska, 2012). In the treatment of AMD, anions such as sulphate can be removed via an anionic resin (R-OH) according to Reaction 2.9 (Haghsheno *et al*., 2009). Where 'R'

is the ion exchange radical, and k_1 and k_2 are the forward and reverse reaction rate constants, respectively (Haghsheno *et al*., 2009).

$$2R - OH + SO_4^{2-} \overset{K_{1,2}}{\longleftrightarrow} SO_4 + 2OH^- \qquad \text{Reaction 2.9}$$

The advantage of ion exchange is that it enables efficient removal of even traces of impurities from solutions. It is therefore particularly useful to treat large volumes of diluted solutions and polish already treated effluents (Simate and Ndlovu, 2014). A similar technology, GYP-CIX, has successfully been applied at the Grootvlei Proprietary Mines Ltd site in South Africa (Everett *et al*.., 1993; Schoeman and Steyn, 2001). This process was used to reduce the feed sulphate concentration by 94% with sulphate concentration between 500 and 900 mg l^{-1} (Haghsheno *et al*., 2009). This is a two stage operation. In the first stage, cations are removed using the cation exchange resins and sulphuric acid is used to regenerate the cation exchange resin. The second stage is the removal of anions using anion exchange resins and lime slurry is used for the anion resin regeneration (Schoeman and Steyn, 2001). The disadvantage of this technology is the large amount of gypsum sludge produced (Simate and Ndlovu, 2014). Also, this process is not suitable to treat large volume of effluents (Everett *et al*.., 1993) and therefore may not be suitable at mine areas with large amounts of effluent.

2.3.1.3 Membrane technology: reverse osmosis

Reverse osmosis (RO) technology employs semi-permeable membranes that allow separation of a solution into two streams, the permeate (which contains the purified water that passes through the membrane) and the concentrate that is retained in the membrane (Pérez-González *et al*., 2012). Naturally, pure water diffuses through a membrane to the solute side until equilibrium is reached. However, the opposite is observed in RO (Mauguin and Corsin, 2005; Kim, 2011). The characteristics of the retentate or brine and concentrate, depend on the type of membrane employed, the quality of the feed water, the quality of the produced water (recovery varies from 35% to 85%), the added chemicals during pre-treatment method and the cleaning procedures used (Kim, 2011; Pérez-González *et al*., 2012; Joo and Tansel; 2015). Various technologies for RO have been developed for the treatment of wastewaters. The selection of the best available technology for further treatment of RO concentrate to achieve zero liquid discharge (ZLD) or near- ZLD depends on several key parameters. These include the identification of pre-treatment requirements and the total dissolves solids (TDS) content of the RO concentrate (Subramani and Jacangelo, 2014). Particulate matter and species such as $CaCO_3$ and $CaSO_4$ may cause scaling or fouling of the membrane (Kim, 2011; Pérez-González *et al*., 2012).

Advanced Reject Recovery of Water (ARROW®) technology can be used to directly treat brackish water (mixture of saltwater and freshwater) to achieve high feed water recoveries of up to 99% (Brandhuber and Burbano, 2012; Subramani and Jacangelo 2014). Unlike in a typical chemical

precipitation before the RO process or in between stages, in ARROW®, precipitation is performed in the back-end. That is, in the reject of second stage RO. This configuration assists in the reduction of the brine volume that needs to be chemically treated. Ions such as calcium and silica are removed by increasing the pH of the brine stream in the chemical precipitation step. After chemical precipitation, the treated brine is then is filtered and recycled back in between the first and second stage RO. ARROW® is a patent technology and is illustrated in Figure 2.2 (Brandhuber and Burbano, 2012).

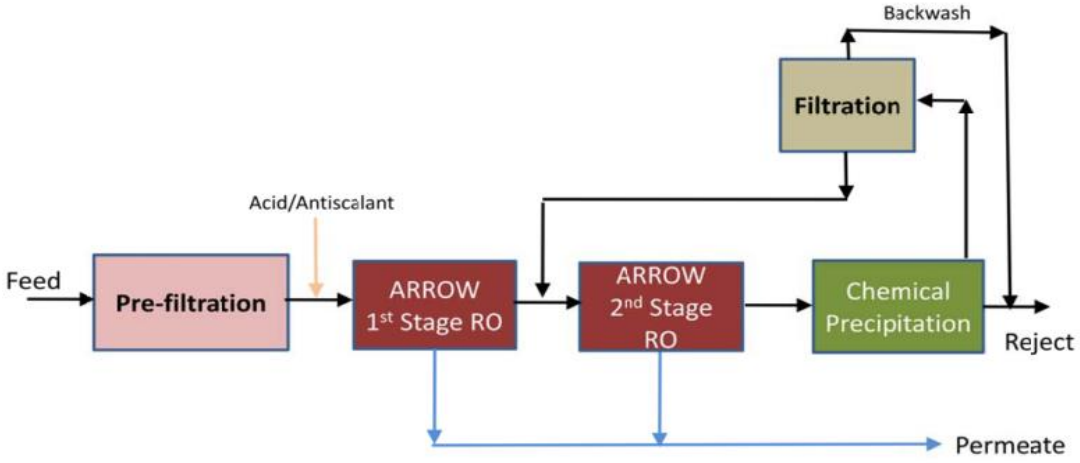


Figure 2.2. Schematic representation of ARROW® technology (Brandhuber and Burbano, 2012, with permission).

2.3.1.4 Biological sulphate reduction

The dissimilatory sulphate reduction is a process whereby sulphate (SO_4^{2-}) is reduced to hydrogen sulphide (H_2S) in the presence of an organic electron donor or H_2, which is coupled to energy conservation and microbial growth (Sánchez-Andrea *et al*., 2014). Sulphate reducing bacteria (SRB) play a crucial role in the remediation of wastewaters (Rose *et al*., 1998; Boshoff *et al*., 2004; Neba, 2006; Sofia *et al*., 2008; Oyekola *et al*., 2010; Choudhary and Sheoran, 2012; MacIngova and Luptakova, 2012; Uster *et al*., 2015). These organisms have been isolated from various environments including extremely acidic mine waters (Ito *et al*., 2002). The products of sulphate reduction, HS^-, H_2S, CO_3^{2-} and HCO_3^-, buffer the system at pH 6 to 8 by neutralising the excess protons in AMD (Reactions 1.1 to 1.3) (Drury, 1999; Liamleam and Annachhatre, 2007; Ndlovu 2014; Sánchez-Andrea *et al*., 2014; Akinwekomi *et al*., 2016).

$$Electron\ donor\ (carbon\ source) + SO_4^{2-} \rightarrow HS^- + HCO_3^- \quad \text{Reaction 1.1}$$

$$Me^{2+} + HS^- \rightarrow MeS\downarrow + H^+ \quad \text{Reaction 1.2}$$

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2 \quad \text{Reaction 1.3}$$

The reduction of sulphate results in the consumption of protons which leads to the increase of the pH. The presence of sulphide as HS^- results in the formation of metal sulphides. Metals can therefore be precipitated out as metal sulphides (Reaction 1.2) (Sánchez-Andrea *et al*., 2014). These are formed

rapidly, even at low pH. Metal sulphides are less soluble than their hydroxide equivalents, allowing lower residual metal concentration in solution (Huisman *et al.*, 2006). These metal sulphides can be recovered and reused in further industrial processes (Veeken and Rulkens, 2003). Alternatively, sulphur can be recovered in its elemental form, for use in the fertiliser industry according to Reaction 1.4 (Marais *et al*., 2018).

$$HS^- + \frac{1}{2}O_2 \rightarrow S^0 + OH^- \qquad \text{Reaction 1.4}$$

Studies have used novel consortia of acidophilic sulphidogenic bacteria or novel reactor configurations or both to remove transition metals selectively from acidic mine waters (Ňancucheo and Johnson, 2012). Full scale sulphidogenic technologies have been developed and widely reported. These include Rhodes BioSURE® (Rose *et al*., 2004; Neba, 2006), SULFATEQ™ (PAQUES, 2016) and Thiopaq® (Janssen *et al*., 2000; Cline *et al*., 2003; Benschop *et al*., 2004) processes. The Rhodes BioSURE® (Figure 2.3) technology consists of a falling sludge bed reactor (FSBR) whereby sewage sludge is degraded to provide electrons donors and a carbon source for SRB (Rose *et al*., 2004). The technology was developed at Rhodes University, South Africa (Rose *et al*., 2004; Neba, 2006). Currently, the technology is used in full scale to treat mine water from Grootvlei Gold Mine high density sludge (HDS) plant by linking with the ERWAT Ancor sewage treatment works (Rose, 2013). Ten Ml/day HDS treated AMD is currently pumped from this process, while 2 Ml/day of the iron hydroxide sludge is pumped in a separate pipeline (Rose, 2013). Investigations revealed that with careful regulation of the mine water and the flow rates of the sewage sludge, sulphate levels could reliably be reduced to below 100 mg/l, at hydraulic retention times as low as 12 h. Incoming mine water is blended with a side stream of sulphide rich water to precipitate heavy metals in the influent (Neba, 2006)

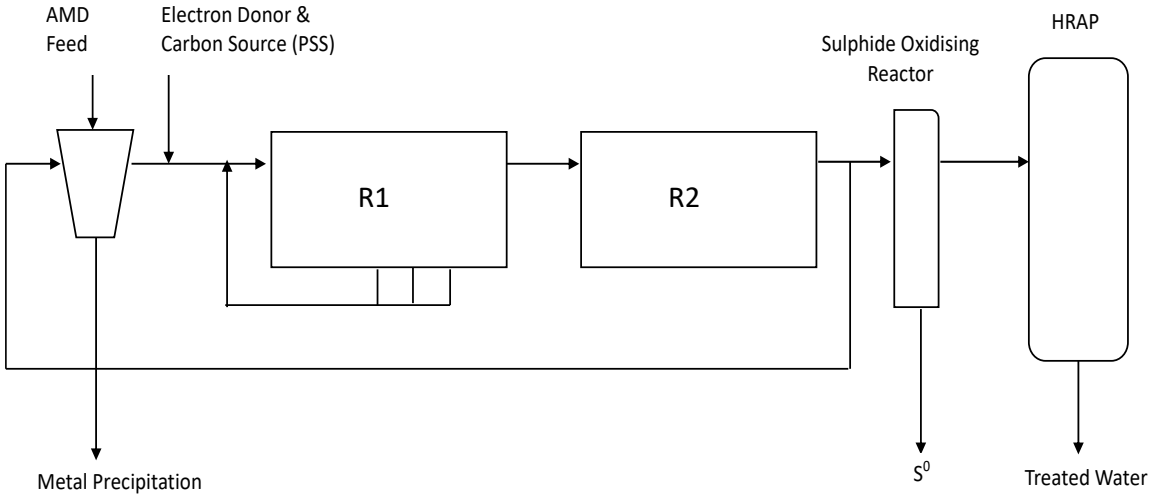


Figure 2.3. Process flow path of the full-scale Rhodes BioSURE® technology applied to the treatment of AMD, showing R1(recycling sludge bed reactor), R2 (baffled reactor) and HRAP (high rate algal ponds). Incoming mine water is blended with a sulphide rich side stream to precipitate heavy metals in the influent (re-drawn from Neba, 2006)

The first THIOPAQ® process was licensed in 1999 to the refining industry for the removal and recovery of sulphur from effluent streams (Janssen *et al*., 2000). The process can be applied to a wide range of biogas streams containing H_2S for the removal of H_2S and recovery of elemental sulphur. In the THIOPAQ® process, in the scrubber, the gas containing H_2S, is absorbed under slightly alkaline conditions (pH 8- 9) enabling a chemical reaction with hydroxide ions (Reaction 2.10). The dissolved sulphide is subsequently oxidized into elemental sulphur according to Reaction 1.4 (Janssen *et al*., 2000) a reaction already described in Chapter 1. The elemental sulphur produced from this process can be used as a high quality fertiliser.

$$H_2S + OH^- \rightarrow HS^- + H_2O \qquad \text{Reaction 2.10}$$

$$HS^- + \frac{1}{2}O_2 \rightarrow S^0 + OH^- \qquad \text{Reaction 1.4}$$

A high H_2S removal efficiency (>99.5%) is obtained in the THIOPAQ® gas desulfurization process, and discharge is safe and easy to manage as both the sulphur produced and the small bleed stream (consisting of sodium salts) are free of sulphide (Janssen *et al*., 2000; Benschop *et al*., 2004). The THIOPAQ® technology is illustrated in Figure 2.4.

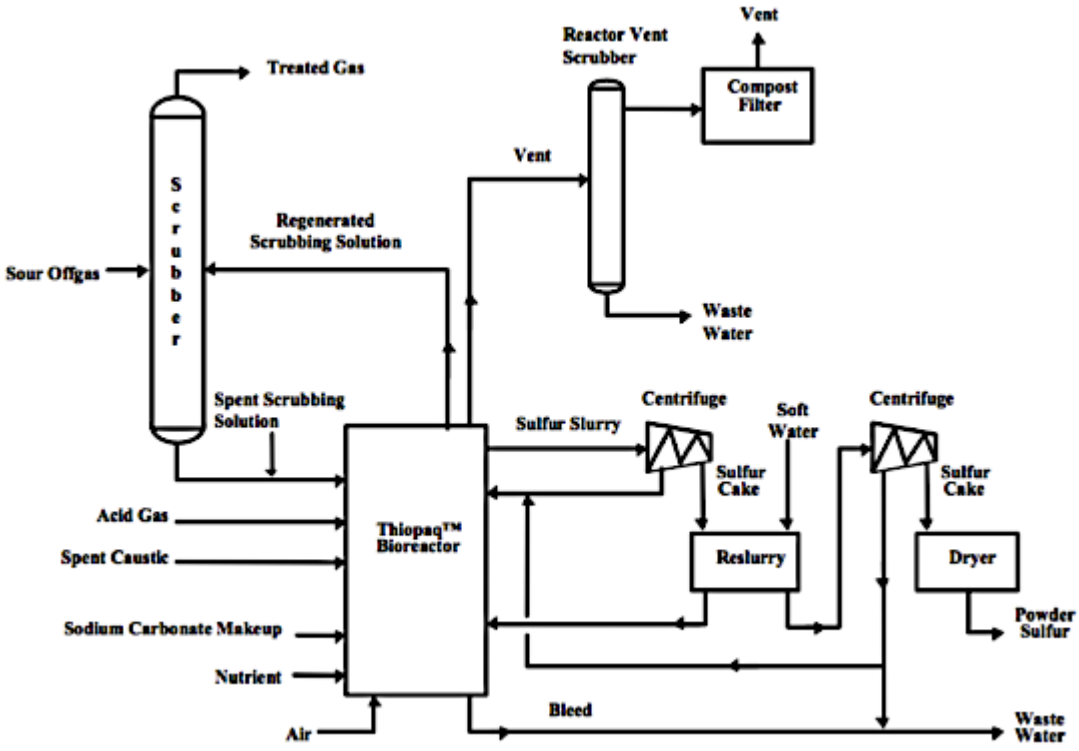


Figure 2.4. Schematic diagram demonstrating the THIOPAQ process (Benschop *et al*., 2004, with permission)

SULFATEQ™ technology was also developed by Paques BV. The technology offers treatment of sulphate-containing effluents with SRB. Sulphate in the effluent is removed to less than 300 mg/l and

is converted into hydrophilic (non-clogging) elemental sulphur (PAQUES, 2016). Additionally, valuable metals such as copper, nickel and zinc are recovered as marketable metal sulphides. This technology circumvents the problems faced with the use of lime treatment, reverse osmosis and ion exchange. The technology is a two-step process. Firstly, the biological sulphate is reduced by converting it into dissolved sulphide in high-rate bioreactors, as demonstrated in Figure 2.5 (PAQUES, 2016). Hydrogen or alcohol serve as electron donors for BSR. Secondly, the sulphide is oxidised to elemental sulphur with air and the elemental sulphur is separated from the liquid. The recovery of valuable metals can be added as an additional step (PAQUES, 2016). The SULFATEQ™ technology is illustrated in Figure 2.5.

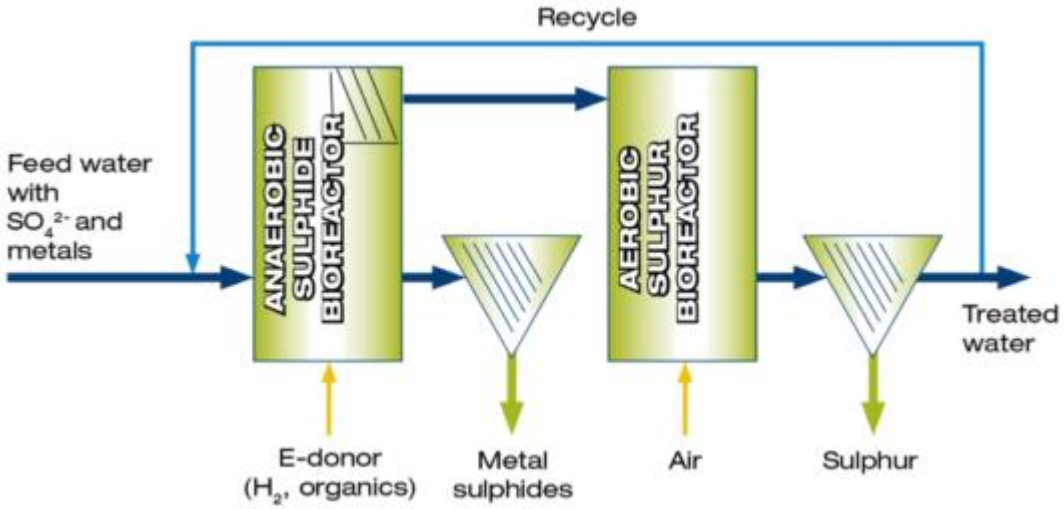


Figure 2.5. Schematic diagram illustrating the process flow of SULFATEQ™ technology (PAQUES, 2016, with permission).

Sulphidogenic reactors such as packed bed reactor (PBR), gas-lift bioreactor (GLB), membrane bioreactor (MBR), fluidised bed bioreactor (FBR), upflow anaerobic sludge blanket reactor (UASB) and expanded granular sludge bed (EGSB) reactor to treat sulphate-laden waters and continuous stirred tank reactor (CSTR) have been largely used to investigate BSR kinetics. The performance of these bioreactor types in treating sulphate-containing and heavy metal-laden waste streams has been studied under different pH, temperature, initial sulphate concentration, hydraulic retention time (HRT) and carbon source and electron donor (Table 2.5).

Table 2.5. Performance of various bioreactors used to treat sulphate-containing and heavy metal-laden waste streams with different electron donor and carbon source

Bioreactor	pH	T (°C)	Electron donor/ carbon source	Source of inoculum	Feed sulphate concentration ($g\ l^{-1}$)	HRT (d)	Sulphate conversion (%)	Volumetric sulphate reduction rate ($g\ l^{-1}\ h^{-1}$)	Reference
CSTR	8 ± 0.2	35	Lactate	Wastewater treatment plant	1-10	0.5 -5	22- 86	0.084-0.041	Oyekola *et al.* (2010)
CSTR	8 ± 0.2	35	Acetate	Wastewater treatment plant	1-10	0.5 -5	60- 93	0.007- 0.17	Moosa *et al.* (2002)
CSTR	6-7.5	35	Ethanol	Wastewater treatment plant	1-12.5	5-9	4.1-93	0.004-0.091	Hansford *et al.* (2000)
CSTR	7.4-7.8		Ethanol	Wastewater treatment plant	2.5	4-6	43-86	0.011-0.015	
CSTR	nr	35	Butyrate	nr	0.068-1.667	5-20	67	nr	Mizuno *et al.*, (1994)
CSTR	7.2-7.5	35	Propionate	nr	0.5-2.5	2.5-5.8	60-95	nr	Uberoi and Bhattacharya (1997)
Packed bed	7.4	23	Volatile fatty acids	Oil fields	0.02	120	nr	nr	Grigoryan *et al.*, (2008)
MBR	4	30	H_2/ CO_2	Acclimated granular sludge	0-5	0.5	0-95	8	Bijmans *et al.* (2010)
FBR	3	35	Ethanol	Methanogenic sludge and mine sediments	2	0.25- 0.88	50- 100	<4.3	Kaksonen *et al.* (2004)
UASB	4.8-6.3	30 ± 1	Domestic water	Acidic sediment	9.2	1-2	>75	0.34	Sánchez-Andrea *et al.* (2012)
EGSB	7.5	65	Methanol	Sludge from sulphate reducing reactor	2.8	0.15- 0.6	nr	0.625	Weijma *et al.* (2000)
UASB	nr	nr	Micro-algal biomass	Sludge from methanogenic reactor		2	70.8- 90.3	0.030-0.058	Boshoff *et al.* (2004)

CSTR = Continuous stirred tank reactor
MBR = Membrane bioreactor
EGSB = Expanded granular sludge bed
UASB = Upflow anaerobic sludge blanket reactor
FBR = Fluidised-bed reactor
nr = not reported

2.3.2 Passive treatment technologies

Unlike their active counterparts, passive treatment technologies require little human interaction, low maintenance and low operation costs (Taylor *et al*., 2005). Remediation of AMD by passive systems was first recorded in the early 1980s by Huntsman and colleagues as well as Wieder and Lang (Gazea *et al*., 1996). This natural passive system employed the use of a natural *Sphagnum* moss wetlands, resulting in the amelioration of water quality without any obvious ecological damage to the wetland system (Gazea *et al*., 1996). Huntsman and colleagues noticed that AMD flowing through the natural wetlands had decreased levels of iron, magnesium, calcium, sulphate, and manganese, while pH increased (Huntsman *et al*., 1978). This sparked the idea that *Sphagnum* moss could be used in constructed wetlands for AMD remediation (Huntsman *et al*., 1978; Gazea *et al*., 1996). However, the use of *Sphagnum* in constructed wetlands was not a viable option as the moss was found to be sensitive to transplanting, abrupt changes in water chemistry and increased accumulation of iron and was not readily available (Hedin *et al*., 1989; Gazea *et al*., 1996). Since then, further progress in passive systems has resulted in the development of technologies such as anoxic limestone drains (ALDs), aerobic wetlands, anaerobic wetlands, open limestone channels and sulphate reduction bioreactors (Taylor *et al*., 2005; Zipper *et al.*, 2014).

2.3.2.1 Anoxic limestone drains and open limestone channels

Anoxic limestone drains (ALDs) consist of a buried bed of crushed limestone ($CaCO_3$) engineered to intercept AMD while in the anoxic state (Watzlaf *et al*., 2000). Therefore, ALDs depend on the dissolution of the limestone as a way to add alkalinity to mine water (Watzlaf *et al*., 2000). The main function of ALDs is to increase the pH of AMD from low pH (≤pH 6) to pH 8, as well as optimising the addition of bicarbonate alkalinity to AMD (Taylor *et al*., 2005; Zipper *et al*., 2014). The reaction is indicated by Reaction 2.10 (Zipper *et al*., 2014).

$$H^+ + CaCO_3 \rightarrow Ca^{2+} + HCO_3^- \qquad \text{Reaction 2.10}$$

Typically, the dimensions for ALDs are 1.5 m in depth and 30 m in length (Johnson and Hallberg, 2005). The level of the resultant degree of neutralisation is affected by the amount of ferric iron (Fe^{3+}), calcium concentration and initial pH of the AMD, the stone size and calcium carbonate content of the crushed limestone, the residence (contact) time and the initial partial pressure of carbon dioxide (Watzlaf *et al*., 2000). The process conditions required to treat AMD effluent successfully by ALDs are indicated in Table 2.4. AMD treatment by ALDs require further treatment by aerobic wetlands to oxidise and remove dissolved iron, manganese and other contaminants (Watzlaf *et al*., 2000). The greatest challenge in the design of ALDs is incorporating sufficient porosity as to make sure plugging of void spaces within the drain does not happen. Effluents that contain high concentrations of aluminium

(>1 mg/l) lead to precipitation of Al as aluminium hydroxide hydrate [$Al(OH)_3.3H_2O$] upon contact with limestone, which may clog the drain (Taylor *et al*., 2005).

Table 2.4. The process conditions required for treating AMD effluent successfully by anoxic limestone drains (Watzlaf *et al*., 2000).

Average acidity load (mg/l)	Average flow rate (l/s)	Dissolved oxygen (mg/l)	Typical pH range	Max pH range
<150	[a]	<1	>2	6-8

[a] maximum flow rate depends on the size of the system

OLCs are open, free-flowing channels lined with coarse limestone (Ziemkiewicz *et al*., 1994) best suited for the remediation of net acidic waters with a large concentration of heavy metals (Zipper *et al*., 2014). The objective of OLCs is to add alkalinity to acidic AMD, while maintaining iron in its reduced form. This is to avoid the oxidation of ferrous iron and precipitation of ferric hydroxide on the limestone, a phenomenon referred to as armouring (Johnson and Hallberg, 2005). Armouring can interfere with the effectiveness of the neutralising agent (Johnson and Hallberg, 2005). OLCs can vary from narrow (0.6–1.0 m) to wide (10–20 m) diameter, typically ca. 1.5-m deep and ca. 30 m in length (Johnson and Hallberg, 2005; Alcolea *et al*., 2012). Their performance depends on variables such as flow velocity, slopes, thickness of the coating of limestone and pH (RoyChowdhury *et al*., 2015). OLCs have been shown to remove 20 % of Al and Mn, 4 to 69 % of acidity and 72 % of Fe from AMD (Skousen *et al*., 2000; Skousen and Ziemkiewicz, 2005; RoyChowdhury *et al*., 2015). Generally, OLCs are constructed in combination of other passive treatment systems (Skousen and Ziemkiewicz, 2005). The major advantage of OLCs is that the alkalinity is produced at a lower cost than constructed anaerobic wetlands (Skousen and Ziemkiewicz 2005). Santofimia and López-Pamo (2016) demonstrated that OLCs can increase the pH of AMD from 3 to 4.5 and resulted in the removal of all dissolved Fe and partially dissolved Al. During a study comparing ALDs with OLCs, drainage ditches constructed with or containing limestone, Kusin *et al*. (2013) demonstrated that ALDs were more effective than OLCs in terms of raising pH, decreasing acidity and producing alkalinity.

2.3.2.2 Aerobic wetlands

Aerobic wetlands are the simplest type of passive treatment but are quite limited in terms of the type of waste water they can effectively remediate (Hedin *et al*., 1994). Aerobic wetlands are used for the treatment of net alkaline waters or slightly acidic waters containing high levels of Fe (Hedin *et al*., 1994; Gazea *et al*., 1996). In these systems, the aeration of water is allowed by letting it flow through vegetation. Metals then precipitate primarily as metal hydroxides, oxyhydroxides and oxides through oxidation. The hydrolysis of metal consumes OH^- ions which generates the resulting acidity. The acidity

generated slows down the oxidation rate. The pH must be maintained between 5.5 and 6.5 to facilitate the precipitation of iron and other metals present (Gazea *et al*., 1996). In order to ensure aerobic conditions and growth of aquatic plants, the water levels in aerobic wetlands must be maintained between 10 and 30 cm. This also ensures that plant diversity is maintained. These plants serve as a physical barrier which causes flowing waters to move more slowly, thus allowing more time for oxidation (Zipper *et al*., 2014). Commonly used plant species include *Typha* sp. (Leto *et al*., 2013), *Scirpus validus* (Fraser *et al*., 2004), *Phragmites australis* (Barbera *et al.*, 2009; Toscano *et al.*, 2015) and *Eriophorum angustifolium* (White *et al*., 2011). Reports by White *et al*. (2011) suggested that plant-derived phenol compounds may interfere with the remediation of Fe from AMD. The study reported that the use of *Eriophorum angustifolium* in wetland for AMD remediation, initially caused the concentrations of Fe to decrease from levels higher than 1100 to 75 mg l^{-1}. However, after 15 days, an increase in Fe to levels higher than 300 mg l^{-1} was reported. This rise of iron was attributed to the rise of plants-derived phenol compounds within the wetland. These findings suggest that together with providing sufficient residence time to allow metal oxidation and hydrolysis in wetlands (Sheoran and Sheoran, 2006), the choice of plant species chosen within the wetlands is also important for them to be effective.

2.3.2.3 Anaerobic wetlands

Anaerobic wetlands are employed for the treatment of net acidic waters (Gazea *et al*., 1996; Zipper *et al*., 2014). A bed of limestone is added beneath or mixed with an organic substrate. This encourages generation of alkalinity as bicarbonate (Zipper *et al*., 2014). Alkalinity is also generated according to Reaction 2.11 (Zipper *et al*., 2014). This process is microbially catalysed biological sulphate reduction as a result of biodegradation of organic material present.

$$SO_4^{2-} + 2CH_2O \rightarrow H_2S + 2HCO_3^- \qquad \text{Reaction 2.11}$$

The bicarbonate (HCO_3^-) can react with H^+ according to Reaction 2.12 resulting in a rise in pH and the precipitation of acid-soluble metals. The formation of hydrogen sulphide also plays a large role in the facilitating the precipitation of metals as metal sulphides according to Reaction 1.2 (Zipper *et al*., 2014).

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2\,(aq) \qquad \text{Reaction 2.12}$$

$$Me^{2+} + HS^- \rightarrow MeS\downarrow + H^+ \qquad \text{Reaction 1.2}$$

Anaerobic wetlands generally contain a 30 to 60 cm layer of organic matter, over 15–30 cm bed of limestone, or a mixture of limestone and organics can be placed to a depth of 50-100 cm (Zipper *et al*., 2014) (Figure 2.6). The advantage of anaerobic wetlands is that they are capable of treating AMD with high metal loading (Fe or Al), high dissolved oxygen and acidity higher than 300 $CaCO_3$ mg/L (Gazea *et al*., 1996). However, these systems usually require long retention times and large surface areas due to the slow mixing of the alkaline substrate water with acidic waters near the surface, therefore limiting

their effectiveness (Hedin *et al*., 1994; Gazea *et al*., 1996). One study demonstrated the anaerobic wetlands can reduce Fe concentration by between 62 to 80% (Faulkner and Skousen., 1994; RoyChowdhury *et al*., 2015).

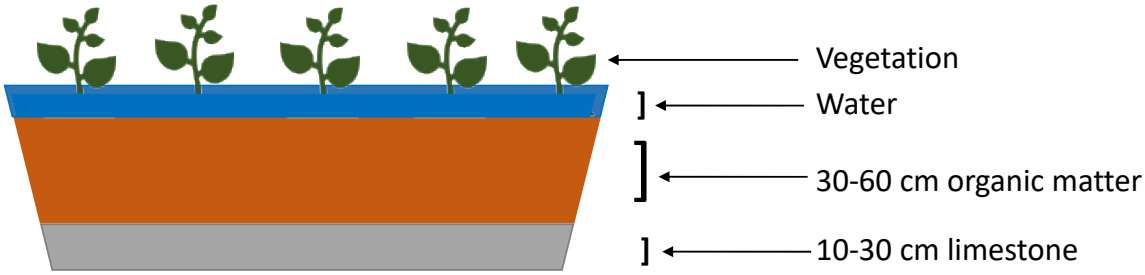


Figure 2.6. Schematic diagram indicating components of an anaerobic wetland.

2.3.2.4 Sulphate reducing bioreactors (SRBRs)

Various studies on sulphate reducing bioreactors (SRBRs) have been conducted as active systems and a few SRBRs are operated as passive systems (Gopi Kiran *et al*., 2017). Early passive SRBRs used mushroom compost and animal manure as substrates, to support microbial growth and metabolism, as they generate significant alkalinity (Dvorak *et al*., 1992). Other studies have used a combination of alfalfa, limestone and sawdust for the generation of initial alkalinity and as carbon source and electron donor for BSR (Gopi Kiran *et al*., 2017). In Canada, two field scale passive SRBRs were successfully installed and operated on the North-western Quebec mine sites between 1999 and 2000. These were known as the Wood Cadillac biofilter and Cadillac molybdenite passive bioreactor (Germain and Cyr, 2003; Tassé *et al*., 2003). A Cadillac biofilter is 50×57 m in area and 1 m in depth. Yellow birch bark chippings served as carbon source and electron donor for BSR. At a pH range between 7.5 and 8.0, sulphate was reduced from 650 to 120 mg/l, while arsenic (As) was reduced from 200 to 20 µg/l (Germain and Cyr, 2003; Tassé *et al*., 2003). Using the same systems with wood chips, manure, hay and limestone as substrates in the Cadillac molybdenite passive bioreactor at 5 days HRT, Kuyucak *et al*. (2006) demonstrated sulphate removal from 887 to less than 360 mg/l. DiLoreto *et al*. (2016) used a mussel shell bioreactor (MSB) that utilises waste from the shellfish industry as an organic source for BSR as a passive bioreactor. Mussel shells have a structure that consists of amorphous $CaCO_3$ consisting of interlamellar sheets of chitin in a “brick and mortar” arrangement, thus providing an effective surface area (Jacob *et al*., 2008). The reactor consisted of three cells or components. The first component was the sediment settling pond used to reduced sediment loads within the MSB. All reactions occurred in the second cell as it contained the mussel shell material and drainage system. The third cell was used as the second settling pond which allowed for aeration and residual sediment settling before discharge (DiLoreto *et al*. 2016). In this reactor, the effluent pH increased from 3.4 to 8.3. Removal of up to approximately 99% of dissolved Al and Fe, and >90% of Ni, Tl and Zn were observed. When searching for the best treatment solutions for AMD and sulphate-rich wastewaters, biological

sulphate reduction (BSR) is posed as a good alternative, however, operating costs and the cost of carbon source and electron donor are crucial (Van Hille *et al*., 1999; Gibert *et al*., 2002; Gopal, 2005; McCarthy, 2011; Sánchez-Andrea *et al*., 2012; Dev and Bhattacharya, 2014; Gopi Kiran *et al*., 2017).

2.3.1 Cost of AMD treatment

Many studies have evaluated BSR kinetics and performance using purchased, commercial or laboratory grade, carbon sources and electron donors (Table 2.5). It is recognised that the availability of a cost-effective carbon source and electron donor still remains a challenge in the treatment of AMD and sulphate-laden wastewaters (Van Hille *et al*., 1999; Gibert *et al*., 2002; Gopal, 2005; McCarthy, 2011; Sánchez-Andrea *et al*., 2012; Dev and Bhattacharya, 2014; DiLoreto *et al*., 2017; Gopi Kiran *et al*., 2017). In 2005, the US Forest Service estimated that the cost of remediating impacted AMD sites on National Forest System land was around $4 billion. The cost of AMD impacted National Forest System land clean-up services, between 1998 and 2003, was reported to be $310 million (RoyChowdhury *et al*., 2015). This emphasises the need for a cost-effective remediation process including a suitable carbon source and electron donor.

Four factors can be considered when evaluating the cost of a remediation process carbon source and electron donor for BSR: capital costs, operating and maintenance costs, cost of carbon source and electron donor and cost of sludge disposal (Gopal, 2005; DiLoreto *et al*., 2017) with the first two dependent on the carbon source selected (Gopal, 2005). Gopal (2005) performed a comprehensive study comparing the economic viability of the use of either ethanol, molasses or primary sewage sludge (used in the BioSURE® process discussed in Section 2.3.1.4) as a carbon source and electron donor for BSR. The study demonstrated that the capital costs for either ethanol, molasses or primary sewage sludge were relatively similar and suggested that the substrate holding tank (required for sewage sludge in the BioSURE® process) will not have a significant effect on the total operating cost of the system as it only accounted for 0.1% of the total capital cost. Although the cost of primary sewage sludge was assumed to be zero, the operating costs and the cost of disposal were higher when primary sewage sludge served as a carbon source and electron donor for BSR. DiLoreto *et al*. (2017) demonstrated that when mussel shell materials were used as a carbon source and electron donor for BSR, the greatest costs were incurred from capital costs and operating and maintenance costs. Similar to the primary sewage sludge, the cost of mussel shell material was assumed to be zero as these are regarded as waste materials. These study indicated that complex carbon sources, which do not need to be purchased, can be utilised as substrate for BSR and make a significant contribution in reducing the cost of AMD treatment. However, the use of some complex carbon substrates may result in slow sulphate reduction kinetics (Waybrant *et al*., 1998) and may therefore not be suitable for use with existing BSR processes. To gain a holistic understanding of the BSR rate kinetics achievable with complex carbon substrates as carbon source and

electron donor studies investigating both the reaction conditions and the mixed microbial communities involved must be performed. These studies are essential to provide answers on the feasibility of the use of these substrates and the operating conditions required to ensure effective BSR of sulphate rich AMD when using these substrates.

2.4 SULPHUR CYCLING AND MICROORGANISMS

2.4.1 Biological sulphur cycle

Sulphur is among the most abundant elements on the Earth (Kellogg *et al*., 1972). The sulphur cycle is complex because sulphur has a broad range of oxidation states, from –2 (completely reduced) to +6 (completely oxidized) (Figure. 2.7), and can be transformed between oxidation states both chemically and biologically (Muyzer and Stams 2008; Sánchez-Andrea *et al*., 2014). The final oxidation product is usually sulphate (SO_4^{2-}) which accumulates in minerals and in the ocean as $CaSO_4$ (Sánchez-Andrea *et al*., 2014). Microorganisms, fungi and plants play critical roles in the cycling of sulphur by catalysing both the oxidation and reduction of sulphur compounds (Sánchez-Andrea *et al.*, 2014). These reactions are reviewed by Sánchez-Andrea *et al.* (2014) and are listed below

(i) oxidation of sulphide by phototrophic bacteria and lithotrophic bacteria with Fe^{3+}, O_2, Mn^{4+} or NO_3^- as electron acceptors, resulting in the production of sulphur and subsequently sulphate

(ii) disproportionation of sulphur compounds (sulphite, sulphur and thiosulphate) to sulphate and sulphide;

(iii) mineralization of organic compounds with hydrogen sulphide release

(iv) assimilatory sulphate reduction whereby the reduced sulphide is assimilated in biomass, proteins, amino-acids and cofactors by microorganisms; fungi and plants

(v) dissimilatory sulphur reduction whereby the electron acceptor is elemental sulphur

(vi) dissimilatory sulphate reduction whereby the reduction of sulphate to sulphide is coupled to growth and conservation of energy (Sánchez-Andrea *et al*., 2014).

The final reaction is of interest for AMD remediation utilising BSR and is explored in this thesis.

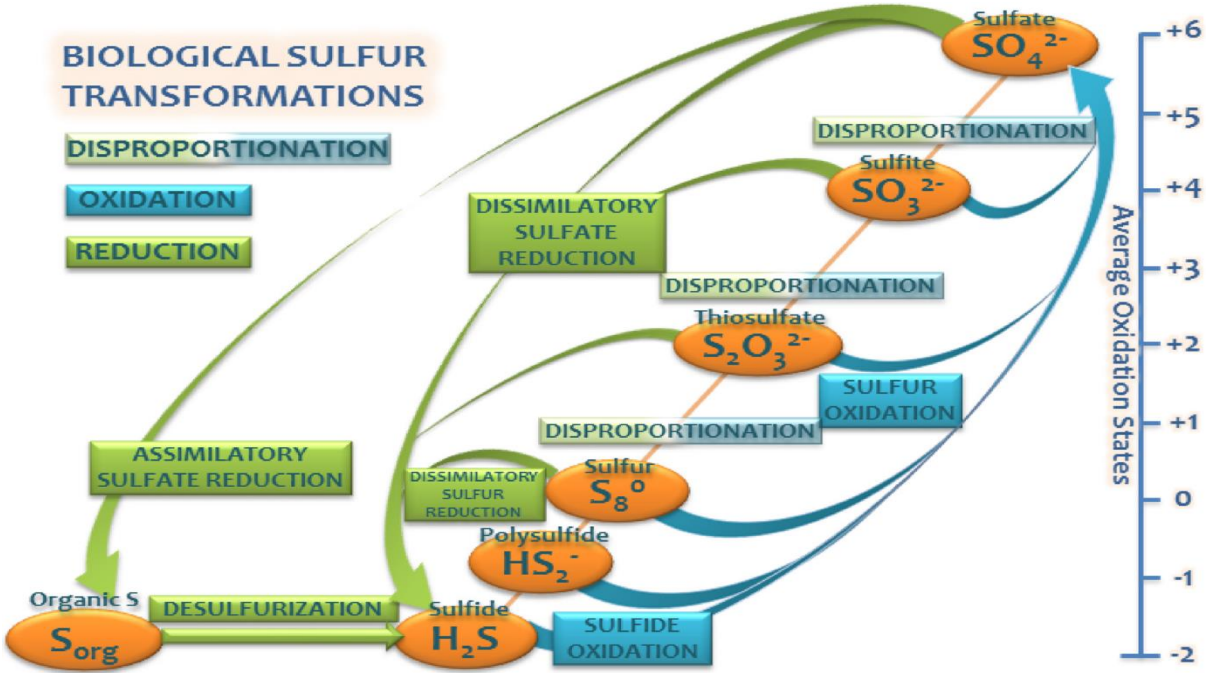


Figure 2.7. The biological sulphur transformations (Taken from Sánchez-Andrea *et al*., 2014 with permission).

2.4.2 Dissimilatory sulphate reduction

Sulphate is a chemically unfavourable electron acceptor for microorganisms (Muyzer and Stams, 2008). The reason for this is that the redox potential couple sulphate to sulphite is –516 mV which is lower than that achievable by the intracellular electron mediators ferredoxin and/or NADH (redox potential of -398 mV and –314 mV, respectively) present in sulphate reducing microorganisms (Fitz and Cypionka 1990; Broco *et al*., 2005; Muyzer and Stams, 2008). SRBs have overcome this by modifying their metabolic pathways required to facilitate sulphate reduction (Fitz and Cypionka, 1990; Broco *et al*., 2005). Before sulphate reduction can happen, ATP sulphurylase activates sulphate, which results in the formation of adenosine-phosposulphate (APS) and pyrophosphate, which in turn is hydrolysed by pyrophosphatase to 2-phosphate (Fitz and Cypionka 1990; Broco *et al*., 2005; Muyzer and Stams, 2008). The redox potential of APS-sulphite plus AMP is -60 mV (Muyzer and Stams, 2008), thus allowing the reduction of APS to be facilitated by reduced NADH. AMP is then phosphorylated by ATP-dependent adenylate kinase into two molecules of Adenosine diphosphate (ADP). This phosphorylation utilises two molecules of ATP, required for sulphate activation (Fitz and Cypionka, 1990; Broco *et al*., 2005). The further steps in the pathway required for the reduction of sulphite to sulphide are however not currently fully understood (Muyzer and Stams, 2008). However, it is postulated that the sulphite formed is either reduced through a series of intermediates such as metabisulphite ($S_2O_5^{2-}$), dithionite ($S_2O_4^{2-}$), trithionate ($S_3O_6^{2-}$), and thiosulphate ($S_2O_3^{2-}$) to form sulphide as demonstrated in step 4 of Figure 2.8 (Postgate *et al*., 1984; Lenler *et al*. 1999). Another theory suggests the formation of the sulphite directly, through a single step which involves the transfer of six-

electrons in the presence of sulphite reductase (Reaction 2.13). The sulphide generated is then excreted through the cell wall into the environment (Menert *et al*. 2004; Shen and Buick, 2004).

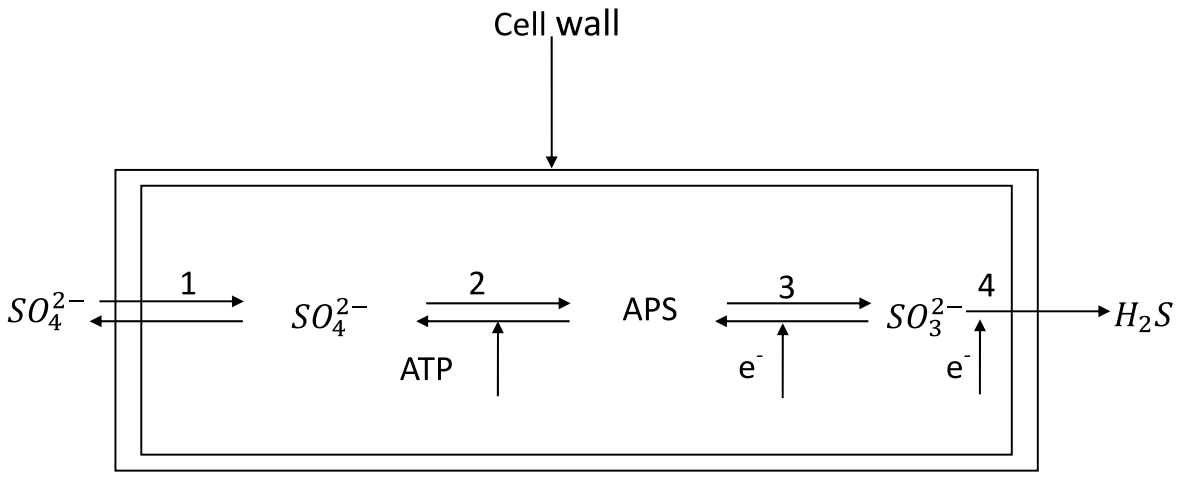


Figure 2.8. The pathway of dissimilatory sulphate reduction (e^- = electron). 1-4 represent the steps involved in the dissimilatory sulphate reduction process (Shen and Buick, 2004).

$$HSO_3^- \xrightarrow{3H_2 6e^-} HS^- + 3H_2O$$

$\Delta G_0 =$ -171 kJ mol^{-1} Reaction 2.13

2.4.3 Sulphate reducing bacteria

Sulphate reducing bacteria (SRB) perform dissimilatory sulphate reduction and their activity was first recorded in 1895 by Beijerinck (Hao *et al*., 2014). He noticed that sulphate could be reduced to sulphide by anaerobic respiration in sediments. SRB consist of a large diverse group of microorganisms that share the exclusive ability to use sulphate as terminal electron acceptor while oxidising various carbon sources (Liamleam and Annachhatre 2007; Miletto et al. 2007; Muyzer and Stams 2008; Hao *et al*., 2014).

A large number of SRB were first isolated and characterised by Widdel (1980). To date, SRB have been isolated from marine environments (Dhillon *et al*., 2003), freshwaters habitats (Holmer and Storkholm, 2001), oxic and anoxic biofilms (Probst *et al.*, 2013), hypersaline microbial mats (Jonkers *et al*., 2005), the digestive tracts of animals and humans (Rey *et al*., 2013), extremely haloalkaline condition (Sorokin *et al*., 2010) and wastewater treatment systems (Ito *et al*., 2002; DiLoreto *et al*., 2016). Traditionally, SRB are viewed as obligate anaerobes that are incapable of tolerating oxygen and thus are restricted to anoxic environments (Baumgartner *et al.*, 2006). However, SRB activity has been shown to occur in oxic environments (Visscher *et al*., 2000; Dupraz *et al.*, 2004), suggesting that some may be characterised as facultative anaerobes.

SRB can be classified into four taxonomic groups (Sheoran *et al*., 2010). Firstly the δ-Proteobacteria subdivision which contains Gram-negative mesophilic SRB, including the genera *Desulfovibrio,*

*Desulfomicrobium, Desulfobulbus, Desulfobacter, Desulfobacterium, Desulfococcus, Desulfosarcina, Desulfomonile, Desulfonema, Desulfobotulus, Desulfoarculu*s and *Desulfocurvus*. The species within this group demonstrate diverse physiological traits and morphologies, with optimal growth temperatures ranging from 20 to 40°C (Castro *et al*., 2000; Moosa *et al*., 2005; Klouche *et al*., 2009; Oyekola *et al*., 2010; Hamdi *et al*., 2013). The second group consists of the spore forming Gram-positive organisms (Algie, 1983). This group is mainly represented by the genera *Desulfotomaculum* (Parshina *et al*., 2005; Sheoran *et al*., 2010; Aüllo *et al*., 2013), *Desulfosporosinus* (Robertson *et al*., 2001), *Desulfurispora* (Kaksonen *et al*., 2007a), *Desulfovirgula* (Kaksonen *et al*., 2007b) as well as *Desulfallas, Desulfofundulus, Desulfofarcimen* and *Desulfohalotomaculum* (Watanabe *et al*, 2018). Most species require a similar growth temperature range as the δ-Proteobacteria, however some can withstand higher temperatures due to the survival of spores (Sheoran *et al*., 2010). The third group consists of the thermophilic SRB. This group contains the genera *Thermodesulfobacterium* and *Thermodesulfovibrio* with optimal growth temperatures between 65–70°C (Jeanthon *et al*., 2002; Sekiguchi *et al*., 2008). The last taxonomic group is the archaeal thermophilic sulphate reducing archaea which thrive at temperatures above 80°C (Castro *et al*., 2000). All members of this group belong to the genus *Archaeoglobus* (Castro *et al*., 2000; Sheoran *et al*., 2010). SRB are diverse in morphology and can be rod, vibrio, oval, filamentous, sphere or curved shaped (Hao *et al*., 2014) and can be classified on their ability to completely oxidise organic compounds to carbon dioxide or incompletely to acetate (Table 2.6). Complete oxidisers, such as *Desulfobacter postgatei*, commonly use acetate as a growth substrate and may employ either the modified citric acid cycle (Brandis-Heep *et al*., 1983; Wood *et al*., 1986; Thauer, 1988) or the acetyl-CoA pathway, such as species of the *Desulfobacterium*, *Desulfotomaculum* and *Desulfococcus* genera and the species *Desulfobacca acetoxidans* (Schauler *et al*., 1986; Bogte *et al*., 1999). Some SRB such as species belonging to the *Desulfotomaculum* genus and some strains of *Desulfofrigus oceanense* have the ability to switch from complete to incomplete oxidation at high concentrations of substrate (Kuever *et al*., 1999). Incomplete oxidisers often lack the biochemical pathway for the oxidation of acetyl-CoA to CO_2 (Postgate and Odom, 2013). However, complete oxidisers will do not always oxidise substrates completely to CO_2. The oxidation of compounds such as propionate, butyrate, ethanol or lactate often leads to a build-up of acetate and thereafter the oxidation of acetate may proceed very slowly (Brysch et al. 1987).

To date, SRB have been classified into at least 48 genera, of which 20 genera are characterised as incomplete oxidisers and 24 genera as complete oxidisers (Table 2.6). The four remaining genera *Desulfomonile, Desulfotomaculum Desulfofundulus* and *Desulfallas,* appear to contain both complete and incomplete oxidising species (Hao *et al.*, 2014, Watanabe *et al.*, 2018). Some of the most common electron acceptors for SRB species are sulphate, sulphite, thiosulphate and elemental sulphur although other compounds such as nitrate, nitrite, ferric, fumarate and dimethyl sulphoxide (DMSO) can also serve as electron acceptors. The diversity in morphology of SRB species and electron donors and

electron acceptors capable of supporting SRB growth is demonstrated in Table 2.6 (Hao *et al*., 2014). When complex polymeric organic compounds such as starch, cellulose, proteins and nucleic acids are used, SRB rely on the activity of hydrolytic and fermentative bacteria to produce compounds accessible to SRB (Neculita *et al*., 2007). The interaction of SRB with other anaerobic microorganisms is discussed in Section 2.5.

Table 2.6. Diversity in morphology, electron donors and acceptors for SRB growth. Adapted from Hao *et al.* (2014).

SRB group	Cell morphology	Electron donor	Electron acceptor	Reference
Incomplete oxidisers				
Desulfovibrio	Vibrio	Bu/ Et/ Fo/ La/ Py	SO_3^{2-}/$S_2O_3^{2-}$/NO_2^- NO_3^-/ O_2/ Fe (iii) MnO_2/ Fumarate	Krekeler and Cypionka (1995), Suzuki *et al.* (2010).
Desulfomicrobium	Oval to rod	H_2/ Et/ Fo/ Fu/ La/ Ma/ Py	SO_3^{2-}/$S_2O_3^{2-}$ NO_2^- Fumarate/DMSO	Dias *et al.* (2008), Genthner *et al.* (1994, 1997), Krumholz *et al.* (1999)
Desulfocurvus	Rod and vibrio	Fo/ La/ Py	SO_3^{2-}/$S_2O_3^{2-}$	Klouche *et al*., 2009
Desulfohalobium	Rod	H_2/ Bu/ Et/ Fo/ La/ Py	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Ollivier *et al*. (1991)
Desulfonatronum	Vibrio	H_2 + Ac/ Et/ La/ Py	SO_3^{2-}/ $S_2O_3^{2-}$/S^0	Pikuta *et al.* (2003), Sorokin *et al*. (2010), Perez-Bernal *et al.* (2017)
Desulfobotulus	Vibrio	Bu/ La/ Pr/ Py	SO_3^{2-}/S^0	Rees and Patel, (2001), Sorokin *et al.* (2010)
Desulfocella	Vibrio		SO_3^{2-}/ $S_2O_3^{2-}$/S^0	Brandt *et al*. (1999)
Desulfofaba	Vibrio	Ac/ Be/ Bu/ Pr/ Pro/ Et/ Fu/ La/ Ma,/Py/ Su	SO_3^{2-}/ $S_2O_3^{2-}$/S^0	Knobtauch *et al.* (1999), Rees and Patel (2001)
Desulforegula	Rod	Ac/ Bu/ Et/ Fu/ La/ Pr	Desulfoviridin, SO_3^{2-}/ $S_2O_3^{2-}$/S^0	Rees and Patel, (2001)
Desulfobulbus	Lemon/onion	Et/ La/ Pr/ Pro/ Pro/ Py	SO_3^{2-}/ $S_2O_3^{2-}$ NO_2^- /NO_3^-/ O_2/ Fe(iii)	Widdel and Pfennig (1982), Holmes *et al.* (2004)
Desulfocapsa	Rod	Ac/ Be/ Bu/ Et/ Fu/ La/ Ma/ Py/ Su	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Brandt *et al.* (1999), Finster *et al*. (2013)
Desulfofustis	Rod	Pr	$S_2O_3^{2-}$/S^0	Barton and Hamilton (2007)
Desulforhopalus	Rod	Bu/ Et/ La/ Pr/ Pro/ Py	SO_3^{2-}/$S_2O_3^{2-}$/NO_3^-	Isaksen *et al*. (1996), Lie *et al*. (1999)
Desulfotalea	Rod	H_2/ Et/ Fo/ Fu/ La/ Ma/ Pr/ Py	SO_3^{2-}/$S_2O_3^{2-}$/S^0/ Fe(iii)Citrate	Knoblauch *et al.* (1999), Barton and Hamilton (2007)
Thermodesulfobacterium	Rod	H_2/ Et/ Fo/ La/ Py	SO_3^{2-}/ $S_2O_3^{2-}$/S^0	Jeanthon *et al.* (2002), Hamilton-Brehm *et al.* (2013)
Desulfosporosinus	Straight/ curved rod	Bu/ Fo/ Fu/ La/ Me/ Pro	SO_3^{2-}/$S_2O_3^{2-}$/S^0/ Fe(iii)	Robertson *et al*. (2001), Vatsurina *et al*. (2008), Ramamoorthy *et al.* (2006)
*Desulfotomaculum**	Vibrio	Ac/ Be/ Bu/ Et/ Me/ Fo/ La/ Py	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Daumas *et al* (1988), Kaksonen *et al.* (2006), Spring *et al*. (2012)
*Desulfomonile**	Rod	Ac/ Py	3-chlorobenzoate/ Fumarate/ SO_3^{2-}/$S_2O_3^{2-}$/S^0/ NO_3^-	DeWeerd *et al*. (1990) Sun *et al*. (2001)
Desulfovirgula	Rod	H_2+CO_2/ Bu/ Fu/ Py	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Kaksonen *et al*. (2007b)

Ac= acetate Be= benzoate Bu= butyrate Et= ethanol Fu= fumarate La= lactate

Ma= malate Me= methanol Pro= propanol Pr= propionate Py= pyruvate Su= succinate

*some species in this group may oxidise the electron donor completely or incompletely to acetate

Table 2.6 continues.

SRB group	Cell morphology	Electron donor	Electron acceptor	Reference
Incomplete oxidisers				
Desulfurispora	Rod	H_2+CO_2/ Bu/ / Et/ Fo/ La/ Me/ Pro/ Py	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Kaksonen *et al.* (2007a)
Desulfohalotomaculum	Rod to curved rods	H_2/ Et/ La/ Ma/ Pro/ Py	SO_3^{2-}/$S_2O_3^{2-}$	Watanabe *et al.* (2018)
Pseudodesulfovibrio	Vibrio	H_2/ Fo/ Ma/ Su	SO_3^{2-}/$S_2O_3^{2-}$/NO_3^-/ Fumarate	Cao *et al*. (2016)
Complete oxidisers				
Desulfothermus	Rod to curved	Ac/ Et/ Fu/ Fo/ Pr/ Py	SO_3^{2-}	Nunoura *et al.* (2007)
Desulfobacter	Rod to ellipsoidal	H_2/ Ac/ Bu/ Et/ La/ Py	SO_3^{2-}/ $S_2O_3^{2-}$	Widdel (1987), Lien and Beeder (1997)
Desulfobacterium	Oval to rod	Ac/ Bu/ La/ Pr/ Pro	SO_3^{2-}/$S_2O_3^{2-}$/ Fumarate	Brenner *et al.* (2005); Amann *et al*. (2010)
Desulfobacula	Oval to curved	Ac/ Be/ Et/ Fu/ Ma/ Su	SO_3^{2-}/ $S_2O_3^{2-}$	Kuever *et al*. (2001)
Desulfococcus	Sphere	Ac/ Bu/ Et/ Fo/ Fu/ Ma/ Pr/ Pro/ Py/ Su	SO_3^{2-}/ $S_2O_3^{2-}$	Brenner *et al.* (2005), Imhoff-Stuckle *et al*. (1983)
Desulfofrigus	Rod		SO_3^{2-}/$S_2O_3^{2-}$/ Fe(iii)-Citrate	Knobtauch *et al.* (1999), Suzuki *et al.* (2008)
Desulfonema	Filaments	Ac/ Be/ Bu/ Fu/ Pr/ Su	SO_3^{2-}/$S_2O_3^{2-}$/NO_3^-	Barton and Hamilton (2007; Icgen *et al.* (2007) Widdel *et al*. (1983)
Desulfosarcina	Irregular shape/ Aggregate	Ac/ Bu/ Be/ Et/ La/ Me/ Pr	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Arendsen (1993), Oyekola (2008), Watanabe *et al.* (2017)
Desulfospira	Curved	H_2/ Bu/ Fo/ Fu/ La/ Su	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Brenner *et al.* (2005), Finster *et al.* (1997)
Desulfotignum	Rod to curved	Ac/ Bu/ Fo/ La/ Ma/ Py/ Su	SO_3^{2-}/$S_2O_3^{2-}$/CO_2	Kuever *et al.* (2001), Brenner *et al.* (2005), Schink *et al*. (2002)
Desulfatibacillum	Rod	Ac/ Bu/ Fu/ Ma/ Pr/ Su	SO_3^{2-}/$S_2O_3^{2-}$	Cravo-Laureau *et al.* (2004), Barton and Hamilton (2007), Callaghan *et a*l. (2012)
Desulfarculus	Vibrio	Ac/ Be/ Fu/ Pr	SO_3^{2-}/ $S_2O_3^{2-}$	An and Picardal *et al*. (2014), Kuever *et al*. (2001), Brenner *et al*. (2005)
Desulforhabdus	Rod to ellipsoidal	H_2	SO_3^{2-}/ $S_2O_3^{2-}$	Brenner *et al.* (2005)
Desulfovirga	Rod	Ac/ Bu/ Et/ Pr/ Py	SO_3^{2-}/$S_2O_3^{2-}$/S^0	Tanaka *et al*. (2000)
Desulfobacca	Oval to rod	Ac	SO_3^{2-}/ $S_2O_3^{2-}$	Oude Elferink *et al.* (1999)

Ac= acetate Be= benzoate Bu= butyrate Et= ethanol Fu= fumarate La= lactate

Ma= malate Me= methanol Pro= propanol Pr= propionate Py= pyruvate Su= succinate

Table 2.6 continues.

SRB group	Cell morphology	Electron donor	Electron acceptor	Reference
Complete oxidisers				
Desulfonatronovibrio	Vibrio	H_2 + [Ac]/ [Fo] + Ac	$SO_3^{2-}/S_2O_3^{2-}/S^0$	Sydow *et al.* (2002), Zhilina *et al.* (1997)
Thermodesulforhabdus	Rod	[Ac]/ [Bu]/ [Et]/ [Fu]/ [La]/ [Ma]/ [Su]	$SO_3^{2-}/S_2O_3^{2-}/S^0/O_2$	Beeder *et al.* (1995), Sievert and Kuever (2000)
Thermodesulfobium	Rod	[Fo]	SO_3^{2-}/	Mori *et al.* (2003)
Thermodesulfovibrio	Curved rod	[La]/ [Py]	SO_3^{2-}/ $S_2O_3^{2-}$ Fe(iii)-Arsenate	Frank et al., (2016), Haouari *et al.* (2008), Sekiguchi *et al.* (2008)
*Desulfallas**	Rod	H_2/ [Be]/ [Bu]/ [Et]/ [Fo]/ [Fu]/ [Ma]/ [Pr]/ [Py]	$SO_3^{2-}/S_2O_3^{2-}$	Watanabe *et al.* (2018)
*Desulfofundulus**	Rod	H_2/ [Be]/ [Bu]/ [Et]/ [Fo]/ [La] [Me]/ [Pr]	$SO_3^{2-}/S_2O_3^{2-}/NO_3^-$	Sousa *et al.* (2018), Watanabe *et al.* (2018)
Desulfofarcimen	Rod	[Ac]/ [Bu]/ [Et]/ / / / /	$SO_3^{2-}/S_2O_3^{2-}/S^0$/ Fumarate	Watanabe *et al.* (2018)
Desulfonauticus	Curved rod	[Fo]	$SO_3^{2-}/S_2O_3^{2-}/S^0$	Mayilraj *et al.* (2009)
Thermodesulfovibrio	Curved rod	[La]/ [Py]	SO_3^{2-}/ $S_2O_3^{2-}$/ Fe(iii)-Arsenate	Frank et al. (2016), Haouari et al. (2008), Sekiguchi et al. (2008)
Archaeoglobus	Irregular coccoid	[Et]/ [Fo]/ [Pro]/ [Py]	SO_3^{2-}/ $S_2O_3^{2-}$	Hartzell and Reed (2006), Stetter (1992)
Desulfacinum	Oval	[Ac]/ [Bu]/ [Et]/ [Fo]/ [Fu]/ [La]/ [Ma]/ [Pr]/ [Py]/ [Su]	$SO_3^{2-}/S_2O_3^{2-}/S^0$	Rees *et al.* (1995), Sievert and Kuever (2000), Rozanova *et al.* (2001)

[Ac]= acetate [Be]= benzoate [Bu]= butyrate [Et]= ethanol [Fu]= fumarate [La]= lactate
[Ma]= malate [Me]= methanol [Pro]= propanol [Pr]= propionate [Py]= pyruvate [Su]= succinate

*some species in this group may oxidise the electron donor completely or incompletely to acetate

2.4.4 Factors affecting biological sulphate reduction

Apart from the SRB communities present, BSR kinetics for the treatment of AMD and sulphate-laden wastewaters is influenced by factors such as sulphate concentration, pH, temperature, redox potential, salinity, the presence of inhibitors such as molybdate, sulphide concentration and the type of electron donor and carbon source (Erasmus, 2000; Hansford, 2007; Laanbroek *et al.*, 1984; Moosa *et al.*, 2002; 2005; Moosa and Harrison, 2006; Oyekola *et al.*, 2010; 2012; Dev *et al.*, 2016). These factors are discussed in the following Sections.

2.4.4.1 Sulphate concentration

In anoxic environments, sulphate reduction is performed by SRB (Postgate 1963; Widdel 1980). The response of SRB and other microorganisms to sulphate loading rate is key to the development of well-functioning AMD treatment processes. Sulphate concentration affects the kinetics of biologically catalysed sulphate reduction (Erasmus, 2000; Hansford, 2007; Laanbroek *et al.*, 1984; Moosa *et al*., 2002; 2005; Moosa and Harrison, 2006; Oyekola *et al*., 2010; 2012; Dev *et al*., 2016) and the SRB community dynamics and their growth rates (Laanbroek *et al*., 1984; Mizuno *et al*., 1994; Uberoi and Bhattacharya 1997; Weijma *et al.*, 2000; Boshoff *et al*., 2004; Kaksonen *et al*., 2004; Van Houten *et al*., 2006; Grigoryan *et al*., 2008; Bijmans *et al*., 2010; Oyekola *et al*., 2012; Sánchez-Andrea *et al*., 2012; Dev *et al*., 2016). To date, only a few studies have investigated the competition between SRB for sulphate. A study by Laanbroek *et al*. (1984), investigating the effect of sulphate concentration on the growth of SRB in chemostats, demonstrated that the hydrogen oxidising SRB *Desulfovibrio baculatus* had the highest affinity for sulphate followed by lactate oxidiser *Desulfobulbus propionicus* and the acetate oxidiser *Desulfobacter postgatei* species, respectively. This suggested that under sulphate limiting conditions SRB utilise ethanol, H_2, and lactate as carbon sources and electron donor but not acetate and propionate (Laanbroek *et al.*, 1984). Therefore, it was postulated that when sulphate is limited, syntrophic groups play a role in the degradation of organic acids, while hydrogen-utilising SRB replace hydrogenotrophic methanogens (Muyzer and Stams, 2008). These observations were supported by O'Flaherty *et al*. (1998) who demonstrated that propionate and H_2 oxidisingSRB communities had higher affinity for sulphate than acetate, butyrate or ethanol degrading SRB.

Comparing sulphate removal rates between different SRB studies can be challenging as generally the initial sulphate concentrations in these studies varies. Some studies also present calculated percentages of sulphate removal and not the initial and final sulphate concentration (Sheoran *et al*., 2010). Nonetheless, these studies have provided insights on how different sulphate concentrations affect SRB communities. Studies with acetate (Moosa *et al*., 2002; 2005), ethanol (Erasmus, 2000; Hansford, 2007) and lactate (Oyekola *et al*., 2010; 2012) demonstrated that the rate of sulphate removal was affected by volumetric sulphate loading rate, mediated through dilution rate and feed sulphate concentration. Using acetate as a carbon source and electron donor for BSR, Moosa *et al*., (2005) reported sulphate inhibition above 0.83 g l^{-1} h^{-1}. These observations were supported by Oyekola *et al*. (2010) who reported that increasing VSLR from 0.15 to 0.21 g l^{-1} h^{-1} results in inhibition of some SRB communities within a particular mixed SRB consortium. Oyekola (2008) demonstrated that an SRB community, dominated by species such as *Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, and *Desulfococcus multivorans*, maintained on lactate in a CSTR was influenced by both sulphate concentration and dilution rate. On the other hand, studies at lower sulphate concentration of 0.6 to 1.0 g l^{-1} with marine waste extract (MWE) as a carbon source and electron donor for BSR demonstrated that increasing sulphate from 0.6 to 1.0 g l^{-1} gradually increased the growth rates

methanoge(μ) of SRB with the highest growth rates achieved at a sulphate concentration of 1.0 g l^{-1} (Dev *et al*., 2016). Taken together, these studies demonstrate that for effective sulphate removal using BSR from AMD treatment, careful consideration of the sulphate concentration of the AMD and the loading rate of these waters into the treatment plant is crucial.

2.4.4.2 Effect of pH

SRB are ubiquitous and have been identified in environments with various pH values (Widdel 1980; Muyzer and Stams, 2008; Hao *et al*., 2014). Studies have been conducted to investigate the activity of SRB in various pH values (Fortin *et al*., 1996; Al-zuhair *et a*l., 2008). Ideal conditions for growth in typical SRB communities is between pH 6 and 8 (Al-zuhair *et al*., 2008; Sánchez-Andrea *et al*., 2014). Growth is often not supported at pH values below 6 and above 9 (Fortin *et al*., 1996; Al-zuhair *et al*., 2008). Studies have reported a decline in microbial sulphate reduction rate and reduction in metal removal capacity beyond this range (Sheoran *et al*., 2010). However, some SRB have been isolated in habitats with extreme pH environments such as that of AMD, pH 2 (Sen, 2001), and soda lakes where the pH can be as high as 10 (Geets *et al*. 2006). A neutral pH allows enhanced SRB activity, production of sulphide, and facilitates metal removal as metal sulphide precipitates. pH also plays an important role in how SRB will compete effectively for substrate with other bacterial groups such as methanogens and therefore on the BSR reactor efficiency (O'Flaherty *et al*., 1998; Cohen 2006).

2.4.4.3 Sulphide

While SRB have a high tolerance for dissolved sulphide, sulphate reduction can be inhibited by high dissolved sulphide concentrations (Postgate *et al*., 1984; Reis *et al*., 1992; Okabe *et al*., 1995; Maillacheruvu and Parkin, 1996; Kaksonen *et al*., 2004a; Moosa and Harrison, 2006; Oyekola *et al*., 2012; Häusler *et al*., 2014). During BSR the state of sulphides produced depends solely on environmental pH (Lens *et al*., 1998; O'Flaherty *et al*., 1998; Sheoran *et al*., 2010). Sulphides are present as hydrogen sulphide in the liquid and gas phase and Henry's law shows the relation between the concentrations of undissociated hydrogen sulphide (Lens *et al*., 1998). Hydrogen sulphide as HS^- may complex with metals present and precipitate as metal sulphides at pH 7, as previously mentioned, however Bharathi *et al*. (1990) postulated that the precipitation of trace element metals as metal sulphides may result in the inhibition of SRB. The hydrogen sulphide in the absence or limited presence of metals may dissociate according to the equilibrium reactions indicated in Reactions 2.14 and 2.15.

$$H_2S \leftrightarrow HS^- + H^+ \qquad \text{Reaction 2.14}$$

$$HS^- \leftrightarrow S^{2-} + H^+ \qquad \text{Reaction 2.15}$$

In the pH range between 6 and 8, hydrogen sulphide exists as a mixture of HS^- and H_2S as demonstrated in Figure 2.9.

The undissociated form, H_2S, dominates below pH 6. The dissociated hydrogen sulphide dissociates further to S^{2-} near pH 12. The total sulphide concentration can be demonstrated by Reaction 2.16,

$$H_2S_{total} = H_2S_{(aq)} + HS^- \qquad \text{Reaction 2.16}$$

or when hydrogen sulphide accumulates from the complete reduction of sulphate to sulphide Reaction 2.17 can be used to calculate the total sulphide generated.

$$H_2S = SO_4^{2-}\ (\text{influent}) - SO_4^{2-}\ (\text{effluent}) \qquad \text{Reaction 2.17}$$

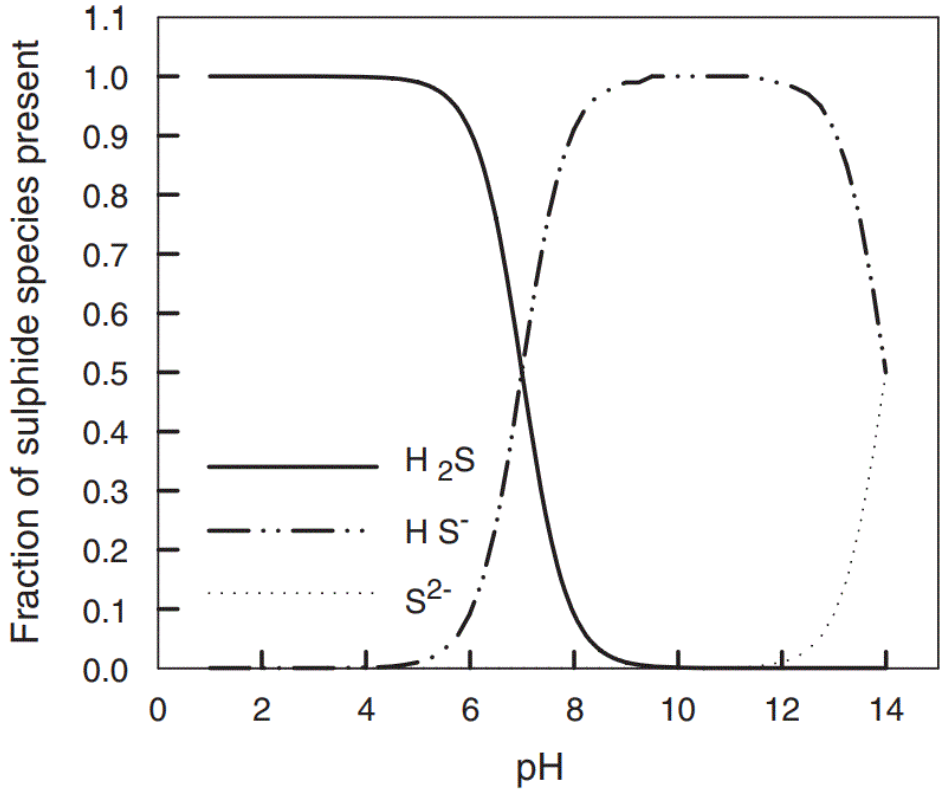


Figure 2.9. Relationship between the species of hydrogen sulphide and pH (Lens *et al.*, 1998, with permission).

Moosa and Harrison (2006) demonstrated that in a BSR CSTR supplemented with acetate, and sulphate at a concentration of 2.5 g l^{-1} (35 °C and pH 7.8), the sulphate removal and volumetric sulphate reduction rates (VSRRs) decreased when sulphide was added in the form of sodium sulphide to achieve concentrations from 0 to 1.25 g l^{-1}. The maximum sulphate removal remained steady (89%) at sulphide concentrations between 0 to 0.75 g l^{-1}, however decreased to 62% as the sulphide concentration reached 1.0 g l^{-1}, indicating a critical sulphide concentration where BSR inhibition started occurring. Once steady-state was reached at the increased feed sulphide concentration and a decrease in the maximum

growth rate (μ_{max}) and the maximum VSRR. The findings by Moosa and Harrison (2006) were consistent with that reported by Okabe *et al*. (1995) for a lactate operated BSR CSTR. Furthermore, the study by Moosa and Harrison (2006) showed that inhibition was mediated by the undissociated H_2S sulphide species, rather than the total sulphide concentration. A study by O'Flaherty *et al*. (1998) reported that inhibition of growth of specific SRB groups that degrade ethanol, acetate, propionate or butyrate was related to the total sulphide concentration, and propionate degrading SRB were the most sensitive group to high concentrations of total sulphide.

Sulphide toxicity originates from the absorption of sulphide into the cell, destroying the protein and thereby rendering it inactive, implying that the cell damage would be irreversible (Postgate *et al.* 1984). However, sulphide inhibition of SRB growth was reported to be reversible by Reis *et al*. (1992). Okabe *et al*. (1995) also reported that *Desulfovibrio desulfuricans* can recover from the shock of high sulphide concentrations, suggesting that SRB may have adapted to periodic high sulphide concentrations. Parkin *et al*. (1990) demonstrated that a sulphide concentration of 60 to 65 mg l^{-1} resulted in process failure in chemostat studies operated on acetate or propionate as carbon source and electron donor for BSR. When lactate served as a carbon source and electron donor for BSR in a chemostat receiving sulphate at a concentration of 2.5 g l^{-1}, the addition of 0.5 g l^{-1} sulphide inhibited lactate fermentation. The μ_{max} of the lactate fermentation culture was reduced from 0.3 to 0.15 h^{-1} while the μ_{max} and Ks of lactate oxidisers (LO) remained unchanged by addition of 0.5 g l^{-1} sulphide (Oyekola *et al*., 2012). The study demonstrated that lactate oxidisers (LO) competed more effectively for lactate at high sulphide concentrations (0.5 g l^{-1}) and low lactate concentrations (≤5 g l^{-1}) while lactate fermenters competed more effectively at excess lactate concentration (≥5 g l^{-1}) and low sulphide concentrations (0.014–0.088 g l^{-1}).

2.4.4.4 Temperature

SRB can grow through a diverse range of temperatures (Moosa *et al*., 2005; Sheoran *et al*., 2010; van den Brand *et al*., 2014b; Marais *et al*., 2018). Mesophilic SRB grow at temperatures below 40°C, with moderate thermophiles growing at 40-60°C, while extreme thermophiles grow at temperatures above 60°C (Widdel, 1988; Sheoran *et al*., 2010). The Gram-positive spore forming subdivision can survive higher temperatures than the δ-Proteobacteria since they form heat resistant spores, while thermophilic SRB such as *Thermodesulfobacterium* and *Thermodesulfovibrio* grow optimally at 65–70°C. Thermophilic sulphate reducing prokaryotes, belonging to the archaeal group, thrive at temperatures above 80°C (Castro *et al*., 2000). Sulphate reducers have also been described at temperatures ≥100°C (Amend and Teske, 2005). The ability of *Desulfovibrio vulgaris* to survive high temperatures has been attributed to the presence of *hrcA* gene which encodes a putative transcriptional regulator of heat shock genes (Chhabra *et al*., 2006). Studies have indicated that temperature can significantly affect the rate of

sulphate reduction (Moosa *et al*., 2005; van den Brand *et al*. 2014a). In general, SRB can tolerate a broad range of temperatures from below -5 to 75°C (Postgate *et al*., 1984). Moosa *et al.* (2005) demonstrated that for a particular environmental SRB community, BSR was improved by increasing the reactor temperature from 20 to 35°C for an acetate fed reactor. However, increasing the temperature further to 40°C resulted in a decrease of BSR. This study and a study by van den Brand *et al*. (2014a), demonstrating that the consumption rates of both acetate and propionate were 1.9 times lower at 10°C than at 20°C, suggest that temperature optimisation is key to ensure effective BSR.

2.4.4.5 Redox potential

Sulphate-reducing bacteria require a low redox potential (<-200 mV) for growth, with optimum growth reported between -390 and -490 mV (White and Gadd, 1996). The associated low oxygen requirement limits their presence to reducing environments. It was previously thought that SRB were strict anaerobes. However, research has indicated that some SRB can tolerate low levels of oxygen. These include members of the *Desulfobulbus*, *Desulfonema*, *Desulfovibrio*, *Desulfococcus* and *Desulfomicrobium* genera, most of which are incomplete oxidisers (Johnson *et al*., 1997; Nagpal *et al*., 2000; Dolla *et al*., 2006; Sheoran *et al*., 2010). Two ways by which *Desulfovibrio desulfuricans* tolerates oxygen is by the formation of aggregates and through aerotaxis (movement of organisms towards or away from oxygen caused by changes in oxygen concentration) (Eschemann *et al*., 1999; Dolla e*t al*., 2006). In the presence of oxygen, *D. desulfuricans* cells will form a ring-shaped band pattern around the air bubble some distance away. This suggests that high oxygen concentrations act as a repellent and that the cells are capable of negative aerotaxis. In the oxic part of the gradient however, *D. desulfuricans* moves in circles, once entering the band, the circular movement would trap the cell in the oxic zone (Eschemann *et al*., 1999; Dolla *et al*., 2006). This circular movement is not observed in *D. vulgaris*, which suggests that SRB have developed several different strategies to respond to the presence of oxygen. Due to their tolerance of oxygen, SRB are able to participate in aerobic wastewater treatment such as the activated sludge process (Johnson *et al*., 1997).

2.4.4.6 Salinity

SRB members that can tolerate low salt have been isolated from various environments. These include *Desulfonatronovibrio hydrogenovorans* (Zhilina *et al*., 1997), *Desulfonatronum lacustre* (Pikuta *et al*., 1998), *Desulfonatronum thiodismutans* (Pikuta *et al*., 2003) and *Desulfonatronum cooperativum* (Zhilina *et al*., 2005). Although it can survive at low-salt, *Desulfonatronovibrio hydrogenovorans* was isolated from Lake Magadi, a hypersaline soda lake in Kenya, by Zhilina *et al*. (1997). Also, *Desulfovibrio vulgaris* Hildenborough, which also possesses osmoprotectants such as ectoine and glycine betaine, was able to survive exposure to excess salt by elongating its cells. These osmoprotectants are used as the primary mechanism by *D. vulgaris* to counter hyperionic stress

(Mukhopadhyay *et al*. 2006). Studies by Foti *et al*. (2007) indicated the presence of *Desulfovibrionales* and *Desulfobacteraceae* at a salt concentration of 475 g l^{-1}. This was unexpected as Oren (1999) formerly hypothesised that complete oxidisers cannot grow at salt concentrations greater than 130 g l^{-1}.

2.4.4.7 Molybdate

Molybdate is a sulphate analogue that enters the cell via the sulphate transport mechanism and compete with the formation of adenosine-5'-phosphosulphate (APS) as indicated in Figure 2.8 (Section 2.4.2). It interferes by preventing the active transportation of sulphate into the cell (Isa and Anderson, 2005).. However, SRB are able to recover from molybdate inhibition once it is removed while methane producing bacteria (MPB) cannot (Isa and Anderson, 2005).

2.4.4.8 Choice of carbon source and electron donor

Both simple and complex carbon sources and electron donors for BSR have been investigated. Simple carbon sources and electron donors include ethanol (Erasmus, 2000; Hansford *et al*., 2007), acetate (Moosa, 2000; Moosa *et al*., 2002; 2005; Moosa and Harrison, 2006; Icgen *et al*., 2007), propionate (van den Brand *et al*., 2014b), butyrate (O'Flaherty *et al*., 1998) and lactate (Oyekola *et al*., 2012) while complex carbon sources and electron donors include sewage sludge (Rose *et al*., 2004; Neba, 2006; Rose, 2013), mushroom compost (Dvorak *et al*., 1992), sea shell material (Masukume *et al*., 2014), hay (Kuyucak *et al*., 2006), municipal compost (Gibert *et al*., 2003), algal extracellular products or algal biomass (Rose *et al*., 1998; Molwantwa *et al*., 2000; Boshoff *et al*., 2004), marine waste extract (Dev *et al*., 2016), sweetmeat waste (Das *et al*., 2015) and grass cuttings (Mulopo; 2016). Oyekola (2008) demostrated that some SRB community members from a BSR culture adapted on lactate differed from a similar SRB mixed consortium adapted on acetate (Icgen *et al*., 2007). Studies have reported different BSR kinetics and microbial community dynamics with different carbon sources and electron donors. However, as highlighted before, comparisons of the reported kinetics and microbial communities between different studies can be a challenge as the initial sulphate concentrations in these studies varies and some studies present calculated percentages of sulphate removal without having presented the initial and final sulphate concentration. While complex carbon sources and electron donors have been explored as a cheap alternative, these can result in slow kinetics (Waybrant *et al.,* 1998) and may not be desirable, demonstrating the need for a cost-effective carbon source that can sustain BSR long term.

2.5 ANAEROBIC DIGESTION AS A MEANS OF PROVIDING ELECTRON DONOR AND CARBON SOURCE FOR BIOLOGICAL SULPHATE REDUCTION

As discussed in Section 2.3.1, the cost of carbon source and electron donor is crucial in BSR, therefore studies have used complex organic materials as cheap alternatives (Dvorak *et al*., 1992; Rose *et al*., 1998; Molwantwa *et al*., 2000; Gibert *et al*., 2003; Boshoff *et al*., 2004; Rose *et al*., 2004; Kuyucak *et al*., 2006; Neba, 2006; Mulopo *et al*., 2011; Rose, 2013; Masukume *et al*., 2014; Das *et al*., 2015; Dev *et al*., 2016; DiLoreto *et al*., 2016; Mulopo; 2016). However, SRB cannot degrade complex organic substrates and therefore rely on the synergistic interaction of hydrolytic bacteria, fermentative bacteria and acetogenic bacteria to breakdown complex organic substrates into volatile fatty acids (VFAs) such as lactate, acetate, propionate and butyrate that are readily available to SRB (Figure 2.10) (Muyzer and Stams, 2008). The reactions involved in the anaerobic digestion of organic matter are demonstrated in Table 2.7 (Reactions 2.18 to 2.29). Methanogens are responsible for catalysing the final step of anaerobic digestion to produce methane and can be classified into two main groups. Firstly, the acetate utilising methanogens called acetoclastic methanogens and secondly the H_2 utilising methanogens called hydrogenotrophic methanogens (Sheoran *et al*., 2010). In excess sulphate conditions, SRB compete with methanogens for acetate and hydrogen (Brysch *et al*., 1987; Van Houten *et al*., 2006; van den Brand *et al*., 2014). Hydrogenotrophic methanogens and acetogenic bacteria easily outcompete SRB for H_2, since SRB have a lower affinity for H_2. The kinetic properties of the interacting microorganisms; the maximum specific growth rate (μmax) and substrate affinity (K_s) define the competition between the interacting microbial organisms (Monod 1942; Contois, 1959; Bailey and Ollis, 1976; Chen and Hashimoto, 1980; Kovarova-Kovar and Egli 1998; Moosa *et al*., 2002; *Weijma et al*., 2002) and are discussed in Section 2.6.

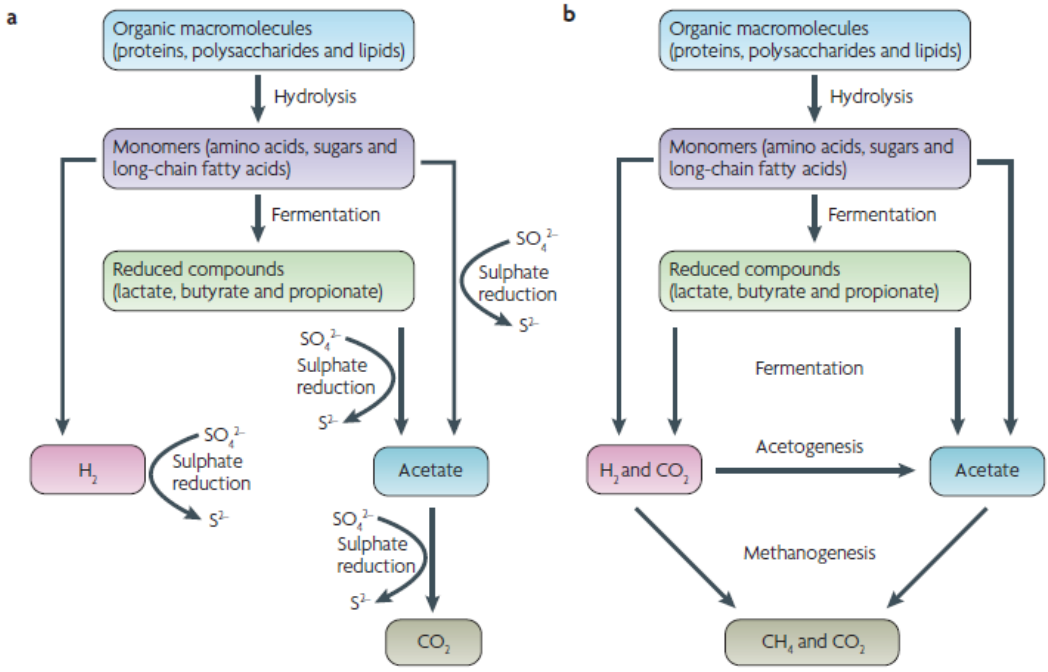


Figure 2.10. The pathway of anaerobic degradation of organic matter by microbial communities in anoxic environments, in the presence (a) and absence (b) of sulphate. Hydrolysis of organic macromolecules is carried out by hydrolytic microorganisms followed by fermentative bacteria. In the presence of sulphate (a), sulphate reducing bacteria utilise the reduced compounds. In the absence of sulphate (b) hydrogen and acetate are utilised by methanogens (Taken from Muyzer and Stams, 2008 with permission).

Table 2.7. General reactions involved in anaerobic reactions (Taken from Muyzer and Stams, 2008).

Reaction	$\Delta G^{o'}$ (kJ/ reaction)	Reactions
Sulphate reducing reactions		
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-151.9	Reaction 2.18
$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-47.6	Reaction 2.19
$Propionate^- + \frac{3}{4}SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + \frac{3}{4}HS^- + \frac{1}{4}H^+$	-37.7	Reaction 2.20
$Butyrate^- + \frac{1}{2}SO_4^{2-} \rightarrow 2Acetate^- + \frac{1}{2}HS^- + \frac{1}{2}H^+$	-27.8	Reaction 2.21
$Lactate^- + \frac{1}{2}SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + \frac{1}{2}HS^-$	-80.2	Reaction 2.22
Acetogenic reactions		
$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+76.1	Reaction 2.23
$Butyrate^- + 2H_2O \rightarrow Acetate^- + H^+ + 2H_2$	+48.3	Reaction 2.24
$Lactate^- + 2H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2H_2$	-4.2	Reaction 2.25
Methanogenic reactions		
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6	Reaction 2.26
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0	Reaction 2.27
Homoacetogenic reactions		
$4H_2 + 2HCO_3^- + H^+ \rightarrow Acetate^- + 4H_2O$	-104.6	Reaction 2.28
$Lactate^- \rightarrow \frac{3}{2}Acetate^- + \frac{1}{2}H^+$	-56.5	Reaction 2.29

2.5.1 Anaerobic digestate as a potential carbon source for AMD treatment

The availability of the carbon source at the site of AMD treatment needs to be considered before choosing it as a suitable carbon and electron donor for BSR (Boshoff *et al*., 2004; Gopal 2005; Liamleam and Annachhatre, 2007). The consideration of microalgae and cyanobacterial species, as a potential cost-effective carbon source for BSR can be attributed to observations that were made by Oswald in 1956. It was observed that the settling of microalgae in high rate oxidation ponds led to active fermentation and the release of toxic substances, such as hydrogen sulphide (H_2S) (Oswald *et al.,* 1963). Decades later, it was reported that degradation of algal biomass resulted in the generation of sulphide in natural systems (Brierley and Brierley, 1983). Nedergaard *et al*. (2002) also reported the generation of sulphide in marine environments resulting from the degradation of macroalgae. Sulphate removal of up to 90.3% was observed when microalgae were used as a carbon source for BSR which was attributed to their high COD content by Boshoff *et al*. (2004). This was higher than sulphate removal efficiencies reported using some single simple carbon sources such as acetate, propionate and butyrate (Bhattacharya *et al.,* 1996; Uberoi and Bhattacharya, 1997). Studies by Inglesby (2011) and Inglesby *et al*. (2015) demonstrated that a cyanobacterial species *Arthrospira platensis*, commonly referred to as Spirulina was a good substrate for anaerobic digestion (AD) due to its high COD content and easily digestible cell walls. Spirulina is a microscopic, spiral, filamentous blue-green cyanobacteria found both in sea and fresh water (Ciferri, 1983). The digestate resulting from the partial digestion of Spirulina (hereafter referred to as anaerobic digestate) had a high COD content and was rich in VFAs. The digestate were found to contain acetate, propionate and butyrate. The VFA concentrations and elemental composition of the digestate are given in Table 2.8 (Inglesby *et al*., 2015).

Table 2.8. Elemental composition of anaerobic digestate originating from an anaerobic digester operated on Spirulina biomass

Constituent	**%**
Elemental composition (%)	
C	41
H	6.1
N	7.7
P	trace
VFAs (mg l^{-1})*	
Acetate	4700
Propionate	1500
Butyrate	2000

*Maximum recorded concentration over a time course of 65 days of a continuously operated anaerobic digester

Spirulina has the potential for on-site cultivation, possibly by using existing water bodies or treated effluent as the basis for the media. This enhances the cost effectiveness of Spirulina production (Boshoff *et al*., 1996; Rose *et al*. 1998). The above observations suggest that anaerobic digestate has the potential to be a cost-effective carbon source for BSR.

2.5.1.1 Acetate as a carbon source for and electron donor for BSR

SRB can utilise acetate according to Reaction 2.19 in Table 2.7 (Omil *et al.*, 1998; Oude Elferink *et al.* 1999). On the other hand, not all SRB can utilise acetate (Table 2.6 in Section 2.4.3). Although *Desulfovibrio* species generally cannot utilise acetate, Phelps *et al.* (1985) demonstrated that *Desulfovibrio vulgaris* can grow on acetate in a co-culture with *Methanosarcina barkeri*. Some SRB species such as *Desulfobacca acetoxidans* specialise its growth on acetate (Omil *et al.*, 1998; Oude Elferink *et al.* 1999).

$$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \qquad \text{Reaction 2.19}$$

$\Delta G^{o'}$ = -47.6 kJ/ reaction

Methanogens can compete with SRB for acetate as indicated by Reaction 2.27 (Rinzema *et al.*, 1988; Muyzer and Stams, 2008). However, SRB have a thermodynamic advantage over methanogens and acetogens as indicated by the standard free energy change of the two acetate oxidation reactions (Rinzema *et al.*, 1988; Muyzer and Stams, 2008).

$$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \text{Reaction 2.27}$$

$\Delta G^{o'}$ (kJ/ reaction) = -31.0

The concentration of sulphate plays a crucial role in the competition between SRB and methanogens (Mccartney and Oleszkiewicz, 1993; Mizuno *et al.*, 1994; Oude Elferink *et al.*, 1994; Omil *et al.*, 1998; Van Houten *et al.*, 2006). Studies by Omil *et al.* (1998) indicated that in long-term operations with excess sulphate in a UASB reactor, where the COD:SO_4^{2-} ratio was lower than 0.67, SRB became dominant. While reports by Harada *et al.* (1994) revealed that in long term operations, SRB outcompete methanogens in sulphidogenic reactors due to their higher affinity for sulphate and higher substrate removal rate. To date no methanogens have been described that can utilise propionate and butyrate for growth.

Acetate oxidation may proceed via the citric acid cycle pathway (Figure 2.11) or the oxidative carbon monoxide dehydrogenase pathway (Figure 2.12) (Möller *et al.*, 1987; Colleran *et al.*, 1995; Postgate and Odom, 2013). In *Desulfobacter* strains, a citric acid cycle is employed which is modified from most anaerobes (Brandis-Heep *et al.*, 1983; Möller *et al.*, 1987). These modifications include: (i) the presence of α-ketoglutarate dehydrogenase that is ferredoxin rather than to NAD-dependent (ii) synthesis of citrate is catalysed by ATP-citrate lyase rather than by citrate synthase and (iii) membrane bound NAD-independent malate dehydrogenase (Möller *et al.*, 1987; Postgate and Odom, 2013). The activation of acetate proceeds via the transfer of CoA groups from succinyl-CoA. ATP-citrate lyase allows for the microorganism to obtain ATP by substrate level phosphorylation through the oxidation of acetate to two CO_2 molecules (Möller *et al.*, 1987; Postgate and Odom, 2013). Not many studies have looked at the metabolism of acetate oxidation. Previous studies such as Schauder *et al.* (1986) and Möller *et al.* 1987 reported that unlike most *Desulfobacter* species, *Desulfobacter acetoxidans* does not possess a citric acid cycle, as evidenced by the absence of the key enzyme, α-ketoglutarate dehydrogenase.

Instead, it uses a non-cyclic pathway which involves the cleavage of the two-carbon unit into a methyl and carbon monoxide moiety, each of which is oxidised independently to CO_2. This pathway is shared with other complete oxidisers including *Desulfobacterium* species, *Desulfococcus* species and *Desulfovibrio baarsi* (Schauder *et al.*, 1986; Möller *et al*. 1987) (Figure 2.11).

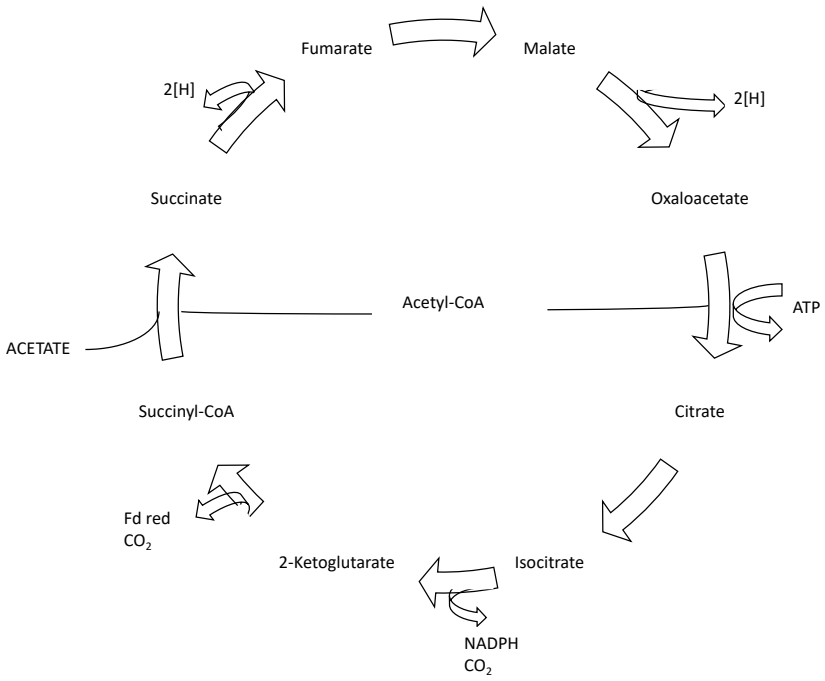


Figure 2.11. Pathway of acetate oxidation in *Desulfobacter postgatei* (Re-drawn from Möller *et al*. 1987).

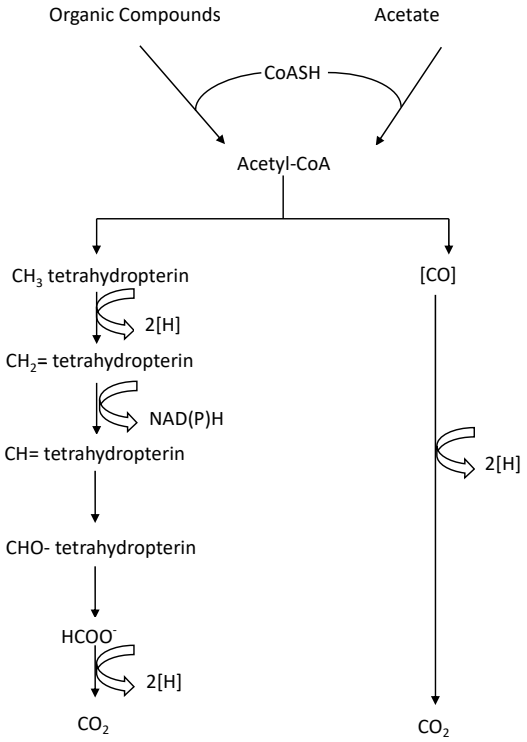


Figure 2.12. Non-cyclic carbon monoxide dehydrogenase pathway for oxidation of acetyl groups by *Desulfotomaculum acetoxidans* (Re-drawn from Colleran *et al*., 1995).

Schauder *et al.* (1986) reported that *DesuIfotomaculum acetoxidans* grew more slowly on acetate than the *Desulfobacter* species, with a doubling time of 48 hours. *Desulfobacter postgatei* had a doubling time of 28 hours on acetate (Schauder *et al.*, 1986). Studies by O'Flaherty *et al*. (1998) and Widdel and Pfennig (1981) reported also reported that *D. postgatei* had greater affinity for acetate than*D. acetoxidans*. Maximum specific growth rates (μ_{max}) and half-saturation constant (K_S) of 0.93 d^{-1} and 20 mg l^{-1} for *D. postgatei* was reported and *D. acetoxidans* was shown to have μ_{max} and K_S of 1.5 d^{-1} and 40 mg l^{-1} (O'Flaherty *et al*., 1998). Studies by Colleran *et al*. (1995) and Widdel (1988) reported specific growth rates in the range of 0.058 and 0.063 h^{-1} for *Desulfotomaculum* genera on acetate. These observation were in agreement with studies by Icgen et al. (2007) who observed more than 3 x 10^6cell/ ml of *Desulfotomaculum* species on acetate at feed sulphate of 2.5 g l^{-1}. Widdel (1988), however, reported that complete oxidisers such as members of the *Desulfobacterium, Desulfosarcina, Desulfonema* and *Desulfococcus* genera, oxidised acetate at much slower rates and sometimes even show no substantial formation of cell mass. As highlighted before, a comparison of microbial growth and microbial communities in different studies can be a challenge as the initial concentrations, pH, temperature, type of reactor, COD:SO_4^{2-} and the mixed SRB inoculum in these studies may vary. Therefore, the elucidation of reactor parameters and the mixed SRB consortium is crucial.

2.5.1.2 Propionate as a carbon source and electron donor for biological sulphate reduction

Propionate is a key intermediate produced during the anaerobic digestion of complex molecules (Gibson, 1990a; Colleran *et al*., 1995; Muyzer and Stams, 2008; Inglesby, 2011; Mulopo *et al*., 2011; Postgate and Odom ,2013). SRB can utilise propionate, either completely to CO_2 or incompletely to acetate (Table 2.6 in Section 2.4.3). Incomplete oxidisers are *Desulfobotulus, Desulfocella, Desulforegula, Desulfobulbus, Desulfofustis, Desulforhopalus* and *Desulfotalea* species. Complete oxidisers include *Desulfothermus, Desulfobacterium, Desulfococcus, Desulfonema, Desulfosarcina, Desulfatibacillum* and *Desulfarculus* (Table 2.6), although most of these species grow slowly when propionate is provided as sole carbon source and electron donor (Hansen, 1993). *Syntrophobacter* species can oxidise propionate to acetate in co-culture with methanogens, while *Desulfobulbus* species cannot. *Desulfobulbus* species can utilise propionate, either completely to CO_2 or incompletely to acetate via a randomising pathway which involves transcarboxylation of propionyl-CoA, using oxaloacetate as donor to methylmalonyl CoA followed by isomerisation to succinyl CoA (Figure 2.13) (Stams *et al*., 1984; Widdel 1988; Gibson 1990a; Colleran *et al*. 1995). Several pathways that occur when an anaerobic system is fed with propionate and sulphate are described in Figure 2.14, and thermodynamic analysis by Maillacheruvu *et al*. (1993) concluded that incomplete oxidation of propionate indicated by Reaction 2.20 was the preferred pathway by SRB (Maillacheruvu *et al*., 1993; Van Wageningen *et al*., 2006; Muyzer and Stams, 2008).

$$Propionate^- + \frac{3}{4}SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + \frac{3}{4}HS^- + \frac{1}{4}H^+$$ Reaction 2.20

$\Delta G^{o'}$ = -37.7 kJ/ reaction

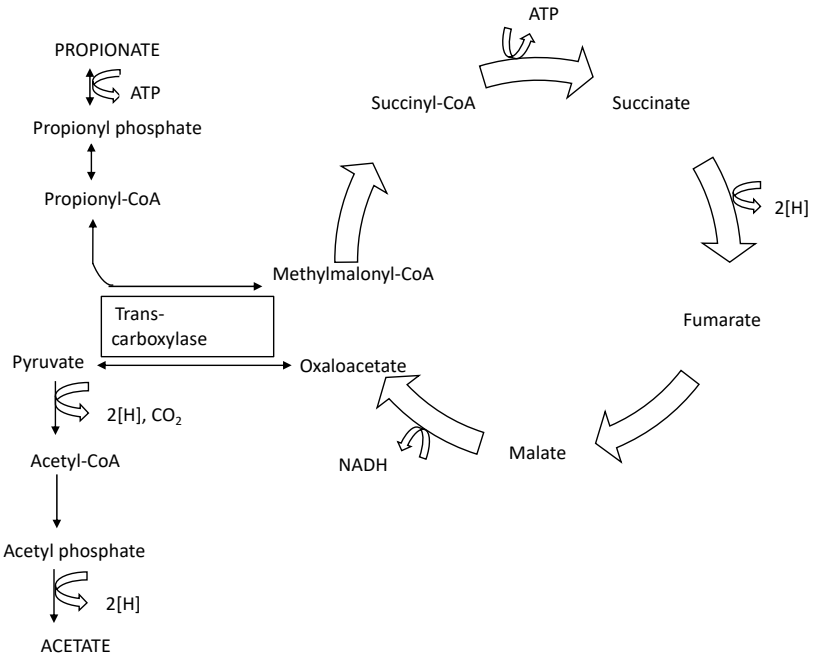


Figure 2.13. Pathway for the incomplete oxidation of propionate by *Desulfobulbus propionicus* (Re-drawn from Stams *et al*., 1984; Colleran *et al*. 1995).

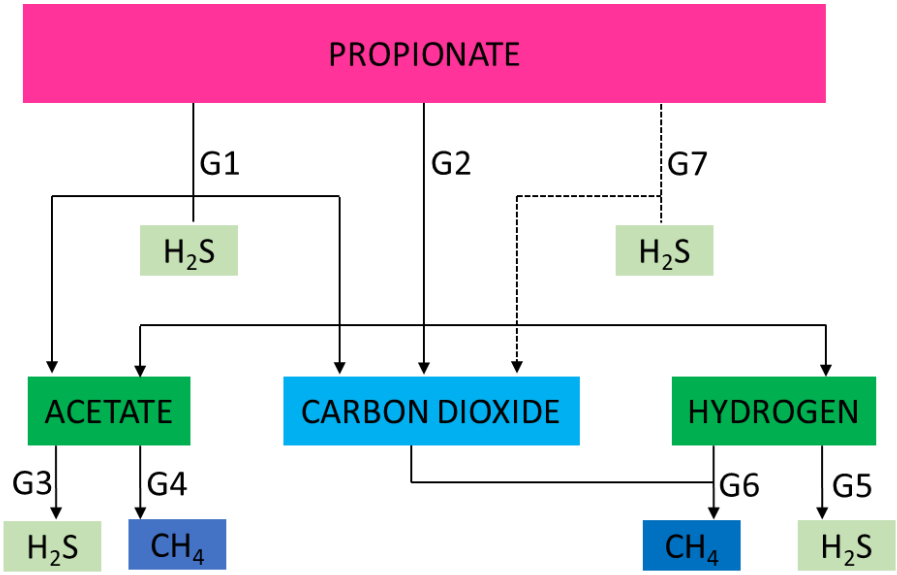


Figure 2.14. Propionate utilising pathway indicating incomplete propionate oxidising SRB (G1), propionate fermenting bacteria (G2), acetate utilising SRB (G3), acetate utilising methanogens (G4), hydrogenotrophic SRB (G5), Hydrogenotrophic methanogens (G6) and complete propionate oxidising SRB (G7) (Re-drawn from Maillacheruvu and Parkin, 1996).

In a sulphidogenic reactor when both acetate and propionate are present in excess, SRB will outcompete methanogens as SRB are capable of oxidising both substrates (Laanbroek *et al*., 1984; Kovarova-Kovar

and Egli; 1998). Oxidation of propionate by SRB is more efficient at high sulphate concentrations, and only at sulphate limiting conditions will syntrophic propionate oxidizers outcompete propionate degrading SBB (Visser *et al*., 1993; Liamleam and Annachhatre, 2007; van den Brand *et al*. 2014). Studies by Rinzema *et al*. (1988) concluded that propionate breakdown was the rate limiting step when methanogenesis was inhibited by H_2S. Under sulphate limiting conditions, SRB preferentially use hydrogen, lactate and ethanol as substrates, but not propionate and acetate (Muyzer and Stams, 2008).

2.5.1.3 Butyrate as a carbon source and electron donor

In the presence of sulphate as an electron acceptor, some SRB can oxidise butyrate completely to CO_2 or incompletely to acetate (Rabus *et al*., 2006; Muyzer and Stams, 2008). The incomplete oxidation of butyrate to acetate by SRB in the presence of sulphate is indicated by Reaction 2.21.

$$Butyrate^- + \frac{1}{2}SO_4^{2-} \rightarrow 2Acetate^- + \frac{1}{2}HS^- + \frac{1}{2}H^+ \qquad \text{Reaction 2.21}$$

$\Delta G^{o'}$ = -27.8 kJ/ reaction

SRB that can utilise butyrate belong to the families *Desulfobacteraceae*, *Desulfohalobiaceae* and *Syntrophobacteraceae* in the class *Deltaproteobacteria* or to the *genus Desulfotomaculum* in the phylum *Firmicutes* (Table 2.6). The oxidation of butyrate is thermodynamically unfavourable in anaerobic conditions. Generally, butyrate is degraded under anaerobic conditions by syntrophic interactions between H_2-utilizing methanogens and H_2-producing acetogenic bacteria (Stams, 1994; Suzuki *et al.*, 2010). Like propionate oxidising SRB, butyrate oxidising SRB grow much faster than syntrophic propionate and butyrate degrading methanogenic or other sulphate reducing communities, which gives these SRB a competitive advantage (Muyzer and Stams, 2008). However, unlike syntrophic propionate oxidisers, syntrophic butyrate oxidisers can compete with butyrate oxidising SRB for the available butyrate, even in excess of sulphate conditions (Liamleam and Annachhatre, 2007). A study by Schmidt *et al*. (2016) reported that in an anaerobic peat, unclassified *Bacteroidetes* and unclassified *Fibrobacteres* may be involved in the oxidation of propionate while *Telmatospirillum*-related species may be involved in the syntrophic oxidation of butyrate. The researchers observed that there was a transient accumulation of acetate in ethanol and butyrate treatments but not in propionate treatments. This was attributed to the thermodynamic constraints as the delta Gibbs free energy (ΔGs) created by the syntrophic oxidation of butyrate and ethanol are more favourable than the ΔGs for the syntrophic oxidation of propionate.

2.5.2 Carbon source and electron donor amendment and effect on SRB growth

To date, no apparent correlation between the presence of specific SRB genera and the carbon source and electron donor used for SRB growth has been identified (Hao *et al*., 2014; Table 2.6). Most SRB

preferentially oxidise acetate, propionate and butyrate as indicated by Table 2.6. At low sulphate concentrations, SRB will compete with each other for the available organic carbon source and electron donor (Muyzer and Stams, 2008). Experiments done by Laanbroek *et al*. (1984) indicated that, at sulphate limiting conditions, *Desulfovibrio* species had the highest affinity for sulphate followed by *Desulfobulbus* species and *Desulfobacter* species, which suggested that at these conditions hydrogen and lactate are used as carbon source and electron donor for metabolism and not acetate, butyrate and propionate (Muyzer and Stams, 2008). Laanbroek *et al*. (1984) also ranked the affinity of SRB for reduced carbon source and electron donor in the order of H_2 > propionate > other organic electron donors. A *Desulfovibrio* species was demonstrated to grow on lactate in the absence of sulphate, and in the presence of a methanogen (Bryant *et al*., 1977). This suggested that this organism produced H_2 that is used by the methanogen, acting as an alternative electron sink in the absence of sulphate (Bryant *et al*., 1977; Muyzer and Stams, 2008). These observations may suggest that in sulphate depleted environments, SRB may be metabolically active by living in association with methanogens instead of reducing sulphate (Bryant *et al*., 1977; Plugge *et al*. 2011). Stolyar *et al*. (2007) also successfully co-cultured *Desulfovibrio vulgari*s with a hydrogen utilising methanogen, *Methanococcus maripaludis,* on lactate in the absence of sulphate. In the current literature, studies have identified SRB communities in reactors supplemented with either acetate, propionate or butyrate but few studies have examined how BSR microbial communities are influenced by a mixture of acetate, propionate and butyrate. Although the current literature has not made an apparent connection, Table 2.9 suggests that certain SRB will be favoured by the electron donor provided. Understanding how these mixed VFAs may affect the dynamics of SRB communities and BSR kinetics is important for decision making on managing a plant using a mixed microbial community when anaerobic digestate or similar carbon sources and electrons donors are used.

Table 2.9. SRB genera identified within sulphate reducing bioreactors fed with either acetate, propionate, butyrate or a combination of either VFA as carbon source and electron donor

SRB	Carbon source and electron donor					References
	Acetate	Propionate	Butyrate	Acetate + Propionate + Butyrate	Acetate + Propionate	
Desulfonema	√	√	√			Brandt *et al*. (1999); Callbeck et al. (2013); van Houten *et al*. (2006); Icgen *et al*. (2007); Imhoff-Widdel *et al*. (1983), O'Flaherty *et al*. (1999); Roest (2007); Stuckle *et al*. (1983)
Desulfosarcina	√		√			Suzuki *et a*l. (2007a Arendsen (1993), Oyekola (2008), Watanabe *et al*. (2017)
Desulfococcus	√	√				O'Flaherty *et al*. (1999); Roest (2007); Stuckle *et al*. (1983)
Desulfomicrobium	√					O'Flaherty *et al*. (1999); Roest (2007); Stuckle *et al*. (1983)
Desulfobacter	√		√	√		Postgate and Campbell (1966); Laanbroek *et al*. (1984); Colleran *et al*. (1995); Widdel (1988); Callbeck *et al*. (2013); O'Flaherty *et al.* (1999)
Desulfotomaculum	√		√			Postgate and Campbell (1966); Laanbroek *et al*. (1984); Colleran *et al*. (1995); Widdel (1988); Callbeck *et al*. (2013); O'Flaherty *et al*. (1999)
Desulfovibrio			√			Laanbroek *et al*. (1984); Callbeck *et al*. (2013)
Desulfobacterium	√	√	√			Widdel and Pfennig (1981); Suzuki *et al.* (2008)
Desulfobulbus	√	√				Widdel and Pfennig (1981); Suzuki *et al.* (2008)
Desulfobotulus				√	√	Visser *et al*. (1993); Icgen *et al*. (2007); van den Brand *et al*. (2014b)
Desulfotalea						Laanbroek *et al*. (1984); Callbeck *et al*. (2013)
Desulfofustis			√		√	Finster *et al.*(1994); Oude Elferink *et al*. (1994; 1999); Omil *et al*. (1998); Callbeck *et al*. (2013); An *et al*. (2015)

Table 2.9 (continues).

SRB	Carbon source and electron donor				References
	Acetate	Butyrate	Acetate + Propionate + Butyrate	Acetate + Propionate	
Desulfuromonas[b]	√			√	Finster *et al.*(1994); Oude Elferink *et al*. (1994; 1999); Omil *et al.* (1998); Callbeck *et al*. Finster *et al.*(1994); Oude Elferink *et al*. (1994; 1999); Omil *et al.* (1998); Callbeck *et al*. (2013); An *et al*. (2015); An *et al*. (2015)
Desulfobacca acetoxidans	√				Grigoryan *et al*. (2008)
Desulforhabdus amnigenus	√	√	√		Oude Elferink *et al*. (1994; 1999); Omil *et al.* (1998)
***Desulfohalobiaceae* spp**			√		Oude Elferink *et al*. (1994)

[b]Sulphur reducing bacteria

2.6 BIOKINETICS OF BIOLOGICAL SULPHATE REDUCTION

2.6.1 Microbial growth kinetics models

Models for the prediction of microbial growth in response to specific carbon sources and electron donors can be useful when deciding on operating conditions for a BSR process when a mixed VFA stream is supplied. The relationship between cell growth and a single substrate can be described using the empirical Monod kinetics model (Equation 2.1), (Monod 1942). The model relates the specific growth rate to the concentration of the limiting substrate and the specific growth rate (Monod 1942; Kovarova-Kovar and, Egli 1998);

$$\mu = \frac{\mu_{max} S}{K_S + S} \qquad \text{Equation 2.1}$$

where μ and μ_{max} are specific and maximum specific growth rates (h^{-1}) of the microorganism respectively; S and K_S are the concentration and half-saturation constant of the limiting substrate. At high S, μ becomes independent of the substrate concentration, which results in zero-order kinetics. At low limiting substrate concentration, μ is directly proportional to S, resulting in first-order reaction kinetics. Therefore, the rate of microbial growth is dependent on the concentration of substrates.

Under conditions where the specific growth rate (μ) is dependent of two limiting substrates, Equation 2.2 can be used to describe this phenomenon,

$$\mu = \mu_{max} \frac{S_1}{K_1 + S_1} \frac{S_2}{K_2 + S_2} \qquad \text{Equation 2.2}$$

where K_1 and K_2 are half saturation constants for the two limiting substrates and where S_1 and S_2 are concentrations of the limiting substrates, respectively (Bailey and Ollis, 1976). The Contois model (Reaction 2.32) (Contois, 1959) and Chen and Hashimoto model (Equation 2.4) (Chen and Hashimoto, 1980), have also been used to describe specific microbial growth rates as a function of residual substrate concentration at both high feed substrate and biomass concentrations;

$$\mu = \frac{\mu_{max} S}{K_S'' X + S} \qquad \text{Equation 2.3}$$

$$\mu = \frac{\mu_{max} S}{K_s S_0 + (1 - K_S) S} \qquad \text{Equation 2.4}$$

where S_o is the initial substrate concentration (mg l^{-1}) and X is the biomass concentration (mg l^{-1}).

Studies by Moosa *et al*. (2002) on BSR using acetate as carbon source and electron donor revealed the dependence of the K_S term on the sulphate concentration in the feed, which resulted in the modification of the Contois model and thereby including the feed sulphate concentration (S_0) in the model (Equation 2.3), resulting in Equation 2.5

$$\mu = \frac{\mu_{max} S}{K_S' S_0 X + S}$$ Equation 2.5

2.6.2 Microbial energetics

The energy obtained from microbial substrate utilisation in the absence of extracellular product formation is used for two main purposes. Firstly, the maintenance energy is utilised for functions essential for the cell's integrity. These non-growth related functions are associated with processes such as internal pH regulation, regulation of cell membrane potential and osmotic pressure, cell mobility and material turnover. Secondly, energy is used for cell growth (Pirt, 1965). The Pirt equation is used to describe the biomass yield. From this equation, it is observed that the biomass yield is dependent on the specific growth rate and the maintenance coefficient under substrate-limited conditions (Equation 2.6) (Pirt, 1965).

$$\frac{1}{Y_{app}} = \frac{1}{Y_{max}} + \frac{m_s}{\mu}$$ Equation 2.6

where Y_{app} (g $_{biomass}$ g^{-1} $_{substrate\ consumed}$) is the observed growth yield, Y_{max} (g $_{biomass}$ g $^{-1}$ $_{substrate\ consumed}$) is the maximum theoretical yield equivalent to the stoichiometric growth yield, μ (h^{-1}) is the observed growth rate, and m_s (g $_{substrate\ consumed}$ g $^{-1}$ $_{biomass}$ h$_{-1}$) is the maintenance coefficient. The slower the growth rate, the larger the percentage of the total substrate utilised for maintenance and subsequent decrease in substrate availability for biomass production. The growth yield observed, Y_{app}, describes the total amount of biomass production as a result of total substrate utilisation by both growth and maintenance activities. In contrast, the maximum theoretical growth yield, Y_{max}, accounts for the substrate assimilated directly into cell biomass only (Nielsen *et al*., 2005).

2.6.3 Environmental effects on BSR growth kinetics

Environmental factors, such as temperature, affect microbial metabolism and growth (Section 2.4.4). Therefore the kinetic constants μ_{max}, K_s and Y_{app} which characterise microbial growth for given growth conditions are also dependent on the temperature of the cell environment (Bailey and Ollis 1976; Okabe and Characklis 1992; Okabe *et al*., 1995) or in the case of BSR the temperature of the process reactor. Previously, Moosa *et al*. (2002; 2005) developed a model based on the kinetics of SRB growth on acetate as an electron donor. Based on goodness of fit, the Contois model was the preferred model. The model included the temperature dependence of the reaction rate by considering the Arrhenius equation. The model also expresses the dependence of the sulphate reduction rate on the inlet sulphate concentration, bacterial concentration and residual sulphate concentration (Equation 2.7);

$$r_s = \left[\frac{(0.061)S}{6.52\ x\ 10^{-35}\ e^{198/RT}\ S_0\ X + S} - 8.8\ X\ 10^{11} e^{-78.7/RT}\right] \frac{X}{0.567}$$ Equation 2.7

where r_s is the rate of sulphate reduction (g l^{-1} h^{-1}), R is the universal gas constant (atm 1 $mmol^{-1}$ K^{-1}), S and S_o are the residual and feed sulphate concentrations (g l^{-1}) respectively and T is the absolute temperature (K). Hansford *et al*. (2007) used the kinetic model in Equation 2.6 to develop a kinetic model which incorporated terms for the effects of initial and residual concentrations of sulphate, as well as biomass concentration (Equation 2.8).

$$r_s = \left[\frac{\mu_{max}\,[S]}{K'_s\,[S_0][X] + [S]} - K_d\right]\frac{[X]}{Y} \qquad \text{Equation 2.8}$$

2.7 CHARACTERISATION OF SULPHATE REDUCING BACTERIA IN MIXED MICROBIAL COMMUNITIES

2.7.1 The importance of characterising SRB in mixed consortia

Performance and robustness of a biological wastewater treatment process depends primarily on the microbial activity of microorganisms involved, emphasising the need and importance of understanding microbial community structure and dynamics in these system (Kim *et al*., 2013). Owing to the complexity of microbial community compositions and a lack of methods for enumerating individual microbial communities in AMD treatment plants and sulphidogenic reactors, wastewater treatment systems have been regarded as a black box for decades (Kim *et al*., 2013). Traditional culture dependent methods, based on isolation and cultivation in a laboratory have been limiting, as a vast majority of microorganisms cannot be cultured due to limited knowledge on their growth requirements(Butlin *et al*., 1949; Postgate, 1963; Amann *et al*., 1995; Johnson, 1995; Muyzer *et al*., 1998; Lewis, 2010; Ňancucheo *et al*., 2016). The sulphide produced by SRB, indicated in Figure 2.9 in Section 2.4.4.7, can inhibit the growth of other microorganisms adding another challenge when culturing SRB (Lens *et al*., 1998; Koschorreck, 2008; Ňancucheo *et al*., 2016). In addition, traditional culturing methods are laborious, time consuming, and may not reflect the real *in situ* existing microbial diversity (Fakruddin and Mannan, 2013; Douterelo *et al*., 2014). Therefore, using only traditional culture-based approaches can lead to a distorted view of microorganisms in biological wastewater treatment systems and sulphate-laden waters (Kim *et al*., 2013).

Molecular techniques offer an opportunity to analyse individual microorganisms, microbial groups and community structure and dynamics in complex microbial communities including SRB (Gilbride *et al*., 2006 ; Kim *et al*., 2013). These methods include fluorescent *in situ* hybridisation (FISH) (Ito *et al*., 2002; Icgen et al., 2007; Lücker *et al*,. 2007; Dar *et al., 2008;* Liu *et al*., 2009), qPCR (Wagner *et al*., 1998; Stubner 2004; Geets *et al*., 2006; Steger *et al*., 2011; Probst *et al*., 2013), denaturing gradient gel electrophoresis (DGGE) (Manefield *et al*., 2005; Miletto *et al*., 2007; Dar *et al*., 2007; Dev *et al*., 2016), terminal restriction fragment length polymorphism (T-RFLP) (Perez-Jimenez *et al*., 2005; Sun *et al*.,

2016) cloning (Castro *et al*., 2002; Bahr *et al*., 2005; Miletto *et al*., 2007), amplified ribosomal DNA restriction analysis (ARDRA) (Vaneechoutte *et al.*, 1993; Sklarz *et al*., 2009; Sklarz *et al*., 2009); microarray screening (Loy *et al*., 2002; Groh *et al*., 2005) and metagenomics (Tyson *et al*., 2004; 2005; Denef *et al*., 2010; Mohapatra *et al*., 2011; Kantor *et al*., 2015; 2017; Hessler *et al*., 2018; Vermote *et al*., 2018;). These molecular techniques are based on the identification of molecular markers such as the 16S rRNA gene (discussed in Section 2.7.2.1), and genes related to important biogeochemical functions such as the *dissimilatory sulphite reductase* (*dsr*) gene (*dsrAB*) (discussed in Section 2.7.2.2) (Wagner *et al*., 1998; Friedrich, 2002; Clarridge 2004; Loy *et al*., 2004; Zverlov *et al.*, 2005; Müller *et al*., 2015). Since the 16S rRNA gene is universal in bacteria (Woese, 1987; Kröber *et al*., 2009) it was used in this thesis to assess the relationships among all bacteria; and the analysis of 16S rRNA gene clone library sequences used to provide insight into the phylogenetic structure of the community examined. Because the *dsrAB* is a key gene in sulphate reduction and its pathway is conserved in all cultivated sulphate reducing microorganisms (Milucka *et al*., 2012; Müller *et al*., 2015) it was used in this study to examine the total SRB present within the BSR communities.

2.7.2 Molecular markers used to characterise SRB

2.7.2.1 The 16S rRNA

Ribosomal RNA (rRNA) molecules are important molecular markers and present in most organisms, excluding viruses (Woese, 1987; Durzyńska and Goździcka-Józefiak, 2015). These rRNA molecules are 5S, 23S and the 16S rRNA molecules (Woese 1987; Clarridge 2004). 16S rRNA is encoded by the 16S rRNA gene and is highly conserved amongst species and therefore can be used as a molecular marker for bacteria in various environments (Clarridge, 2004). In the 1960s, Dubnau *et al*. (1965) reported that there was conservation in the 16S rRNA gene sequences of *Bacillus* species. The 16S rRNA gene sequence is about 1,550 bp long and is comprised of variable and conserved regions (Clarridge, 2004). Therefore, universal primers can be designed complementary to the conserved regions to the 16S rRNA (Chen *et al*., 1989). Of the 20 million sequences deposited in GenBank (the largest database of sequences), over 90 000 are of 16S rRNA. Therefore, one can compare 16S rRNA sequences of unknown strains to the previously deposited 16S rRNA sequences (Clarridge, 2004). Pioneering work by Giovannoni *et al*. (1990) on clone library assays targeting the 16S rRNA gene has made it possible for the 16S rRNA gene to be widely used for studying and categorising microorganisms from various environments including wastewater treatment systems (Blackall *et al*., 1998; Pellegrin *et al*., 1999; Gich Batlle *et al*. 2000). 16S rRNA sequences have been used to design FISH probes (Amann *et al*., 1990; Loy *et al*., 2002; Dar *et al*., 2007) and PCR primers (Wagner *et al*., 1998; Stubner 2002; Loy *et al*., 2004; Stubner, 2004; Geets *et al*., 2006) to also identify SRB.

2.7.2.2 The *dsrAB* gene

The dsrAB-type dissimilatory (bi)sulphite reductase is a key enzyme involved in both the reductive and oxidative steps of the biogeochemical sulphur cycle in microorganism. These enzyme contains the α and the β subunits that are encoded by the paralogous genes *dsrA* and *dsrB*, respectively (Dahl *et al*., 1993; Wagner *et al*., 1998; Müller *et al*., 2015). The *dsrAB* gene has remained a specific molecular target for SRB as it is the key enzyme catalysing the last and main energy-generating step during sulphate reduction (Wagner *et al*., 1998; Loy *et al*., 2004; Zverlov *et al*., 2005). This pathway is conserved in all four bacterial phyla: *Proteobacteria*- class *Deltaproteobacteria, Nitrospirae, Firmicutes, Thermodesulfobacteria* and two archaeal phyla: *Euryarchaeota*, and *Crenarchaeota* (Milucka *et al*., 2012; Müller *et al*., 2015). Although the anaerobic syntrophs of the spore-forming *Firmicutes* genera *Pelotomaculum* and *Sporotomaculum* possess and transcribe *dsrAB*, they are incapable of metabolising sulphide, sulphate or organosulphonates. Therefore the physiological role of the *dsrAB* in these organisms is currently unknown (Brauman *et al*. 1998; Imachi *et al*., 2006; Müller *et al*., 2015). Primers targeting the *dsrAB* gene have been designed and published in literature (Suzuki *et al*., 2005; Geets *et al*. 2006; Pester *et al*. 2010a; Steger *et al*. 2011; Lever *et al*. 2013; Müller *et al*., 2015) which has allowed the quantification of SRB in soil (Miletto *et at.*, 2007) and biofilms (Probst *et al*., 2013). However, not many PCR primers have been reported in literature for the quantitative analysis of individual SRB genera and species which makes it challenging to analyse and temporally track specific SRB species in mixed microbial communities. This poses the need for the development of molecular tools, specifically genus and species specific primers, for the elucidation of microbial organisms in sulphate reducing environments.

2.7.3 Molecular tools used for the characterisation of SRB

2.7.3.1 Amplified ribosomal DNA restriction analysis

Amplified ribosomal DNA restriction analysis (ARDRA) was developed by Vaneechoutte *et al*. (1993) as a method to characterise *Mycobacterium* species. ARDRA is a tool commonly used to study microbial diversity that relies on DNA polymorphisms (Sklarz *et al*., 2009). Clones that contain the 16S rRNA gene fragments, obtained by applying either universal or genus-specific primer sets, are amplified and restricted by restriction endonucleases (REs) (Vaneechoutte *et al*., 1993; Sklarz *et al*., 2009). This is followed by separating the resulting fragments on high-density agarose or acrylamide gels. The DNA fragment profiles that emerge are then used either to cluster the community into genotypic groups or for strain typing (Tiedje *et al*., 1999). Studies have used ARDRA to identify species from particular genera in wastewater treatment systems (Blackall *et al*., 1998; Pellegrin *et al*., 1999; Gich Batlle *et al*., 2000) suggesting that this technique can be applied in this thesis to identify unique ribotypes for further analysis. However, careful choice of REs must be considered according to whether bacteria or archaea

are being assessed. ARDRA technique can discriminate among genus, but not always species (Sklarz *et al*., 2009). Restriction enzymes that have been used in ARDRA include *AluI* and *HaeIII* as well as *AluI* and *HhaI* with resulting patterns used to characterise bacteria and archaea, respectively (Sangeetha *et al*., 2016). These restriction enzymes recognise four base pair sequences and will therefore restrict the 16S rRNA gene more frequently than restriction enzymes with longer sequence recognition sequences. ARDRA can be used as a quick assessment of genotypic changes in the environmental community over time, or to compare microbial communities subject to different environmental parameters (Sklarz *et al*., 2009). Careful DNA extraction, PCR and cloning techniques are crucial for the accuracy reflection of a microbial community by ARDRA (Gilbride *et al*., 2006).

2.7.3.2 Fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) combines the visual information given by the use of microscopy and the precision provided by molecular genetics. Individual microbial cells can be visualised and identified within their natural microhabitats (Moter and Göbel, 2000). In 1969, two research groups independently developed *in situ* hybridisation (John *et al*., 1969; Pardue and Gall, 1969). The researchers hybridised radioactively labelled DNA or 28S RNA to cytological preparations of *Xenopus* oocytes and these were detected by microautoradiograpy. This technique offered the advantage of the nucleic acid being examined inside a cell without altering the cell morphology or the integrity of various cell compartments (Moter and Göbel, 2000). Later Giovannoni *et al*. (1988) introduced FISH into bacteriology by using radioactively labelled rRNA-directed oligonucleotide probes for the microscopic detection of bacteria. With time, radioactive labels were steadily superseded and replaced by non-isotopic dyes through the development of fluorescent labels (Landegent *et al*., 1984; Pinkel *et al*., 1988). Fluorescently labelled oligonucleotide probes were first used in 1989 by DeLong to detect single microbial cells (DeLong *et al*., 1989). Typical FISH probes are 5' end-labelled with fluorochrome reporters such as fluorescein (Cy3) or sulfoindocyanine (Cy5) (Hugenholtz *et al*., 2002). These offered the advantage of being safer than radioactive probes providing better resolution and not needing additional detection steps (Moter and Göbel, 2000). Fluorescent probes can be labelled with dyes of different emission wavelength, thus enabling detection of several target sequences, and therefore species, within a single hybridisation step (DeLong *et al*., 1989; Moter and Göbel, 2000).

In FISH, 16S rRNA probes are utilised as they hybridise to the high copy number of RNA in microbial cells (Rogers *et al*., 2000). Theoretically, each ribosome within a bacterial cell, containing one copy 16S is stained by one probe molecule during the hybridisation (Amann *et al*., 1990; Pernthaler *et al*., 2001; Loy *et al*., 2002), therefore allowing for the quantification and identification at the level of populations and even single cells (Amann *et al*., 1995; Dar *et al*., 2008). Total cell counts are conducted using the DAPI stain (4,6-diamidino-2-phenylindole) stain (Zarda *et al*., 1997). Both dead and inactive

cells are included in the DAPI stain. This may result in the overestimation of the total number of living bacteria (Hesham and Alamri, 2012). To some extent, the FISH technique can circumvent this problem. Because FISH labels only bacteria with a certain content of ribosomal RNA only active cells or cells that have recently been active will be enumerated (Bouvier and Del Giorgio, 2003; Hesham and Alamri, 2012). The FISH protocol often involves the staining of cells by DAPI to visualise all organisms and thereafter FISH is performed with the specific probe. FISH has become a powerful molecular tool for the identification of bacteria, including SRB (Loy *et al*., 2005; Icgen *et al*., 2007; Oyekola, 2008) and was chosen in this thesis to visualise specific SRB genera and total eubacteria. However, in order to ensure the correct enumeration and identification, FISH probes must be chosen wisely, and optimised for the specific system being assessed (Hugenholtz *et al*., 2002; Roest 2007)).

2.7.3.3 Quantitative real time PCR

Quantitative real-time PCR (qPCR) allows the quantification of DNA in real time, yielding a quantitative measurement of PCR products accumulated from the specific amplification of selected DNA sequences during the course of the reaction (Gibson *et al*.,1996; Bustin, 2000; Bustin *et al*., 2005; VanGuilder *et al*., 2008). The reactions are carried out in a thermocycler that allows measurement of a fluorescent molecule which intercalates with double stranded PCR products. This direct measurement technique decreases post-processing steps and thus minimises potential experimental error (Riedy *et al*., 1995; Gibson *et al*., 1996). Intercalating fluorescent dyes such as SYBR Green is most commonly used for qPCR. SYBR Green fluoresces upon intercalation into double-stranded DNA. Therefore the fluorescence emitted following the primer-mediated replication of the target sequence during PCR is directly related to the amount of DNA product (Zipper *et al*., 2004). qPCR is highly sensitive, has a wide dynamic range whereby threshold cycles (C_t) values obtained from samples containing hugely different levels of DNA can be compared and is more resistant to non-specific amplification (Gibson *et al*.,1996; Bustin, 2000; Bustin *et al*., 2005). The DNA used must be of high quality free of nucleases for extended storage and must be free from contamination with other DNA as this can result in false positives (Bustin e*t al*., 2005; Bustin *et al.*, 2009). qPCR have been successfully used to quantify different bacterial groups (mostly using primers based on the 16S rRNA gene) and SRB groups (using primers based on the *dsrAB* gene) in rice fields soils (Stubner 2002; Stubner 2004; Liu *et al*., 2009), soda lakes (Foti *et al*., 2007), oil fields (Agrawal and Lal, 2009), low sulphate peat-lands (Pester *et al*., 2010b; Steger *et al*. 2011), sulphidic aquifers (Probst *et al*., 2013), industrial anaerobic biogas digesters (Moestedt *et al*., 2013), mature fine tailings (Sun *et al.*, 2016), marine sediments (Besaury *et al*., 2012) and sulphidogenic reactors (Dar *et al*., 2007; Callbeck *et al*., 2013). Further, qPCR can provide a rapid diagnostic for decision making on managing a plant using a mixed microbial community (Gilbride and Beaudette, 2006; Helbling *et al*., 2012, Kim *et al*., 2013) and was therefore chosen in this thesis.

qPCR primers are vital to the specificity, sensitivity, and efficiency of the reaction (Bustin *et al.*, 2005; Bustin *et al.*, 2009; Müller *et al.*, 2015). The primer-template association and dissociation kinetics, possible formation of secondary structure and the primer template complementarity (Watson-Crick base-pairing) are the most important primer characteristic contribution to a successful amplification (Arnheim and Erlich, 1992; Cha and Thilly, 1993). The stability of the primer-template duplex and the efficiency with which the polymerase extends the primer, are affected by mismatches between primers and template (Kwoks *et al.*, 1990; Arnheim and Erlich, 1992; Bru *et al.*, 2008). These mismatches, irrespective of their location within the primer sequence, will result in a decreased thermal stability in the primer-template duplex which could potentially lead to bias results or even PCR failure (Klein, 2002; Whiley and Sloots 2005). Mismatches located in the '3 end region of a primer have a significantly larger effects on priming efficiency than 5' located mismatches, since 3' end mismatches can disrupt the nearby polymerase active site (Whiley and Sloots 2005; Stadhouders *et al.*, 2010). Therefore, when designing qPCR primers, it is imperative that there are no mismatches located in the 3' end region.

2.7.3.4 Metagenomics

Metagenomics refer to the study of all the genetic information directly extracted from the environmental or process sample has become the gold standard when researchers would like to obtain a holistic view of all microorganism present within that sample. Since the inception of metagenomic approaches, an array of metagenomic DNA-related techniques have been developed. Companies such as Macrogen (Seoul, South Korea) now offer multiple next-generation sequencing services to researchers worldwide.

The general and traditional pipeline for performing metagenomic studies involves the extraction and purification of DNA from environmental samples. These DNA samples are then separated by size and cloned into a vector in order to develop a metagenomic library. The purified metagenomic DNA and or clone libraries, or both, can be sequenced directly. Together with the generation of large amounts of DNA sequence information, the metagenomic library can also be used to identify novel genes by screening for the library clones for specific metabolic functions. This allows us to link the function of microorganisms in the target environment, highlighting the specific role of individual microbial groups in their related processes (Tyson *et al*., 2004; 2005; Mohapatra *et al*., 2011; Kantor *et al*., 2015; 2017).

Metagenomics has been used to successfully elucidate the functions of some microorganisms in AMD (Tyson *et al*, 2004; 2005; Denef *et al*., 2010). The study by Tyson *et al*. (2004) was the first to use the metagenomic sequenced-based approach to elucidate the microbial diversity and ecophysiology of a natural acidophilic biofilm growing on the surface of flowing AMD at an abandoned mine in Iron Mountain California. This was achieved by preparing a metagenomic plasmid library of approximately 3.2 kb and sequencing the library in both directions of the 3.2 kb insert. A total of 76.2 Mbp sequence

was gathered from 103 462 high qualities reads with an average of 737 bp per read. The sequences revealed nearly complete genome sequences for two previously uncultured microorganisms; *Leptospirillum* group II and *Ferroplasma* group II. These were shown to be the most dominant. The researchers assigned the less abundant organisms to two more *Ferroplasma*-related species and one other species of *Leptospirillum*. The presence of the enzymes CO dehydrogenase and formate hydrogen lyase were shown in all the organisms by analysing the open reading frames. This suggested that these microorganisms can fix carbon for their survival in environments with high concentration of heavy metals and extreme acidity (Tyson *et al*., 2004). The *Leptospirillum* III (*Leptospirillum ferrodiazotrophum*) was shown to be the only microorganism that possesses a nitrogen fixation operon (*nif*), indicating the survival of the AMD biofilm depends on this non-dominating bacteria (Tyson *et al*., 2005). Metagenomic analysis of an AMD biofilm community enabled Denef *et al*. (2010) to reconstruct the 12 near-complete archaeal and bacterial genomes from metagenomic sequences obtained.

The time and cost associated with the sequencing has always been the limitation of the use of this technique. When performing whole genome or shotgun metagenomic sequencing we gain an understanding of the metabolic potential of the specific species sequenced or through sequencing of all the genes present in all the species within the sample. However, the lack of robust binning methods for assignment of metagenomic sequence fragments to microbial taxa also remains a challenge (Sedlar *et al*., 2017). It is predicted that advances with the advent of high-throughput DNA sequencing and bioinformatics tools is will help improve the robustness of metagenomics methods (Mohapatra *et al*., 2011; Vermote *et al*., 2018). Amplicon metagenomic sequencing offers a platform where all 16S rRNA gene sequences within samples can be captured through the amplification of a specific variable region (between V3 and V4). Sequencing of this region allows the taxonomic analysis of the species present mostly to the phylum, family or genus level. This is a powerful tool for the characterisation of microbial community dynamics within BSR systems as various process and operational changes are applied. In this thesis, 16S rRNA gene amplicon sequencing was used to validate qPCR and FISH results for a small subset of samples. 16S rRNA gene amplicon sequencing has however extensively been used in numerous reactor studies to investigate changes in the microbial population (Miao *et al*., 2015; Fykse *et al*., 2016; Mosbæk *et al*., 2016; Delforno *et al*., 2017a; Delforno *et al*., 2017b; Hessler *et al*., 2018)

2.8 RESEARCH MOTIVATION

Previous studies within our research centre have used acetate (Moosa, 2000; Moosa *et al*., 2002; 2005; Moosa and Harrison, 2006), ethanol (Erasmus, 2000; Hansford *et al*., 2007) and lactate (Oyekola; 2008; Oyekola *et al*., 2009; 2010; 2012) to demonstrate that biological sulphate reduction (BSR) reaction

kinetics are influenced by feed sulphate concentration, biomass concentration, residence time, residual sulphate concentration and its volumetric loading rate. While acetate does not leave COD or organics behind during BSR, there is high competition for acetate between SRB and methanogens, and methanogens can outcompete SRB (McCartney and Oleszkiewicz, 1993; Bhattacharya *et al*., 1996; Moosa *et al*., 2002; Dar, S.A. *et al*., 2008). Also, some studies reported that low biomass yields of SRB were observed with acetate (Widdel and Hansen, 1992), making it none ideal carbon source and electron donor for BSR at these conditions. Lactate is a more favourable substrate for BSR, Oyekola *et al*. (2012) demonstrated that there is competition for substrate between lactate oxidisers (LO) and lactate fermenters (LF), with lactate oxidisers outcompeting fermenters for lactate at low lactate concentrations ($\leqslant$5 g l^{-1}) and high sulfide concentrations (0.5 g l^{-1}). Also, lactate is an expensive carbon source for use in long term processes. Ethanol is a more economically favourable carbon source and electron donor for BSR than lactate (Nagpal, *et al*., 2000) and can support the growth of a diverse SRB community (Kaksonen *et al*., 2004; 2006), the BSR treatment costs are elevated by operational costs due to transportation costs (Dijkman *et al*., 1999; Gopal, 2005; Kaksonen and Puhakka, 2007). An even more attractive carbon source and electron donor for BSR would be a suitable waste stream. Although certain industrial effluents may contain COD or organic matter, they may not be available in isolated or remote areas or in sufficient quantities or concentrations to sustain a BSR process for effective AMD treatment (Whittington-Jones *et al*., 2002). This would therefore require the addition of external carbon sources and electron donors such as formate or lactate to support sufficient BSR (Alvarado, 2016) which would increase operational costs.

The above reasons demonstrate the need for a cost-effective carbon source with continuous availability which does not rely on the industrial activity of the region, and can sustain efficient BSR. Anaerobic digestate has a high COD content, primarily due to the presence of VFAs such as acetate, propionate and butyrate (Inglesby, 2011; Inglesby *et al*., 2015). Hence anaerobic digestate is proposed as a cost-effective carbon source and electron donor for BSR.

As indicated in Section 2.4.3 (Table 2.6) and Section 2.5.2 (Table 2.9), the type of carbon source (simple or complex) influences the composition of microbial communities in mixed culture operations (and in). The performance and robustness of a reactor rely on the interactions and activities of SRB with other present microbial communities in the systems (Pol *et al*., 1998). Quantitative analyses of microbial community dynamics across conditions have not been performed for reactors receiving acetate, ethanol or lactate as a carbon source and electron donor due to the lack of affordable molecular tools. Although metagenomic approaches, such as 16S rRNA gene amplicon sequencing, can now be applied to generate large microbial community datasets, molecular tools such as FISH, ARDRA and qPCR are important in the rapid assessment of mixed cultures. In wastewater management situations where a plant has collapsed, qPCR can be used as a rapid diagnostic tool to allow quick decisions to be made based on

rapid quantification (Gilbride and Beaudette, 2006; Helbling *et al*., 2012, Kim *et al*., 2013). Although microbial community dynamics are critical in understanding and optimising BSR systems, there is limited information in the currently available literature on how different microbial communities and SRB genera and species respond to changes in the sulphate loading as a result of carbon source and electron donor provided, making it difficult to link the relationship between the microbial community structure and the performance of BSR.

Based on the literature reviewed in the previous sections, the limitations and gaps in knowledge of the current body of literature, and requirement for its extensions are as follows:

- Anaerobic digestate, based on its composition, has the potential to be a cost-effective carbon source but has not been investigated in terms of its efficacy to drive BSR for AMD remediation
- The study of the microbial kinetics when a cost-effective carbon source, that can sustain BSR long term, is utilised for BSR
- Studies that compare microbial kinetics and microbial community structure across carbon sources are limited
- There is lack of genus specific qPCR primers for the quantitative analysis of SRB in mixed consortia available in the current literature
- There is limited knowledge on the use of combined VFAs (acetate, propionate and butyrate) as a carbon source for BSR and the resulting microbial community dynamics when operating parameters such as residence time and sulphate loading are changed
- Few studies linking microbial community and performance of sulphate reducers across carbon sources and electron donors are available

Research statement

An understanding of the microbial community structure and dynamics is critical in understanding the biological sulphate reducing (BSR) system. This can inform possible optimisation and assist in avoiding system failure. While most studies on microbial community dynamics of SRB to date have been qualitative, molecular tools are developing to allow quantitative analysis of SRB. Hence opportunity exists to link microbial community dynamics and performance of sulphate reducers across carbon sources and electron donors. The aim of this thesis was to evaluate anaerobic digestate of blue-green algae (*Arthrospira platensis*) as a sole carbon source and electron donor for BSR relative to lactate, to study the microbial community structure and dynamics as a function of the carbon source and electron donor, operating environment and to relate this to process performance. This thesis also aimed to develop quantitative or semi-quantitative molecular tools to characterise the mixed microbial communities for BSR. The system was sustained in a CSTR receiving anaerobic digestate as a complex carbon source which allowed rapid response to changes in sulphate loading and residence time. The

results obtained were compared to those from a previous BSR study performed using the same reactor configuration with lactate as carbon source and electron donor. Furthermore, the thesis aimed to investigate the relationship between the BSR process and the SRB community dynamics as a result of carbon source and electron donor. The contribution from this study will build and extend the research initiative on BSR and AMD treatment at the Department of Chemical Engineering, UCT, centred to date on BSR kinetic studies using acetate, ethanol and lactate, and associated microbial community dynamics. This thesis further offered the opportunity to add to the understanding of microbial ecology in sulphate reducing systems allowing recommendations to be made for the optimisation of industrial and remediation processes through the manipulation of the community structure.

2.8.1 Research hypotheses

- Using the 16S rRNA gene sequences from a clone library generated from metagenomic DNA obtained from a lactate fed SRB reactor, genus specific qPCR primers can be designed and modified from existing primers to allow the investigation of microbial community dynamics within an anaerobic digestate operated SRB reactor
- Anaerobic digestate is expected to contain a mixture of VFAs suitable for use as electron donor for BSR by a mixed SRB consortium, therefore the kinetics observed with anaerobic digestate will match the kinetics observed when pure VFAs (acetate or lactate) or ethanol is used as carbon source and electron donor for BSR
- Anaerobic digestate is a biologically derived mixture of VFAs (acetate, propionate and butyrate) therefore it will support a diverse community which will results in a robust BSR process

2.8.2 Research objectives

Based on the research hypotheses described above and previous research reviewed in this chapter, the following objectives were developed for this thesis:

- To develop and optimise molecular tools (FISH, ARDRA and qPCR) for quantitative and qualitative analysis of the mixed microbial community in a CSTR whereby lactate or anaerobic digestate is served as carbon sources and electron donors for BSR
- To use the optimised molecular tools to determine microbial communities across carbon sources and electron donor as a factor of sulphate concentration and residence time
- To evaluate the performance of the reactor by characterising the kinetics of a mixed SRB community on complex organic carbon source (anaerobic digestate) as a factor of sulphate loading and residence time

- To use mathematical models previously used for acetate, ethanol and lactate to describe the effect of feed sulphate concentration on the biological sulphate reduction kinetics when anaerobic digestate is used as a carbon source and electron donor
- To correlate observations with respect to microbial community structure and dynamics with those centred on process performance to inform the structure-function relationship in the mixed microbial community in BSR

CHAPTER 3
MATERIALS AND METHODS

3.1 GENERAL INFORMATION AND APPROACH

This chapter presents the detailed experimental material and methodologies employed to achieve the objectives outlined in Chapter 2. All chemical and media components used in this study were of analytical grade. For speciality chemicals manufacturer details are given in specific sections. Prepared solutions and media were sterilised by autoclaving at 120°C and 103 kPa for 20 minutes when required. Figure 3.1 illustrates the approach taken in this thesis, highlighting the key components.

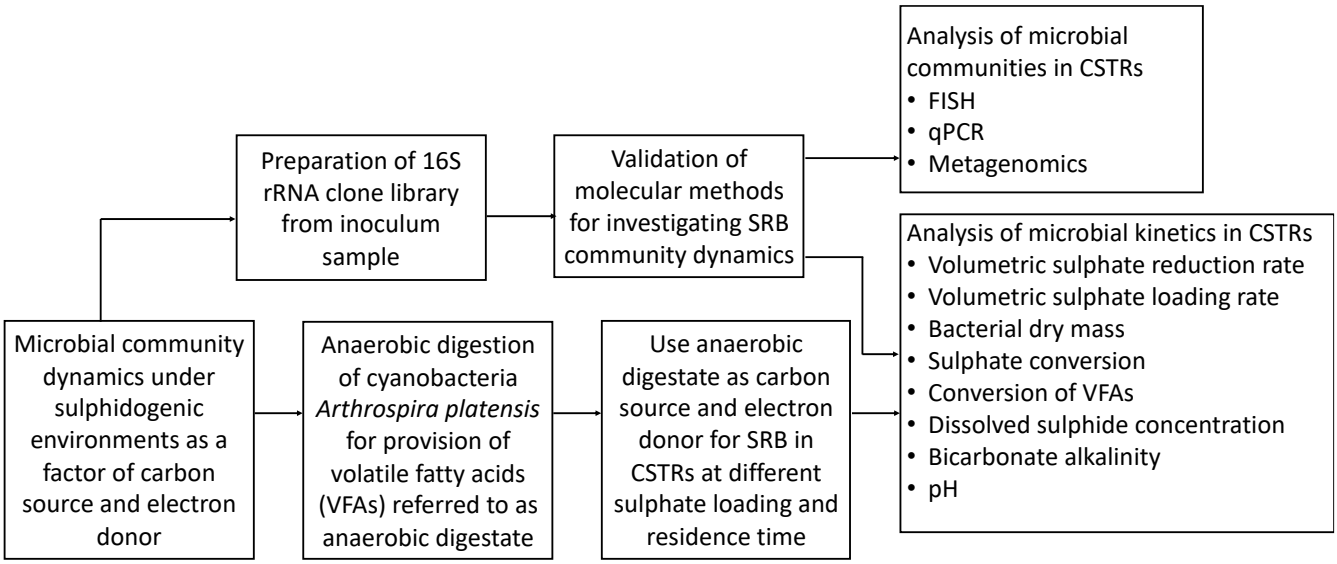


Figure 3.1. Schematic diagram illustrating approach and methods undertaken in this study.

3.2 CYANOBACTERIAL CULTURE, HARVESTING AND PARTIAL ANAEROBIC DIGESTION

Stock cultures of a cyanobacterial species from the *Arthrospira* genus, commonly referred to as Spirulina, were originally isolated from an abandoned wastewater treatment pond at Western Tanning Company outside Wellington, South Africa. The stock culture is maintained at the Centre of Bioprocessing Engineering Research (CeBER) in the Department of Chemical Engineering at the University of Cape Town (UCT). Stock cultures were cultivated in 200 mL bottles containing 150 mL culture in Zarrouk's medium (Zarrouk, 1966). Cultures were aerated with humidified air supplied after filtration through a 0.22 µm syringe filter. Cultures were illuminated by fluorescent lamps (approx. 120 µmol photons.m^{-2}.s^{-1}). The Zarrouk's media consisted of 18 g l^{-1} $NaHCO_3$, 2.5 g^{-1} $NaNO_3$, 0.5 g l^{-1} K_2HPO_4, 1 g l^{-1} K_2SO_4, 0.04 g l^{-1} $CaCl_2.2H_2O$, 1 g l^{-1} NaCl, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.01 g l^{-1} $FeSO_4.7H_2O$ and 0.08 g l^{-1} EDTA), metal solution A5 (2.86 g l^{-1} H_3BO_3, 1.81 g l^{-1} $MnCl_2.4H_2O$, 0.22 g l^{-1}

$ZnSO_4.7H_2O$, 0.08 g l^{-1} $CuSO_4.5H_2O$ and 0.0124 g l^{-1} Na_2MoO_4) at a concentration of 1 ml l^{-1} and metal solution B6 (56.6 mg l^{-1} K_2CrO_7, 47.8 mg l^{-1} $NiSO_4.7H_2O$ and 4.2 mg l^{-1} $CoSO_4.7H_2O$) at a concentration of 1 ml l^{-1}. The first round of the cyanobacterial biomass used in this study was grown in hanging plastic bag reactors (8 litre) illuminated by fluorescent lamps (providing approx. 120 µmol photons.m^{-2}.s^{-1}) in Zarrouk's medium. Further upscaling was required to generate the required cyanobacterial biomass for digestion, thus cultures were cultivated in a covered indoor raceway pond (80 litre), or harvested from an outdoor 50 000 litre raceway pond operated at Biodelta (Simondim, Cape Town, South Africa) as described by Mogale (2006). Harvesting was carried out every second day, or as required, using a nylon cloth with pore size 100 µm. The collected biomass was removed and partially digested using an approx. 2 litre upright anaerobic digester shown in Figure 3.2 and described by Inglesby (2011). The resulting effluent, hereafter referred to as anaerobic digestate, was collected and the digestate was filtered through a nylon cloth (pore size 100 µm) to remove large particulates and undigested cyanobacterial biomass. The resulting filtrate was transferred to 500 ml Beckman centrifuge tubes and centrifuged at 6026 g force for 10 minutes to remove bacteria and any further undigested cyanobacterial biomass (Beckman Coulter Inc., California, USA). The pH, redox potential and chemical oxygen demand (COD) of the resulting supernatant was then analysed.

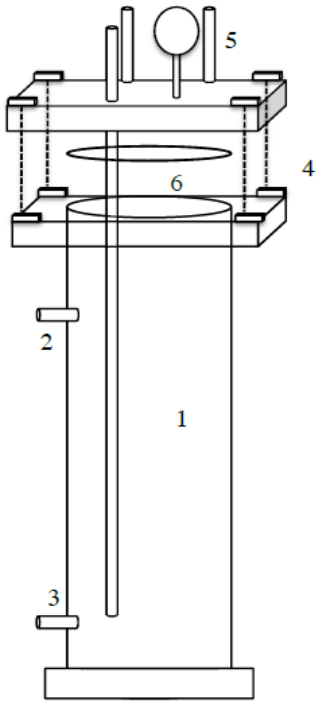


Figure 3.2. Schematic representation of the 2 litre upright semi-continuous anaerobic digestion unit used for the generation of digestate from cyanobacterial biomass for use in the biological sulphate reduction (BSR) system. On the figure (1) is the 110 mm thick Perspex tube fitted with (2) an overflow 510 mm from base and (3) continuous feed port. (4) indicates the Perspex tube, (5) is the lid and (6) 105 mm outer diameter silicone o-ring used to seal the lid (taken from Inglesby, 2011).

3.3 SRB CULTURE AND GROWTH MEDIA

The SRB culture used in the study was originally obtained from the Department of Microbiology, Biochemistry and Biotechnology at Rhodes University, South Africa (Prof. John Duncan's laboratory) and has been maintained at UCT since 2001. The culture was previously adapted for growth on lactate as electron donor by Oyekola (2008). The effluent from this CSTR at a feed sulphate concentration of 1.0 g l^{-1} was used as inoculum for the current study. For this study, modified Postgate B medium which contained 0.5 g KH_2PO_4; 1.0 g NH_4Cl; 2.0 g $MgSO_4.7H_2O$; 1.0 g Na_2SO_4 and 0.3 g sodium citrate (Postgate, 1984) was supplemented with 1.0 g l^{-1} sulphate ions (from either sodium sulphate or magnesium sulphate) and anaerobic digestate as the major carbon source and electron donor. Prior to the cultivation of the SRB in continuous reactors, 3.2 g l^{-1} bromo-ethane-sulphonicacid (BESA) was added to the batch flow culture in order to inhibit methanogenic activity (Visser, 1995).

3.4 SRB CONTINUOUS STIRRED TANK REACTORS (CSTRS)

The three bioreactors used in this study were initiated in batch culture using a 10% (v/v) inoculum from a CSTR receiving lactate as carbon source and electron donor with sulphate concentration of 1.0 g l^{-1} operated at a 5 day residence time (Oyekola, 2008). Three identical 1 litre CSTRs, operated anaerobically, equipped with overhead stirrers driving Rushton impellers for continuous mixing at 400 rpm (tip speed of 1.05 m s^{-1}) were used to carry out the CSTR studies. Reactors were placed in a water bath to maintain a constant temperature at 35°C. The pH was not actively maintained but remained at pH 8± 0.2 by the concomitant generation of bicarbonate (described in Chapter 5, Section 5.3.2). A schematic representation of the continuous bioreactor set-up is given in Figure 3.3. Once a stable sulphate reduction and viable microbial population were established, the reactors were converted to continuous operation. A variable speed peristaltic pump was used to introduce the medium into the bioreactor, while an overflow tube was used to discharge the effluent by gravity (Figure 3.3). In order to maintain strict anaerobic conditions, vacuum grease was applied to seal glass lids of reactors and other glass fittings. Three feed sulphate concentrations (1.0, 2.5 and 5.0 g l^{-1}) were used to investigate the effects of sulphate concentration and its volumetric loading on the biological sulphate reduction kinetics, community structure and the stoichiometry of biological sulphate reduction. At each flow rate, steady-state was achieved and the resulting data was used to estimate the kinetics of sulphate reduction and bacterial growth. Flow rates, 0.0083 to 0.083 h^{-1}, to obtain residence times of 0.5 to 5.0 days were increased in accordance to recommendations by Oyekola (2008). Steady-state conditions were assumed to be established when both the residual sulphate and bacterial concentrations varied by <10% for a duration of operation equal to three retention times. Anaerobic digestate and the modified Postgate B medium were fed from separate feed bottles at a ratio of 0.7 COD:SO_4^{2-} (g g^{-1}).

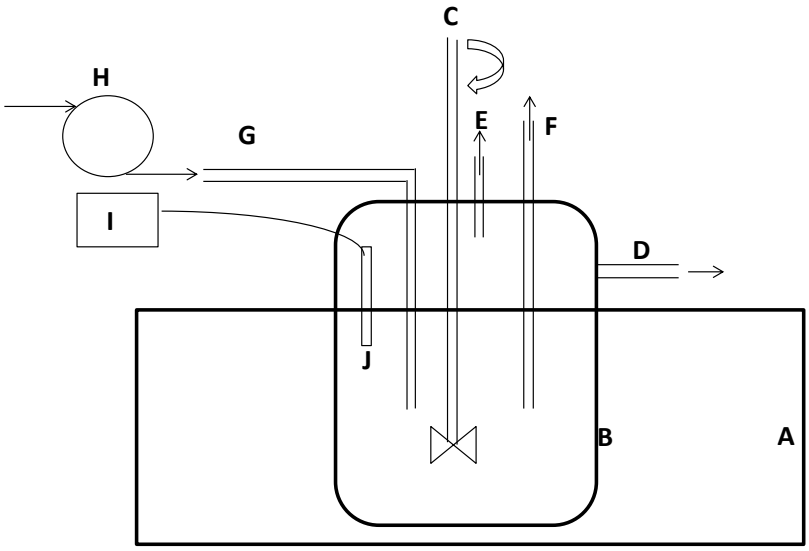


Figure 3.3. Schematic diagram of experimental set-up: (A) thermoregulated waterbath; (B) anaerobic bioreactor; (C) overhead stirrer; (D) overflow port; (E) gas vent; (F) sampling port; (G) feed inlet; (H) feed pump; (I) pH meter; (J) pH probe.

Disposable syringes under air-tight conditions were used to collect triplicate samples every second day from the reactors after the steady state conditions were established. These samples were analysed for dissolved sulphide, sulphate concentration, volatile fatty acids (VFAs), chemical oxygen demand (COD), pH, alkalinity and biomass concentration was determined. Genomic DNA was also extracted from these samples.

3.5 ANALYTICAL METHODS

3.5.1 Sulphide assay

Total dissolved sulphide was determined spectrophotometrically using a colorimetric assay as described by Cline and Richards (1969). A 2 ml sample volume was centrifuged using 2 ml Eppendorf Safe lock tubes for 10 minutes at 11420 g force. It was assumed that the Eppendorf Safe lock tubes would minimise the loss of hydrogen sulphide during centrifugation. The supernatant (20 µl) was added to 200 µl of zinc acetate (10 g l^{-1}) in a test tube. Deoxygenated deionised water was then added to the test tube to achieve a final volume of 5 ml. This was followed by the addition of 500 µl of the colorimetric reagent, *N*,*N*-dimethyl-*p*-phenylenediamine (4 g l^{-1} prepared in 6 M HCl solution) and 500 µl 16 g l^{-1} ferric chloride solution prepared in 6 M HCl. The test tube was vortexed for 30 seconds and left on the bench top for 10 minutes to allow development of the methylene blue product (Cline and Richards, 1969). The absorbance of the resultant product was measured spectrophotometrically at 670 nm. Standard sulphide solutions (0 to 1.0 mg l^{-1}) were assayed in the same way as the samples to generate

a standard curve. The standard curve and more detail on reagent preparation are given in *Appendix A1.4* and *A1.2*, respectively. Standard deviations and errors were carried out on the resulting data.

3.5.2 Sulphate analysis

The sulphate concentration in samples was determined by high performance liquid chromatography (HPLC) (Waters System), using a Waters IC-Pak HR Anion Column (4.6 x 75 mm, 5 µm) and conductivity detector (Waters System). Sodium-borate gluconate as was used as mobile phase at a flow rate of 1 ml min^{-1}. A 50 µl sample was injected. Each sample was run for 12 minutes in order to ensure that the sulphate ion and other ions were completely eluted. Samples were prepared by centrifugation (13000 x g for 10 minutes), the supernatant diluted with an equal volume of dH_2O and filtering through a 0.22 mm hydrophilic Durapore (PVDF) Millex syringe filter. Dilution of the sample ensured the chromatography column was not overloaded with a high sulphate concentration (Waters, 2015). The standard curve generated with 0-10 mM sulphate and details on mobile phase preparation is given in Figure A.1 in *Appendix A*. Standard deviations, errors and the coefficience of variances were calculated for the resultant data.

3.5.3 Volatile fatty acid analysis

The feed and effluent concentrations of acetic, lactic, propionic, butyric, valeric, iso-butyric and iso-valeric acid were determined by HPLC (Thermo Scientific System) using an ultra-violet (UV) detector and a Biorad organic acids column (Aminex HPX-87H, 30 cm x 7.8 mm, 9 µm). Acidified distilled water (0.01 M H_2SO_4) was used as the mobile phase at a flow rate of 0.6 ml min^{-1}. A sample (100 µl) was injected and the VFAs were detected at a wavelength of 210 nm. A runtime of 35 minutes (Table A1 and Figure A4 in *Appendix A*) was found to be sufficient to allow separation of the VFAs present within the samples. Prior to HPLC analysis, the sample was subjected to centrifugation (13000 xg for 10 minutes). The supernatant was filtered using a 0.22 µm hydrophilic Durapore (PVDF) Millex syringe filter. The filtrate was then diluted (1:1) with dH_2O prior to analysis (Biorad, 2012). The standard curves were generated for the required range (0-1.0 g l^{-1}) for each VFA. Standard curves and retention times are shown in (Table A1, Figure A3, and Figure A4 in *Appendix A*). Standard deviations and errors were calculated for the resultant data.

3.5.4 Chemical oxygen demand assay

The measurement of chemical oxygen demand (COD) is based on the oxidation of the sample in a hot sulphuric acid and potassium dichromate solution, containing silver sulphate as a catalyst. Chloride ions are masked by the mercury sulphate in solution A. A standard curve for the COD analysis was generated using potassium hydrogen phthalate (Figure A5 in *Appendix A*). This compound is known to have a COD of 10 g COD l^{-1} at a concentration of 8.5 g l^{-1}. The 8.5 g l^{-1} potassium hydrogen phthalate solution

was used to prepare a standard curve equating a COD range from 0 to 10 g l^{-1} as required. Low range and high range COD solutions were used as required according to the manufacturer's instructions (Merck, South Africa). For the low range COD analysis, 0.3 ml COD solution A (Cat No. 114538), 2.3 ml COD solution B (Cat No. 114539) and 3 ml of sample were used. For the high range analysis, 2.2 ml COD solution A (Cat No. 114679), 1.8 ml COD solution B (Cat No. 114680) and 1 ml of sample were mixed together within a COD tube. The COD tubes were heated at 150°C for two hours (HANNA thermoreactor, United Kingdom). The tubes were allowed to cool down to room temperature and the COD concentration was determined using a spectrophotometer at 610 nm (Geneys Spectrophotometer, Thermo Scientific, Netherlands).

3.5.5 pH and Redox potential measurements

All pH testing was done on a Cyberscan 2500 micro pH meter and probe. The meter was calibrated daily using standard (pH of 4.0 and 7.0) buffer solutions (Thermo Scientific, Netherlands). Redox analyses were done using a Metrohm Redox platinum-ring electrode probe (Model 6.0451.00, Herisau, Switzerland).

3.5.6 Alkalinity assay

The method described by Snoeyink and Jenkins (1980), was used to measure alkalinity within samples from the biological sulphate reduction (BSR) CSTR. A 20 ml volume of sample was used and titrations were performed using 0.05 M (0.1 N) sulphuric acid (H_2SO_4). Firstly, the initial pH of the sample and the volume of acid in a burette were recorded. In order to allow for temperature fluctuations during the addition of acid to the sample, a temperature probe connected to the pH instrument was used. Samples were then placed on a magnetic stirrer plate, with slow continual agitation, and the change in pH monitored during the addition of acid. Two phases of titrations were performed. Firstly the addition of acid to the sample until the pH was between 4.3 and 4.6 and followed by the slow addition of acid until a pH of 3.6 was reached. Five pH value points were recorded between pH 3.5 and 3.0. The volume of acid used was also recorded. The recorded values were plotted and the resulting linear equation was used to calculate the volume (ml) of titrant at equivalence point (A). The resulting equation (Equation3.1) where A is the volume of standard acid (H_2SO_4) or titrant at equivalence point (ml), N is the normality of the acid used and V is the volume of the sample (ml), was used to calculate alkalinity.

$$\text{Alkalinity } \frac{Eq}{L} = \frac{A\ x\ N}{V} \qquad \text{Equation 3.1}$$

3.5.7 Determination of biomass growth in CSTR by dry weight measurement

Increase in bacterial biomass within the BSR CSTR was determined by dry mass. Samples from the bioreactors, 4.5 ml, were centrifuged (16 000 xg, 15 minutes), using an Eppendorf® centrifuge (model

5415 D) in pre-dried (80°C, 48 h) and pre-weighed microfuge tubes by performing a few rounds of centrifugation to allow pelleting of the complete volume. The supernatant was decanted after each centrifugation and the final pellet washed twice with distilled water. The pellets were dried at 80°C for 48 h, allowed to cool in a desiccator and the microfuge tubes containing the dried pellets were then weighed to determine the dry biomass.

3.6 MOLECULAR TECHNIQUES

The following section describes the molecular methods used in this study.

3.6.1 Extraction of total genomic DNA

Total genomic DNA was extracted from the inoculum sample prior to initiation of the cyanobacterial digestate operated BSR CSTRs and from steady state samples from the CSTRs operated with 2.5 and 5.0 g l^{-1} sulphate. Steady state reactor samples, when residual sulphate and bacterial concentrations varied by <10% over a period equating three retention times, were collected for 5, 4, 3, 2, 1 and 0.5 day residence times from SRB reactors. Sampling was performed by drawing 2 ml from the sampling port, labelled F on Figure 3.2. Cells were harvested by centrifugation at 10 000 xg for 10 minutes using a Beckman centrifuge (AVANTI® J-25 Model). The pellet recovered was washed with 1x PBS solution (pH 7.2) followed by centrifugation at 8 000 rpm for 5 minutes. The cell pellet was then re-suspended in an appropriate volume of the same buffer and total DNA extracted using the Roche High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche, Germany). The extracted DNA was quantified using a NanoDrop™ ND-2000 Spectrophotometer (Thermo Scientific, South Africa).

3.6.2 Amplification of 16S rRNA and *dsr* genes

PCR amplification of the 16S rRNA and *dsr* genes within the inoculum samples was performed using KAPA 2G Fast HotStart ReadyMix DNA Polymerase (KAPA Biosystems Cape Town, SA). For the amplification of the 16S rRNA genes the PCRs were carried out in a total volume of 25 µl, containing 1x KAPA2G Fast HotStart ReadyMix and 0.3 µM of 27F (5′-GAGAGTTTGATCTGGCTCAG -3′) forward and 1492R (5′-GTACGGTACCTTGTTAGCACTT-3′) reverse primer, respectively. These primers were specific for bacteria. The following PCR conditions were applied to the reaction: DNA was denatured at 96°C for 6 minutes; followed by 25 cycles of 96°C for 15 seconds denaturation, annealing at 55°C for 20 seconds and extension at 72°C for 35 seconds. Amplification of the *dsr* gene was carried out with forward primer DSRp2060F (5'-CAACATCGTYCAYACCCAGGG-3') (Geets *et al*., 2006) and reverse primer DSR4R (5'-GTGTAGCAGTTACCGCA-3') (Wagner *et al*., 1998). Amplification was achieved by initial denaturation of DNA at 95 °C for 3 minutes followed by 40

cycles of denaturing at 95 °C for 10 seconds, annealing at 60 °C for 20 seconds and extension at 72 °C for 20 seconds.

3.6.3 DNA electrophoresis, excision and purification from agarose gels

The PCR products were separated by electrophoresis on a 1.2% (w/v) agarose gel in tris-acetate-EDTA (TAE; 40 mM tris, 20 mM acetate and 1 mM EDTA; pH 8.6) at 60 V for approximately 45 minutes. The gels were stained with 0.1% (w/v) ethidium bromide and destained with distilled water. The amplified DNA fragments were visualised with a G-Box (SYNGENE, Germany) under long wavelength UV light (365 nm). The approximately 1.5 kb DNA fragment for the 16S rRNA product and 350 bp *dsr* gene product, were excised from agarose gels and purified using either the QIAquick® Gel Extraction Kit (QIAGEN, Netherlands) or the NucleoSpin® Gel and PCR Clean Up kit (Machery-Nagel, Germany), according to the manufacturer's instructions.

3.6.4 Cloning of DNA fragments

Cloning of DNA fragments was performed using either the pGEM®-T Easy Vector (Promega, South Africa) for the 16S rRNA gene products or the pJET 1.2 vector system (Fermentas, South Africa) for the *dsr* gene products as per manufacturer's description. Five µl of the ligation reaction was used to transform chemically competent *Escherichia coli* (*E. coli*) DH5α cells.

3.6.5 Preparation of *Escherichia coli* strain DH5α chemically competent cells for cloning of 16S rRNA and *dsr* gene fragments

The preparation of *E. coli* used for transformation was carried out using the $CaCl_2$ method modified from Dagert and Ehrlich (1979). *E. coli* DH5α were plated from glycerol stocks (maintained in 16% (v/v) glycerol at -60°C) onto Luria-Bertani (LB) agar plates. Plates were incubated for approximately 16 hours at 37°C. A single colony was selected and used to inoculate 10 ml LB liquid medium and subsequently incubated on a shaking platform (160 rpm) for approximately 16 hours at 37°C. The resultant culture was used to inoculate 100 ml LB medium. The culture was incubated at 37°C with shaking at 160 rpm until an OD (measured at 600 nm) of approximately 0.4 was achieved. The resultant culture was then cooled on ice, transferred to pre-chilled sterile 500 ml Beckman centrifuge tubes and centrifuged at 3000 xg for 5 minutes at 4°C (AVANTI® J-25 Model). A volume of 100 ml (pre-chilled) 0.1 M $MgCl_2$, was used to re-suspend the cells. The pellet was incubated on ice for 1 minute and centrifuged as before. Pre-chilled 0.5 M $CaCl_2$ (50 ml) was used to suspend the pellet by swirling it gently and incubating it on ice for 1 hour. The cells were centrifuged as described above and the resultant cell pellet re-suspended in 10 ml 0.5 M $CaCl_2$ supplemented with 15% glycerol. Finally, cells were aliquoted into volumes of 100 µl into sterile Eppendorf® centrifuge tubes and stored at -60°C until use.

3.6.6 Heat-shock transformation of *E. coli* DH5α cells

Five µl of the ligation mix was added to the 100 µl of $CaCl_2$ competent *E. coli* DH5α cells. Cells were incubated on ice for 20 minutes and then heat shocked at 42°C for 50 seconds, before being placed on ice for 2 minutes. A volume of 300 µl ψ-broth was added to each transformation reaction and cells incubated at 37°C for 45 minutes. A volume of 100 µl of the resulting expression mixture was spread plated on Luria-Bertani (LB) agar plates containing 0.1 mM IPTG, 20 µg/ml X-gal and 100 µg/ml ampicillin, and incubated at 37°C overnight. *E. coli* DH5α transformants capable of growth on ampicillin were presumed to be harbouring plasmid DNA. Ten white *E. coli* DH5α transformants, containing an insert, were individually picked onto fresh LB agar plates containing 100 µg/ml ampicillin and incubated at 37°C.

3.6.7 Colony PCR amplification of the cloned 16S rRNA and the *dsr* gene fragments

A colony PCR was performed, in order to amplify the cloned DNA fragment. Briefly, a small amount of biomass from an individual colony was picked with a sterile tip and suspended in 20 µl sterile water. The mixture was subjected to two freeze (-20°C)/thaw cycles, to lyse the cells before 1 µl was used as the template in a PCR reaction. PCR amplification was performed with KAPA 2G Fast HotStart ReadyMix DNA Polymerase (KAPA Biosystems Cape Town, SA). PCR reactions were carried out in 25 µl reaction volumes containing 1x KAPA2G Fast HotStart ReadyMix and 0.5 µM primers. The cloned insert was amplified using M13 forward (5′-GTAAAACGACGGCCAGT-3′) and reverse (5′-GCGGATAACAATTTCACACAGG-3′) primers in the case of pGEM-T easy cloned 16S rRNA gene fragments and the pJET1.2 Forward (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2 Reverse (5'-AAGAACATCGATTTTCCATGGCAG-3') primers in the case of the pJET1.2 cloned *dsr* fragments. The PCR cycling conditions were as follows, the template DNA was initially denatured at 96°C for 8 minutes. This was followed by 25 cycles of 95°C for 10 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 15 seconds. This was followed by a final extension at 72°C for 10 minutes. Approximately 5 µl of the PCR reactions were separated by electrophoresis on 1.2 % (w/v) TAE agarose gels at 60 V for approximately 45 minutes, in order to confirm the amplification of single products of approximately 1.5 kb. The gels were stained with 0.1% (w/v) ethidium bromide and destained with distilled water, before visualisation on a G-Box (SYNGENE, Germany) UV transilluminator. Thereafter, the amplified PCR products were individually purified using the NucleoSpin® Gel and PCR clean up kit, according to the manufacturer's instructions (Machery-Nagel, Germany), and the purified PCR products quantitated using a NanoDrop™ ND-2000 (Thermo Scientific, South Africa).

3.6.8 Amplified ribosomal DNA restriction analysis (ARDRA) of the 16S rRNA gene PCR products

Approximately 500 ng of purified PCR product was digested using *Hae*III, *Hha*I and *Alu*I, respectively (Fermentas, South Africa), at 37°C overnight. The digested DNA fragments were separated by electrophoresis on a 1.2% (w/v) TAE agarose gel at 55 V for approximately 60 minutes. The gels were stained with 0.1% (w/v) ethidium bromide and destained with distilled water, before visualisation on a G-Box (SYNGENE, Germany) UV transilluminator. Putative unique ribotypes were identified on the basis of unique *Hae*III, *Hha*I and *Alu*I restriction patterns.

3.6.9 Plasmid extraction

Extraction of plasmids was carried out using the GenElute™ HP Plasmid miniprep kit according to the manufacturer's instructions (Sigma Aldrich, Germany). The extracted plasmid DNA was quantified using a Nanodrop™ ND-2000 (Thermo Scientific, South Africa).

3.6.10 Sequencing of unique 16S rRNA ribotype and dsr plasmid insert gene fragments

Recombinant *E. coli* cells harbouring unique 16S rRNA gene sequences in the pGEM-T easy vector and the pJET1.2 vector with *dsr* gene insert, were selected from colony PCR results and incubated overnight at 37°C with shaking (160 rpm). Plasmids were extracted as described in section 3.6.9 and were sent for sequencing using the M13 forward and reverse primers (Macrogen, Korea) for pGEM-T vectors and pJET1.2 Forward and Reverse primers for pJET vectors harbouring *dsr* gene fragments (Inqaba Biotech, South Africa).

3.6.11 Analysis of 16S rRNA and *dsr* gene sequences

The sequences obtained from the 16S rRNA gene clone library were edited and assembled using Chromas version 2.01 (Technelysium Pty Ltd., Australia) and DNAMAN for windows version 4.13 (Lynnon Biosoft, Canada). Homology and similarity searches of DNA sequences were performed using the basic local alignment search tool (BLAST) (Altschul *et al.*, 1989; Altschul *et al.*, 1997), provided by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

For phylogenetic analysis the 16S rRNA sequences obtained from the 16S rRNA gene clone library and reference sequences obtained from the NCBI database were aligned using CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Thompson *et al*., 1997) and manually edited to a common length of 1300 nucleotides. Phylogenetic analyses were conducted using MEGA version 5.1 (Kumar *et al*., 2004) and a neighbour-joining (Saitou and Nei, 1987) tree constructed. Bootstrap values were based upon 1000 re-sampled data sets (Felsenstein, 1985) and only bootstrap values greater than 40% were indicated.

3.6.12 Fluorescence *in Situ* hybridisation

Samples were collected from respective reactors and cells were harvested (10000 xg, 10 minutes). These were washed (8000 xg, 5 minutes) in 1 ml 1x PBS followed by resuspension in 375 µl fresh 1x PBS and three volumes (1.125 ml) of freshly prepared paraformaldehyde (4% [w/v] in 1x PBS) added to fix the cells. Cells in fixative were incubated at 4°C overnight. Overnight samples were subsequently washed in fresh 1x PBS and stored in 1x PBS and ice cold absolute ethanol at a 1:1 ratio, and stored at -20°C until hybridisation (Amann *et al*., 1995). A volume of 5 µl of resuspended fixed cells was mounted onto Teflon-coated glass slides and oven-dried at 46°C for 20 minutes. To increase the permeability of the target cells for the oligonucleotide probes, 5 µl of lysosyme (10 mg ml^{-1}) was added on to the cell spot, followed by incubation on ice for 20 minutes. The lysosyme was then washed off using sterile Millipore water. The samples were dehydrated using an increasing ethanol series (50, 80 and 98% ethanol) for 3 minutes each. The slides were allowed to air dry and stored at -20°C prior to hybridisation (Li *et al*., 1997). Hybridisation buffer was prepared as follows: 360 µl 5 M NaCl; 40 µl 1 M Tris-HCl (pH 7.2); varying volumes of 60% stock solution formamide to achieve formamide concentrations for different probes (Table 3.1) and 2 µl SDS (10% [w/v]). Sterile ddH_2O was added to a final volume of 2 ml. A volume of 8 µl hybridisation buffer (prepared as described above) was added to the fixed cell spots. Paper towel was folded into a rectangle slightly larger than the slide and placed into a 50 ml polypropylene tube. The remaining hybridisation buffer was poured onto the paper towel. This prevented evaporation of buffer in the wells during hybridisation (Hugenholtz *et al*., 2002). A volume of 0.5 µl of each probe at a working concentration of 50 ng $µl^{-1}$ was added to the cell spots and mixed carefully with a pipette tip while avoiding touching the surface of the slide (Hugenholtz *et al*., 2002). Cover slips were placed on top of the slides to ensure that the entire sample was exposed to the hybridisation mix. The slide was gently placed in the 50 mL tube containing the moistened towel. The cap was then replaced and the tube incubated horizontally in a hybridisation oven at 46°C for 2 h.

During hybridisation, 50 ml of washing buffer was prepared in a sterile 50 ml polypropylene tube by addition of components in the following order: appropriate volume of 5 M NaCl to achieve required NaCl concentrations for each probe (Table 3.1); 1 ml 1 M Tris-HCl (pH 7.2); appropriate volume of sterilised ddH_2O to obtain a final volume of 2 ml and 50 µl SDS (10% [w/v]). Following hybridisation, the wells were immediately rinsed with washing buffer pre-warmed to 48°C pipetted into the hybridisation tube. The slides were carefully removed from the hybridisation tube, and placed into wash buffer tube, and held at 48°C for 15 minutes. Rapid transfer of slides during these steps prevented cooling which can lead to nonspecific probe binding (Hugenholtz *et al*., 2002). The slides were then removed from the wash buffer, rinsed briefly in a beaker of ice-cold ddH_2O water, and thoroughly dried using compressed air in the dark. Samples were counterstained by addition of 5 µl of 4′, 6-diamidino-2-phenylindole (DAPI) (1 µg ml^{-1} in ddH2O) on to the slides. Cover slips were placed on top of the slides. Slides were incubated at ambient temperature for 5 minutes, rinsed with ddH_2O and thoroughly

dried in the dark. Aliquots of antifade solution, AFI (Citiflour Ltd., London UK) were added to the wells, and the cover slips gently pressed down to remove excess antifade, prior to microscopy. Microscopy was performed using an Olympus model BX40 epifluorescence microscope and images captured using an attached Analysis® Five Digital Imaging Solution

Table 3.1. 16S rRNA gene-targeted oligonucleotide probes used in this study

Probe[a]	Probe Name	Target	Position or target site[b]	[Formamide][c] (%)	[NaCl] (M)[d]	Sequence (5'-3')	Reference
EUB338 (FAM)	S-D-Bact-0338-a-A-18	Most bacteria	338-355	40	0.046	GCTGCCTCCCGTAGGAGT	Amann *et al.* (1990)
DSV827 (FAM)		*Desulfovibrio*	827 - 844	30	0.102	GGTCGCCCCCCGACA	Dar *et al*. (2007)
DELTA495a (FAM)	S-C-dProt-0495-a-A-18	Most Deltaproteobacteria and Gemmatimonadetes	495 - 512	35	0.070	AGTTAGCCGGTGCTTCCT	Loy *et al*. (2002)
DSM213 (FAM)	S-G-Dsm-0213-a-A-18	*Desulfomicrobium*		15	0.318	CATCCTCGGACGAATGCA	Loy *et al*. (2007)
SRB660 (FAM)	S-G-Dsbb-0660-a-A-20	*Desulfobulbus*	660-679	30	0.071	GAATTCCACTTTCCCCTCTG	Devereux *et al*. (1992)

[a] nomenclature according to Alm et al., (1996)
[b] 16S rRNA position according to *E.coli* 16S rRNA sequence numbering (Brosius *et al*., 1981)
[c] formamide concentration in the hybridisation buffer
[d] sodium chloride concentration in the washing buffer

3.6.13 Real-time quantitative PCR (qPCR)

The qPCR standards used for validation of primers and 16S rRNA gene copy number determination were taken from the 16S rRNA gene clone library plasmids: LB1, LB3, LB5, LB7, LB8, LB12, LB19, LB22, LB28, LB31, LB36, LB43, LB44, LB48, LB49, LB52, LB53, LB55, LB62, LB64, LB71, LB89 and LB90. These plasmids carried 16S rRNA genes sequences identified by BLASTn analysis representing the SRBs targeted in this study. The positive control prepared for the *dsr* gene, also identified by BLASTn analysis was used to quantify the number of *dsr* genes within the total gDNA extracted from samples. Following plasmid extraction and quantification of the DNA a 10 ng μl^{-1} stock of each standard was prepared. Tenfold serial dilutions (10^{-1} to 10^{-7}) were prepared from these stocks and used to generate standard curves. The copy number represented by 1 µl of the plasmid standard for each of the prepared standards was calculated based on the molecular weight of the plasmid with insert. All qPCR reactions were performed using the RotorGene 6000 Real-Time PCR system (Corbett Life Science, Concorde, NSW, Australia) and carried out in 15 µl with 1x SYBR® Green FAST qPCR Master Mix (Kapa Biosystems, Cape Town, South Africa). For amplification, 1 µl of a 1 ng μl^{-1} stock solution prepared from extracted DNA was used for the amplification with all the primers tested. The suitable amount of DNA template for qPCR was tested by performing qPCR with increasing amounts of DNA and determining the linearity of the reaction. This was indicative of no inhibition due to presence of possible inhibitors co-extracted with the DNA. A concentration of 0.3 µM for each primer was used. Amplification of total bacteria was carried out with Total bacteria-specific primers TotalF (5'-TCCTACGGGAGGCAGCAGT-3') and TotalR (5'-GGACTACCAGGGTATCTAATCCTGTT-3') (Nadkarni *et al*., 2002). Cycling conditions were as follows: hold at 95 °C, 3 minutes; cycles (40 repeats): 95 °C (5 minutes), 60 °C (30 s) and 71 °C (20 s). All the above standards were used in this experiment.

The *Desulfomicrobium* species were amplified using forward primer DSM442F (5'-CCACACTGGGACTGGAACAC-3') and reverse primer DSM632R (5'-AGATATCTACGGATTTCACTCCTACACCT- 3') designed and validated in this study (more details are given in Section 4.3.3.3) The amplification conditions optimised in this study and used for routine qPCR analyses were as follows: hold at 95 °C, 3 minutes; cycles (40 repeats): 95 °C (10 s), 60 °C (20 s) and 72 °C (20 s). The plasmid DNA of LB1 was used as standard. A similar amplification protocol was followed to quantify *Desulfovibrio* species in the reactors. The primers used for this experiment were the forward primer DSV-II-312f[b] (5'-CCACACTGGGACTGGAACAC-3') and reverse primer DSV681R[b] (5'-AGATATCTACGGATTTCACTCCTACACCT-3'). Both primers were modified from Stubner (2004), as indicated in Table 3.2. Plasmid DNA of LB5 was used to prepare a standard for this qPCR reaction. Amplification of the *dsr* gene was carried out with forward primer DSRp2060F (5'-CAACATCGTYCAYACCCAGGG-3') (Geets *et al*., 2006) and reverse primer DSR4R (5'-

GTGTAGCAGTTACCGCA-3') (Wagner *et al*., 1998). Amplification was achieved by initial denaturation of DNA at 95 °C for 3 minutes followed by 40 cycles of denaturing at 95 °C for 10 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s. A pJET1.2 plasmid harbouring the *dsr* gene was used as a standard for this qPCR.

Further information on the validation of qPCR conditions and primer specificity is given in Section 4.3.3. Table 3.2 details primer sequences, annealing temperatures (Tm), either computationally determined or taken from references, and % GC content.

3.6.14 Metagenomic analysis of bacterial diversity by 16S rRNA Illumina® MiSeq® sequencing

Selected genomic DNA samples were subjected to 16S rRNA V3 to V4 Illumina® MiSeq® sequencing (Macrogen, Seoul, South Korea). Sample preparation, sequencing, pre-processing of reads, clustering of sequence data and the taxonomic assignment was performed by the service provider. Amplification of the V3 to V4 variable regions of the 16S rRNA gene was performed using FwOvAd_341F and ReOvAd_785R dual-index barcoded primers. Paired-end sequence reads were merged using Fast Length Adjustment of Short reads (FLASH 1.2.11; http://ccb.jhu.edu/software/FLASH/) (Magoč, and Salzberg, 2011). Raw reads trimming and filtering, and OTU picking was formed by CD-HIT-OTU (http://weizhongli-lab.org/cd-hit-otu/) (Li *et al.*, 2012) at a difference distance cut-off of 0.03. the OTUs taxonomy was then assigned using the ribosomal database project (RDP) 16S rRNA classifier algorithm (http://rdp.cme.msu.edu/index.jsp) (Edgar, 2010) by QIIME, UCLUST (Langille, 2013).

Table 3.2. Primers for the amplification of bacterial species and SRB species.

Primer name	Sequence (5′-3′)	Target	Product size	Tm (°C)	Length (bp)	GC content (%)	Reference
TotalF	TCCTACGGGAGGCAGCAGT	All Bacteria	466	59.4	19	63.2	Nadkarni *et al*. (2002)
TotalR	GGACTACCAGGGTATCTAATCCTGTT	All Bacteria	466	58.1	26	42.2	Nadkarni *et al.* (2002)
DSM442F	GGCATTGGTCTAATAGGCCTTTGTT	*Desulfomicrobium*	190	64.7	25	44	This study
DSM632R	TGGGATTTCACCCCTGACTTACAA	*Desulfomicrobium*	190	65.1	24	45.8	This study
[m]DSV-II-312f[b]	CCACACTGGGACTGGAACAC	*Desulfovibrio*	399	58.1	20	41.4	Stubner (2004)
[m]DSV681R[b]	AGATATCTACGGATTTCACTCCTACACCT	*Desulfovibrio*	399	58.1	29	60	Stubner (2004)
DSR4R	GTGTAGCAGTTACCGCA	*dsrB* gene	350	52	17	52.9	Wagner *et al.* (1998)
DSRp2060F	CAACATCGTYCAYACCCAGGG	*dsrB* gene	350	55.6 to 60.5	21	57.1	Geets *et al*. (2006)

[m]Modified primer from Stubner 2004

3.7 RESEARCH STRATEGY

The inoculum (10%) from a reactor receiving lactate as a carbon source an electron donor at 1 g l^{-1} SO_4^{2-} concentration (Oyekola 2008) was used to inoculate the CSTRs receiving digestate as feed operated at a sulphate concentration of 1.0, 2.5 and 5.0 g l^{-1} used in this study. The effect of the feed sulphate concentration and residence time in the presence of digestate which consists of acetate, propionate and butyrate at digestate COD:SO_4^{2-} of 0.7 (g g^{-1}), on biological sulphate reduction kinetics and the community structure dynamics were investigated. To achieve this, three CSTRs were initiated receiving sulphate feed concentrations of 1.0, 2.5 and 5.0 g l^{-1} respectively. The bioreactors were maintained at previously determined optimal conditions of 35°C and pH of 8.0 ± 0.2 by a concomitant generation of bicarbonate throughout the experiment. Residence times were varied from 5 to 0.5 days to vary the specific growth rate and the volumetric sulphate loading rate. Data was collected on residual sulphate and VFA concentrations, resulting bicarbonate alkalinity and sulphide, conversion rates and the microbial communities were analysed. FISH and 16S rRNA gene clone library preparation were used to characterise the microbial community in the inoculum and the information was used to validate the molecular techniques used for further microbial characterisation in the study. Mixed microbial community dynamics in the reactors receiving digestate as feed, and comparative samples from a lactate operated reactor, as a result of sulphate loading and residence time were analysed using FISH and qPCR. The relationship between the reaction kinetics and community structure was explored using the data obtained and conclusion around these are presented. The data obtained from study was used to conclude on the feasibility of using digestate as a sole carbon source and electron donor for BSR systems. The approach taken in this thesis is illustrated in Figure 3.4.

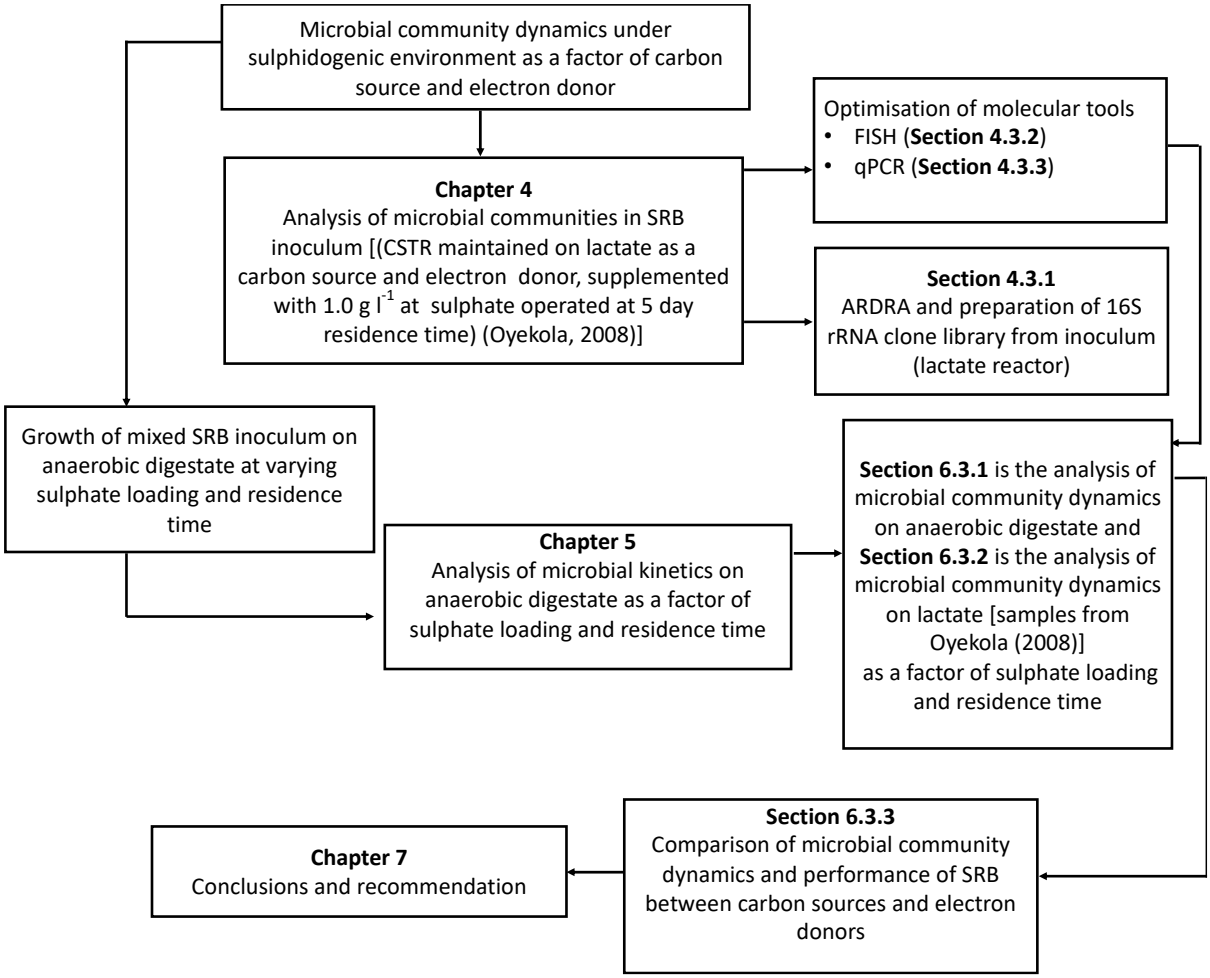


Figure 3.4. Schematic diagram illustrating the approach taken during the current study.

CHAPTER 4
DEVELOPMENT OF MOLECULAR TOOLS FOR THE CHARACTERISATION OF MICROBIAL COMMUNITIES INVOLVED IN BIOLOGICAL SULPHATE REDUCTION

4.1 INTRODUCTION

Despite playing a primary role in the performance of wastewater remediation, the diversity of microbial communities associated with the remediation process kinetics, has in most cases not been regarded (Manefield *et al*., 2005; Kapley *et al*., 2007a; Kapley *et al*. 2007b; Helbling *et al.*, 2012; DiLoreto *et al*., 2016; Gopi Kiran *et al*., 2017; Kantor *et al*., 2015; 2017). If the equilibrium of the microbial community is disturbed, the ability to operate the biological treatment plant optimally may be compromised (Lefebvre and Moletta 2006; Chowdhury *et al*., 2010). A comprehensive understanding of the microbial community is therefore necessary to avoid conditions that may result in bioreactor failure and poor performance. In biological sulphate reduction (BSR) systems, the addition or presence of different carbon sources and electron donors have shown to select for different microbial community members thus affecting the reaction kinetics (Widdel, 1988; Oude Elferink *et a*l., 1994; Stams *et al*., 2003; Kaksonen, *et al*., 2004a; Stams *et al*., 2005; Oyekola *et al*., 2012, Callbeck *et al*., 2013; Fadhlaoui *et al.*, 2018). Additionally, factors such as sulphate loading and residence time also play a major role in microbial community dynamics (Erasmus, 2000; Moosa *et a*l., 2002; Hansford *et al*, 2007; Oyekola *et al*., 2010). An understanding of the microbial community composition and shifts in species abundance as a result of reactor operating changes in many complex microbial communities remains limited (Amann *et al.*, 1995; Muyzer *et al.*, 1998; Kantor *et al*., 2015; 2017).

As discussed in Section 2.4.3, anaerobic organisms, particularly sulphate reducing bacteria (SRB), are difficult to isolate using traditional cultivation techniques (Butlin *et al*., 1949; Postgate, 1963; Amann *et al*., 1995; Johnson, 1995; Muyzer *et al*., 1998; Lewis, 2010; Ňancucheo *et al*., 2016). To allow the monitoring of species involved in BSR, culture independent tools are required that accurately reflect the microbial diversity and changes in the community structure when operating conditions such as sulphate loading and residence time are changed. Molecular techniques such as amplified ribosomal DNA restriction analysis (ARDRA) and fluorescent *in situ* hybridisation (FISH) (Ito *et al*., 2002; Icgen et al., 2007; Lücker *et al*,. 2007; Dar *et al.*, *2008;* Liu *et al*., 2009) as well as quantitative real-time PCR (qPCR) (Wagner *et al*., 1998; Stubner 2004; Geets *et al*., 2006; Steger *et al*., 2011; Probst *et al*., 2013) offer an opportunity for the monitoring and analysis of the structure and dynamics of microbial communities. These techniques can be used to assess the abundance of the 16S rRNA genes associated with specific species, when parameters are changed (Moter and Göbel, 2000; Gilbride *et al*., 2006 Sklarz *et al*., 2009; Müller *et al.*, 2015). The use and benefits of these molecular techniques and metagenomic approaches are discussed in Sections 2.6.1 to 2.6.2. Due to the diversity of sulphate

reducers, the 16S rRNA gene based approaches have been limited to qualitative or semi-quantitative analyses such as FISH highlighting the need to development molecular tools for both the semi-quantitative and qualitative characterisation of SRB present within the bacterial communities (Castro *et al*., 2000, Dhillon *et al.*, 2003 Miletto *et al*., 2007). To investigate the microbial diversity involved in BSR and to be able to link these to the process performance of wastewater treatment, requires the optimisation of these molecular tools (Hugenholtz *et al*., 2002).

This chapter details how 16S RNA library construction followed by ARDRA, FISH and qPCR were optimised and used to characterise the mixed SRB inoculum, used to initiate the reactors reported on during this study. The inoculum was obtained from a mixed microbial community associated with a continuous stirred tank reactor (CSTR) maintained on lactate as a sole carbon source, supplemented with 1 g l^{-1} sulphate operated at a 5 day residence time (described in Section 3.3 and 3.4). The optimised methods from this chapter were applied to study the changes in BSR community structures of CSTRs maintained on anaerobic digestate as a carbon source and electron donor at sulphate concentrations of 2.5 and 5.0 g l^{-1} (reported in Chapter 6).

The specific objectives of the molecular study presented in this chapter were as follow:

i. To characterise the microbial community comprising the inoculum used to initiate reactors receiving anaerobic digestate as a carbon source.

ii. To optimise FISH techniques for this community through the investigation of conditions for probe hybridisation stringency. The aim was to improve the technique for the characterisation of the BSR microbial communities in CSTR reactors receiving anaerobic digestate as a sole carbon and electron donor.

iii. To design and test genus or species-specific qPCR primer sets, or both, to allow the quantitative assessment of changes in the BSR community structure in response to operational changes.

4.2 EXPERIMENTAL APPROACH

Total genomic DNA (gDNA) from the inoculum was successfully extracted using the method described in Section 3.6.1. Three samples, taken over three separate days, from the BSR CSTR receiving lactate as carbon source and electron donor were used for this extraction (Figure B1.A in *Appendix B*). DNA from these extractions were combined and used as template for the amplification of the 16S rRNA genes from the total gDNA. Following 16S rRNA gene library construction; ARDRA analyses of the resultant 16S rRNA gene clones were performed by restriction with *Hae*III, *Hha*I and *Alu*I (Fermentas, South Africa). Putative unique ribotypes were identified on the basis of unique restriction patterns visualised

following gel electrophoresis. A total of 49 unique 16S rRNA gene clones were chosen for Sanger sequencing and their 16S rRNA gene sequences analysed according to Section 3.7.10. The 16S rRNA gene sequences of the unique clones together with 36 reference sequences were subjected to phylogenetic analysis and the tree was generated in MEGA 7.018 using the neighbour joining method. Bootstrap values were based upon 1,000 re-sampled data sets and only bootstrap values greater than 40% are indicated. The branch lengths indicate the number of nucleotide substitutions per site.

Fluorescence *in situ* hybridisation (FISH), methodology detailed in Section 3.7.11, was used as a visual confirmation of the different microbial communities present within the inoculum. The FISH probes used were EUB338 (specific for domain bacteria, Amann *et al.*, 1990a), ARCH915 (specific for domain Archaea), DELTA495a (specific for *Deltaproteobacteria*, Loy *et al*., 2002), DSM213 (specific for *Desulfomicrobium* species, Loy *et al*., 2007), DSV827 (specific for *Desulfovibrio* species, Dar *et al*., 2007) and SRB660 (specific for *Desulfobulbus* species, Devereux *et al*., 1992). The positive signals obtained from the hybridised samples were compared with the results from DAPI (4',6-diamidino-2-phenylindole) staining, a fluorescence stain that binds strongly to A-T rich regions within DNA and thus targets all microorganisms present in the consortia (Hesham and Alamri, 2012). Therefore, the cells which stains with DAPI but do not show a positive signal when hybridised to the SRB specific probes (DELTA495a, DSM213, DSV827 and SRB660) were taken as representing non-SRB cells or "other SRB" that were not targeted by the SRB genus specific probes applied. Formamide concentration for each probe was optimised accordingly. The pure culture of *Desulfobulbus propionicus* DSM 2056 was used to validate FISH conditions for SRB660.

qPCR can be applied as a quantitative tool for analysing microbial communities (Nadkarni *et al*., 2002; Horz *et al.*, 2005). The primer pair, TotalF (5'- TCCTACGGGAGGCAGCAGT -3') and TotalR (5'GGACTACCAGGGTATCTAATCCTGTT- 3') from Nadkarni *et al*. (2002) were used to amplify total bacteria. *In silico* analysis of the 16S rRNA gene sequences captured from the clone library was used to validate the universal total bacterial primers applied in this study. *The Desulfovibrio* species were amplified using the forward primer DSV-II-312f^{b} (5'- CCACACTGGGACTGGAACAC -3') and reverse primer DSV681R^{b} (5'- AGATATCTACGGATTTCACTCCTACACCT- 3'), where superscript b indicates the primer was modified from Stubner (2004), while the *Desulfomicrobium* species were amplified using the novel forward primer DSM442F (5'CCACACTGGGACTGGAACAC-3') and novel reverse primer DSM632R (5'AGATATCTACGGATTTCACTCCTACACCT- 3') according to Section 3.7.12. The specificity of these primers was determined using 16S rRNA gene clones with high sequence similarity to the target organisms and inclusion of the appropriate 16S rRNA gene clones to test the specificity. The total sulphate reducing potential of the mixed microbial community was captured by performing qPCR with *dissimilatory sulphite reductase* (*dsr*) specific primers. Literature suggests that BSR relies on the presence of the *dsr* gene within the genomes of SRB, and therefore

qPCR performed with these primers should allow for the quantification of all SRBs represented in the community (Kim *et al*., 2013; Müller *et al.*, 2015). A *dsr* standard for the qPCR was prepared by performing PCR using the DSR4R (5'-GTG TAGCAGTTACCGCA- 3'), and DSRp2060F (5'-CAACATCGTYCAYACCCAGGG- '3) (Geets *et al*., 2005) primer pair and the total genomic DNA extracted from the inoculum was as template. The pair was chosen according to Müller *et al.* (2015) recommendations (Section 4.3.3.4). The 378 bp product was cloned into the pJET cloning vector (Section 3.6.4) and three recombinant *E. coli* clones were subjected to Sanger sequencing according to Section 3.6.10.

4.3 RESULTS AND DISCUSSION

4.3.1 Microbial diversity associated with a lactate operated BSR CSTR used as inoculum for this thesis

To gain access to the microbial community associated with the lactate operated BSR CSTR used as inoculum for the current study, the 16S rRNA gene population was amplified from the total gDNA extracted from the reactor operated at a 1 g l^{-1} sulphate loading and a steady state residence time of 5 days. A 86.3% ± 0.5% sulphate conversion was maintained at the time of sampling (Oyekola *et al*., 2010). Figure B1.1 in *Appendix B* shows the successful extraction of high molecular weight and intact DNA from three independent extraction events from the reactor, while Figure B1.B shows the 1.5 kbp PCR products indicating the successful amplification of the 16S rRNA genes from these gDNA samples. These products were cloned into the commercial pGEMT cloning vector to create a library of 16S rRNA gene clones in the *E. coli* host system. A subset of 96 recombinant bacterial clones were subjected to ARDRA. Of these, 49 clones were identified to have unique ribotypes on the basis of their *Hae*III and *Alu*I restriction profiles. Figure 4.1 shows an example of the ARDRA performed on these recombinant clones, while the remaining gels are shown in *Appendix B* (Figure B2). The notation given above the gel images, R#, indicate unique ribotypes identified from their restriction patterns, and the numbers indicate the lane of the gel.

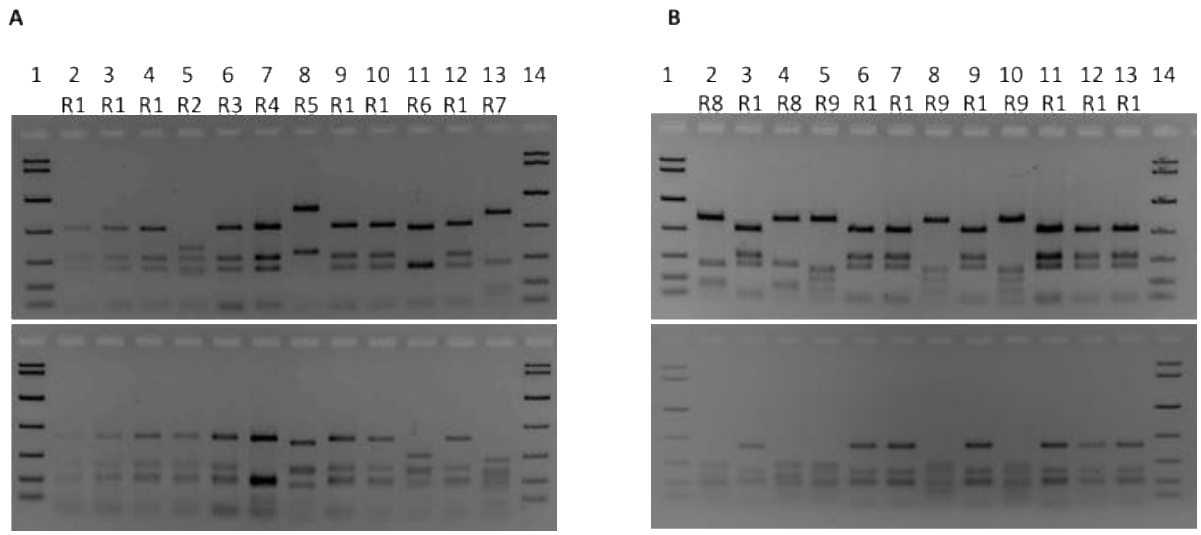


Figure 4.1. Restriction enzyme digestion analyses of PCR product from the inoculum reactor supplemented with lactate and 1.0 g l^{-1} sulphate at residence time of 5 days using *Hae*III (top) and *Alu*I (Bottom). Gel A: Lanes 2-13 contains LB1 to LB12; Gel B: Lanes 2-13 contains LB13 to LB24. Lane 1 and Lane 14 on both Gel A and Gel B contain molecular weight marker (KAPA^{TM} Express ladder, Kapa Biosystems (both gels).

The 16S rRNA gene clones representing the 49 unique ribotypes were sequenced and resultant 16S rRNA gene sequences subjected to BLASTn analysis (Altschul *et al*., 1997). To provide insight into the phylogenetic relatedness of the bacterial community within the mixed BSR inoculum, a phylogenetic tree was generated (Figure 4.2A). A total of 36 reference 16S rRNA gene sequences, based on BLASTn results, and the 49 16S rRNA gene sequences from the clone library were used to generate the phylogenetic tree (Table 4.1). The recombinant plasmids were given the prefix "LB" and numbered. The phylogenetic analysis of the mixed BSR inoculum revealed that the 16S rRNA gene sequences of LB1, LB3, LB19, LB25, LB33, LB43, LB50, LB53, LB57 and LB64 clustered together with 16S rRNA gene sequences from *Desulfomicrobium* species. LB89 showed the highest homology (93%) to *Desulfomicrobium apsheronum* (AF228136.2) (Table 4.1) and clustered with the other *Desulfomicrobium* species but appears to be an outlier within this group (Figure 4.2A). The 16S rRNA gene sequences of LB22 showed high homology (99%) to *Desulfovibrio* species, *Desulfovibrio desulfuricans* (EU980606.1) and *Desulfovibrio desulfuricans* (EU980606.1), while LB5 showed high homology (99%) to *Desulfovibrio aminophilus* (NR_024916.1) and LB9 showed 97% homology to *Desulfovibrio aminophilus* (NR_024916.1). These sequences clustered with 16S rRNA gene sequence of LB62 which showed 94% similarity to *Desulfocurvus vexinensis* (NR_043981.1). The number of unique ribotypes and bacterial sequences obtained from 16S rRNA gene library construction, revealed a more diverse community for the lactate fed reactor than previously reported by Oyekola (2008). From the phylogenetic tree, it can be observed that sequences with the 16S rRNA gene sequences captured by the clone library represented a diverse number of species from the *Desulfomicrobium* genus (Figure 4.2 A). The frequency at which ribotypes were observed from ARDRA was used to calculate the relative abundance of these species within the inoculum sample (Figure 4.2 B). Ribotypes representing *Desulfomicrobium* species represented 52% of the ribotypes captured during the ARDRA analyses,

suggesting that members of this genus were abundant in the lactate operated BSR CSTR bacterial community. Although we admit that PCR and cloning bias may skew these results, it may still provide an indication of the species abundance.

The 16S rRNA gene sequence of LB6 showed high phylogenetic relatedness to members of the *Desulfobulbus* genus and clustered with LB5 and LB9 (Figure 4.2 A). LB49 clustered with the 16S rRNA gene sequences of the sulphur reducing *Desulfuromonas* species (Grigoryan *et al*., 2008) (Figure 4.2 A). The 16S rRNA gene sequence of LB48 showed 96% homology to the uncultured *Firmicutes* (AB678373.1). *Firmicutes* contain the *dsrAB* gene encoding the key enzyme catalysing the last and main energy-generating step during sulphate reduction (Wagner *et al*., 1998; Loy *et al*., 2004; Zverlov *et al*., 2005) within their genomes. Further analysis of other banding patterns observed from ARDRA indicated that the frequency at which ribotypes representing SRB species (*Desulfomicrobium, Desulfovibrio, Desulfobulbus, Desulfocurvus* and *Firmicutes*) were captured during the ARDRA analyses was 66% (Figure 4.2B). The remaining proportion (34%) of the clones captured by the 16S rRNA gene library shared sequence similarity with non-SRB species (Figure 4.2B), also reported by Oyekola *et al.,* (2012) for this community and by other authors for sulphate reducing environments (Baker and Banfield, 2003; Grabowski *et al.*, 2005; Probst *et al*., 2013; Zhang *et al*., 2016). The non-SRB species are known lactate fermenters which may be contributing to the lactate fermentation observed within the CSTRs examined.

Of the 16S rRNA gene sequences that showed phylogenetic relatedness to non-SRB, the majority showed high homology to members of *Bacteriodetes* and B*acteriodes.* The frequency at which the ribotype was encountered during ARDRA suggested these organisms account for 14% of the inoculum (Figure 4.2B). The second most abundant group comprised 16S rRNA gene sequences that showed high homology to members of the *Acholeplasma* genus (LB10, LB21, LB29, LB40, LB46 and LB55), followed by 16S rRNA gene sequences that showed high phylogenetic relatedness to members of the *Mesotoga* genus (LB7, LB20, LB28, LB32 and LB41). These species accounted for 6 and 5% of the total microbial community, respectively (Figure 4.2B). The remaining group of 16S rRNA gene sequences revealed phylogenetic relatedness to members of the *Sphaerochaeta, Citrobacter, Synergistetes* and *Chloroflexi* genera (Figures 4.2).

Table 4.1 The unique 16S rRNA gene clone library sequences from the inoculum reactor receiving lactate as carbon source and electron donor and corresponding NCBI nucleotide sequence 3 entries determined by means of BLASTn.

Genus/ Phylum	Clone name	Closest Match	Max Score	Total score	Query cover (%)	E - value	Identity (%)	Accession number	Date Retrieved
Desulfomicrobium	LB1*	*Desulfomicrobium baculatum* DSM 1742	2565	2565	100	0.0	99	AR277896.1	28/01/2019
	LB3*	*Desulfomicrobium baculatum* DSM 4028	2562	2562	100	0.0	99	NR_042019.1	28/01/2019
	LB19*	*Desulfomicrobium norvegicum* DSM 1741	2478	2478	99	0.0	97	NR_025407.1	28/01/2019
	LB25	*Desulfomicrobium baculatum* DSM 4028	2557	2557	100	0.0	99	NR_042019.1	28/01/2019
	LB33	*Desulfomicrobium baculatum* DSM 4028	2571	2571	100	0.0	99	NR_042019.1	28/01/2019
	LB43*	*Desulfomicrobium norvegicum* DSM 1741	2606	2606	100	0.0	99	NR_025407.1	28/01/2019
	LB50	*Desulfomicrobium baculatum* DSM 4028	2572	2572	100	0.0	99	NR_042019.1	28/01/2019
	LB53*	*Desulfomicrobium norvegicum* DSM 1741	2422	2422	96	0.0	98	NR_025407.1	28/01/2019
	LB57	*Desulfomicrobium norvegicum* DSM 1741	2606	2606	100	0.0	99	NR_025407.1	28/01/2019
	LB64*	*Desulfomicrobium escambiense* DSM 10707	2405	2405	95	0.0	98	NR_042018.1	28/01/2019
	LB89*	*Desulfomicrobium apsheronum*	1900	1970	93	0.0	93	AF228136.2	28/01/2019
Desulfovibrio	LB5*	*Desulfovibrio aminophilus* strain ALA-3	2567	2567	100	0.0	99	NR_024916.1	28/01/2019
	LB9	*Desulfovibrio aminophilus* strain ALA-3	2460	2460	100	0.0	97	NR_024916.1	28/01/2019
	LB22*	*Desulfovibrio desulfuricans* strain Ser-2	2637	2637	100	0.0	99	EU980606.1	28/01/2019
Desulfocurvus	LB56	*Desulfocurvus thunnarius strain* Olac 40	1999	1999	100	0.0	90	NR_109747.1	28/01/2019
	LB62*	*Desulfocurvus vexinensis* strain VNs36	2448	2448	100	0.0	97	NR_043981.1	28/01/2019
Desulfobulbus	LB6	*Uncultured Desulfobulbus* clone OTU-X2-12	2131	2131	100	0.0	97	JQ668557.1	28/01/2019
Desulfuromonas	LB49*	*Desulfuromonas michiganensis* strain BB1	2361	2361	100	0.0	96	NR_114607.1	28/01/2019
Sphaerochaeta	LB36*	*Sphaerochaeta associate* strain GLS2	2586	2586	100	0.0	99	NR_145842.1	28/01/2019
	LB39	*Sphaerochaeta globosa* strain Buddy	2527	2527	99	0.0	98	NR _114608.1	28/01/2019
Citrobacter	LB52*	*Citrobacter freundii* strain IG1	2607	2607	100	0.0	99	KY435705.1	28/01/2019
Synergistetes	LB7*	Uncultured *Synergistetes* bacterium clone	2527	2527	100	0.0	99	JX575962.1	28/01/2019
	LB12*	*Aminivibrio pyruvatiphilus* strain 4F6E	1799	1799	100	0.0	88	NR_113331.1	28/01/2019
Firmicutes	LB44*	Uncultured *Firmicutes* bacterium clone	2397	2397	92	0.0	99	CU922072.1	28/01/2019
	LB48*	Uncultured *Firmicutes* bacterium clone	2327	2327	96	0.0	96	AB678373.1	28/01/2019
Chloroflexi	LB71*	Uncultured *Chloroflexi* bacterium clone	2536	2536	100	0.0	99	AB853917.1	28/01/2019
Mesotoga	LB20	*Mesotoga infera* strain VNs100	2599	2599	100	0.0	99	NR_117646.2	28/01/2019
	LB28*	*Mesotoga infera* strain VNs100	2599	2599	100	0.0	99	NR_117646.2	28/01/2019
	LB32	*Mesotoga infera* strain VNs100	2584	2584	100	0.0	99	NR_117646.2	28/01/2019
	LB41	*Mesotoga infera* strain VNs100	2590	2590	100	0.0	99	NR_117646.2	28/01/2019

Table 4.1 (continues).

Genus/ Phylum	Clone name	Closest Match	Max Score	Total score	Query cover (%)	E - value	Identity (%)	Accession number	Date Retrieved
Acholeplasma	LB10	*Acholeplasma* species enrichment clone 12-7C	2510	2510	100	0.0	99	EU517562.2	28/01/2019
	LB21	*Acholeplasma* species enrichment clone 12-7C	1804	1804	100	0.0	88	EU517562.2	28/01/2019
	LB29	*Acholeplasma* species enrichment clone 12-7C	2515	2515	100	0.0	96	EU517562.2	28/01/2019
	LB40	*Acholeplasma* species enrichment clone 12-7C	2510	2510	100	0.0	99	EU517562.2	28/01/2019
	LB46	*Acholeplasma* species enrichment clone 12-7C	2510	2510	100	0.0	98	EU517562.2	28/01/2019
	LB55*	*Acholeplasma* species enrichment clone 12-7C	2497	2497	100	0.0	98	EU517562.2	28/01/2019
Bacteriodetes	LB2	Uncultured *Bacteriodetes* bacterium clone H44	2476	2476	95	0.0	99	KC352324.1	28/01/2019
	LB8*	Uncultured *Bacteriodetes* bacterium clone QEDN4AA05	1985	1985	92	0.0	92	CU9255258.1	28/01/2019
	LB30	Uncultured *Bacteriodetes* bacterium clone De114	2424	2424	100	0.0	97	HQ183935.1	28/01/2019
	LB34	Uncultured *Bacteriodetes* bacterium clone De114	2529	2529	100	0.0	99	HQ183935.1	28/01/2019
	LB35	Uncultured *Bacteriodetes* bacterium clone De114	2569	2569	100	0.0	99	HQ183935.1	28/01/2019
	LB38	*Paludibacter* species clone 2C058	1982	1982	100	0.0	91	JN713554.1	28/01/2019
	LB51	*Paludibacter* species clone 2C058	2105	2105	100	0.0	92	JN713554.1	28/01/2019
	LB63	*Paludibacter* species clone 2C058	2096	2096	100	0.0	92	JN713554.1	28/01/2019
	LB88	Uncultured *Bacteriodetes* bacterium clone De114	2582	2582	100	0.0	99	JN713554.1	28/01/2019
Bacteroides	LB18	*Bacteroides* species 22C	2611	2611	99	0.0	99	AY554420.1	28/01/2019
	LB31*	*Bacteroides* species 22C	2577	2577	99	0.0	99	AY554420.1	28/01/2019
	LB45	*Bacteroides* species 22C	2615	2615	99	0.0	99	AY554420.1	28/01/2019
	LB90*	*Bacteroides* species 22C	1955	1955	99	0.0	90	AY554420.1	28/01/2019

The 16S rRNA plasmid of 23 clones, highlighted with an asterisk (*) were chosen to validate primer sets used in this thesis.

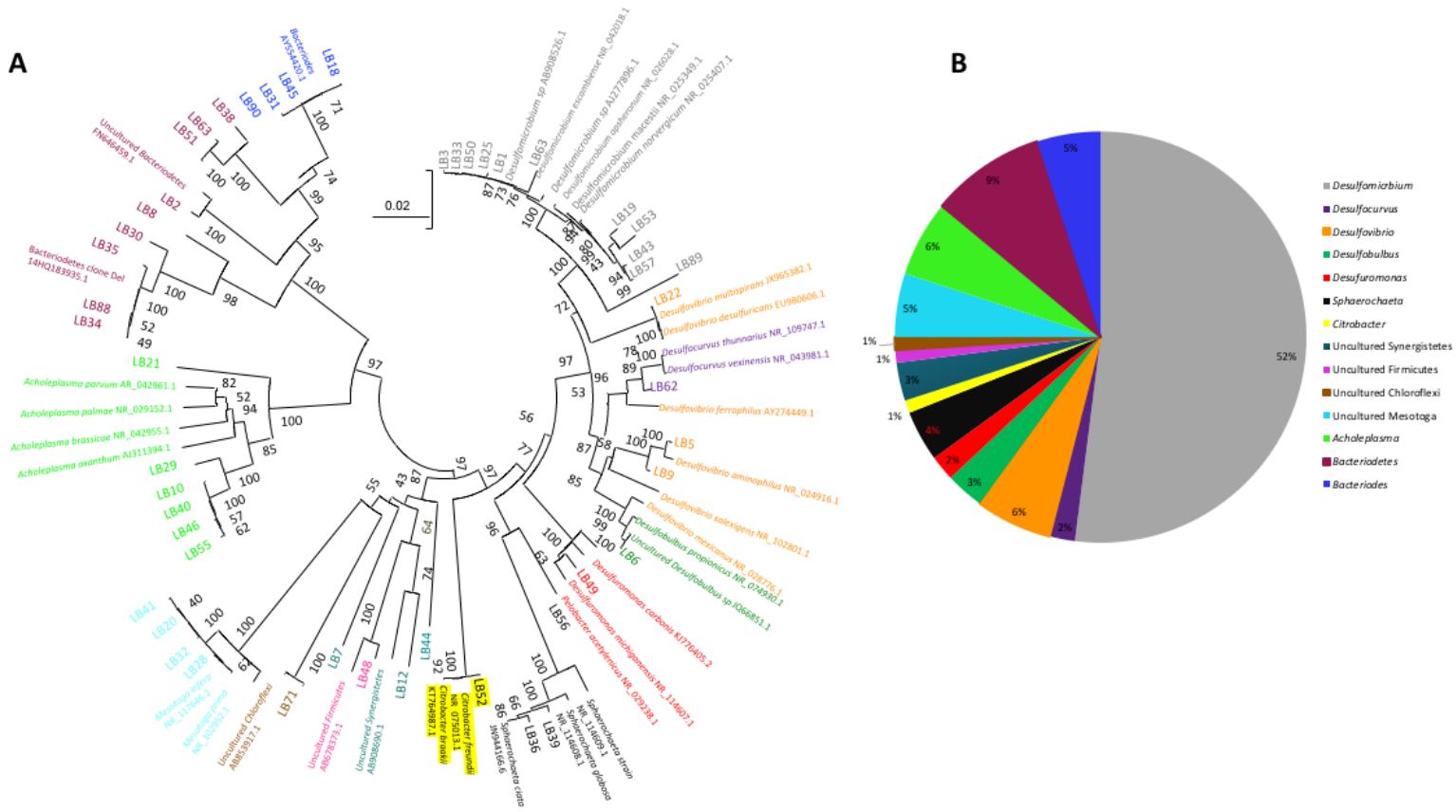


Figure 4.2. Microbial diversity from inoculum reactor supplemented with 1.0 g l^{-1} sulphate and lactate. **A**16S rRNA gene phylogenetic tree developed from the clone library of the inoculum reactor supplemented with 1.0 g l^{-1} sulphate and lactate. The16S rRNA gene sequences and reference sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The tree was generated in MEGA 7.018 using neighbour joining method. Bootstrap values were based upon 1,000 re-sampled data sets and only bootstrap values greater than 40% are indicated. The scale bar indicates 0.02 changes per sequence position. Nucleotide sequence accession numbers are given in parentheses. **B**. Genus level abundance of bacteria identified from ARDRA analysis in the CSTR receiving lactate which was used as an inoculum in this thesis. A total of 96 clones were analysed.

The 16S rRNA gene sequences from the inoculum had a high percentage homology to SRB members from the *Desulfomicrobium, Desulfovibrio, Desulfobulbus*, *Desulfuromonas* and *Desulfocurvus* genera and non-SRB members belonging to the *Sphaerochaeta, Citrobacter*, *Synergistetes*, *Firmicutes, Chloroflexi, Mesotoga, Acholeplasma, Bacteriodetes, Petrimonas* and *Bacteriodes* genera (Table 4.1). A study performed on the microbial community associated with the lactate fed CSTR used as inoculum for this thesis, suggested the presence of five key SRB species namely *Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* and *Desulfococcus multivorans*, and the lactate fermenters *Clostridium homopropionicum, Veillonella parvula, Pelobacter propionicus* (Oyekola, 2008). The molecular techniques employed in the above study involved restriction enzyme digestion of 16S rRNA gene PCR products obtained from the total gDNA, followed by *in silico* analysis to obtain matches for the restriction patterns and infer species identity from expected compared to obtained patterns. By constructing a 16S rRNA gene clone library we have increased the depth to which species could be identified. However, using only ARDRA in the hope of capturing the complete microbial community associated with BSR should be reported with caution as restriction profiles may not be unique to each sequence which may result in different species sharing fragmentation profiles on agarose gels (Sklarz *et al.*, 2009). The recombinant 16S rRNA plasmid clones generated from this library were used to further validate and develop a set of molecular tools to assess the dynamics of microbial diversity in sulphate reducing environments.

4.3.2 Evaluation and validation of FISH probes

The optimisation of FISH probes is required to ensure that they specifically hybridise to the targeted organisms (Hugenholtz *et al*. 2002; Roest 2007). Varying ratios of sample volume, hybridisation buffer volume, probe concentration and formamide concentration have been reported in the literature (Amann *et al*., 1990; Poulsen *et al.*, 1993; Daims *et a*l., 1999; Pernthaler *et al.*, 2001; Hugenholtz *et al*., 2002; Loy *et al*., 2002). For instance, Oyekola, (2008) reported using 9 µl of hybridisation buffer to l µl of the Gram negative mesophilic SRB probe (SRB385), (final probe concentration of 5.0 ng μl^{-1}), while Hugenholtz *et al*., (2002) recommended 8 µl of hybridisation buffer to 0.5 µl of any probe, (final probe concentration of 2.9 ng μl^{-1}). Therefore, both mixes were tested using a eubacterial probe labelled with general fluorescein (EUB338-Fam, Amann *et al.*, 1990a; Manz *et al*., 1992) at varying formamide concentrations (10, 20, 40 and 50%). The 8 µl of hybridisation buffer and 0.5 µl of the probe, final probe concentration of 2.9 ng μl^{-1} showed the best results and was therefore used for further studies (Figure B3 in *Appendix B*).

The hybridisation stringency conditions with the EUB338-Fam probe were tested using formamide concentrations between 10 and 50%. The most stringent conditions (50%) showed a similar signal as that obtained with the general DAPI stain (Figure 4.3) and was therefore selected as formamide

concentration for all further analyses. The absence of archaea within the inoculum was confirmed by the lack of hybridisation signal when FISH was performed with the ARCH915 probe (data not shown). The ARCH915 probe is a general archaeal probe designed to hybridise to a wide range of archaeal species (Stahl, 1991). The lack of archaea within the inoculum sample is possibly due to the addition of 3.2 g l^{-1} bromo-ethane-sulphonic acid (BESA), shown to inhibit methanogenic activity (Visser, 1995), during the enrichment of the lactate inoculum BSR CSTR (Section 3.3).

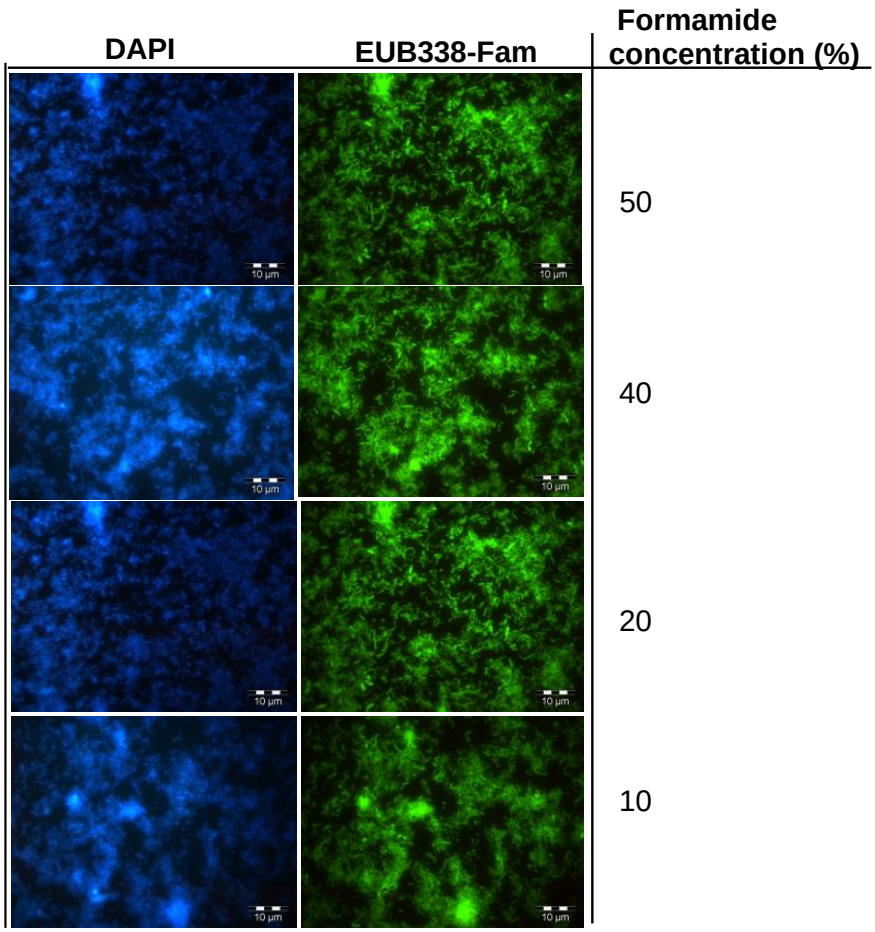


Figure 4.3. Epifluorescence micrographs of inoculum obtained from reactors receiving lactate as feed at sulphate (S_o) concentrations of 5.0 g l^{-1}(RT = 5 d). DAPI staining on the left column is compared FISH performed with general bacteria probe EUB338-Fam at a series of formamide concentrations on the right column (same microscopic field). Final probe concentration of 2.9 ng μl^{-1} was used.

In silico analyses of 16S rRNA gene sequences with high similarity to species belonging to known BSR genera: *Desulfomicrobium, Desulfovibrio, Desulfobulbus, Desulfocurvus* and *Firmicutes*, confirmed the presence of the sequence to which the DELTA495a (Loy *et al*., 2002) probe would hybridise (Figure B4 in *Appendix B*). This sequence was absent from 16S rRNA gene sequences that displayed homology to non-SRB. Therefore, the DELTA495a-Fam probe was used to verify the prevalence of SRB species amongst the microbial community in the inoculum. Hybridisation conditions for the DELTA495a probe

have been well documented and validated (Loy *et al*., 2002; Lücker *et al*., 2007; Macalady *et al.*, 2006). When compared to the DAPI stain, the FISH results indicated the presence of a large proportion of SRB cells in the inoculum (Figure 4.4A). These observations were in line with the 16S rRNA gene clone library results which suggested the abundance of SRB species (66% from ARDRA ribotype frequencies) in the inoculum sample.

To validate the prevalence of *Desulfomicrobium, Desulfovibrio* and *Desulfobulbus* in the inoculum, cells were stained with DAPI and FISH performed with the DSM213-Fam probe (Loy *et al*., 2007), DSV827-Fam probe (Dar *et al*., 2007) and SRB660-Fam probe (Devereux *et al*., 1992). Comparison of the micrographs of the DAPI stain and hybridisation probes confirmed the presence of these species within the inoculum (Figure 4.4). FISH with the DSM213-Fam probe was performed at 15% formamide concentration (Loy *et al*., 2007) while FISH with the DSV827-Fam and SRB660-Fam probes was performed at a 30% formamide concentration as suggested by literature (Dar e*t al*. 2007; Devereux *et al*. 1992) (Figure 4.4C). These observations from the FISH results indicated the presence of *Desulfomicrobium, Desulfovibrio* and *Desulfobulbus* species within the SRB inoculum.

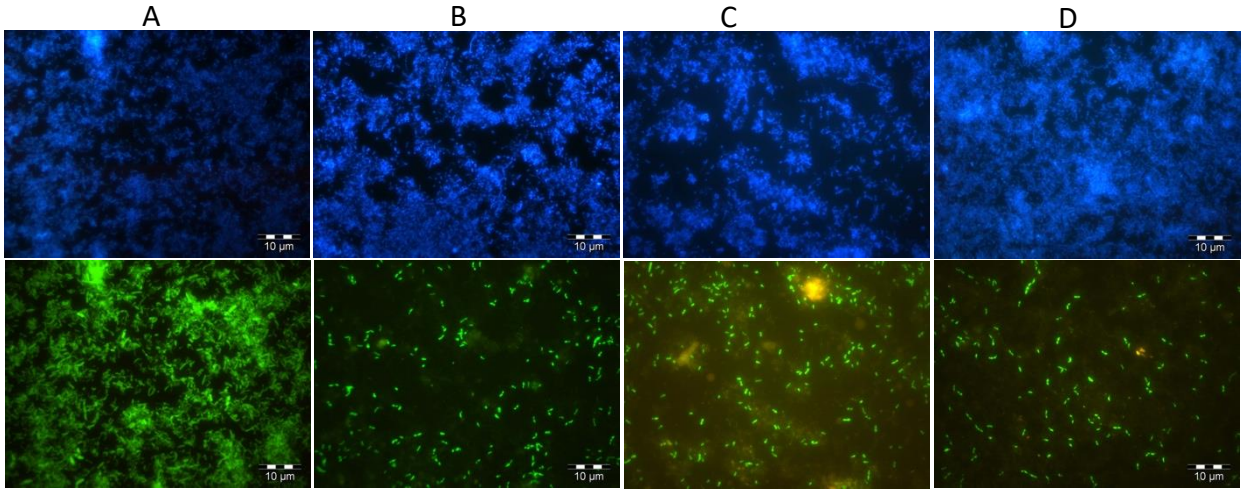
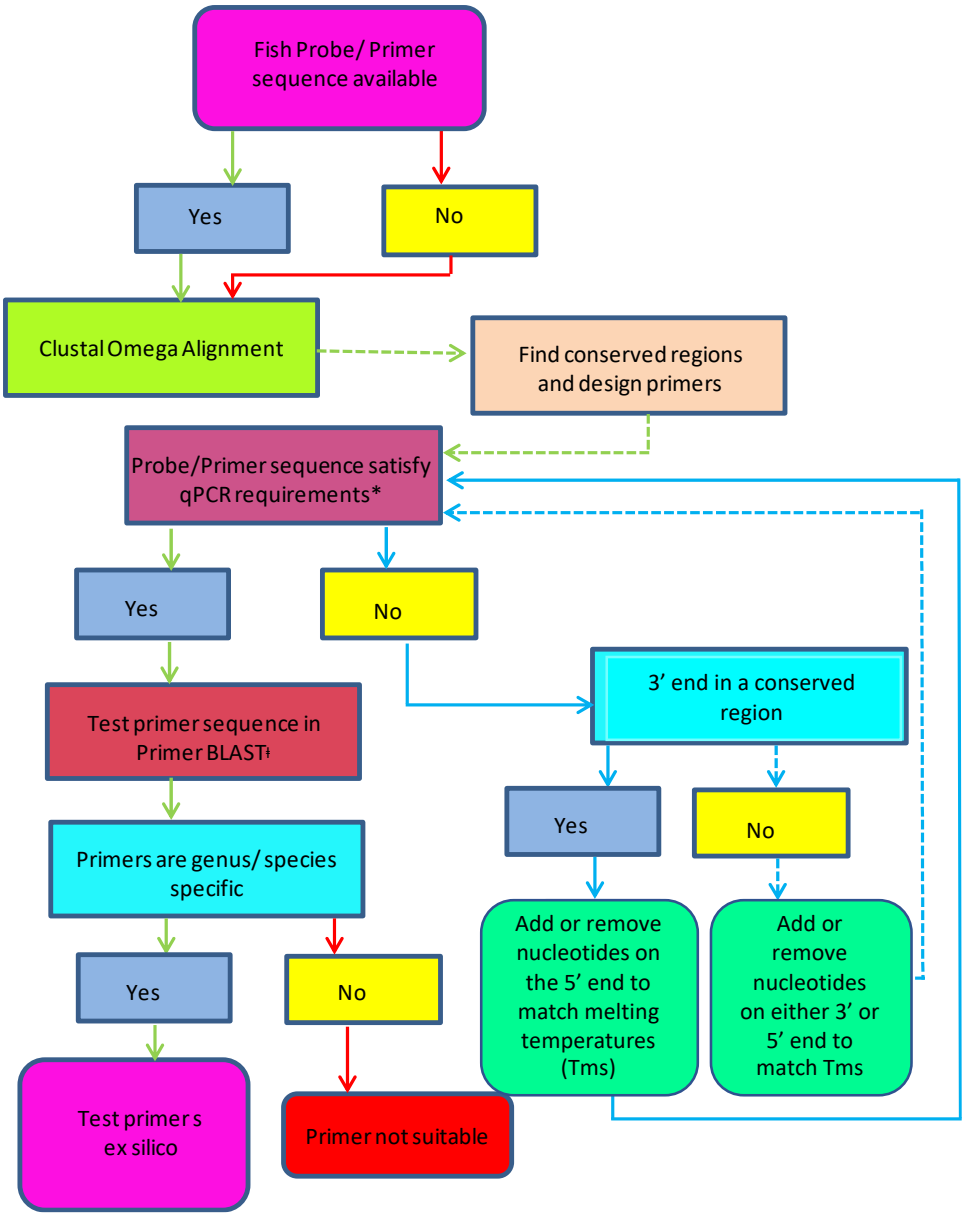


Figure 4.4. Epifluorescence micrographs of inoculum obtained from reactors receiving lactate as feed at sulphate (S_o) concentrations of 5.0 g l^{-1}(RT = 5 d). DAPI staining results (images in top row) were compared to FISH performed with SRB probe DELTA495a-Fam (A), *Desulfomicrobium* probe, DSM213-Fam (B), *Desulfovibrio* probe, DSV827-Fam, (C) and (D) *Desulfobulbus* probe, SRB660-Fam (images in bottom row).

4.3.3 qPCR primers design and testing

Quantitative real time PCR (qPCR) can be used as a quick assay method, utilising only small sample volumes, to assess the microbial community involved in remediation processes. However, an understanding of the expected microorganisms is required before this technique can be applied. Additionally, some knowledge of the role of specific species within the process is beneficial. To achieve this, 16S rRNA gene library preparation and phylogenetic analysis of the organisms present within the process, as applied in this study, or other metagenomic sequencing approaches is required before qPCR techniques can be designed and routinely employed. Diverse microbial communities have been described for BSR environments (Erasmus, 2000, Moosa *et al*., 2007; Oyekola *et al*., 2012), however not many qPCR primers have been reported for SRB. Therefore, in this thesis, qPCR primers were designed and chosen based on 16S rRNA gene sequence data obtained from the 16S rRNA gene clone library constructed from the metagenomic DNA obtained from the inoculum sample.

The design and testing of qPCR primers involved two steps. The first step was *in silico* testing whereby a multiple sequence alignment of 16S rRNA gene sequences from the 16S rRNA gene library, with sequence similarity to known SRB, was performed using Clustal Omega (version 1.2.1, https://www.ebi.ac.uk/Tools/msa/clustalo/). These alignments were used to identify regions of homology between these 16S rRNA gene sequences where genus and group specific primers could be designed. The second step involved performing *ex silico* qPCR experiments with the appropriate controls to validate the chosen primers. The approached taken in this thesis for the design of SRB species and genus specific qPCR primers is illustrated in Figure 4.5.



***** Bustin *et al*., 2009

Figure 4.5. A systematic approach for SRB genus specific primer design for qPCR.

4.3.3.1 Validation of total bacteria in SRB

The 16S rRNA genes representing all the bacteria present within the SRB inoculum sample was amplified using universal 16S rRNA gene primers. The universal primers, TotalF and TotalR, were taken from a study by Nadkarni *et al*. (2002). A later study by Horz *et al.* (2005) evaluated this primer pair in comparison to nine other previously published primer sets to assess the total bacterial load in clinical samples and found it provided the greatest coverage of the domain bacteria. This primer set provided 86% coverage for *Proteobacteria*, 81% coverage for *Firmicutes*, and 83% coverage for *Bacteroidete*s. This primer set was therefore tested as a universal total bacterial primer pair for this study. To test the suitability of the primers to 'capture' the majority of bacteria present within the SRB

inoculum, the corresponding sequences which would allow binding of both the TotalF and TotalR primers were investigated for the 16S rRNA gene clone library sequences obtained from the inoculum study.

In silico analyses revealed the presence of single nucleotide mismatches at the 3' end of the TotalF primer and the 16S rRNA gene sequences of 16S rRNA clones LB36 (99% similarity to *Sphaerochaeta associate*), LB44 (99% similarity to uncultured *Firmicutes* species) and LB71 (99% similarity to uncultured *Chloroflexi* species, AB853917.1). Similar mismatches were observed for 16S rRNA gene sequences from related organisms. For example, the mismatch observed between TotalF and the 16S rRNA gene sequence of LB39 (bacterial clone with 16S rRNA gene sequence with 99% similarity to *Sphaerochaeta globosa*, NR_114608.1) was also observed in the 16S rRNA gene sequence of LB36 (bacterial clone with 16S rRNA gene sequence with 99% similarity to *Sphaerochaeta associate*, NR_145842.1). Mismatches between 16S rRNA gene clone library sequences and the TotalF primer sequences are given in Table 4.2 and Figure B5 in *Appendix B*. Hence only one representative is shown (Table 4.2 and Figure B5 in *Appendix B*).

Table 4.2. Mismatches between 16S rRNA gene clone library sequences and the TotalF primer sequences. Mismatches are highlighted in red text and underlined.

Name	**Sequence (5 to 3')**	**Closest match**
TotalF	TCCTACGGGAGGCAGCAGT	
LB36	TCCTACGGGAGGCAGCAG**C**	*Sphaerochaeta associate* (NR_145842.1)
LB44	TCCTACGGG**G**GGCAGCAGT	Uncultured *Firmicutes* sp. (CU922072.1)
LB71	TCCTACGGG**T**GGCAGCAGT	uncultured *Chloroflexi* sp. (AB853917.1)

In the case of the reverse primer (TotalR), *in silico* analysis revealed the presence of two mismatches in the 16S rRNA gene sequence of LB7 (bacterial clone with 16S rRNA gene sequence with 99% similarity to uncultured *Synergistetes* species, JX575962.1) and 16S rRNA sequence of LB36 (bacterial clone with 16S rRNA gene sequence with 99% similarity to *Sphaerochaeta associate*, NR_145842.1). Three mismatches between TotalR and 16S rRNA gene sequence of LB71 (the bacterial clone with 16S rRNA gene sequence with 99% similarity to uncultured *Chloroflexi* species, AB853917.1) were observed. Mismatches between 16S rRNA gene clone library sequences and the TotalR primer sequence are given in Table 4.3 and Figure B6 in *Appendix B*.

Table 4.3. Mismatches between 16S rRNA gene clone library sequences and the TotalR primer sequences. Mismatches are highlighted in red text and underlined.

Name	Sequence (5 to 3')	Closest match
TotalR	GGACTACCAGGGTATCTAATCCTGTT	
LB7	GGACTACC**G**GGGTATCTAATCCTGTT	uncultured *Synergistetes* sp. (JX575962.1)
LB36	GGACTACC**T**GGGTATCTAATCC**A**GTT	*Sphaerochaeta associate* (NR_145842.1)
LB71	GGACTACC**C**GGGT**G**TCTAATCC**G**GTT	uncultured *Chloroflexi* sp. (AB853917.1)

To determine the suitability of the Total Bacterial primers (TotalF and TotalR) to amplify the 16S rRNA gene sequences from the bacterial consortium represented in the inoculum, a qPCR reaction was performed using plasmid DNA isolated from the 16S rRNA of the 23 bacterial clones highlighted with asterisks in Table 4.1. These bacterial clones represented one member from each genus 'captured' in the 16S rRNA gene clone library. A plasmid containing the archaeal 16S rRNA gene of the acidophile *Acidiplasma cupricumulans* was included as a negative control. Mismatches between the 16S rRNA gene sequence of *A. cupricumulans* and the total bacterial primer pair, TotalF and TotalR, are indicated in Table 4.4. While only two nucleotide mismatches were observed between the 16S rRNA gene sequence of *A. cupricumulans* and the TotalR primer sequence, several nucleotide mismatches were observed (both at the 3' end and 5' end) between the 16S rRNA gene sequence of *A. cupricumulans* and the TotalF primer sequence (Table 4.4).

Table 4.4. Mismatches between the 16S rRNA gene sequence of the archaeal species *Acidiplasma cupricumulans* and universal primers, TotalF and TotalR. Mismatches are highlighted in red text and underlined.

Sequence name	Sequence (5 to 3')
TotalF	TCCTACGGGAGGCAGCAGT
A. cupricumulans	**GGCCCT**GGGAGG**GGTAGCC**
TotalR	GGACTACCAGGGTATCTAATCCTGTT
A. cupricumulans	GGACTACC**C**GGGTATCTAATCC**G**GTT

Despite the observed mismatches in the 16S rRNA gene sequences of LB7, LB44 and LB71, the 16S rRNA genes from these bacterial clones were successfully amplified. Also, the level of detection with this primer set was equally efficient at amplifying the different 16S rRNA clones, when they are applied as single templates in individual qPCR reactions. However, the 16S rRNA gene sequence from the plasmid DNA of the bacterial clone that showed 99% similarity to *Sphaerochaeta associate*, NR_145842.1 (LB36) and the 16S rRNA gene sequence from the plasmid DNA of *A. cupricumulans* were not amplified (Figure 4.6). Both the 16S rRNA gene sequences from these clones exhibited

nucleotide mismatches at the primer binding site, especially the 3' end (Tables 4.3 and 4.4), known to result in a decreased thermal stability of the primer-template duplex which could potentially lead to no amplification (Klein *et al.*, 2001; Whiley and Sloots 2005; Stadhouders *et al.*, 2010).

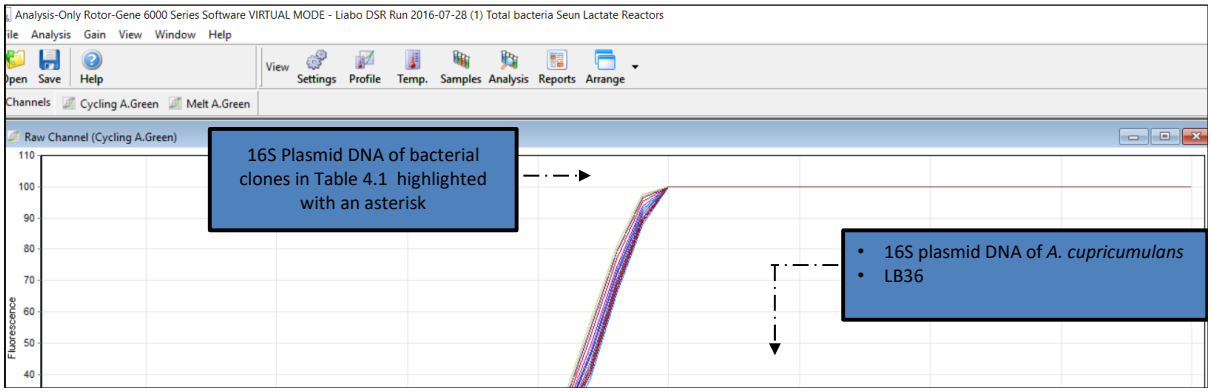


Figure 4.6. qPCR fluorescence curves generated using the total bacterial primers (TotalF and TotalR) and representative 16S rRNA plasmid clones from the clone library. A plasmid containing the 16S rRNA gene from the archaeal acidophile *Acidiplasma cupricumulans* was used as negative plasmid control.

ARDRA results suggested that the percentage of the 16S rRNA gene sequences with high similarity to *Sphaerochaeta* species represented 4% of the bacterial community associated with the inoculum sample (Figure 4.2 B). Therefore, although the TotalF and TotalR primers were selected to be used as universal bacterial primers for qPCR analysis in this study, it must be kept in mind that a small percentage of bacterial groups (such as the *Sphaerochaeta* species) may not be "captured".

4.3.3.2 Validation of *Desulfovibrio* genus specific primers

A previously published *Desulfovibrio* genus specific primer set was evaluated for use in this study (Stubner, 2004). The forward and reverse primers, renamed DSV-III-312f[b] and DSV618R[b] (Stubner, 2004), were modified following *in silico* analyses. The 16S rRNA gene sequences that had high similarity to *Desulfovibrio* species, revealed by BLASTn analysis, were used to validate the suitability of the primer set. These included the 16S rRNA plasmids from bacterial clones LB5 (99% similarity to *Desulfovibrio aminophilus*, NR_024916.1), LB9 (97% similarity to *Desulfovibrio salexigens*, NR_024916.1) and LB22 (99% high similarity to both *Desulfovibrio desulfuricans*, EU980606.1).

Similar to the total bacterial primer set, the validation of the *Desulfovibrio* primers was performed using both *in silico* analyses and qPCR assays using the appropriate controls. *In silico* studies involved performing a ClustalX alignment of the 16S rRNA gene sequences of the all the bacterial clones that showed high similarity with *Desulfovibrio* species [(LB5, LB9 and LB22, (Table 4.1)] and the forward

primer (DSV-III-312f[b]) and reverse primer (DSV681R[b]) respectively. The alignment results prompted the modification of the forward primer from Stubner (2004) by adding one nucleotide (C) on the 5' end of the primer. This resulted in the new primer sequence DSV-III-312f[b] 5'-CCACACTGGGACTGGAACAC- 3' with a melting temperature (T_m) of 58.1°C. No nucleotide mismatches between the 16S rRNA sequences of LB5 and LB9 and the forward primers were observed, however, two mismatches were observed between the primer and the 16S rRNA sequence of LB22 (Table 4.5 and Figure B7 in *Appendix B*).

Table 4.5. Mismatches between 16S rRNA gene clone library sequence of LB22 and the DSV-III-312f[b] primer sequences. Mismatches are highlighted in red text and underlined.

Sequence name	Sequence (3' to 5')
DSV-III-312f[b]	CCACACTGGGACTGGAACAC
LB22	CCACACTGG**A**ACTG**A**AACAC

The reverse primer DSV681R[b], was generated by adding the 5'- TCCGTAGATATCT -3' sequence towards the 3' end of DSVIB679r [5'-AGGTGTAGGAGTGAAA-3' (Stubner, 2004)]. The resulting primer, DSV681R[b] (5'– AGGTGTAGGAGTGAAATCCGTAGATATCT -3) had a T_m of 58.1°C, thus matching the forward primer. The multiple sequence alignment of the resultant reverse primer (DSV681R[b]), with the 16S rRNA gene sequences that showed high similarity with *Desulfovibrio* species (LB5, LB9 and LB22) and the 16S rRNA gene sequences of 23 bacteria clones highlighted with asterisks in Table 4.1 indicated that this modification resulted in the increased selectivity of the primer to members of the *Desulfovibrio* genus and allowed detection of all the *Desulfovibrio* species captured in the 16S rRNA gene clone library. No nucleotide mismatches were observed between the resultant reverse primer (DSV681R[b]) and the 16S rRNA gene sequences exhibiting high homology to *Desulfovibrio* species. All other 16S rRNA gene sequences from bacterial clones that did not show high similarity to *Desulfovibrio* species exhibited nucleotide mismatches at the primer binding site, including the 3' end (Figure B8 in *Appendix B*).

qPCR reactions were performed to experimentally validate the specificity of the *Desulfovibrio* primer set using the purified 16S plasmid DNA from bacterial clones LB5 (99% similarity to *Desulfovibrio aminophilus*, NR_024916.1) and LB22 (99% similarity *Desulfovibrio desulfuricans*, EU980606.1). The 16S rRNA plasmid DNA from the 20 clones highlighted with asterisks in Table 4.1 were included as negative controls. The results indicated that only plasmid DNA from LB5 and LB22 could be amplified when DSV-III-312f[b] and DSV681R[b] primers were used, and the detection of LB22 was equally efficient to that of LB5. This demonstrated that the primer set was specific to *Desulfovibrio* species and was able to tolerate the discrepancies in the 16S rRNA genes of different *Desulfovibrio* organisms under the

qPCR conditions applied (Figure 4.7). Therefore, these primers were selected to be used for qPCR analysis.

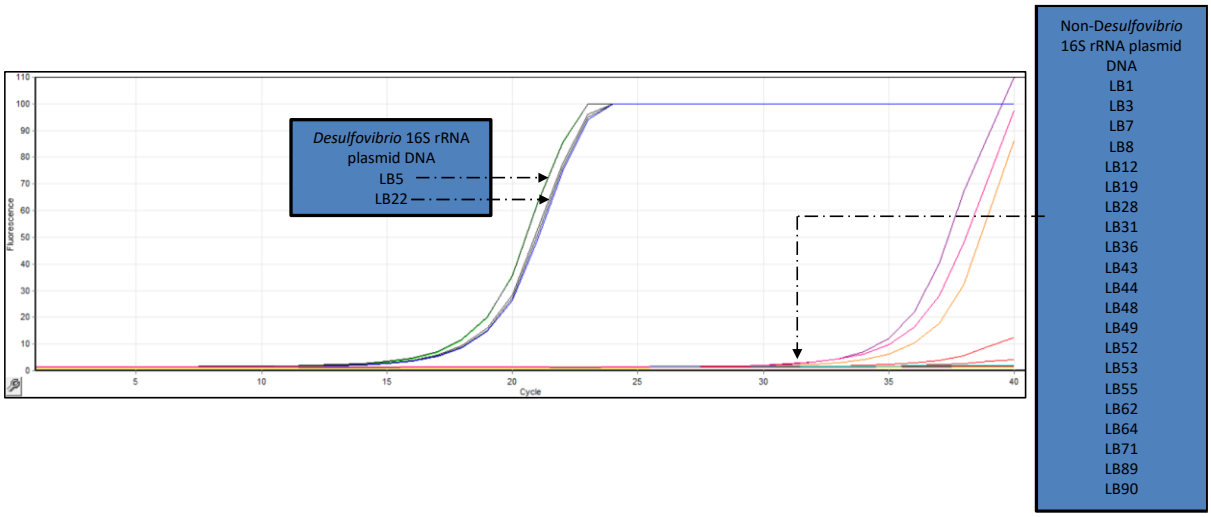


Figure 4.7. qPCR fluorescence curves generated using the *Desulfovibrio* primers (DSV-III-312f[b] and DSV681R[b]). 16S rRNA gene plasmid clones with high similarity with *Desulfovibrio* species (LB5 and LB22) and 16S rRNA gene plasmid clones representing unrelated species from the clone library was included as negative controls.

4.3.3.3 Validation of *Desulfomicrobium* genus specific primer

For the quantification of the 16S rRNA genes representing members of the genus *Desulfomicrobium*, a novel primer set DSM442F (5'- GGCATTGGTCTAATAGGCCTTTGTT -3') and DSM632R (5'- TGGGATTTCACCCCTGACTTACAA -3') was designed. Primer annealing positions were identified following a multiple sequence alignment of the 16S rRNA gene sequences with high similarity to *Desulfomicrobium* species and other 16S rRNA gene sequences captured in the clone library constructed from the inoculum sample. Regions of dissimilarity to other SRBs were identified and these were included in the 3' region of the primer sequences to ensure selective amplification of the *Desulfomicrobium* species. *In silico* studies revealed that no mismatches were observed for the 16S rRNA gene sequences of LB1, LB3, LB19, LB25, LB33, LB43, LB50, LB53, LB57 and LB64 which are the 16S rRNA sequences with high similarity to *Desulfomicrobium* species, and the forward primer, DSM442F. However, up to 11 mismatches were observed between the DSM442F sequence and the primer binding site on the 16S rRNA gene sequence of LB89 (93% similarity to *Desulfomicrobium apsheronum*) (Table 4.6 and Figure B9 in *Appendix B*). The phylogenetic analysis of the 16S rRNA gene sequence of LB89 also revealed that it formed an outlier within the *Desulfomicrobium* clade (Figure 4.2). A multiple sequence alignment between the 16S rRNA sequences and the reverse primer, DSM623R revealed that no mismatches were observed for the 16S rRNA sequence with high similarity to *Desulfomicrobium* species (LB1, LB3, LB19, LB25, LB33, LB43, LB50, LB53, LB57 and LB64)

while one mismatch was shown between the DSM623R and 16S rRNA gene sequence of LB89. Up to 12 mismatches were observed between the reverse primer and other non-*Desulfomicrobium* related 16S rRNA sequences captured in the clone library (Table 4.6 and Figure B10 in *Appendix B*).

Table 4.6. Mismatches between 16S rRNA gene clone library sequences and the *Desulfomicrobium* primers, DSM442F and DSM632R. Mismatches are highlighted in red and underlined.

Name	**Sequence (5' to 3')**	**Closest match**
DSM442F	GGCATTGGTCTAATAGGCCTTTGTT	
LB89	GG**T**ATT**A**GTC**CT**ATAGGC**ACCGAAG**	*Desulfomicrobium apsheronum* (AF228136.2)
DSM632R	TGGGATTTCACCCCTGACTTACAA	
LB89	TGGG**G**TTTCACCCCTGACTTACAA	*Desulfomicrobium apsheronum* (AF228136.2)

Experimental validation of the suitability of the DSM primer pair for the quantification of the *Desulfomicrobium* species present within the BSR community was performed. qPCR reactions with purified 16S plasmid DNA of LB1, LB3, LB19, LB43, LB53, LB64 and LB89 (high similarity to *Desulfomicrobium* species) and of the 13 bacterial clones highlighted in Table 4.1 with asterisks (LB5, LB7, LB8, LB12, LB22, LB28, LB31, LB36, LB44, LB48, LB49, LB52, LB55, LB62, LB71 and LB90) indicated that the primer set (DSM442F and DSM623R) could only amplify *Desulfomicrobium* species. However, this primer set was not equally efficient in detecting the 16S plasmid DNA of LB89 (Figure 4.8). This could be due to the high dissimilarity observed between the 16S rRNA gene sequence and the forward primer sequence including the 3' end (Table 4.6 and Figure B9 in *Appendix B*). Also, this sequence was shown to have only 93% similarity to its closest match (*Desulfomicrobium apsheronum*) in the BLASTn database (Table 4.1) and was a clear outlier to the other members of the *Desulfomicrobium* clade (Figure 4.2A). The sequence captured in LB89 may be an anomaly possibly introduced through amplification and cloning of the 16S rRNA gene (Dean *et al*., 2002; Ashelford *et al*., 2006; Haas *et al.*, 2011). None of the other SRB [*Desulfovibrio* (LB5 and LB22), *Desulfocurvus* (LB62), *Desulfuromonas* (LB49)] *Firmicutes* (LBLB44 and LB48) and non-SRB (LB7, LB8, LB12,LB28, LB31, LB36, LB52, LB55, LB71 and LB90) was amplified, indicating that this primer set was specific to the *Desulfomicrobium* genus.

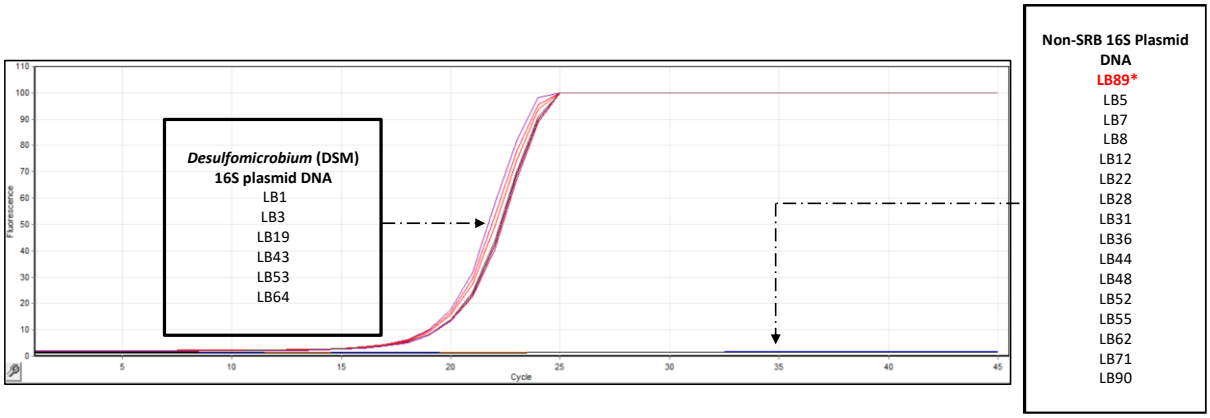


Figure 4.8. qPCR fluorescence curves generated using the *Desulfomicrobium* primers (DSM442F and DSM632R), 16S rRNA gene plasmid clones with high similarity to *Desulfomicrobium* species (LB1, LB3, LB19, LB43, LB53, LB64 and LB89) and 16S rRNA gene plasmid clones from the clone library representing non-*Desulfomicrobium* species used as negative controls. The 16S rRNA sequence of plasmid DNA from LB89 (highlighted in red) was not amplified by the primer pair.

4.3.3.4 Validation of *dsr* gene primers

The primer pair selected for the qPCR amplification of the *dsrAB* gene was DSR4R (5'- GTGTAG CAGTTACCGCA- 3'), and DSRp2060F (5'- CAACATCGTYCAYACCCAGGG -'3) (Geets *et al*., 2005) as suggested and detailed by Müller *et al.* (2015). The use of this primer pair has successfully been applied to quantify SRB in diverse microbial community associated with oil fields (Callbeck *et al*., 2013; Priha *et al.*, 2013), industrial anaerobic biogas digesters (Moestedt *et al*., 2013), paddy soil (Liu *et al*., 2009), and mature fine tailings (Liu *et al*., 2015).

The *dsr* gene was amplified from gDNA isolated from the inoculum sample and the resultant 378 bp amplicon cloned into the pJET1.2 vector system and used to transform $CaCl_2$ competent *E. coli* cells. Plasmids from three randomly selected clones were subjected to sequencing and the BLASTn results indicated that the *dsr* gene sequence from all three clones were similar and showed 99% homology to the *dsrB* gene (KC466047.1) from an uncultured bacterium (Table 4.8). The sequences also shared percentage identity with *Desulfobaculum xiamenense* with 98% homology. One of these clones was used to validate the cycling conditions for the *dsrAB* primer set and was also used as a standard with known copy number. Experimental validation of the suitability of the *dsr* gene primer pair for the quantification of the SRB species present within the BSR community was performed. qPCR reaction with A pJET1.2 plasmid harbouring the *dsr* gene from Clone 1 (Table 4.8) indicated that the primer set (DSRp2060F and DSR4R) could successfully amplify SRB species within the inoculum reactor (Figure 4.8). Therefore, the DSRp2060F and DSR4R primer pair was selected to be used for further qPCR analysis.

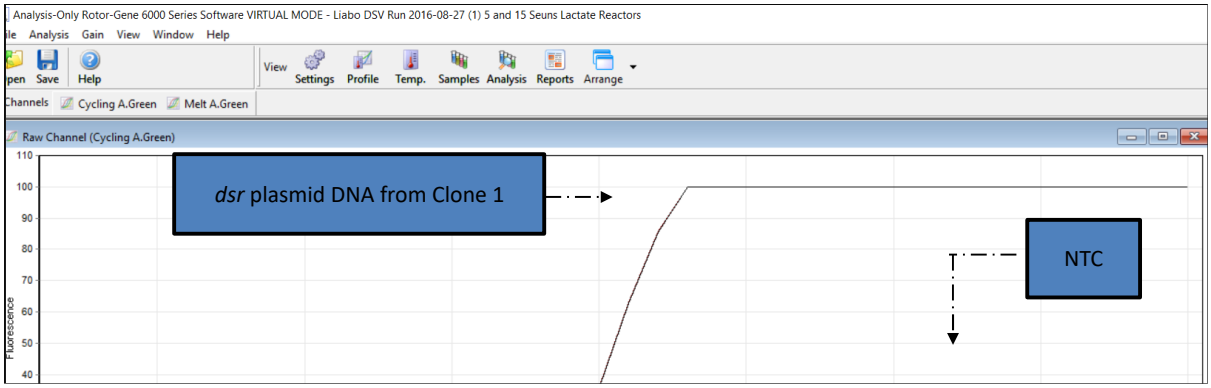


Figure 4.9 qPCR fluorescence curves generated using the *dsr* primers (DSRp2060F and DSR4R) and *dsr* plasmid DNA of clones from the clone library

4.4 GENERAL DISCUSSION

The DELTA495a-Fam probe, which targets 16S rRNA gene sequences with high homology SRB within the inoculum, showed a clear hybridisation signal. Similar amount of fluorescence was observed from the DAPI stain and the DELTA495a-Fam probe, suggesting that the SRB species were abundant in the sample used as an inoculum for this study. The presence of *Desulfomicrobium*, *Desulfovibrio* and *Desulfobulbus* species within the inoculum confirmed by FISH with DSM213-Fam, DSV827-Fam and SRB660-Fam probes, respectively was in line with the observations from ARDRA and the phylogenetic tree which suggested that the prevalence of these species within the inoculum. The 16S rRNA gene sequences of bacterial clones that showed high similarity to *Desulfomicrobium* species (LB1, LB3, LB19, LB25, LB33, LB43, LB50, LB53, LB57 and LB64), with the exception of LB89, showed no nucleotide mismatches with the novel *Desulfomicrobium* primer set (DSM442F and DSM632R) designed for use in this study, possibly due to anomalies introduced through PCR amplification and cloning. Similarly, no mismatches were observed between these sequences and the SRB probe, DELTA495a and the *Desulfomicrobium* probe, DSM213.

No nucleotide mismatches were observed between the 16S rRNA gene sequences of species that showed high similarity to *Desulfovibrio* species (LB5 and LB9) and the DSV827 probe. One mismatch was observed between the 16S rRNA sequence of LB22 and the DSV827 probe. Ideally, FISH probes should hybridise with fully complementary RNA or DNA sequences and fail to hybridise with sequences that contain one or more mismatches (Yan *et al*., 2012; Silvia *et al*., 2015). However, in the current study, this was not validated for the16S rRNA sequence of LB22. By modifying previously published *Desulfovibrio* primers, all the *Desulfovibrio* species represented in the 16S rRNA gene clone library were captured. The *dsr* primers allows for the quantification of the total BSR potential of the microbial community.

4.5 CONCLUSION

This chapter describes the validation and optimisation of molecular tools to characterise the mixed microbial community associated with BSR systems. A 16S rRNA gene survey performed by constructing a 16S rRNA gene clone library and analysis of the diversity of clones by ARDRA, revealed a more diverse microbial community in the inoculum than previously reported. The microbial community comprised SRB such as members of the genera *Desulfomicrobium*, *Desulfovibrio* and *Desulfobulbus* and non-SRB groups. A phylogenetic analysis revealed that the *Desulfomicrobium* group of SRB were the most diverse of the SRB groups represented in the bacterial community. Conditions for the improved specificity of previously reported FISH probes are reported and can be applied to allow the visualisation of the eubacteria, total SRB and individual SRB genera, *Desulfobulbus*, *Desulfomicrobium* and *Desulfovibrio* present within BSR systems. This chapter describes the design, validation and modification of primer pairs for the selective amplification of two of the key genera involved in BSR, *Desulfomicrobium* and *Desulfovibrio*. By utilising these primers together with the total bacterial primers and *dsrAB* primers, a more holistic view of changes in species abundance can be obtained using a quantitative and rapid assay, qPCR. These methods were applied to report the changes in the BSR community operated on anaerobic digestate as sole carbon source and electron donor in response to changes in the operating conditions such as retention time and sulphate loading. The kinetics of these reactors are reported in Chapter 5, while the microbial community analyses in relation to the results of Chapter 5 is reported in Chapter 6.

CHAPTER 5
KINETIC STUDY OF BIOLOGICAL SULPHATE REDUCTION USING ANAEROBIC DIGESTATE AS A SOLE CARBON SOURCE

5.1 INTRODUCTION

The optimisation of the sulphate reduction process includes finding a carbon source and electron donor that can sustain biological sulphate reduction (BSR) long term and whose availability is not limited to industrial activity of the region (Rose *et al*., 1998). Previous studies used acetate (Moosa, 2000; Moosa *et al*., 2002; 2005; Moosa and Harrison, 2006) and ethanol (Erasmus, 2000; Hansford *et al*., 2007) to demonstrate that BSR reaction kinetics are influenced by feed sulphate concentration, biomass concentration, residence time (RT), residual sulphate concentration and its volumetric loading and to describe these relationships kinetically. The study with lactate as a sole carbon source and electron donor for BSR by Oyekola (2008) and Oyekola *et al*. (2009; 2010; 2012) built on the acetate and ethanol work, and supported the above observations showing differing kinetics and sensitivity to operating conditions as a function of carbon source and electron donor. In the current thesis, the kinetic studies were extended to the use of anaerobic digestate from partial anaerobic digestion of the cyanobacterial species *Arthrospira platensis*, commonly known as Spirulina (hereafter referred to as anaerobic digestate) as the sole carbon source and electron donor for biological sulphate reduction (BSR). This anaerobic digestate contains a mixture of acetate, propionate and butyrate and is described in Section 2.5.1.

As discussed in Section 2.5 microalgae and the cyanobacterial species *Arthrospira platensis* have the potential for on-site cultivation, possibly by using existing water bodies or treated effluent as the basis for the media. This enhances cost-effectiveness (Boshoff *et al*., 1996; Rose *et al*. 1998). The research conducted by Inglesby (2011) on the anaerobic digestion of the cyanobacterial species indicated that anaerobic digestate had a high carbon oxygen demand (COD), primarily due to volatile fatty acids (VFAs). Furthermore, it was determined that the major VFAs in this anaerobic digestate are acetate, propionate and butyrate, suggesting that this anaerobic digestate can serve as an ideal substrate for BSR, and remediation could be implemented in remote locations (Inglesby *et al*., 2015). This is of particular importance, owing to the significant contribution of transportation of the carbon source and electron donor to the mine site (Gopal, 2005). It is against this background that the investigation of anaerobic digestate as a potential cost-effective carbon source and electron donor for BSR is proposed, based on the potential to be cost-effective and readily available. In this Chapter, the potential for anaerobic digestate formed from cyanobacterial anaerobic digestate is explored, with particular focus on the performance of the sulphate reducing bacteria (SRB) reactors and associated process kinetics.

The specific objectives of the kinetic study were as follows:

i. To investigate anaerobic digestate (a mix of propionate, butyrate and acetate) as a carbon source for BSR, in terms of process performance and kinetics.
ii. To investigate the effect of sulphate concentration, volumetric sulphate loading rate and residence time on the kinetics of anaerobic BSR when anaerobic digestate of cyanobacterial biomass is used as a sole carbon source and electron donor for a mixed SRB culture.

It is hypothesised that, due to the high VFA profile of anaerobic digestate from cyanobacterial biomass (with major VFAs identified as acetate, propionate and butyrate) supporting a wide spectrum of SRB, a high sulphate reduction rate and high sulphate conversion would be observed. Since different SRB prefer different carbon sources and electron donors, the present VFA will be utilised according to the SRB species present within the bioreactors. It is further hypothesised that the kinetics expressions used to describe BSR using acetate, ethanol or lactate as a carbon source and electron donor are applicable with anaerobic digestate.

5.2 REACTOR METADATA AND APPROACH TO DATA ANALYSIS

Three BSR reactors were operated anaerobically as CSTRs using suspension culture reported in Section 4.3.1. On set-up, these reactors were inoculated from a BSR reactor supplemented with lactate as a carbon source and electron donor and operated at a residence time (RT) of 5 days with feed sulphate of 1.0 g l^{-1}. The lactate reactor had been shown to be robust and able to recover after major perturbations which was attributed to high microbial diversity (Oyekola *et al.*, 2012). Anaerobic digestate was introduced continuously as a carbon source and electron donor to provide the stoichiometric carbon requirement (Figure 5.1).

The three laboratory scale chemostat cultures of 1 litre supplemented with the new carbon source and electron donor (anaerobic digestate) were operated at RTs of 0.5, 1, 1.5, 2, 3, 4, and 5 days and feed sulphate concentrations of 1.0, 2.5 and 5.0 g l^{-1} as described in Section 3.4. The steady-state at a RT of 5 days was maintained for five retention times before reducing the RT to 4 days. This allowed for culture adaptation to higher sulphate loading rates. The reactors were operated at 35 °C and pH 8 ± 0.2. The kinetic data (Figure 5.1) were collected and analysed according to the stoichiometric reactions in Table 5.1. A schematic experimental approach is give in Figure 5.2. The detailed experimental approach and analytical methods used are provided in Sections 3.2 to 3.5.

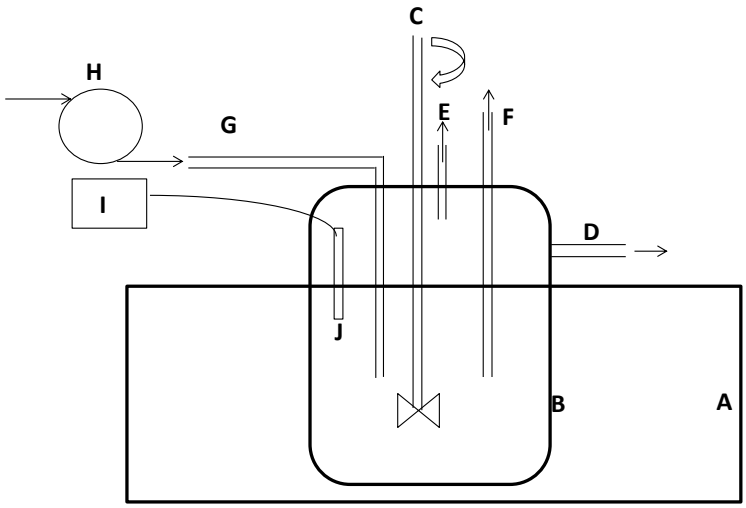


Figure 5.1 Schematic diagram of experimental set-up: (A) thermoregulated waterbath; (B) anaerobic bioreactor; (C) overhead stirrer; (D) overflow port; (E) gas vent; (F) sampling port; (G) feed inlet; (H) feed pump; (I) pH meter; (J) pH probe.

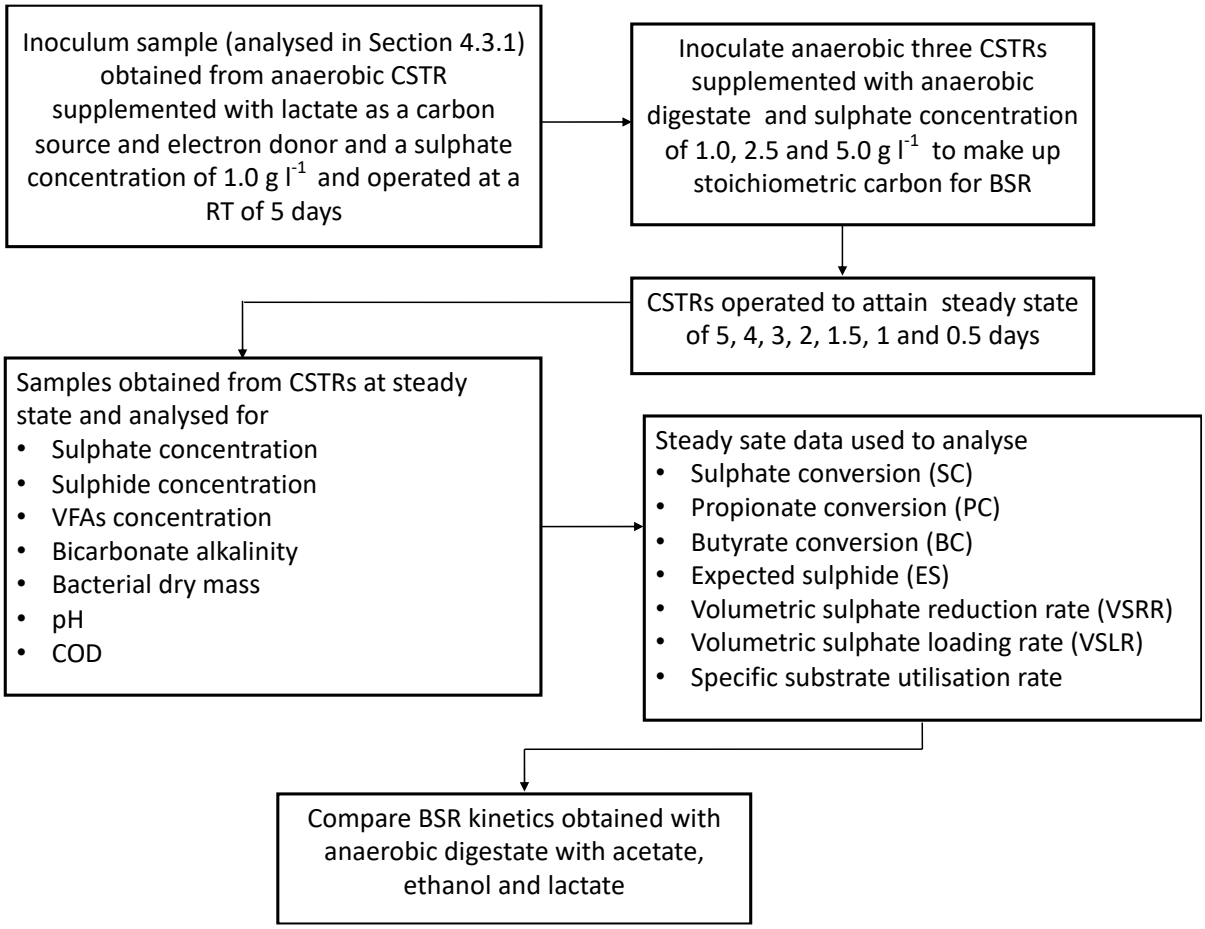


Figure 5.2.Schematic diagram illustrating approach undertaken in the kinetics of the BSR.

Table 5.1. Reactions and free-energy changes for reactions involving anaerobic metabolism of acetate, propionate and butyrate (From Oude Elferink *et al.*, 1998 and Muyzer and Stams, 2008).

VFA Metabolic pathway	Reactions	$\Delta G^{0'}$ (kJ/Reaction)	Reaction number
Acetate reactions			
Complete oxidation	$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-47.6	2.20
Generation of methane	$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-151.9	2.28
Propionate reactions			
Incomplete oxidation	$Propionate^- + 0.75SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.75HS^- + 0.25H^+$	-37.7	2.21
Complete oxidation	$Propionate^- + 1.75SO_4^{2-} \rightarrow 3HCO_3^- + 1.75HS^- + 0.5H^+ + 0.25OH^-$	-88.9	5.1
Acetogenesis	$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+76.1	2.24
	$Propionate^- + 2HCO_3^- \rightarrow Acetate^- + 3HCO_2^- + H^+$		5.2
Butyrate reactions			
Incomplete oxidation	$Butyrate^- + 0.5SO_4^{2-} \rightarrow 2Acetate^- + 0.5HS^- + 0.5H^+$	-27.8	2.22
	$Butyrate^- + 1.5SO_4^{2-} \rightarrow Acetate^- + 2HCO_3^- + 1.5HS^- + 0.5H^+$		5.3
Complete oxidation	$Butyrate^- + 2.5SO_4^{2-} + 0.25H_2O \rightarrow 4HCO_3^- + 2.5HS^- + 0.75H^+ + 0.25OH^-$		5.4
Acetogenesis	$Butyrate^- + 2H_2O \rightarrow Acetate^- + H^+ + 2H_2$	+48.3	2.25
	$Butyrate^- + 2HCO_3^- \rightarrow Acetate^- + 2HCO_2^- + H^+$		5.5

5.3 DATA COLLECTION AND HANDLING

5.3.1 Approach to sampling

Analytical measurements were carried out in triplicate from samples obtained when each bioreactor reached a steady state at the specified operating conditions (Section 3.5). Steady-state operation was confirmed following three residence times (RTs) whereafter a minimum of three readings were taken during proposed steady state operation and confirmed when residual sulphate and bacterial concentrations yielded measurements were within a 10% deviation. All raw data obtained in these experiments are given in *Appendix C*.

5.3.2 Overview of time course data for the CSTR receiving sulphate concentration of 1.0 g l^{-1}

The experiment with the reactor receiving anaerobic digestate as carbon source and electron donor and a sulphate concentration of 1.0 g l^{-1} was carried out for 131 days. Sulphate concentration, bacterial dry mass concentration and dissolved sulphide concentration were measured at least every second day. When bacterial dry mass concentration and sulphate concentration varied by <10%, it was assumed that steady-state conditions were achieved in the reactor. Samples used to analyse data for 5 d residence time (RT) were collected between day 21 and day 45. Samples for RTs of 4, 3, 2, 1.5, 1 and 0.5 d were collected from day 64 to 79, 92 to 100, 107 to 112, 118 to 122, 125 to 127 and 131 to 133, respectively (Figure 5.3).

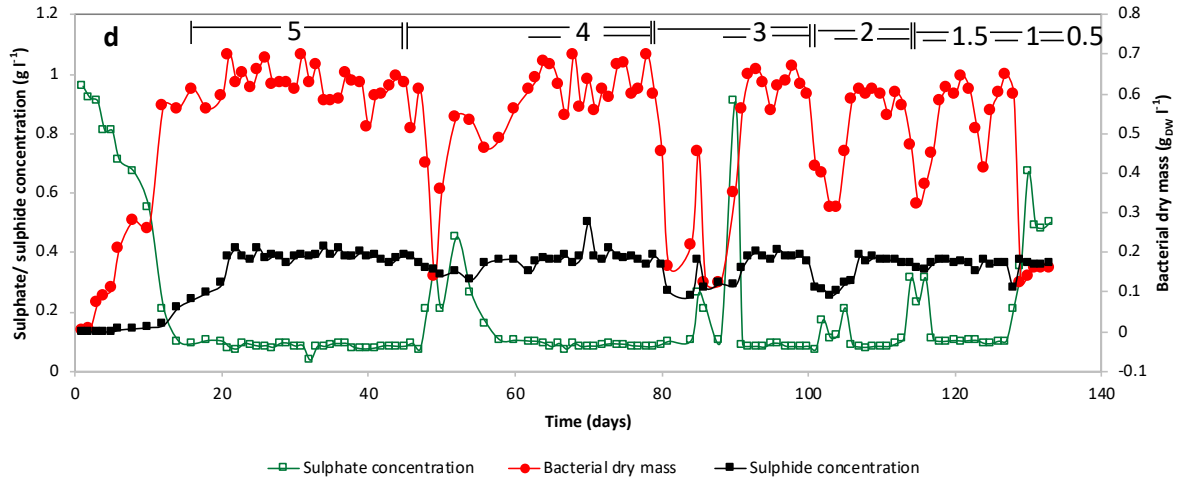


Figure 5.3. Time course data for the CSTR receiving anaerobic digestate at a feed sulphate concentration of 1.0 g l^{-1} The sulphate concentration, dissolved sulphide concentration and bacterial dry mass (g_{DW} l^{-1}) were monitored over a period of 133 days (d) indicating where steady-state was achieved with double lines (=).

5.3.3 Data handling and calculations

Sulphate conversion (SC), reported as a percentage (%), was calculated using Equation 5.1

$$SC = \frac{S_0 - S}{S_0}\ 100 \qquad \text{Equation 5.1}$$

where S_0 and S represent the feed and residual sulphate concentration (g l^{-1}) respectively. Propionate and butyrate conversions were calculated in a similar manner.

Expected sulphide concentration (ES), reported in g l^{-1}, was calculated based on the amount of sulphate removed. Molecular weights of sulphur, HS^- and SO_4^{2-} are 32, 33 and 96 g mol^{-1}, respectively. Because ES is equivalent to hydrogen sulphide formed, ES was calculated according to Equation 5.2

$$ES = (S_0 - S)\left(\frac{32}{96} * \frac{33}{32}\right) = (S_0 - S)\frac{33}{96} \qquad \text{Equation 5.2}$$

where S_0 and S represent the feed and residual sulphate concentration (g l^{-1}) respectively.

Volumetric sulphate reduction rate (VSRR= r_s), reported in g l^{-1} h^{-1}, was calculated based on the initial sulphate concentration (S_0), residual sulphate concentration (S), the feed flowrate (F) (l h^{-1}) and the reactor working volume (V) (l). At steady state, $\frac{F}{V}$ is equivalent to the dilution rate (D) (h^{-1}) of the medium in the bioreactor. The relationship leading to VSRR is shown by Equation 5.3.

$$VSRR = r_s = (S_0 - S) * \frac{F}{V}$$

$$= (S_0 - S) * D \qquad \text{Equation 5.3}$$

Volumetric sulphate loading rate (VSLR), reported in g l^{-1} h^{-1}, represented the load of sulphate fed into the reactor and was calculated as the product of the feed sulphate concentration (S_0) and the dilution rate (D) according to Equation 5.4

$$VSLR = S_0 * \frac{F}{V} \qquad \text{Equation 5.4}$$

$$= S_0 * D$$

The specific substrate utilisation rate (q_s) is defined as the volumetric rate of substrate S utilisation (r_s) (given in g l^{-1} h^{-1}) per unit biomass (X) (g l^{-1}). Hence q_s is given in units of $\frac{g\ substrate}{g\ biomass\ h}$ and calculated according to Equation 5.5

$$q_s = \frac{r_s}{X} \qquad \text{Equation 5.5a}$$

where X represents all the bacterial groups present under the experimental conditions. Volumetric and specific substrate utilisation rates can be calculated for each measured substrate.

$\frac{dS}{dt}$ or $\frac{dC_s}{dt}$ = volumetric substrate utilisation rate Equation 5.5b

$$q_s = \frac{dS/dt}{X}$$

$$= \frac{\Delta g SO_4^{2-}}{\Delta l\ h}\ g\ biomass$$ Equation 5.5c

Biomass yield and maintenance

The Pirt Equation (Equation 5.6a) (Pirt, 1965) was used to obtain the bacterial yield ($Y_{x/s}$) reported as $\boldsymbol{g_{DW}\ g^{-1}_{SO_4^{2-}}}$ and maintenance coefficients (m_s) reported as $\boldsymbol{g_{SO_4^{2-}}\ g^{-1}_{DW}\ h^{-1}}$

$$r_s = r_x \frac{1}{Y_{x/s}} + m_s X$$ Equation 5.6a

where r_s is the volumetric rate utilisation of substrate S (g l^{-1} h^{-1}) and r_x is the rate of biomass formation (g l^{-1} h^{-1}). Since $r_x = \mu X$, Equation 5.6a can be written to introduce the specific growth rate in steady state continuous culture, μ (h^{-1}), to give Equation 5.6b.

$$r_s = \mu X \frac{1}{Y_{x/s}} + m_s$$ Equation 5.6b

Since $q_s = \frac{r_s}{X}$ (Equation 5.5), Equation 5.6b can linearised, yielding Equation 5.6c. Plotting the specific substrate utilisation rate as a function of the specific growth rate,μ, (h^{-1}) gives a straight line demonstrated by Equation 5.6c,

$$\frac{r_s}{X} = \mu \frac{1}{Y_{x/s}} + m_s$$ Equation 5.6c

where $\frac{1}{Y_{x/s}}$ is the slope and m_s is the intercept.

5.3.4 Reproducibility experiments

The reproducibility of the kinetic experiments was assessed from the duplicate experiments at the feed sulphate concentration of 5.0 g l^{-1} at 35°C and pH 8 ± 0.2, as well as by taking multiple steady-state data points. Under this feed sulphate concentration, the two kinetics experiments showed a good reproducibility (Figure 5.4).

Reproducibility of each measured parameter is represented as the coefficient of variance (CV), calculated as the standard deviation expressed as a percentage of the mean. This was done across multiple steady-state points and in each run and across runs and are shown in Table 5.2. Standard deviations for the measurements of volumetric sulphate reduction rate (VSRR) and sulphate conversion across duplicate runs were less than 15% of their respective mean values. This illustrates acceptable reproducibility of the kinetics experiments.

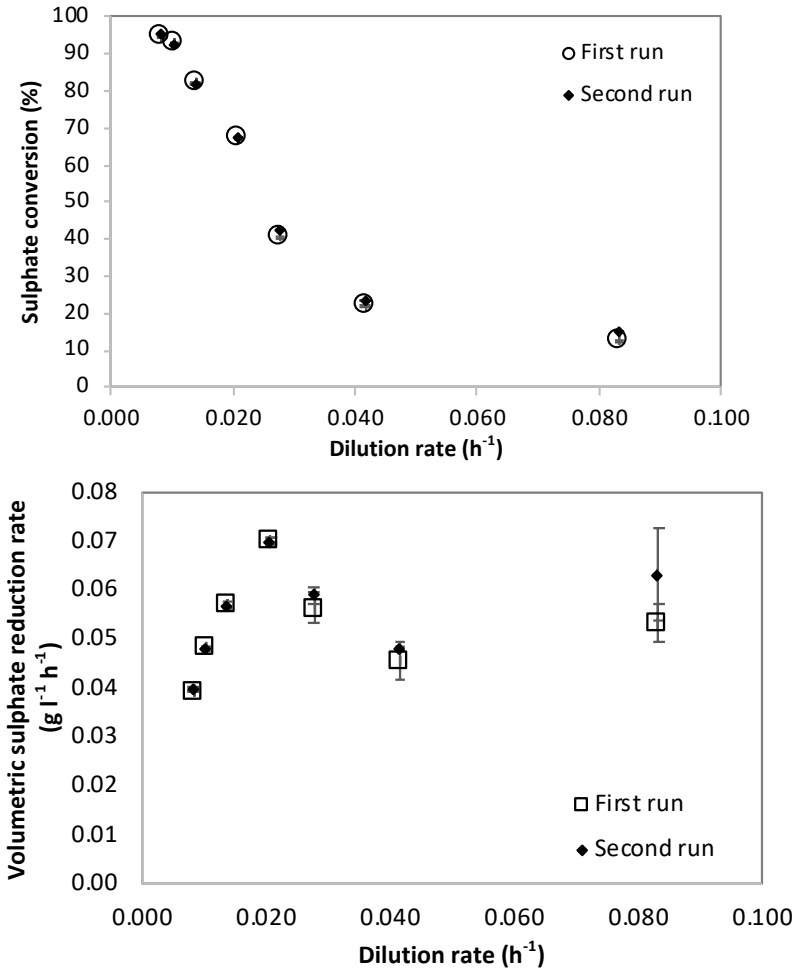


Figure 5.4. The steady-state profiles of volumetric sulphate reduction rate and sulphate conversion as a function of dilution rate at feed sulphate concentration 5.0 g l^{-1} for two datasets obtained from two replicate experimental runs.

Table 5.2. Test of experimental reproducibility as measured by coefficient of variance

Datasets	Coefficient of variance (%) range		
	First run	Second run	Across runs
Sulphate conversion	0.017-0.431	0.14-14.83	0.017-14.83
Volumetric sulphate reduction rate	0.33-8.62	0.14-14.83	0.14-14.83

5.4 STEADY-STATE RESULTS AND DISCUSSION

5.4.1 Steady-state kinetic profiles of CSTRs

The steady-state profiles of sulphate conversion, propionate conversion, butyrate conversion, bacterial dry mass concentration and volumetric sulphate reduction rate (VSRR) as a function of volumetric sulphate loading rate (VSLR) and dilution rate for the different feed sulphate concentrations (1.0, 2.5 to 5.0 g l^{-1}) are shown in Figures 5.3a, 5.4a and 5.5a. Steady-state conditions were assumed to be established when the bacterial dry mass concentration and residual sulphate varied by <10% for three consecutive samples taken after a period of operation equal to at least three retention times since system perturbation. The corresponding concentrations of residual acetate, residual propionate, residual butyrate and residual sulphate are indicated in Figures 5.3b, 5.4b and 5.5b. Results shown in Figures 5.3c, 5.4c and 5.5c indicate the change of dissolved sulphide concentration and bicarbonate alkalinity for the different feed concentrations over the range of dilution rates studied.

5.4.1.1 Reactor performance at feed sulphate concentration of 1.0 g l^{-1}

At a feed sulphate concentration of 1.0 g l^{-1}, the volumetric sulphate reduction rate (VSRR) increased from 0.008 to 0.042 g l^{-1} h^{-1} with increasing dilution rate from 0.0083 to 0.042 h^{-1} [residence times (RT) of 5 to 1 d, Figure 5.5a]. On increasing the dilution rate from 0.042 to 0.083 h^{-1} (RT of 1 to 0.5 d), little further increase in VSRR resulted, demonstrating that microbial community was at full metabolic capacity. Sulphate conversion remained steady over the dilution rate of 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d) at an average of 91 ± 0.4% sulphate conversion. The resulting sulphide concentration also remained steady (0.191 to 0.171 g l^{-1}) across the dilution rates of 0.0083 to 0.042 h^{-1} (5 to 1 d RT) (Figure 5.5c). Sulphate conversion reduced to 50% on further increase in volume sulphate loading rate (VSLR) to 0.083 g l^{-1} h^{-1}, while the corresponding VSRR increased to 0.042 g l^{-1} h^{-1}, confirming the ability of the microbial community to reduce sulphate. The propionate and butyrate conversion at dilution rates of 0.0083 to 0.042 h^{-1} were 100% when anaerobic digestate served as a carbon source and electron donor for BSR. The trends observed in propionate and butyrate conversion in this reactor were similar, with a sharp decrease in both propionate and butyrate conversion observed, to 8 and 7% respectively, upon the increase of the dilution rate from 0.042 to 0.083 h^{-1} (RT of 1 d to 0.5 d). Degradation of propionate and butyrate were accompanied by an increase in residual acetate at dilution rates of 0.0083 to 0.042 h^{-1}.

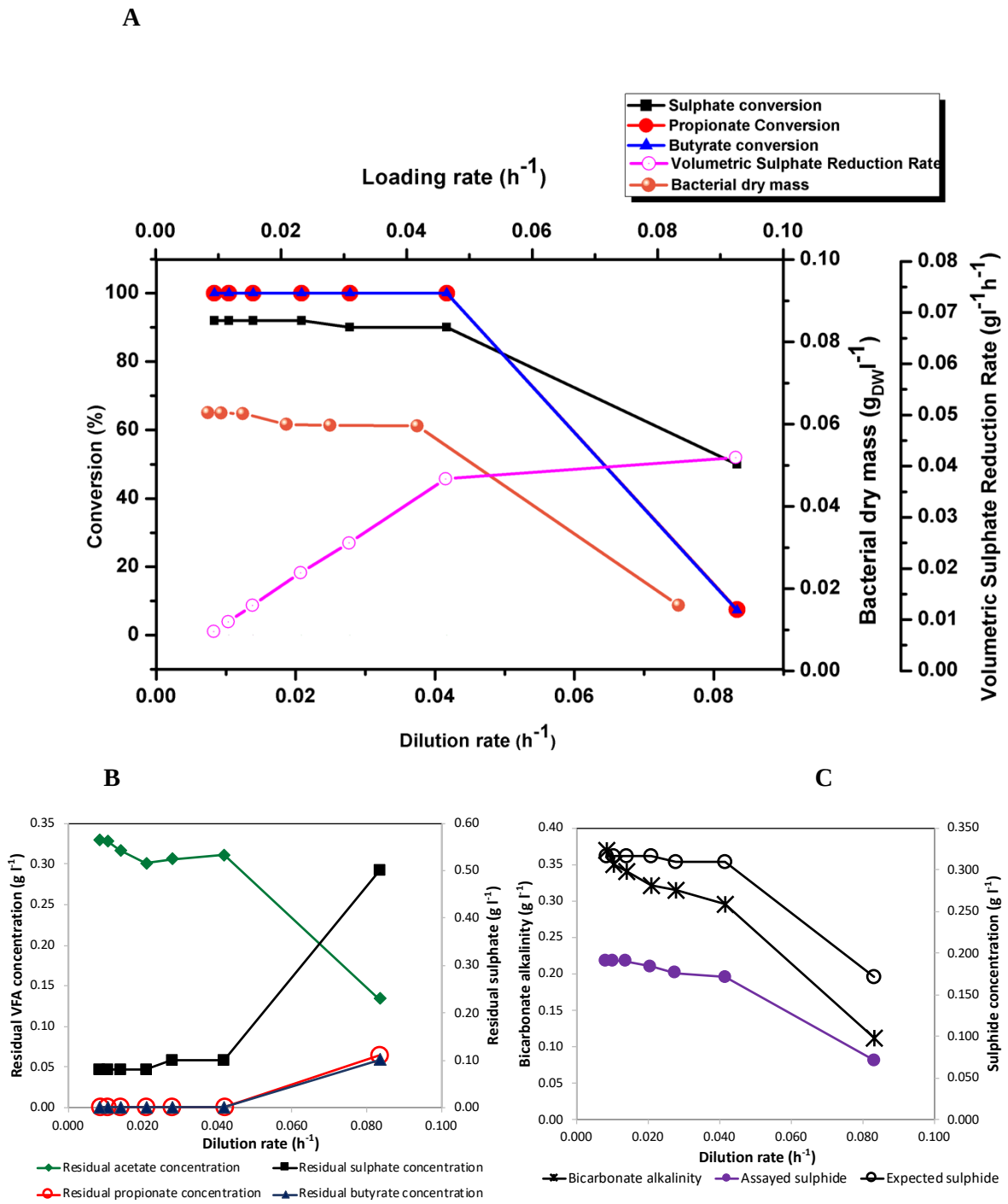


Figure 5.5. Steady-state kinetics of continuous reactor with a feed sulphate concentration of 1.0 g l^{-1}. Steady-state profiles of sulphate conversion, propionate conversion, butyrate conversion, volumetric sulphate reduction rate and bacterial dry mass (A), residual acetate, residual propionate, residual butyrate, and residual sulphate (B) and bicarbonate alkalinity and sulphide concentrations (C) are represented.

According to Reactions 2.20, 2.21, 5.1 and 5.3 in Table 5.1, oxidation of propionate or butyrate and concomitant sulphate reduction are accompanied by acetate production, indicating that acetate is both formed and utilised in the reactor. Observations by Maillacheruvu *et al*. (1996) showed that the incomplete oxidation of propionate was the preferred pathway by SRB (Reaction 2.20). Oude Elferink *et al*. (1998) indicated that the percentage of butyrate conversion via sulphate reduction would decrease drastically if the butyrate conversion mainly proceeded via Reaction 5.3 and not via Reaction 2.21. Therefore Reactions 2.20 and 2.21 were used for stoichiometry calculations of total acetate in each reactor.

$$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$$ Reaction 2.19

$\Delta G^{o'}$ = -47.6 kJ/Reaction

$$Propionate^- + 0.75SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.75HS^- + 0.25H^+$$ Reaction 2.20

$\Delta G^{o'}$ = -37.7 kJ/Reaction

$$Butyrate^- + 0.5SO_4^{2-} \rightarrow 2Acetate^- + 0.5HS^- + 0.5H^+$$ Reaction 2.21

$\Delta G^{o'}$ = -27.8 kJ/Reaction

The stoichiometry ratios were quantified as moles of acetate utilised per mole of sulphate reduced (A:S), moles of acetate used per mole of bicarbonate produced (A:HC), moles of propionate used per mole of sulphate reduced (P:S), moles of propionate used per mole of bicarbonate produced (P:HC), moles of butyrate used per mole of sulphate reduced (B:S) and moles of butyrate used per mole of bicarbonate produced (B:HC). The P:HC and P:A ratios can be combined as indices of both propionate oxidation and propionate acetogenesis; the B:HC and B:A ratios can be combined as indices of both butyrate oxidation and butyrate acetogenesis, while the A:S, P:S and B:S represent propionate and butyrate oxidation only. Since no methanogenic activity was detected (Section 4.3.2) the A:HC was assumed to represent acetate oxidation only (Table 5.3).

In order to get a clear picture of formation or utilisation of acetate, the mole ratio calculations were carried out based on complete utilisation of propionate or butyrate or both [(dilution rates of 0.0083 to 0.042 h^{-1}) (Table 5.4)] and incomplete utilisation of both propionate and butyrate (Table 5.4). Across the lower dilution rates of 0.0083 to 0.042 h^{-1} (RTs of 5 to 1 d), the experimental stoichiometry ratios of A:S and A:HC in a reactor receiving feed sulphate concentration of 1.0 g l^{-1} was 0.90 ± 0.01 and 1.6 ± 0.13, respectively (Table 5.4a). Both values largely concurred with the theoretical value of 1 and 2 respectively (Table 5.3), indicating that almost all the acetate used went to the provision of electrons for sulphate reduction. The experimental stoichiometric ratios P:S and P:HC were 0.20 ± 0.022 and 0.30 ± 0.024, respectively (Table 5.4a). These values are closer to the theoretical values of incomplete propionate oxidation (Reaction 2.20, Table 5.3) which indicated that this was the preferred pathway by propionate utilising SRB. These observations are in agreement with studies by Maillacheruvu *et al*.

(1996) who showed that the incomplete oxidation of propionate was the preferred pathway by SRB (Reaction 2.20). The experimental stoichiometric ratio B:S was 0.13 ± 0.0015 (Table 5.4a) which was closer to the theoretical value of 0.5 from the incomplete oxidation of butyrate via Reaction 2.21 (Table 5.3).

Table 5.3. Dependency of molar ratio of VFA used to other substrates involved in BSR on feed sulphate concentration using anaerobic digestion as a carbon source and electron donor. Theoretical values of total moles of acetate used per mole of sulphate used (A:S), total moles of acetate used per mole of bicarbonate produced (A:HC), total moles of propionate used per mole of sulphate used (P:S), total moles of propionate used per mole of bicarbonate produced (P:HC), total moles of butyrate used per mole of sulphate used (B:S) and total moles of butyrate used per mole of bicarbonate produced (B:HC) are indicated.

VFA	Reaction kinetics			
Acetate	**Reaction number**	**A: S**	**A:HC**	
	2.19	1	2	
	2.27		1	
Propionate	**Reaction number**	**P:S**	**P:A**	**P:HC**
	2.20	0.75	1	1
	5.1	1.75		3
	2.23		1	1
	5.2		1	2
Butyrate	**Reaction number**	**B:S**	**B:A**	**B:HC**
	2.21	0.5	2	
	5.3	1.5	1	2
	5.4	2.5		4
	2.24		1	
	5.5		1	2

Table 5.4a. Experimental ratios of total moles of acetate used per mole of sulphate used (A:S), total moles of acetate used per mole of bicarbonate produced (A:HC), total moles of propionate used per mole of sulphate used (P:S), total moles of propionate used per mole of bicarbonate produced (P:HC), total moles of butyrate used per mole of sulphate used (B:S) and total moles of butyrate used per mole of bicarbonate produced (B:HC) across the dilution rate of 0.0083 to 0.042 h^{-1} indicating standard deviations.

Experimental ratios obtained	Feed sulphate concentration (g l^{-1})		
	1.0	**2.5**	**5.0**
A:S	0.90 ± 0.01	1.2 ± 0.29	1 .3 ± 0.48
A:HC	1.6 ± 0.13	1.7 ± 0.015	2.9 ± 0.50
P:S	0.20 ± 0.022	0.23 ± 0.040	0.2 ± 0.11
P:HC	0.30 ± 0.24	0.33 ± 0.052	0.40 ± 0.16
B:S	0.13 ± 0.0015	0.1 ± 0.018	0.1 ± 0.009
B:HC	0.23 ± 0.018	0.11 ± 0.024	0.18 ± 0.026

Table 5.4b. Experimental ratios of A:S, A:HC, P:S, P:HC, B:S and B:HC at the dilution rate of 0.083 h^{-1} indicating standard deviations.

Experimental ratios obtained	Feed sulphate concentration (g l^{-1})		
	1.0	**2.5**	**5.0**
A:S	0.92 ± 0.02	2.3 ± 1.2	4.3 ± 1.6
A:HC	2.6 ± 0.6	4.2 ± 1.2	9.9 ± 0.2
P:S	0.023 ± 0.002	0.12 ± 0.075	0.15 ± 0.02
P:HC	0.066 ± 0.001	0.21 ± 0.085	0.43 ± 0.02
B:S	0.017 ± 0.003	0.015 ± 0.0055	0.013 ± 0.004
B:HC	0.049 ± 0.0002	0.031 ± 0.019	0.039 ± 0.002

Similar to high dilution rates, at the highest dilution rate of 0.083 h^{-1} (RT of 0.5 d), the experimental stoichiometric ratios of A:S and A:HC concurred with the theoretical value of 1 and 2 respectively. The stoichiometric ratios of A:S observed was 0.92 and the ratio of A:HC observed was 2.6 (Table 5.4b). This was in agreement with Characklis *et al*. (1989) who stated that the stoichiometry of BSR should not be affected by sulphate concentration. Contrarily, the stoichiometry ratios observed for P:S, P:HC, B:S and B:HC were significantly low at the highest dilution rate of 0.083 h^{-1} (Table 5.4b). These observations are supported by the lower sulphate, propionate and butyrate conversions [(50, 8 and 7%, respectively), Figure 5.5a], higher residual concentrations of sulphate, propionate and butyrate (Figure 5.5b) and lower sulphide concentration [(0.071 g l^{-1}), Figure 5.5c). The decline of bacterial dry mass from 0.612 ± 0.01 g l^{-1} across the dilution rates of 0.0083 to 0.042 h^{-1} to 0.160 g l^{-1} at this dilution rate (0.083 h^{-1}) suggested washout of propionate and butyrate oxidisers possibly due to their inability to proliferate. However, the high metabolic efficiency for acetate (90 ± 1.2%) observed across the dilution rates of 0.0083 to 0.083 h^{-1} suggested the presence of acetate oxidising SRB with higher maximum growth rate (μ_{max}) and the half saturation constant of sulphate (K_S) that were able to proliferate at high VSLRs and low retention times.

These results show that, across the lower dilution rate of 0.0083 to 0.042 h^{-1} (RTs of 5 to 1 d), there was simultaneous acetate, propionate and butyrate oxidation in the reactor receiving 1.0 g l^{-1}. Contrarily, in batch reactors mimicking oil fields in terms of VFA composition of acetate, propionate and butyrate, Chen *et al*. (2017) demonstrated that, when these VFAs are present in excess (3 mM each), propionate was preferentially used for sulphate reduction. They also demonstrated that *Desulfobulbus* species were the dominant SRB in the batch reactors. Oude Elferink (1998) demonstrated that propionate and butyrate degrading SRB grow much faster than other SRB communities or syntrophic propionate and butyrate degrading methanogenic communities (Table 5.5); however a maximum specific growth rate of <0.06 h^{-1} was reported for the fastest growing of these, supporting their washout from the reactor at a dilution rate of 0.083 h^{-1} while efficient propionate and butyrate use was shown at 0.042 h^{-1}.

Table 5.5. Specific growth rates (h^{-1}) of SRB and acetogenic bacteria in co-culture with hydrogenotrophic methanogens / sulphate reducers, growing on butyrate or propionate (Oude Elferink, 1998)

Microbial strains	Sulphate reducing culture (h^{-1})	Syntrophic coculture without sulphate (h^{-1})	Syntrophic coculture with sulphate (h^{-1})
Butyrate-degrading strains			
SRB			
Desulfoarculus baarsii	0.0167		
Desulfobacterium autotrophicum	0.0279-0.0463		
Desulfococcus multivorans	0.00708-0.0100		
Desulfotomaculum acetoxidans	0.0463		
Desulfotomaculum strain Grol11	0.0500-0.0542		
Non-SRB			
Syntrophomonas sapovorans		0.025	
Syntrophomonas wolfei		0.0083	0.0125
Syntrophospora (Clostridium) bryantii		0.01	
Spore forming strain FMS2		0.0129	
Spore forming strain FSS7			0.0142
non-spore forming strain FM4		0.01	
non-spore forming strain B1		0.0042	
Propionate degrading strains			
Desulfobulbus longatus	0.0579		
Desulfobulbus propionicus	0.0371-0.0417		
Desulfococcus multivorans	0.0071-0.0100		
NonSRB			
Syntrophobacter strain MPOB	0.00083	0.00625-0.0071	
Syntrophobacter pfennigii	0.00292	0.00292	
Syntrophobacter wolinii	0.0025	0.00083-0.0042	0.0075-0.0875

When lactate was used as a carbon source and electron donor for BSR, it was indicated that oxidation of lactate and concomitant sulphate reduction was the mechanism preferred by SRB and not propionate or acetate (Oyekola *et al*., 2010; 2012). Moosa *et al*. (2002) reported the experimental stoichiometry ratios of moles of acetate used per mole of sulphate reduced (A:S) by a mixed SRB consortium across dilution rates of 0.006 to 0.024 h^{-1} was 0.84 ± 0.15, when acetate was provided as a sole carbon source and electron donor for BSR at a feed sulphate concentration of 1.0 g l^{-1}. The current study indicated that similar metabolic efficiency for acetate was observed with anaerobic digestate as when acetate was a sole carbon source and electron donor for BSR, suggesting the ability of anaerobic digestate to support a robust SRB consortium as demonstrated for acetate. The assayed sulphide (AS) concentration in the liquid phase is indicated Figure 5.5c. These values are lower than the expected sulphide (ES) from the amount of sulphate reduced (Figure 5.5c). This can be attributed to the loss of some of the sulphide produced to formation of polysulphides and loss of H_2S through chemical sulphide oxidation. In spite of high sulphate conversion observed at lower dilution rates 0.0083 to 0.042 h^{-1}, lower sulphide levels were recorded within this range. The loss of sulphide to other forms ($\frac{ES-AS}{ES}$ x 100) varied between 2.3 to 66% as shown in Table C1 in *Appendix C*. Studies have revealed that, at high sulphide and limited

oxygen conditions, chemical sulphide oxidation becomes more prominent, resulting in a large amount of sulphide being converted to thiosulphate (Buisman *et al*., 1990; Janssen *et al.*, 1995). The formation of thiosulphate is increased at low oxygen concentration, exceedingly high sulphide concentration (10 S_{tot}^{2-} : 1.1 O_2) and an alkaline pH 8 (Buisman *et al*., 1990). The visual observation of some white sulphur on the walls of the CSTRs indicated that at least part of the sulphide was partially oxidised to elemental sulphur. This could have been a result of minor oxygen leakage that occurred during sampling. The average pH of the effluent from the reactors in this study was 8.0 ± 0.02 which was ideal for chemical sulphide oxidation.

The impact of polysulphide formation on aqueous sulphide produced

The visual observation of green-yellowish to orange coloured precipitates on the inner walls of the reactors in this study suggested the formation of polysulphides. Inorganic polysulphides (Sn^{2-}) were first described in 1977 by Carl and Scheele (Lens and Kuenen, 2001). They are produced by the oxidation of aqueous hydrogen sulphide at an alkaline pH. The hydrogen sulphide can react with inorganic or organic sulphur (Lens *et al*., 2000; Kamyshny *et al.*, 2004). The reaction of hydrogen sulphide and elemental sulphur (S_8) results in the formation of a polysulphane, which may dissociate to form a long chain polysulphide and proton (Reaction 5.11).

$$HS^- + S_8 \leftrightarrow HS_9^- \leftrightarrow S_9^{2-} + H^+ \qquad \text{Reaction 5.11}$$

The strong nucleophile (HS^-) initiates the reaction by causing the S_8 ring to open and form the polysulphane. Consequently, the nine 'S' polysulphide chain reacts further with hydrogen sulphide to form two polysulphides of chain length five which then dissociate into shorter polysulphide ions (Reaction 5.12) (Steudel, 2003; Kleinjan *et al.*, 2005).

$$S_9^{2-} + HS^- \leftrightarrow 2S_5^{2-} + H^+ \qquad \text{Reaction 5.12}$$

$$2S_5^{2-} \leftrightarrow S_4^{2-} + S_6^{2-} \qquad \text{Reaction 5.13}$$

Under mildly alkaline conditions and in equilibrium with excess inorganic sulphur the average polysulphide chain length (x) can vary from 4.39 - 5.5 (Reactions5.12 to5.13) (van den Bosch, 2008). Polysulphide ion oxidation occurred more rapidly than sulphide oxidation. Polysulphide ions therefore act as a catalyst to sulphide oxidation (Chen and Morris, 1972). The reaction indicated by Reaction 5.14 therefore occurs at a much faster rate than reaction indicated by Reaction 5.11.

$$S_2^{2-} + S_8 \leftrightarrow S_{10}^{2-} \qquad \text{Reaction 5.14}$$

Polysulphides can also be oxidised to thiosulphate and zero-valent sulphur according to Reaction 5.15 (Kleinjan *et al*., 2005a).

$$S_x^{2-} + \frac{3}{2}O_2 \rightarrow S_2O_3^{2-} + (x-2)S^0 \qquad \text{Reaction 5.15}$$

Under alkaline conditions, formation of polysulphide may also occur according to Reaction 5.16.

$$HS^- + (x-1)\,S^0 \leftrightarrow S_x^{2-} + H^+ \qquad \text{Reaction 5.16}$$

Therefore, sulphide chemistry involves a possibility of multiple reactions occurring simultaneously to produce various oxidation products, resulting in an extremely complex chemistry. Chemical sulphide oxidation and formation of polysulphides would account for part of the discrepancy observed in the assayed dissolved sulphide levels observed in Table C1 in *Appendix C*. Sulphides are comprised of three chemical species $H_2S(aq)$, HS^- and S^{2-}. The H_2S which is within the SRB system occurs predominantly in an aqueous or dissociated state. The equilibrium between the three species is strongly dependent on the pH, temperature, sulphide and oxygen concentrations (Chen and Morris, 1972; Broderius and Smith, 1977; Kuhn *et al.*, 1983).

5.4.1.2 Reactor performance at feed sulphate concentration of 2.5 g l^{-1}

At a feed sulphate concentration of 2.5 g l^{-1}, the steady-state profile indicated that the volumetric sulphate reduction rate (VSRR) increased from 0.019 to 0.051 g l^{-1} h^{-1} with increasing dilution rates from 0.0083 to 0.042 h^{-1} (Figure 5.6a). A slight decrease in the VSRR, from 0.051 to 0.045 g l^{-1} h^{-1}, was observed with increasing dilution rate from 0.042 to 0.083 h^{-1} (RT of 1 to 0.5 d). These reduction rates correlated to a steady conversion of sulphate to sulphide at lower dilution rates (0.083 to 0.014 h^{-1}, RT of 5 to 3 d) with a maximum sulphate conversion of 90% observed at 0.083 and 0.010 h^{-1}. Sulphate conversion decreased to 60% at the dilution rate of 0.021 h^{-1} (RT of 2 d) and 0.028 h^{-1} (RT of 1.5 d). Further increase of dilution rate to 0.042 h^{-1} (RT of 1 d) and associated VSLR resulted in the decrease of sulphate conversion to 49.4% and with further decrease to 21.6% with increasing dilution rate to 0.083 h^{-1} (RT of 0.5 d). The bacterial dry mass concentration decreased gradually with increasing dilution rate. A value of 1.45 ± 0.03 g_{DW} l^{-1} bacterial dry mass concentration was maintained over the dilution rates of 0.0083 to 0.014 h^{-1} (RT of 5 to 3 d). This decreased to 0.974 g_{DW} l^{-1} at the dilution rate of 0.021 h^{-1} (RT of 2 d) and plateaued until 0.028 h^{-1} (RT of 1.5 d). Further increase of dilution rate resulted in the decrease of bacterial dry mass concentration to 0.761 g_{DW} l^{-1} at 0.042 h^{-1} (RT of 1 d) and 0.160 g_{DW} l^{-1} at 0.083 h^{-1} (RT of 0.5 d). The latter was associated with decrease in sulphate reduction, suggesting wash out of some sulphate reducers.

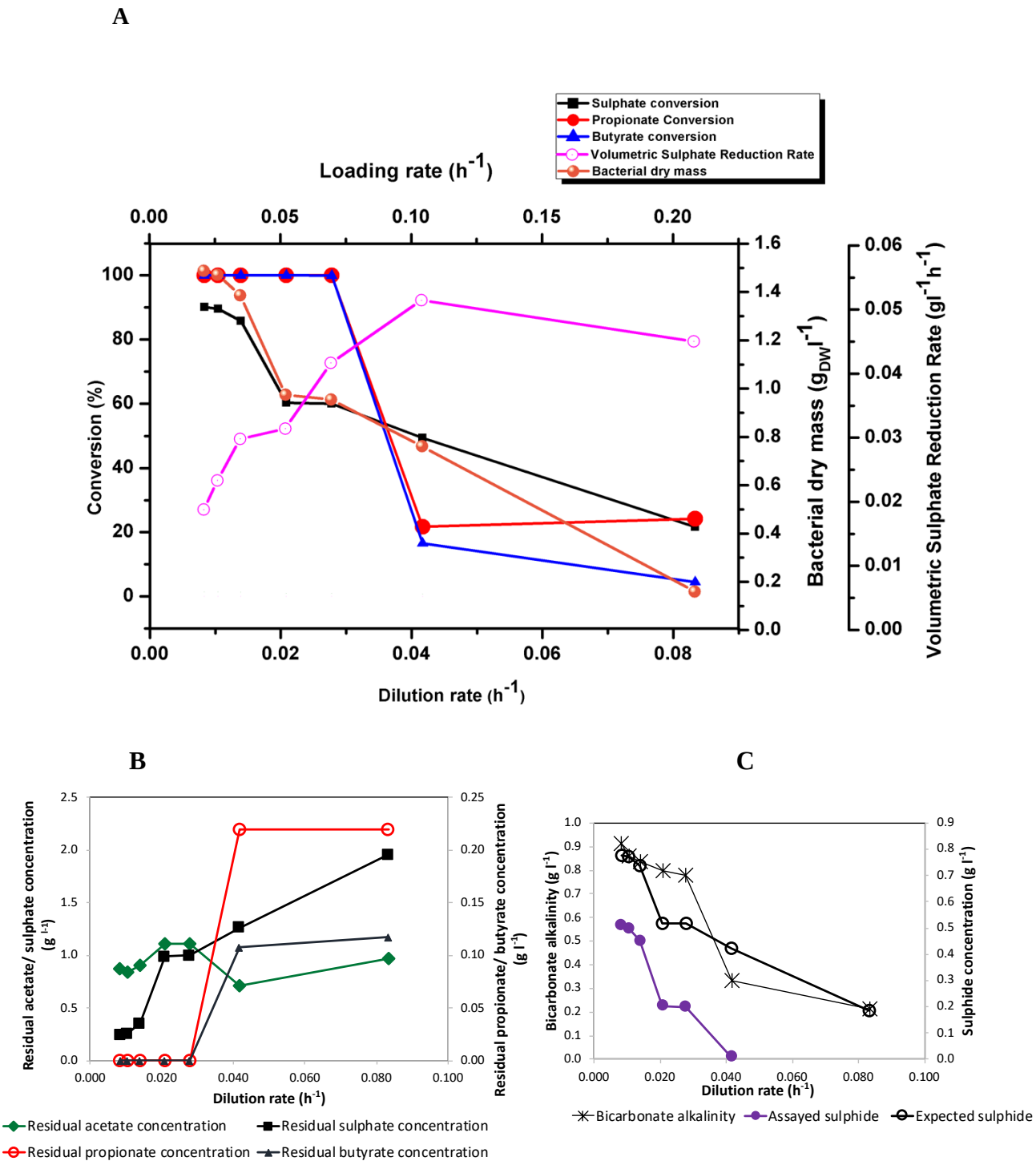


Figure 5.6. Steady-state kinetics of continuous reactor with a feed sulphate concentration of 2.5 g l^{-1}. Steady-state profiles of sulphate conversion, propionate conversion, butyrate conversion, volumetric sulphate reduction rate and bacterial dry mass (A), residual acetate, residual propionate, residual butyrate, and residual sulphate (B) and bicarbonate alkalinity and sulphide concentrations (C) are represented.

On considering the utilisation of propionate and butyrate conversion, complete utilisation of these VFAs was observed across the dilution rates of 0.0083 to 0.028 h^{-1} (RTs of 5 to 1.5 d), suggesting proliferation of propionate and butyrate oxidisers. This was followed by a sharp decline in both propionate and butyrate conversion on the decrease of the RT from 1.5 d (dilution rate of 0.028 h^{-1}) to 1 d (dilution rate of 0.042 h^{-1}). The percentage of propionate conversion was 21.7% at RT of 1 d and 24.2 % at RT of 0.5 d. Butyrate conversion was 16.5 and 4.4% at 1.0 d and 0.5 d RT (dilution rate of 0.042 to 0.083 h^{-1}), respectively. A decrease in propionate and butyrate conversion from 0.028 to 0.042 h^{-1} (RT of 1.5 to 1 d) was accompanied by a slight increase in the VSRR from a value of 0.042 to 0.051 g l^{-1} h^{-1} and a continuing decrease in sulphate conversion (49.4%) at dilution rate of 0.042 h^{-1}, 1 d RT). Consequently, there was a build-up in the residual sulphate, propionate and butyrate at higher dilution rates (0.042 to 0.083 h^{-1} shown in Figure 5.6b).

Based on the stoichiometry estimations according to Reaction 2.19, Reaction 2.20, and Reaction 2.21, across the lower dilution rates of 0.0083 to 0.028 h^{-1} (RTs of 5 to 1.5 d), the experimental stoichiometry ratios of A:S and A:HC in a reactor receiving feed sulphate concentration of 2.5 g l^{-1} were 1.2 ± 0.29 and 1.7 ± 0.15, respectively (Table 5.3b). Both values largely concurred with the theoretical values of 1 and 2 respectively (Table 5.3a), indicating that almost all the acetate used went to the provision of electrons for sulphate reduction in this reactor. The experimental stoichiometry ratio P:S and P:HC were 0.23 ± 0.040 and 0.33 ± 0.052, respectively (Table 5.3b). These values are closer to the theoretical values of incomplete propionate oxidation (Reaction 2.20, Table 5.3a) suggesting that this was the preferred pathway by propionate utilising SRB. The experimental stoichiometry ratio B:S was 0.10 ± 0.018 and B:HC was 0.11 ± 0.0015 (Table 5.3b) which were closer to the theoretical values from the incomplete oxidation of butyrate via Reaction 2.21 (Table 5.3a).

The experimental stoichiometry ratios of A:S and A:HC at the higher dilution rate of 0.042 h^{-1} (RT of 1 d) were 1.4 and 3.4, respectively. A further increase in the dilution rate to 0.083 h^{-1} (RT of 0.5 d), resulted in the experimental stoichiometry ratio of 3.1 for A:S and 5.0 for A:HC, which was a further deviation from the theoretical value of acetate oxidation and concomitant sulphate reduction in Reaction 2.19. The experimental stoichiometry ratios of B:S at the higher dilution rate of 0.042 and 0.083 h^{-1} were 0.02 and 0.04, respectively, and the ratios of B:HC were 0.01 and 0.02, respectively. Consequently, a decrease in butyrate and sulphate conversions (Figure 5.6a) and build-up in residual butyrate concentration (Figure 5.6b) were observed. The deviation of experimental stoichiometry ratios from the theoretical stoichiometry ratios of VFA oxidation and concomitant sulphate reduction suggested the occurrence of acetogenic reactions and lower fraction of VFAs available for sulphate reduction process. Furthermore, the deviation increased with increasing dilution rate from 0.042 to

0.083 h^{-1} (RT of 1 to 0.5 d) and corroborated with a decrease in bacterial dry mass concentration (Figure 5.6a), suggesting washout of key SRB species at the lower dilution rates.

5.4.1.3 Reactor performance at feed sulphate concentration of 5.0 g l^{-1}

At a feed sulphate concentration of 5.0 g l^{-1}, the volumetric sulphate reduction rate (VSRR) increased from 0.040 g l^{-1} h^{-1} to an optimum value of 0.070 g l^{-1} h^{-1} with increasing dilution rate from 0.0083 to 0.021 h^{-1} (RTs of 5 to 2 d, Figure 5.7a). A decline in the VSRR was observed when the dilution rate was changed to 0.042 h^{-1} (RTs of 1 d) resulting in the VSRR of 0.047 g h^{-1} l^{-1}. A further increase of the dilution rate to 0.083 h^{-1} (RTs of 0.5 d) resulted in a slight increase of the VSRR to 0.052 g l^{-1} h^{-1}. Sulphate conversion decreased from a maximum of value of 95% to a minimum value of 13% across the dilution rates of 0.0083 to 0.083 h^{-1} (RTs of 5 to 0.5 d). The trend observed for bacterial dry mass concentration was similar to that of sulphate conversion, with a maximum value of 3.161 g_{DW} l^{-1} observed at the dilution rate of 0.0083 h^{-1} (RT of 5 d) and a minimum value of 0.211 g_{DW} l^{-1} observed at the dilution rate of 0.083 h^{-1} (RT of 0.5 d). Propionate conversion of 100% was maintained across the dilution rates of 0.0083 to 0.028 h^{-1} (RTs of 5 to 1.5 d) while butyrate conversion of 100% was maintained across the dilution rates of 0.0083 to 0.021 h^{-1} (RTs of 5 to 2 d).

Across the lower dilution rates of 0.0083 to 0.028 h^{-1} (RTs of 5 to 1.5 d), the experimental stoichiometry ratios of A:S and A:HC in a reactor receiving feed sulphate concentration of 5.0 g l^{-1} was 1.3 ± 0.48 and 2.9 ± 0.50, respectively (Table 5.3b). Both values largely concurred with the theoretical value of 1 and 2 respectively (Table 5.3a), indicating that almost all the acetate used went to the provision of electrons for sulphate reduction. The experimental stoichiometry ratio P:S and P:HC were 0.20 ± 0.11 and 0.40 ± 0.16, respectively (Table 5.3b) which are closer to the theoretical values of incomplete propionate oxidation (Reaction 2.20, Table 5.3a). This suggested that similar to the reactors receiving feed sulphate concentration at 1.0 and 2.5 g l^{-1}, this was the preferred pathway by propionate utilising SRB at these dilution rates. The experimental stoichiometry ratio B:S was 0.1 ± 0.009 (Table 5.3b) which was closer to the theoretical value of 0.5 from the incomplete oxidation of butyrate via Reaction 2.21 indicated in Table 5.3a, suggesting incomplete oxidation of butyrate by butyrate oxidising SRB across these dilution rates. On increasing dilution rate to 0.083 h^{-1} (RTs of 1 d), experimental stoichiometry ratios of A:S, A:HC, P:S, P:HC, B:S and B:HC deviated from experimental stoichiometry ratios (Table 5.3c). These deviations were associated with decrease in propionate, butyrate and sulphate conversions (5.8, 0.3 and 12.5% respectively), as well as loss of bacterial dry mass concentration to 0.211 g_{DW} l^{-1} (Figure 5.7a), suggesting washout of key SRB species at higher dilution rates (higher VSLR).

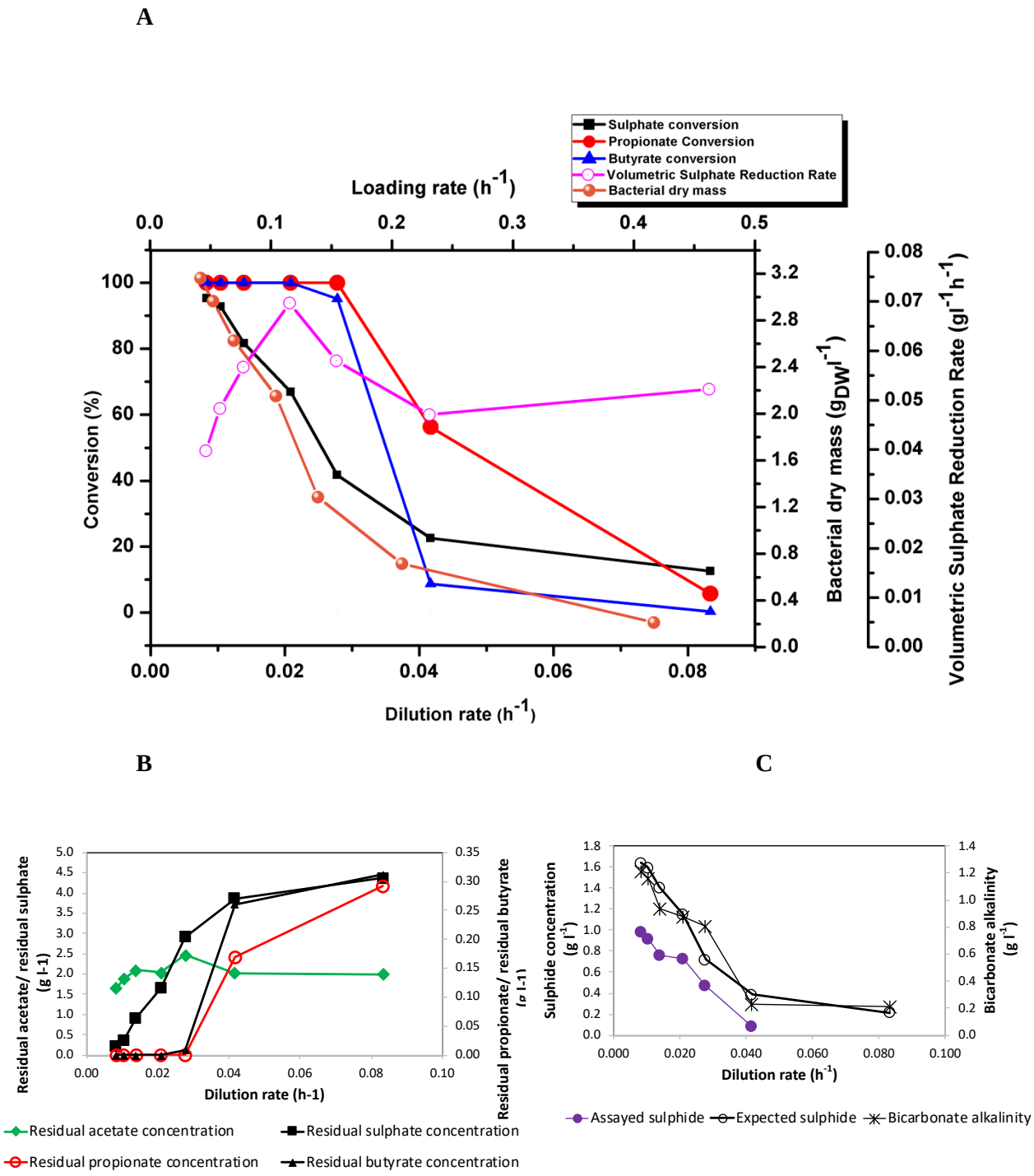


Figure 5.7. Steady-state kinetics of continuous reactor with a feed sulphate concentration of 5.0 g l^{-1}. Steady-state profiles of sulphate conversion, propionate conversion, butyrate conversion, volumetric sulphate reduction rate and bacterial dry mass (A), residual acetate, residual propionate, residual butyrate, and residual sulphate (B) and bicarbonate alkalinity and sulphide concentrations (C) are represented.

5.4.2 Dependency of biological sulphate reduction kinetics on feed sulphate concentration and residence time and sulphate loading rate

In this section, the volumetric sulphate loading rates and resultant sulphate conversions are assessed across both dilution rate and feed sulphate concentration to investigate the effect of these components contributing to volumetric sulphate loading rate on process performance using anaerobic digestate as carbon source and electron donor. Further, the performance of the biological sulphate reduction using this mixed VFA stream is compared to previous studies carried out in the CeBER laboratory using a similar reactor setup and single component electron donors; namely, acetate, ethanol and lactate.

5.4.2.1 Volumetric sulphate reduction rate

The results in Table 5.6 suggest that feed sulphate concentration has an influence on the maximum volumetric sulphate reduction rate (VSRR) owing to its proportionality to volumetric sulphate loading rate (VSLR). This was demonstrated using anaerobic digestate as a carbon source and electron donor for biological sulphate reduction (BSR) as previously demonstrated with acetate (Moosa, 2000; Moosa *et al*., 2002; 2005), ethanol (Erasmus, 2000; Hansford *et al*., 2007) and lactate (Oyekola *et al*., 2009; 2010; 2012). These observations indicated that high initial sulphate concentrations resulted in high maximum VSRR, up to a maximum of 0.070 g l^{-1} h^{-1}. For a feed medium containing sulphate concentration of 1.0 g l^{-1}, the maximum VSRR was 0.042 g l^{-1} h^{-1} achieved at a VSLR of 0.083 g l^{-1} h^{-1} (dilution rate of 0.083 h^{-1}) with the corresponding sulphate conversion of 50% (Table 5.6). For a feed medium containing 2.5 g l^{-1} sulphate, the maximum VSRR was 0.051 g l^{-1} h^{-1} achieved at a VSLR of 0.104 g l^{-1} h^{-1} (dilution rate of 0.042 h^{-1}). The corresponding sulphate conversion was of 49% (Table 5.5). A similar trend was observed for a feed medium containing sulphate concentration of 5.0 g l^{-1}, whereby the VSRR reached a maximum value of 0.070 g l^{-1} at sulphate loading of 0.104 g l^{-1} h^{-1} (dilution rate of 0.021 h^{-1}) followed by a substantial decrease in the VSRR when the VSLR was increased (Figures 5.8a and 5.8b). For both a sulphate feed concentration of 2.5 and 5.0 g l^{-1}, applying higher VSLR led to a slight decrease in the sulphate reduction rate (Figure 5.6a and 5.6b). On increasing dilution rate, an increasing VSRR was observed until a maximum, beyond which the associated VSLR exceeded the metabolic activity of the culture (Figure 5.6a). Since VSLR is the product of dilution rate and feed sulphate concentration, VSRR was higher at equivalent dilution for higher feed sulphate concentration (Figure 5.8a).

The results obtained with anaerobic digestate as carbon source and electron donor were compared with results obtained when ethanol (Hansford *et al*, 2007), acetate (Moosa *et al*., 2002) and lactate (Oyekola *et al*.,2010) were used as carbon source and electron donor for BSR (Figures 5.8c to 5.8h) as similar reactor systems and experimental conditions (sulphate concentration, temperature, pH and COD: SO_4^{2-} ratio) were used. For the feed containing sulphate concentration of 1.0 g l^{-1} for different carbon sources and electron donor, similar performances between anaerobic digestate, lactate and ethanol were

observed. However, ethanol did not support as high VSRR (Figures 5.8c and 5.8d) as sulphide inhibition was experienced sooner (Erasmus *et al*., 2007). Maximum VSRR demonstrated for acetate at 1.0 g l^{-1} sulphate was lower than for the other electron donors (Moosa *et al*., 2002). At higher feed sulphate concentrations (2.5 and 5.0 g l^{-1}), the VSRR reached a maximum at an intermediate VSLR in the range 00083 to 0.415 g l^{-1} h^{-1} and dilution rate in the range 0.0083 to 0.083 h^{-1} and then decreased as the VSLR was further increased for anaerobic digestate, lactate, ethanol and acetate (Figures 5.8e to 5.8h).

Table 5.6. Effect of feed concentration and carbon source on maximum volumetric sulphate reduction rate.

Feed sulphate (g l^{-1})	Carbon source	Maximum volumetric sulphate reduction rate (g l^{-1} h^{-1})	Corresponding volumetric sulphate loading rate (g l^{-1} h^{-1})	Corresponding Dilution rate (h^{-1})	Corresponding Sulphate conversion (%)	Reference
1.0	Anaerobic Digestate	0.042	0.083	0.083	50	This study
2.5	Anaerobic Digestate	0.051	0.104	0.042	49	This study
5.0	Anaerobic Digestate	0.070	0.104	0.021	67	This study
1.0	Lactate	0.041	0.083	0.083	50	Oyekola *et al.* (2010)
2.5	Lactate	0.015	0.035	0.014	42	Oyekola *et al.* (2010)
5.0	Lactate	0.032	0.104	0.021	31	Oyekola *et al.* (2010)
1.0	Acetate	0.006	0.011	0.011	86	Moosa *et al*. (2002)
2.5	Acetate	0.032	0.042	0.017	90	Moosa *et al*. (2002)
5.0	Acetate	0.075	0.104	0.028	54	Moosa *et al*. (2002)
1.0	Ethanol	0.045		0.010	80	Hansford *et al.* (2007)
2.5	Ethanol	0.047	0.052	0.021	90	Hansford *et al.* (2007)
5.0	Ethanol	0.091	0.104	0.021	87	Hansford *et al.* (2007)

Lower VSRRs were observed with ethanol due to sulphide inhibition (Erasmus *et al*., 2007). Anaerobic digestate, acetate and lactate demonstrated similar trends, however, competition with lactate fermenters resulted in lower VSRR at higher dilution rates and higher VSLRs when feed concentrations of 2.5 and 5.0 g l^{-1} were used (Oyekola *et al*., 2012). The study by Oyekola *et al*. (2012) demonstrated competition for lactate between lactate oxidisers (LO) and lactate fermenters (LF), with LO competing more effectively for lactate at low lactate concentrations ($\leqslant$5 g l^{-1}) and high sulphide concentrations (0.5 g l^{-1}). However, at excess lactate concentrations (>5 g l^{-1}) and low sulphide concentrations (0.014–0.088 g l^{-1}), LF outcompeted the LO. Such competition was not observed with acetate or ethanol as a carbon source and electron donor for BSR (Moosa *et al*., 2002; Hansford *et al*, 2007). Using batch studies, Mohanty *et al*. (2000) reported that an increase of sulphate concentration from 1.3 to 3.6 g l^{-1} resulted in the decrease of VSRR which was associated with sulphate toxicity. Contrarily, the dependency of maximum VSRR on feed sulphate concentration did not show this trend in this study

(Table 5.6); however, residual sulphate concentration to which SRB were exposed remained below 4.4 g l^{-1} (Table C6 in *Appendix C*).

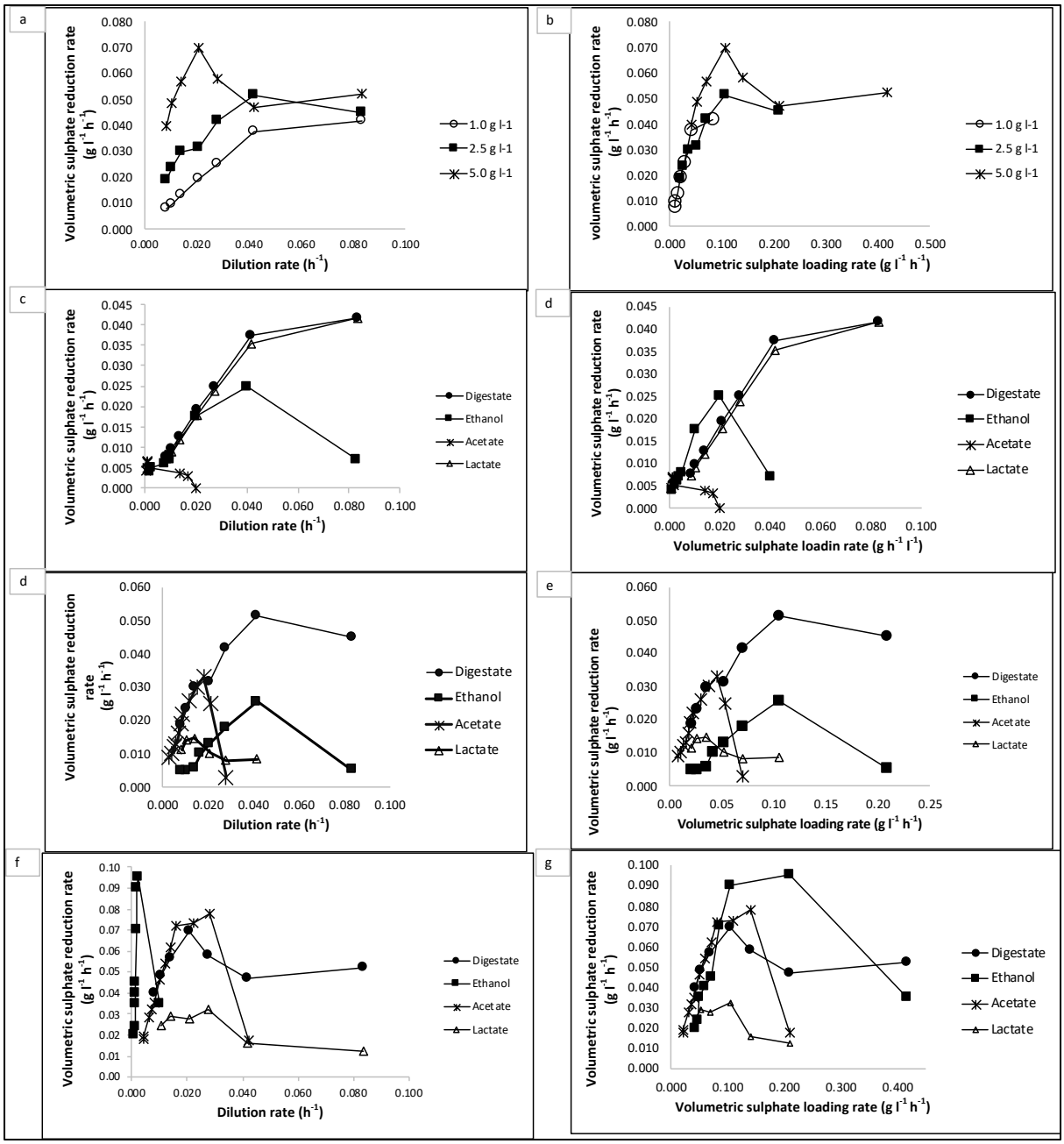


Figure 5.8. Effect of feed sulphate concentration, dilution rate and volumetric sulphate loading on volumetric sulphate reduction rate with various carbon source and feed sulphate concentrations: anaerobic digestate across feed sulphate concentration range (a and b); 1.0 g l^{-1} sulphate concentration (c and d); 2.5 g l^{-1} sulphate concentration (e and f); 5.0 g l^{-1} sulphate concentration (g and h).

The results in Figure 5.8 demonstrate that at ideal conditions, similar performance of acetate, lactate and digestate can be attained. Best performance of ethanol was observed at lower VSLRs as it is unable to sustain BSR at higher VSLR due to sulphide toxicity (Erasmus 2000; Hansford *et al*., 2007). The

combined VFA profile of the anaerobic digestate consisting of acetate, propionate and butyrate results in the demonstrated resilience and, therefore, is expected to support a more diverse microbial community. The microbial diversity as a function of dilution rate and feed sulphate concentration is investigated in **Chapter 6**.

Sulphate toxicity associated with high redox potential may affect the BSR using anaerobic digestate. Observations made from Figures 5.5b, 5.6b and 5.7b indicate that the residual sulphate concentration increased with increasing dilution rate. Figure 5.9 indicates that an increase in residual sulphate concentration resulted in the increase in redox potential. This increase was pronounced in the reactors receiving 2.5 and 5.0 g l^{-1} sulphate. For the reactor receiving a feed medium containing 2.5 g l^{-1}, the redox potential across the lower dilution rate of 0.0083 to 0.028 h^{-1} (RTs of 5 to 1.5 d) was between -401 mV and -350 mV. Since SRB require a low redox potential (<-200 mV) for growth, with optimum growth between -390 and -490 mV (Postgate, 1984; White and Gadd, 1996), these low redox potential values allowed the SRB within the bioreactor to thrive (Section 2.4.4.4). This is corroborated by high sulphate conversions (77.2 ± 15.6%) and high concentrations of dissolved sulphide (0.37 ± 0.16 g l^{-1}) observed across these dilution rates, as indicated in Figures 5.5a, 5.6a and 5.7a and 5.5c, 5.6c and 5.7c, respectively.

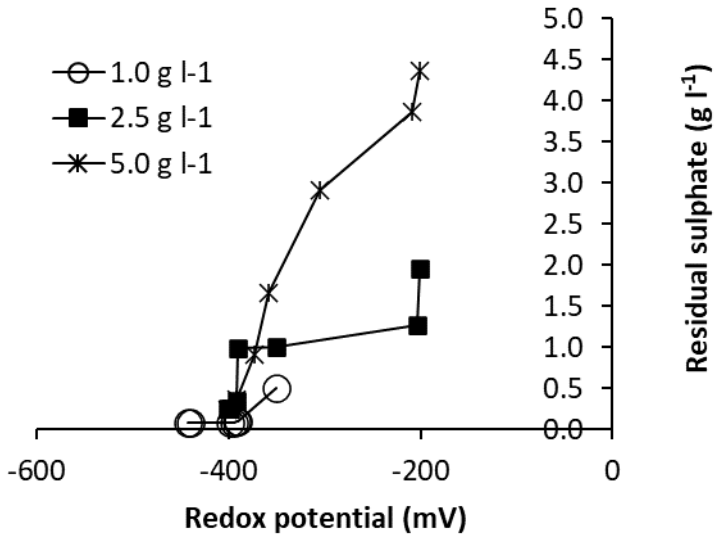


Figure 5.9. Effect of residual sulphate concentration on the redox potential in reactors maintained on anaerobic digestate as carbon source and electron donor, supplemented with 1.0, 2.5 and 5.0 g l^{-1} sulphate. Redox analyses were done using a Metrohm Redox platinum-ring electrode probe (Model 6.0451.00, Herisau, Switzerland).

Contrarily, higher redox potential value of -203 and -201 mV observed at the higher dilution rates of 0.042 and 0.083 h^{-1} (RTs of 1 and 0.5 d) in the reactors fed with 2.5 and 5.0 g.l^{-1} sulphate are likely to have selected for non-SRB species and thus result in the poor performance of the reactor (Section 2.4.4.4). This is supported by higher residual sulphate concentrations (1.2 – 4.4 g l^{-1}) indicated in Figure

5.9, lower sulphate conversions lying in the range 13 to 50% (Figures 5.6b and 5.7b) and lower sulphide concentrations observed at these dilution rates (0.19 – 0.99 g l^{-1}) (Figures 5.6c and 5.7c). The compromised sulphate conversion at these higher redox conditions thus indicated reduced sulphate reduction relative to sulphate available in the feed. While the performance at high feed concentrations and intermediate dilution rates demonstrated the potential for high VSRRs with increased VSLR, at the maximum dilution rate used, all VSSR tended to the same concentration consistent with similar biomass loadings.

The following hypotheses are made to extrapolate the trends of VSRR and anaerobic digestate utilisation observed above:

1. <u>The combined effect of acetate, propionate and butyrate on different SRB communities</u>

The high VSRRs observed when anaerobic digestate is provided as a carbon source and electron donor are possibly due to the diverse SRB communities established within the reactor, following seeding with a diverse inoculum (Table 4.1 and Figure 4.2), which could utilise one or more of the available VFAs, therefore resulting in a resilient SRB system. Contrarily, if a single VFA is provided as a sole carbon source and electron donor for BSR, only the SRB which can utilise the available VFA thrive. This notion is supported by observations in Figure 5.8 where the reactor receiving anaerobic digestate as a as a sole carbon source and electron donor for BSR was demonstrated to sustain higher VSLRs as compared to acetate. The simultaneous use of the mixed VFAs in anaerobic digestate allowed for a robust SRB community. This was contrary to observations made with lactate (Oyekola *et al*., 2012) where it was demonstrated that lactate, and not propionate nor acetate, was preferentially used by the mixed SRB community. Studies have reported methanogens outcompeting SRB in acetate fed reactors (Harada *et al*. 1994; Mizuno *et al.* 1994; Oude Elferink *et al.* 1994; Bhattacharya *et al*., 1996; Omil *et al.* 1998; van den Brand *et al* 2014). To date, no methanogens have been reported to grow on either propionate or butyrate. No methanogens were detected within the inoculum (Section 4.3.2) and the reduced sulphate conversion observed across the lower dilution rates of 0.042 to 0.083 h^{-1} (RTs of 1 to 0.5 d) in the reactors receiving feed sulphate concentrations of 2.5 and 5.0 g l^{-1} (Figures 5.6a and 5.7a, respectively) indicated washout of significant SRB communities in these reactors.

2. <u>Influence of sulphide concentration on microbial populations</u>

Although SRB have the highest tolerance for sulphide, sulphate reduction can be inhibited by sulphide (Postgate *et al*., 1984; Reis *et al*., 1992; Okabe *et al.*, 1995; Maillacheruvu and Parkin, 1996; Kaksonen *et al*., 2004a; Moosa and Harrison, 2006; Oyekola *et al*., 2012; Häusler *et al*., 2014) and this inhibition depends on the sulphide speciation. In this study, the pH was maintained at pH8.0 ± 0.2 (Section 3.4) thus resulting in HS^- being the predominant sulphide species, as discussed in Section 2.4.4.7. A concentration between 0.4 and 1.04 g l^{-1} HS^- (total sulphide concentration between 0.57 and 1.11 g l^{-1}) was demonstrated to inhibit 50% of SRB as the pH ranged from 7.2 and 8.5 (Visser *et al*., 1995;

O'Flaherty *et al*., 1998). Studies with acetate indicated that on increasing total sulphide concentration in the range of 0.75 to 1.45 g l^{-1} (undissociated hydrogen sulphide concentrations of 0.07 to 0.16 g l^{-1}), a decrease of volumetric sulphate reduction rates resulted (Moosa and Harrison, 2006). Studies with mixed acetate and butyrate demonstrated that dissolved sulphide concentrations of 0.15 to 0.20 g l^{-1} (0.06 to 0.075 g l^{-1} of undissociated hydrogen sulphide) resulted in the inhibition of some SRB communities (Kuo and Shu, 2004). From Figures 5.5, 5.6 and 5.7, it is seen that the highest sulphide concentrations correlate with high sulphate conversions, as anticipated. Typically, on reaching maximum VSRR, the sulphate conversion falls off with further increase in feed flowrate and hence dilution rate. This is accompanied by a reduction in sulphide concentration owing to more rapid removal of sulphide in the effluent while it is formed at a constant or decreasing VSRR. The consideration of VSRR as a function of dissolved sulphide concentration, shown in Figure 5.10, supports the lack of correlation between these and indicates that the highest VSRR of 0.070 g l^{-1} h^{-1} was obtained at a high dissolved sulphide concentration of approximately 0.7 g l^{-1}.

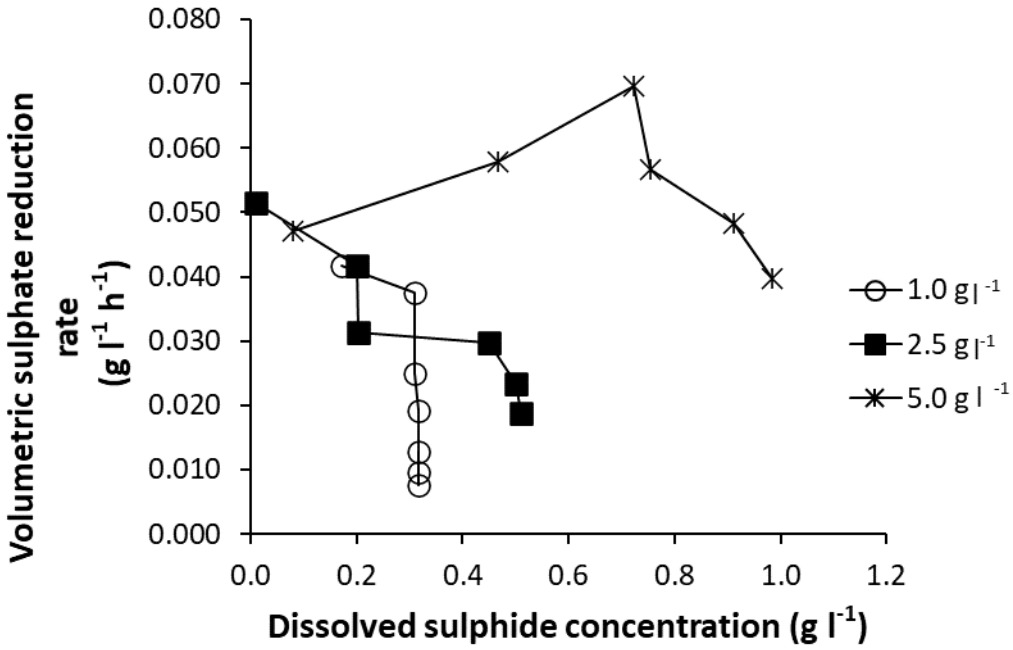


Figure 5.10. Effect of dissolved sulphide concentration on the volumetric sulphate reduction rate in reactors maintained on anaerobic digestate carbon source and electron donor, supplemented with 1.0, 2.5 and 5.0 g l^{-1} sulphate.

5.4.2.2 Sulphate conversion

In all the three reactors receiving anaerobic digestate as a carbon source and electron donor for BSR, the maximum sulphate conversion achieved was at the lowest dilution rates (Table 5.7). These observations were in agreement with studies with acetate (Moosa, 2000; Moosa *et al*., 2002; 2005),

ethanol (Erasmus, 2000; Hansford *et al*., 2007) and lactate (Oyekola *et al*., 2009; 2010; 2012) (Table 5.6 and Figure 5.11). For the reactor receiving sulphate at the concentration of 1.0 g l^{-1}, an average of 91 ± 1.0% sulphate conversion was observed at dilution lower rates (0.0083-0.042 h^{-1}, RTs of 5 to 1 d) when digestate was used as a carbon source and electron donor for BSR. Increasing the dilution rate to 0.042 h^{-1} resulted in the decrease of sulphate concentration to 50% (Figures 5.10a and 5.10b). The decrease in sulphate conversion was associated with a decrease in propionate and butyrate conversion (Figure 5.5a). Contrarily, the metabolic efficiency of acetate remained at 90 ± 1.2% across the dilution rates of 0.0083-0.083 h^{-1} (RTs of 5 to 0.5 d) (Section 5.4.1.1). This indicated that propionate and butyrate oxidisers were more successful at lower dilution rates, while acetate oxidisers with higher μ_{max} and K_S were able to proliferate even at a higher dilution rates and associated volumetric sulphate loading rates (VSLRs) in this reactor. Similarly, high sulphate conversions were associated with high propionate and butyrate conversions in reactors receiving feed sulphate concentrations at 2.5 and 5.0 g l^{-1} (Figures 5.6a and 5.7a). However, unlike in the reactor receiving sulphate concentration of 1.0 g l^{-1}, the stoichiometry ratios observed for A:S (acetate: sulphate) deviated from the theoretical ratio at the highest dilution rate of 0.083 h^{-1} (RT of 0.5 d, Table 5.3c.).

Table 5.7. Effect of feed sulphate concentration and carbon source on maximum sulphate conversion.

Feed sulphate concentration (g l^{-1})	Carbon source	Maximum sulphate conversion (%)	Corresponding volumetric sulphate loading rate (g l^{-1} h^{-1})	Corresponding dilution rate (h^{-1})	Reference
1.0	Anaerobic Digestate	91 ± 1.0	0.008-0.038	0.0083-0.042	This study
2.5	Anaerobic Digestate	89 ± 2.3	0.019-0.030	0.0083-0.014	This study
5.0	Anaerobic Digestate	90 ± 7.3	0.040-0.070	0.0083-0.014	This study
1	Lactate	87 ± 0.14	0.0072-0.010	0.0083-0.042	Oyekola *et al.* (2010)
2.5	Lactate	54 ± 0.07	0.011-0.014	0.0083-0.010	Oyekola *et al.* (2010)
5.0	Lactate	57 ± 2.0	0.024-0.029	0.0083-0.010	Oyekola *et al.* (2010)
1.0	Acetate	85	0.006	0.006	Moosa *et al*. (2002)
2.5	Acetate	90	0.015	0.006	Moosa *et al*. (2002)
5.0	Acetate	92	0.023	0.004	Moosa *et al*. (2002)
1.0	Ethanol	86± 3	0.004 to 0.021	0.004 to 0.021	Hansford *et al*. (2007)
2.5	Ethanol	93 ± 2	0.010 to 0.052	0.004 to 0.021	Hansford *et al*. (2007)
5.0	Ethanol	93 ± 5	0.021 to 0.104	0.004 to 0.021	Hansford *et al*. (2007)

The results obtained with anaerobic digestate as a carbon source and electron donor for BSR were compared with results obtained with ethanol (Hansford *et al*, 2007), acetate (Moosa *et al*., 2002) and lactate (Oyekola *et al*., 2010) in similar reactor systems and under similar experimental conditions (sulphate concentration, temperature, pH and COD: SO_4^{2-} ratio). Similar trends were observed for all the four carbon sources and electron donors with sulphate conversion decreasing as dilution rates increased from 0.0083 to 0.083 h^{-1} (Figures 5.10b to 5.10d). For the reactors receiving feed containing

a sulphate concentration of 1.0 g l^{-1}, similar performances between anaerobic digestate and lactate were observed with a conversion of 85 – 95% maintained across dilution rates of 0.0083 to 0.042 h^{-1}. While high sulphate conversions were observed for both acetate (85%) and ethanol (86 ± 3%), these high conversions were not supported at the higher dilution rates tested (b). For a feed medium containing 2.5 and 5.0 g l^{-1} sulphate, similar trends were observed between anaerobic digestate, acetate and ethanol with maximum conversions of 85 to 95% achieved at low dilution rates and conversion falling off with increasing dilution rate; however, the dilution rate at which onset of reduced conversion was achieved varied. The results obtained with lactate as a carbon source and electron donor demonstrated lower sulphate conversions (≤ 60%) even at lower dilution rates (0.0083 h^{-1}) (Figures 5.10c and 5.10d) which was attributed to lactate fermentation limiting available electron donor for BSR (Oyekola *et al*., 2012).

The results in Table 4.1 and Figure 4.2 indicated that the mixed SRB inoculum used in this thesis was more diverse than previously reported by Oyekola (2008). The use of acetate, propionate or butyrate as a sole carbon source and electron donor for BSR using SRB communities and syntrophic microbial communities is well established in literature (Parkin *et al*., 1990; Visser *et al*., 1993; Oude Elferink *et al*., 1994; Maillacheruvu and Parkin; 1996; Omil *et al*., 1998 ; O'Flaherty and Colleran 1999; O'Flaherty *et al*., 1999;Moosa *et al*., 2002; Greben *et al*., 2004; Kaksonen *et al*., 2004b; Stams *et al*., 2005 NB; Grigoryan *et al*., 2007; Das *et al*., 2008; Mulopo *et al*., 2010; Sorokin *et al*., 2010; van den Brand *et al*., 2014a; Ozuolmez *et al*., 2015; Chen *et al*., 2017) but there is fairly limited literature on propionate and butyrate. The use of these electron donors is discussed in Section 2.5.1. Speece (1996) reported that the presence of SRB in a sulphidogenic bioreactor substantially enhances the degradation of propionate. Greben *et al*. (2004) reported 78% sulphate removal using propionate as a sole carbon source and electron donor and 55.5% sulphate removal when acetate was used as a sole carbon source and electron donor for BSR at a feed medium containing 1.2 g l^{-1} sulphate. Uberoi and Bhattacharya (1995) were able to achieve 90% sulphate removal with propionate. Experiments by Mizuno and Noike (1994) demonstrated that up to 67% sulphate removal can be achieved when butyrate served as a carbon source for BSR. The results in Table 5.7 and Figure 5.11 indicate that anaerobic digestate (primarily acetate, propionate and butyrate) resulted in high sulphate conversions over an extended range of dilution rates and VSLRs, thus showing potential a good substrate for BSR.

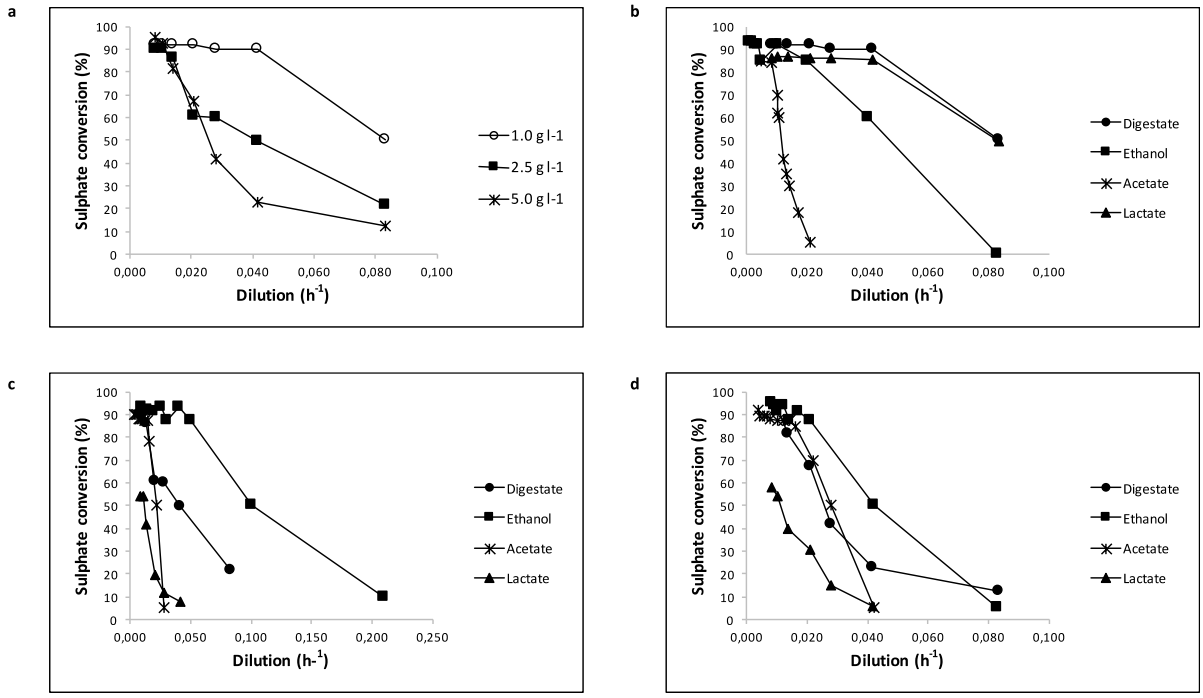


Figure 5.11. Steady-state sulphate conversion of continuous reactors with various carbon source and feed sulphate concentrations: anaerobic digestate (a), 1.0 g l^{-1} sulphate concentration (b), 2.5 g l^{-1} sulphate concentration (c), 5.0 g l^{-1} sulphate concentration (d).

Although the effective sulphate reduction on the utilisation of the mixed VFAs (acetate, propionate and butyrate) by SRB of interest has been reported previously, the resulting BSR kinetics have not been considered rigorously in the current literature. In this thesis, simultaneous utilisation of acetate, propionate and butyrate by the mixed SRB consortium was observed in all the reactors tested which suggests a robust SRB community. This was contrary to the observations made on using lactate as electron donor (Oyekola *et al*., 2012) where it was demonstrated that lactate was preferentially used over the products of lactate metabolism: propionate or acetate. The results in Table 5.7 and Figure 5.11 support the notion that under ideal conditions, anaerobic digestate can result in similar or higher sulphate conversions when compared to acetate, ethanol and lactate. Comparison of sulphate removal rates (both conversions and VSRR) obtained with anaerobic digestate and other complex cost-effective carbon sources and electron donors from different studies is presented in Table 5.8. While the initial concentrations in some of these studies varied, these studies provide insight into sulphate utilisation by mixed SRB communities when complex cost-effective carbon sources and electron donors are provided. Compared to other complex carbon sources, anaerobic digestate demonstrated higher sulphate conversions and higher VSRR at similar hydraulic retention times (HRTs) (Table 5.8), demonstrating that anaerobic digestate is a good substrate for sulphate reduction and can sustain BSR long term. Its cost-effectiveness has potential to be enhanced by using existing water bodies or treated effluent for on-site cultivation of *Arthrospira platensis*. Furthermore, the results presented in this study are generated in a CSTR with no biomass retention, whereas many of the systems to which it was compared

Table 5.8. Comparison of sulphate conversions (SC) and volumetric sulphate reduction rates achieved with different complex carbon sources and electron donor for BSR

Carbon source and electron donor	Reactor type	[Feed SO_4^{2-}] ($g\ l^{-1}$)	COD/SO_4^{2-}	HRT (h)	pH	Temp (°)	SC (%)	VSRR ($g\ l^{-1}h^{-1}$)	Reference
Whey	UAPB	2	NR	192	NR	14-24	98	0.0048	Drury (1999)
Micro-algal biomass	UASB	0.56	8.1	NR	NR	NR	90 ± 10.0	0.013	Boshoff *et al.*(2004)
		0.30	11.2	NR	NR	NR	74.5 ± 17.0	0.015	
		0.16	15.0	NR	NR	NR	71 ± 17.2	0.024	
		3.6	4.5	10.6	NR	35	39	0.023	
Wine waste	DFAFBR	2.5	NR	192	7.5	22	91	0.011	Costa *et al*. (2009)
Reed canary grass hydrolyzate	FBR	4	NR	9	7.5	35	31	0.092	Lakaniemi *et al*. (2010)
Landfill leachate	FBR	2.0	1	25	7	35	43	0.038	Sahinkaya *et al*. (2013)
Sweetmeat waste	UAPB	1.4 ± 0.003	1	24	7.2-7.4	25-28	61	0.036	Das *et al*. (2015)
		2.9 ± 0.006	2	24	7.2-7.4	25-28	76	0.045	
		5.7 ± 0.011	4	24	7.2-7.4	25-28	99	0.059	
		11.4 ± 0.08	8	24	7.2-7.4	25-28	97	0.055	
Marine waste extract	UPBR	4	0.281	120	8	35	97	0.012	Dev *et al*. (2014)
Grass cuttings	UAPB	2	NR	2	7-7.7	25	57	NR	Mulopo (2016)
Anaerobic digestate	CSTR	1.0	0.7	24-120	8.0 ± 0.2	35	91 ± 1.0	0.042	This study
		2.5	0.7	72-120	8.0 ± 0.2	35	89 ± 2.3	0.051	
		5.0	0.7	72-120	8.0 ± 0.2	35	90 ± 7.3	0.070	

CSTR = Continuous stirred tank reactor
COD = Chemical oxygen demand
DFAFBR = Down flow anaerobic packed bed reactor
HRT = Hydraulic retention time
FBR = Fluidised-bed reactor
UAPB = Upflow anaerobic packed bed reactor
UASB = Upflow anaerobic sludge blanket reactor
UPBR = Upflow packed bed reactor
VSRR = volumetric sulphate reduction rate
NR = Not reported

include biomass retention. It is well recognised that biomass retention allows biomass washout to be minimised such that hydraulic and biomass retention times are uncoupled (Marais 2019; Hessler 2020). This facilitates sustained specific sulphate reduction rates at high hydraulic retention times. Enhanced VSRRs can be expected on using the microbial communities and electron donor of this study in combination with biomass retention.

5.4.3 Bacterial dry mass

An increase in feed sulphate concentration in the range 1.0 to 5.0 g l^{-1} resulted in an increase in the maximum bacterial dry mass concentration when anaerobic digestate was used as carbon source and electron donor for BSR (Table 5.9). These observations are corroborated by studies under similar conditions (sulphate concentration, temperature, pH and COD: SO_4^{2-} ratio) with ethanol (Erasmus, 2000; Hansford *et al*., 2007) acetate (Moosa *et al*., 2002) and lactate (Oyekola *et al.*, 2010). In all experiments conducted with anaerobic digestate as a carbon source and electron donor, maximum bacterial dry mass concentration was obtained at the lowest dilution rate (0.0083 h^{-1}) (Table 5.9, Figure 5.12). Maximum sulphate conversions were associated with maximum bacterial dry mass. At all feed sulphate concentrations, the experimental stoichiometry ratios observed with maximum bacterial dry mass for oxidation of acetate and concomitant sulphate reduction was 0.9, which concurred with the theoretical ratio of 1.0. Table 5.9 suggests that incomplete oxidation of propionate and butyrate occurred when the maximum bacterial dry mass was present in the reactor. Increasing volumetric sulphate loading rate (VSLR) negatively influenced the bacterial dry mass concentration in each bioreactor (Figures 5.5a, 5.6a and 5.7a). Similar observations were made with lactate (Oyekola et al., 2010). Contrarily, experiments with ethanol (Erasmus, 2000; Hansford *et al*., 2007) and acetate (Moosa *et al*., 2002) indicated that VSLR positively influenced the bacterial dry mass concentration in the range 1.0 to 5.0 g l^{-1}.

Table 5.9. Effect of feed sulphate concentration and maximum bacterial dry mass on sulphate reduction and volatile fatty acid utilisation when anaerobic digestate was used as a carbon source and electron donor

Feed sulphate concentration (g l^{-1})	Maximum bacterial dry mass (g_{DW} l^{-1})	VSLR (g l^{-1} h^{-1})	VSRR (g $l^{-1}h^{-1}$)	Sulphate conversion (%)	Mole acetate used per mole of sulphate	Mole propionate used per mole of sulphate	Mole butyrate used per mole of sulphate
1	0.628	0.083	0.0077	92.0	0.9	0.2	0.1
2.5	1.488	0.021	0.019	90.1	0.9	0.3	0.1
5.0	3.161	0.042	0.040	95.4	0.9	0.1	0.1

VSRR= Volumetric sulphate reduction rate VSLR= Volumetric sulphate loading rate

Maximum bacterial dry mass was not associated with maximum volumetric sulphate reduction rates (VSRRs). Figure 5.12, correlating biomass concentration and VSRR across a dilution rate in the range 0.0083 to 0.083 h^{-1}, indicates that, at all three reactor feed concentrations, as bacterial dry mass declined from its maximum starting value, an increase in VSRR was observed with increasing dilution rate and VSLR until a maximum or plateau was reached. These observations suggest, firstly, that some of the SRB communities were not able to be sustained in the reactors at high dilution rates due to having lower specific growth rates (μ) and were therefore washed out of the CSTRs. Secondly, the inverse relationship between bacterial concentration and VSRR shown in Figure 5.12 suggests an increase in the specific activity of the biomass with increasing dilution rate such that the specific VSRR increased with dilution rate. Thirdly, at low dilution rates, the increased biomass concentration supported by increasing feed sulphate concentration resulted in an increased VSRR. This is in agreement with Meulepas *et al*. (2009) who showed that VSRR can be increased by further increasing the biomass concentration when acetate was used as a carbon source and electron donor for BSR. Studies with butyrate as a sole carbon source and electron donor for BSR demonstrated that an increase in biomass concentration of *Desulfobotulus alkaliphilus* at pH10 resulted in an increase of sulphate reduction Sorokin *et al*. (2010). Fourthly, increasing the dilution rate to 0.083 h^{-1} (RT of 0.5 d) resulted in all three reactors showing similar bacterial dry mas of 0.18 ± 0.03 g l^{-1} and a similar VSRR of 0.046 ± 0.0023 g l^{-1} h^{-1}, suggesting that despite washout of some SRB in these reactors, enhanced activity supported a VSRR of 65% of the maximum reported across all reactors and the residual sulphate concentration across these reactors suggested that the consistent VSRR resulted from operation in the zero order region with respect to kinetic rates and sulphate concentration.

In Table 5.10, the values of maximum bacterial dry mass concentrations obtained in this study using digestate are compared with those obtained when acetate (Moosa et al., 2002), ethanol (Hansford et al., 2007) and lactate (Oyekola et al., 2010) were used as single carbon source and electron donor for BSR in the feed sulphate concentration range 1.0 to 5.0 g l-1. Increasing feed sulphate concentration in this range resulted in the increase in the maximum bacterial dry mass concentrations with acetate (Moosa et al., 2002) and lactate (Oyekola et al., 2010), but not ethanol (Hansford et al., 2007) (Table 5.10). At a feed sulphate concentration of 1.0 and 2.5 g l-1, acetate resulted in the highest bacterial concentration followed by anaerobic digestate and ethanol, respectively. Overall, lactate resulted in the lowest bacterial dry mass. However, at a feed sulphate concentration of 5.0 g l-1, the values obtained for anaerobic digestate and acetate were comparable (Table 5.10).

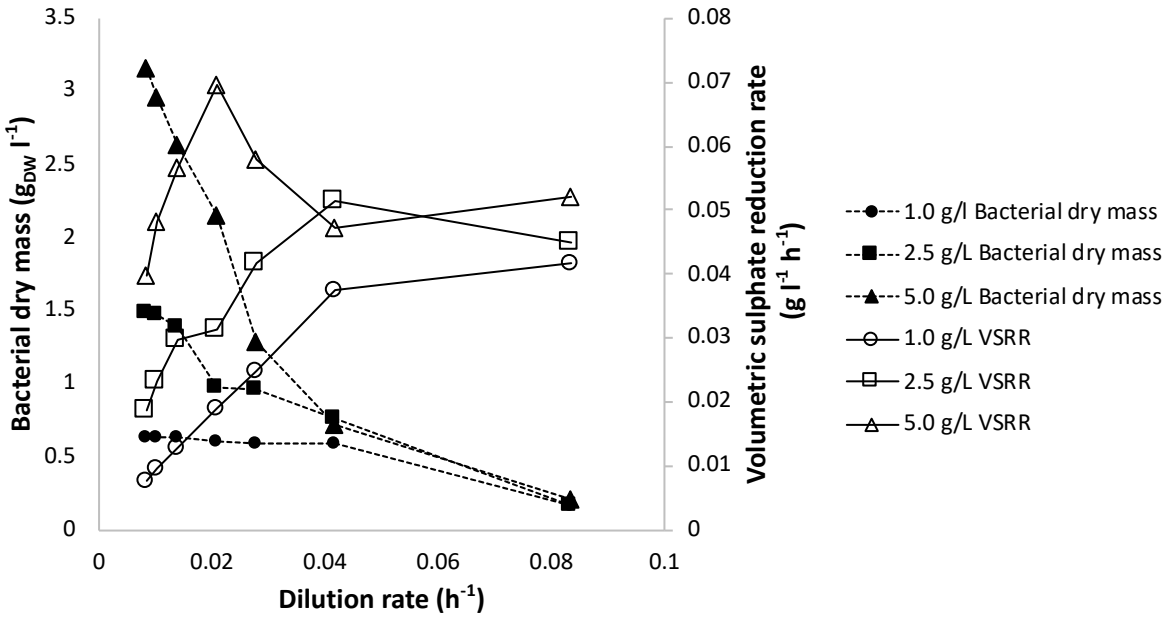


Figure 5.12. Relationship between bacterial dry mass (g_{DW} l^{-1}) and volumetric sulphate reduction rate (VSRR) across the dilution rate in the range 0.0083 and 0.083 h^{-1} in reactors maintained on anaerobic digestate carbon source and electron donor, supplemented with 1.0, 2.5 and 5.0 g l^{-1} sulphate.

Table 5.10. Comparison of maximum bacterial dry mass (g_{DW} l^{-1}) obtained with various carbon sources and electron donors

Sulphate concentration (g l^{-1})	**Maximum bacterial dry mass (g_{DW} l^{-1})**			
	Anaerobic digestate	**Acetate** Moosa *et al*. (2002)	**Ethanol** Hansford *et al*. (2007)	**Lactate** Oyekola *et al*. (2010)
1.0	0.628	0.980	0.500	0.267
2.5	1.488	2.600	0.420	0.350
5.0	3.161	3.100	0.420	0.458

Previous studies have reported that with regards to energy and biomass produced, lactate was a superior electron donor compared to propionate, butyrate, ethanol and acetate (Nagpal *et al*., 2000, Sheoran *et al*., 2010). The high bacterial dry mass observed in the presence of anaerobic digestate could suggest a diverse SRB consortium due to the presence of more than one carbon source and electron donor (Table 2.6 in Section 2.4.3). The presence of nitrogen (7.7%) observed in anaerobic digestate (Table 2.8 in Section 2.5.1) may have also contributed in the bacterial growth. Dev *et al*. (2014) demonstrated that MSRB medium (which was developed by replacing a commercial nitrogen source of standard SRB growth media with marine waste extract with additional nitrogen) resulted in better SRB growth.

5.4.4 Determination of yield coefficients and kinetic constants

Analysis of bacterial yield ($Y_{x/s}$) and the maintenance coefficient (m_s) were based on the relationship between the bacterial growth and sulphate utilisation rate (VSRR) (r_s) as described by the Pirt equation (Equation 5.6c).

$$\frac{r_s}{X} = \mu \frac{1}{Y_{x/s}} + m_s \qquad \text{Equation 5.6c}$$

As indicated in Table 5.11, the biomass yields estimated based on the sulphate utilisation (0.655, 0.608 and 0.632 $g_{DW}\ g_{SO4}^{-1}$) were similar at feed concentrations of 1.0, 2.5 and 5.0 g l^{-1}, respectively when anaerobic digestate was used as a carbon source and electron donor for BSR. The yields obtained with acetate (Moosa *et al*., 2002) were also similar across this feed sulphate concentration range. The yields obtained with anaerobic digestate were some 10% higher that the yields obtained with acetate (Table 5.10). This indicated that the mixed VFAs resulted in slightly better growth yields than when a single VFA (acetate) was provided. This is in agreement with results in Table 5.10. The values obtained for the maintenance coefficient were negative and significantly smaller than $\frac{\mu}{Y_{x/s}}$, therefore approximated as negligible.

Table 5.11. The values of biomass yields and maintenance coefficients when anaerobic digestate or acetate was used as a carbon source and electron donor for BSR (the correlation of coefficient value, R^2, is included)

Sulphate concentration (g l^{-1})	**Anaerobic digestate** (This study)			**Acetate** (Moosa *et al*., 2002)
	$Y_{x/s}$ $g_{DW}\ g_{SO_4^{2-}}^{-1}$	**m_s** $g_{SO_4^{2-}}\ g_{DW}^{-1}\ h^{-1}$	**R^2**	**($Y_{x/s}$)** $g_{DW}\ g_{SO_4^{2-}}^{-1}$
1.0	0.655	-0.0005	0.9997	0.584
2.5	0.608	-0.0014	0.9995	0.573
5.0	0.623	-0.0006	0.9992	0.567

Previously employed kinetic models, Monod (Equation 2.1), Contois (Equation 2.3) and a model previously developed by Moosa *et al*. (2002) (Equation 2.4) which was based on the Contois model, were used to describe BSR kinetics with anaerobic digestate as a carbon source and electron donor. In a continuous culture at steady state where feed is sterile, biomass retention is negligible and cell death is negligible, μ= D (dilution rate). Moosa *et al*. (2002) tested Equation 2.1 and Equation 2.3 to describe BSR kinetics when acetate was used as a carbon source and electron donor, and based on the goodness of fit, the Contois equation was preferred to describe the BSR kinetics in the reactor. In their study, Moosa *et al*. (2002) assumed sulphate to be the dominant limiting substrate.

$$\mu = \frac{\mu_{max} S}{K_S + S}$$ Equation 2.1

$$\mu = \frac{\mu_{max} S}{K_S'' X + S}$$ Equation 2.3

$$\mu = \frac{\mu_{max} S}{K_S' S_0 X + S}$$ Equation 2.5

In this thesis, the above kinetic models were used to describe the microbial coefficients maximum growth rate (μ_{max}) and the half saturation constant of sulphate (K_s) using the steady state data, assuming sulphate as the limiting substrate. Based on the results from Table 5.3 in Section 5.4.1.1, in all the three reactors, the observed experimental ratios of acetate oxidation (A:S and A:HC), incomplete oxidation of propionate (P:S and P:HC), incomplete butyrate oxidation (A:S) and concomitant sulphate reduction concurred with theoretical ratios across the lower dilution rates of 0.0083 and 0.028 h^{-1} (RTs of 5 to 1.5 d) (Tables 5.3b). Contrarily, at the highest dilution rate of 0.083 h^{-1} (RT of 0.5 d), the experimental ratios deviated largely from the theoretical ones (Table 5.3c). Therefore, data across the lower dilution rates of 0.0083 to 0.028 h^{-1} was used to describe sulphate utilisation under conditions where BSR reaction and concomitant VFA oxidation was dominant. The observed kinetic constants (μ_{max} and K_s) are indicated in Table 5.12.

Table 5.12. Kinetic constants obtained using the Monod, Contois kinetic and Moosa *et al*. (2002) models assuming sulphate is limiting substrate. The correlation coefficient (R^2) is also indicated.

Kinetic model	Kinetic constant	Feed sulphate concentration (g l^{-1})		
		1.0	**2.5**	**5.0**
Monod	μ_{max}	0.29	0.080	0.015
	K_s	0.87	1.90	0.49
	R^2	0.791	0.935	0.990
Contois	μ_{max}	0.12	0.044	0.019
	K_s	0.50	0.64	0.41
	R^2	0.774	0.935	0.956
Moosa *et al*. (2002	μ_{max}	0.12	0.044	0.055
	K_s	0.50	0.26	1.2
	R^2	0.774	0.939	0.956

In order to determine the goodness of fit of these models to describe the dependence of microbial growth on sulphate concentration, the microbial growth constants (μ_{max} and K_s) in Table 5.12, the calculated specific growth (μ) values were compared with the actual μ values (experimental dilution rates) in parity plots (Figure 5.13). The Monod, Contois and Moosa *et al*. (2002) models indicated similar fit across

the dilution rates of 0.0010 to 0.042 h^{-1} from the reactor receiving feed sulphate of 2.5 $g1^{-1}$ (Figures 5.13a, 5.13b and 5.13c respectively).

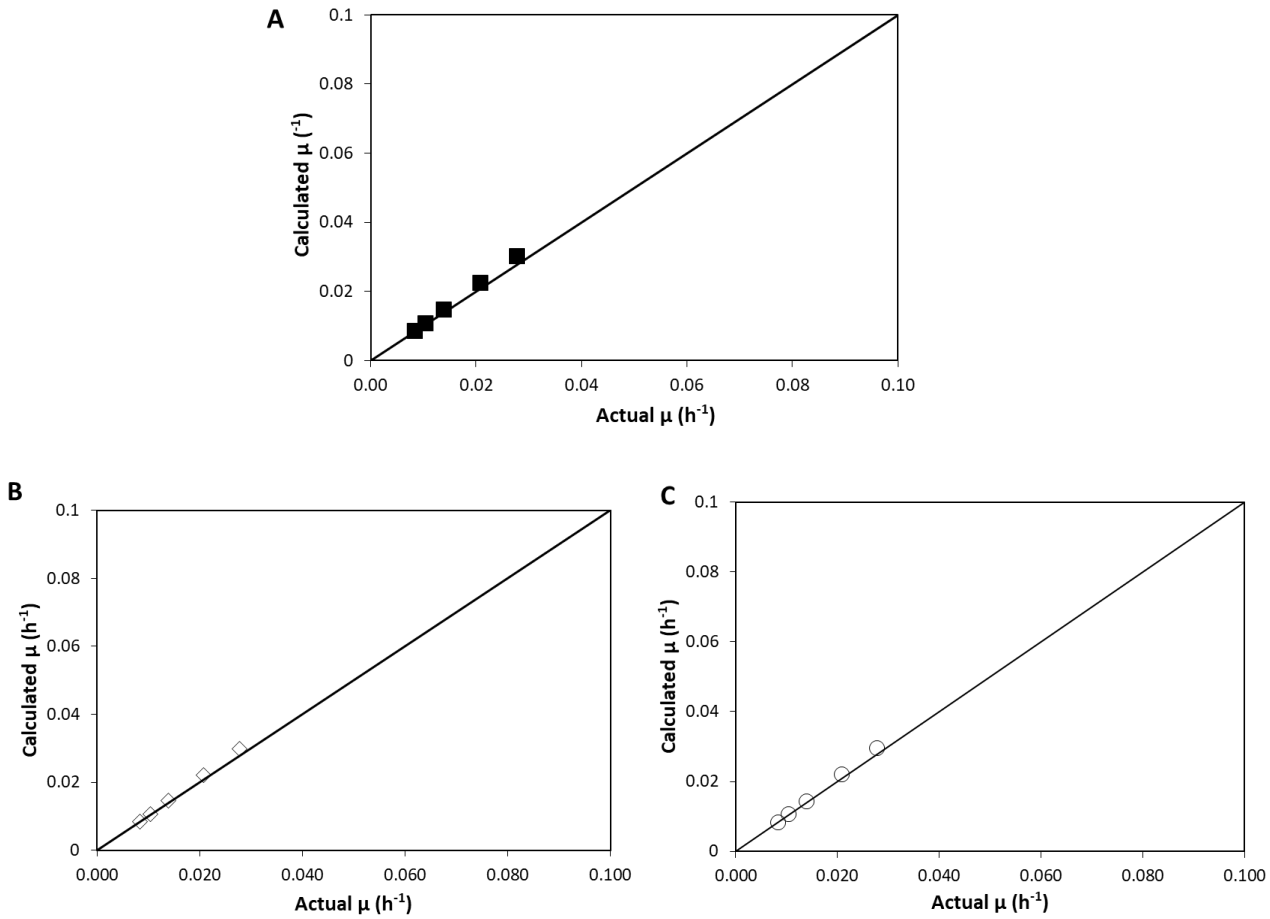


Figure 5.13. Comparison of the actual specific growth (μ) values, presented as h^{-1}, and the predicted μ values using parity charts using the Monod model (A) Contois model (B) and Moosa *et al*. (2002) model (C) using data from the reactor receiving feed sulphate concentration of 2.5 g 1^{-1}.

A further test on the goodness of fit was based on the sum of squared error (SSE) based on μ. The SSE is defined as the sum of square of the difference between the observed μ (y_i) and the predicted μ ($\hat{y}_i$) values as indicated by Equation 5.7.

$$=\sum(y_i - \hat{y}_i)^2 \qquad \text{Equation 5.7}$$

The Moosa *et al*. (2002) model was characterised by the smallest values of the sum of squared error (SSE) based on μ across all the sulphate concentrations which supported its goodness of fit (Table 5.12). Therefore, based on the goodness of fit, as demonstrated by the parity chart (Figure 5.13b)

and the value of SSE (Table 5.13), the Moosa *et al*. (2002) model was preferred to describe the sulphate utilisation kinetics when anaerobic digestate served as a carbon source and electron donor for BSR.

Table 5.13. The sum of squared errors based on Monod, Contois and Moosa *et al*. (2002) kinetic models for reactors receiving feed sulphate concentration of 1.0, 2.5 and 5.0 g l^{-1}.

Feed sulphate concentration (g l^{-1})	Monod	Contois	Moosa *et al*. (2002)
1.0	0.0063	0.00072	0.00072
2.5	0.00014	0.00014	0.00014
5.0	0.0011	0.00088	0.00087

The maximum specific growth rate (μ_{max}) obtained for the reactor receiving feed sulphate concentration of 1.0 g l^{-1} was the higher (0.12 h^{-1}) than the μ_{max} obtained for the other two reactors (Table 5.14). The value of K_s, which is defined by the residual sulphate concentration at which the specific microbial growth rate is half the maximum specific growth rate was the highest in the reactor receiving feed sulphate concentration of 5.0 g l^{-1} (Table 5.14). This (K_s = 1.2 g l^{-1}) indicated that the affinity of the microorganisms for sulphate decreased when the feed sulphate concentration was increased from 1.0 g l^{-1} to 5.0 g l^{-1}.

Table 5.14. Kinetic constants based on sulphate utilisation using the Moosa *et al*. (2002) model.

Feed sulphate concentration (g l^{-1})	μ_{max}	K_s
1.0	0.12	0.50
2.5	0.044	0.26
5.0	0.055	1.2

In Table 5.15, the kinetic constants obtained in this study are compared with kinetic constants based on sulphate utilisation obtained when acetate (Moosa *et al*., 2002) and ethanol (Hansford *et al*., 2007) were used as single carbon sources and electron donors for BSR.

In the reactors receiving feed sulphate concentration of 1.0 g l^{-1}, the value of μ_{max} was higher when anaerobic digestate used than when either acetate or ethanol was used as a carbon sources and electron donor for BSR, meaning that washout of SRB will happen sooner when acetate or ethanol is provided. This a μ_{max} value was higher than the μ_{max} value of 0.085 h^{-1} demonstrated for SRB consortium growing on feed sulphate of 1.0 g l^{-1} and marine waste extract in a Upflow packed bed reactor (Dev *et al*., 2016). However, the study did not report the VFA composition in the reactor. At the highest sulphate concentration tested (5.0 g l^{-1}) the value determined for μ_{max} was comparable (0.06 h^{-1}) for all the three carbon sources and electron donors (Table 5.15). These results indicate that the mathematical kinetics

models previously used to describe acetate, ethanol and lactate were used successfully to describe BSR kinetics when anaerobic digestate was used as a carbon source and electron donor for BSR.

Table 5.15. Comparison of kinetic constants based on sulphate utilisation from the reactors receiving anaerobic digestate, acetate and ethanol as carbon sources and electron donors for BSR.

Feed sulphate concentration (g l^{-1})	Anaerobic digestate This study		Acetate Moosa *et al.* (2002)		Ethanol Hansford *et al.* (2007)	
	μ_{max}	K_s	μ_{max}	K_s	μ_{max}	K_s
1.0	0.12	0.50	0.058	0.027	0.058	0.069
2.5	0.044	0.26	0.061	0.038	0.061	0.093
5.0	0.055	1.2	0.063	0.125	0.059	0.124

5.5 CONCLUSION

The use of acetate, propionate or butyrate as a sole carbon source and electron donor for BSR on SRB communities and syntrophic microbial communities has been presented in literature. While this has been studied in detail for acetate, reports on propionate and butyrate are limited in terms of kinetic performance. This study demonstrated that anaerobic digestate from treatment of Spirulina biomass, containing a mixture of acetate, propionate and butyrate, can serve as carbon source and electron donor for BSR to sustain BSR long term. Its potential cost-effectiveness can be enhanced by using existing water bodies or treated effluent for on-site cultivation of *Arthrospira platensis*.

On using the Spirulina digestate as electron donor for BSR, the mixed VFAs were used simultaneously which allowed a robust SRB community to develop. The results indicated that the volumetric sulphate loading rate (VSLR), the product of the inverse of residence time (dilution rate) and feed sulphate concentration significantly influenced the kinetics and the stoichiometry of BSR. The kinetic profiles of the volumetric sulphate reduction rate (VSRR) resembled previous studies with acetate (Moosa *et al*., 2002) and ethanol (Erasmus, 2000; Hansford *et al*., 2007) and lactate (Oyekola *et al*., 2009; 2010; 2012). The high VSRR observed on use of anaerobic digestate as a carbon source and electron donor for BSR was attributed to combined use of acetate, propionate and butyrate. Maximum VSRRs of 0.04, 0.05 and 0.07 g l^{-1} h^{-1} were recorded at 1.0, 2.5 and 5.0 g l^{-1} sulphate and were maintained over an extended dilution rate range compared with single VFA feeds. Lower sulphate conversions observed at high dilution rates were attributed to the loss of some SRB species with lower μ_{max} values as well as the increased VSLR. Particularly, SRB capable of oxidation of propionate or butyrate appeared to be washed at dilution rates of 0.042 h^{-1} and above, resulting in greatly reduced propionate and butyrate conversion. Higher yield coefficients were observed with anaerobic digestate than with acetate as a carbon source and electron donor for BSR. These yield coefficients remained largely constant at 0.629

± 0.02 $g_{DW}\, g_{SO_4^{2-}}^{-1}$ across feed sulphate concentrations of 1.0, 2.5 and 5.0 g l^{-1}, respectively. Using a kinetic model previously developed by Moosa *et al*. (2002) and assuming sulphate as a limiting substrate, determination of the growth parameters μ_{max} and K_S of the mixed SRB culture were demonstrated for each feed sulphate concentration. At feed sulphate concentration of 1.0 g l^{-1}, μ_{max} and K_S were determined to be 0.12 h^{-1} and 0.50 g l^{-1} respectively. Lower μ_{max} values of 0.044 and 0.055 h^{-1} were observed at feed sulphate concentrations of 2.5 and 5.0 g l^{-1} respectively. The affinity for sulphate (K_s) decreased from 0.26 g l^{-1} to 1.2 g l^{-1} when feed sulphate concentration was increased from 2.5 g l^{-1} to 5.0 g l^{-1}. These observations indicates that in biological sulphate treatment of sulphate laden waters, anaerobic digestate has the potential to be used as a cost-effective carbon source and electron donor and remediation could be implemented in remote locations. To further corroborate the hypotheses and observations made in this Chapter, the microbial community dynamics are investigated in **Chapter 6**.

CHAPTER 6
MICROBIAL COMMUNITY DYNAMICS OF BIOLOGICAL SULPHATE REDUCTION REACTORS OPERATED ON ANAEROBIC DIGESTATE OR LACTATE

6.1 INTRODUCTION

Successful biological sulphate reduction (BSR) relies on a microbial community capable of the reduction of sulphate to soluble sulphide and concomitant generation of alkalinity in the presence of an electron donor under specified process conditions (Reaction 1.1) (Drury, 1999; Liamleam and Annachhatre, 2007; Ndlovu 2014; Akinwekomi *et al.*, 2016).

$$Electron\ donor\ (carbon\ source) + SO_4^{2-} \rightarrow HS^- + HCO_3^- \qquad \text{Reaction 1.1}$$

During BSR, the microbial ecology associated with the reactor determines the efficacy of sulphate reduction, and wash out of the SRB community from the reactor results in process failure (Erasmus, 2000; Moosa *et al*., 2002; 2005; Moosa and Harrison, 2006; Hansford *et al*., 2007; Oyekola *et al*. 2009; 2010; 2012). Microbial community analysis of the inoculum applied in this study, reported in Chapter 4, suggested the presence of sulphate reducing bacteria (SRB) and non-SRB species. 16S rRNA sequences, captured from the inoculum community, showed high similarity to well-known SRB species belonging to the *Desulfomicrobium, Desulfovibrio, Desulfobulbus* and *Desulfocurvus* genera and. These species contain the *dissimilatory sulphite reductase* gene (*dsrAB*), a specific molecular target for the detection of SRBs, within their genomes. DsrAB is the key enzyme catalysing the last and main energy-generating step during sulphate reduction (Wagner *et al*., 1998; Loy *et al*., 2004; Zverlov *et al*., 2005). Other "non-traditional SRB" species belonging to the *Firmicutes* and *Citrobacter* genus, also containing the *dsrAB* within their genomes were also detected (Wagner *et al*., 1998; Loy *et al*., 2004; Zverlov *et al*., 2005; Qiu *et al*., 2009; Zhou *et al.*, 2015). Other species present were identified as members of the *Sphaerochaeta, Synergistetes, Chloroflexi, Mesotoga, Acholeplasma, Bacteriodetes, Petrimonas, Bacteriodes* genera and the elemental sulphur (S^0) reducing genera *Desulfuromonas* (Section 4.3.1). The SRB community used as inoculum for this study was obtained from a lactate operated CSTR receiving 1.0 g l^{-1} sulphate at a residence time of 5 d operating at a SO_4^{2-} conversion efficiency of 86.3 % and volumetric sulphate reduction rate of 0.0072 g l^{-1} h^{-1} at the time of sampling. This community was therefore considered an efficient BSR community, suitable for use as inoculum in this study.

The carbon source, electron donor and sulphate loading supplied also play a critical role in the microbial dynamics and performance of the bioreactor (Table 2.6 and Table 5.7). Oyekola *et al*. (2012) demonstrated that when lactate was used as a carbon source and electron donor for BSR, lactate oxidisers (LO) and lactate fermenters (LF) competed for the available electron source and the competition was influenced by volumetric sulphate loading rate (VSLR). Incomplete lactate oxidation

and concomitant sulphate reduction were dominant at dilution rates of 0.0083 to 0.083 h^{-1} in CSTRs receiving lactate at feed sulphate concentrations of 1.0 and 10.g l^{-1}. On the other hand, the reaction stoichiometry and kinetics of BSR utilising anaerobic digestate (a mixture of acetate, propionate and butyrate), reported in Chapter 5, demonstrated simultaneous use of the mixed VFAs in anaerobic digestate. Therefore, compared to lactate, higher VSRRs were observed in CSTRs receiving anaerobic digestate. It is hypothesised that when applying anaerobic digestate as carbon source and electron donor for BSR, the complex carbon source will support a more diverse group of SRBs compared to a single VFA source such as lactate (as suggested in Table 2.6 and Section 2.4.3). This chapter investigated the microbial community dynamics linked to the performance of BSR reactors in response to applied carbon source and electron donor (either anaerobic digestate or lactate) and VSLR, mediated through dilution rate and feed sulphate concentration using molecular techniques.

The specific objectives of the molecular study presented in this chapter were as follows

i. To use FISH to qualitatively characterise the microbial community dynamics in reactors receiving a complex carbon source and electron donor (anaerobic digestate) at feed sulphate concentrations of 2.5 and 5.0 g l^{-1} in response to changes in residence time
ii. To quantitatively characterise the microbial communities in reactors receiving a simple (lactate) compared to a complex (anaerobic digestate) carbon source and electron donor at different VSLRs, mediated through dilution rate and feed sulphate concentration, using qPCR and 16S rRNA metagenomics
iii. To investigate the link between BSR reactor performance, community dynamics and diversity as a function of the carbon source and electron donor applied

6.2 EXPERIMENTAL APPROACH

FISH analysis was carried out on reactor samples collected from BSR CSTRs receiving anaerobic digestate as carbon source and electron donor using the methods and conditions detailed and validated in Chapter 4 (Section 4.3.2). qPCR analyses were performed on genomic DNA (gDNA) extracted from reactors receiving anaerobic digestate at feed sulphate concentrations of 2.5 and 5.0 g l^{-1}. Samples were unfortunately not collected for reactors operated at 1.0 g l^{-1} sulphate. gDNA extractions were performed as described in Chapter 3 (Section 3.6.1). Comparative quantitative analysis of the bacterial consortium in reactors receiving lactate as a carbon sources was performed at four different sulphate concentrations: 1.0; 2.5, 5.0 and 10 g l^{-1}. These samples were obtained from a study performed and reported by Oyekola *et al.* (2010 and 2012). qPCR analyses included the quantitative determination of the total bacteria (using TotalF and TotalR primers), total SRB species by amplification of the *dsrAB* gene (using DSR4R and DSRp2060F primers), *Desulfomicrobium* species (using novel DSM442F and DSM632R primes) and *Desulfovibrio* species (using modified DSV-III-312f[b] and DSV681R[b] primers) represented in the

gDNA. The specificities of these primers were evaluated in Chapter 4, Section 4.3.3, and the PCR conditions applied were reported in Chapter 3, Section 3.6.13. The percentages of SRB present within the samples were calculated taking into account that SRB on average contain 4.5 copies of the 16S rRNA gene within their genomes (Lee *et al*., 2009), while the *dsrAB* gene is present as a single copy (Müller *et al.*, 2004). Metagenomic analysis of the 16S rRNA variable region from V3 to V4 was carried out by Illumina® MiSeq® sequencing was performed by Macrogen (Seoul, South Korea) and the data analysis to obtain operational taxonomic units (OTUs) performed by the service provider according to Section 3.6.14.

6.3 RESULTS AND DISCUSSIONS

6.3.1 Microbial community structure and dynamics of anaerobic digestate operated BSR CSTRs

The following section discusses the quantitative assessment of microbial communities observed in the BSR CSTRs.

6.3.1.1 Qualitative assessment of microbial community using fluorescence *in situ* hybridisation

Four different FISH probes were used to qualitatively evaluate microbial community shifts in reactors receiving anaerobic digestate as a carbon source and electron donor at increasing dilution rates and at feed sulphate concentrations of 2.5 and 5.0 g l^{-1}. Cells were stained with DAPI and FISH performed with the EUB338-Fam probe (Amann *et al.*, 1990) to capture all eubacteria within the anaerobic digestate fed SRB reactors. The *Deltaproteobacterial* probe, DELTA495a-Fam (Loy *et al*., 2002), was used to capture all SRB as the probe sequence is absent from the 16S rRNA sequences of non-SRB (Section 4.3.2). The following species specific probes were used: DSM-Fam (Loy *et al*., 2007), DSV287-Fam (Dar *et al*., 2007) and SRB660-Fam (Devereux *et al*., 1992). These probes were specific to species from the *Desulfomicrobium, Desulfovibrio* and *Desulfobulbus* genera respectively. The optimisation and validation of FISH with these probes were described in Chapter 4, Section 4.3.2.

In reactors receiving anaerobic digestate at feed sulphate concentrations of 2.5 and 5.0 g l^{-1}, comparison of the micrographs obtained from the DAPI stain and hybridisation with the domain-level probe, EUB338-Fam, indicated the presence of eubacteria at all residence times (RTs) tested. These hybridisation events are shown in Figures 6.1 and 6.2 (panels A and B). For these reactors, very similar images were achieved using the DAPI stain when compared to the EUB338-Fam probe indicating an active bacterially dominated culture. Some researchers have demonstrated the staining of both live and dead cells with DAPI which may overestimate the total amount of living cells (Bouvier and Del Giorgio, 2003). However, this may not be relevant in the SRB cultures reported here, most possibly due to the

continuous operation of the reactors allowing the removal of dead bacterial cells. Although similar results were achieved, the FISH technique and use of EUB338-Fam probe rather than DAPI stain was prefered when investigating the eubacterial community as only cells with sufficient amounts of ribosomal RNA will successfully emit a hybridisation signal upon excitation of the fluorophore (Bouvier and Del Giorgio, 2003; Hesham and Alamri, 2012). The number of cells emitting a hybridisation signal with the EUB338-Fam probe decreased with decreasing RTs at both sulphate concentrations (2.5 and 5.0 g l^{-1}). The lowest hybridisation signals at both 2.5 and 5.0 g l^{-1} were observed at the lowest RTs tested, 1 d (dilution rate of 0.042 h^{-1}) as demonstrated in Figures 6.1 panels A and B and Figures 6.2 panels A and B respectively.

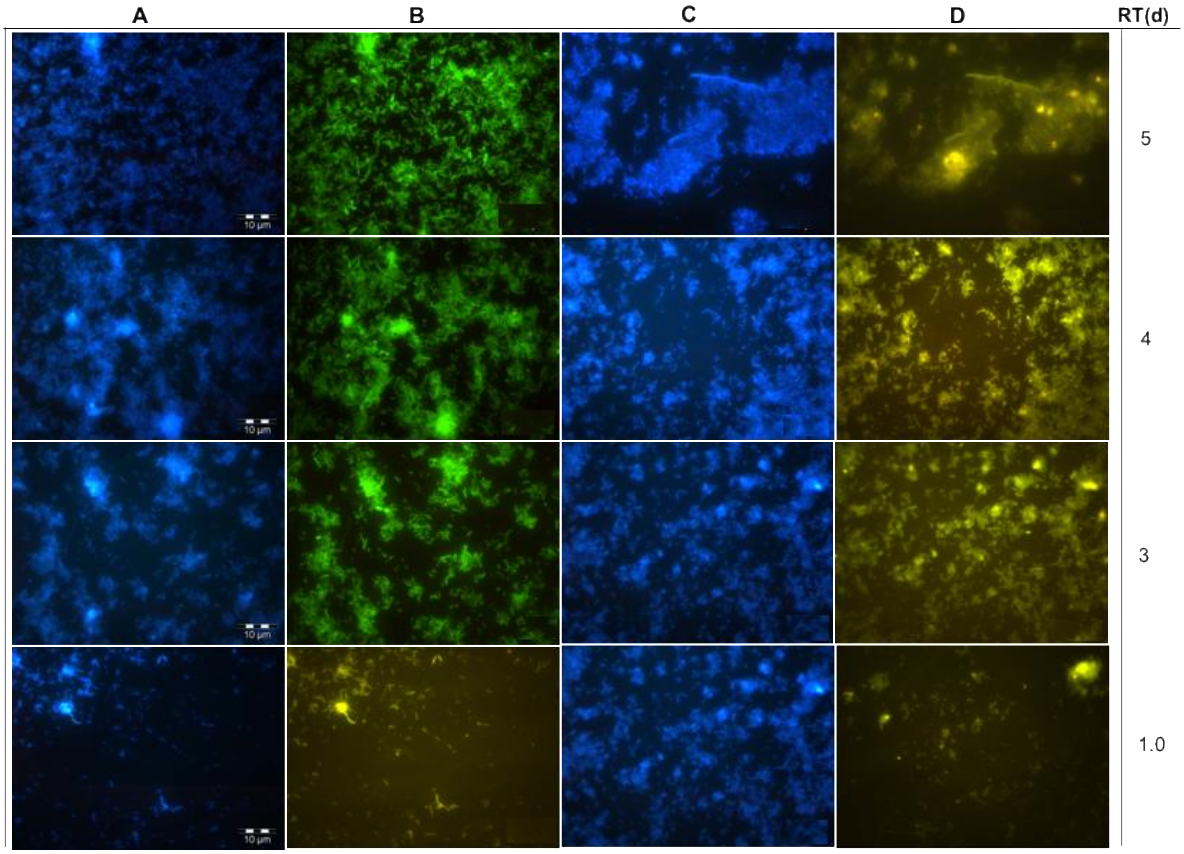


Figure 6.1. Epifluorescence micrographs of samples obtained from reactors receiving anaerobic digestate feed at a sulphate concentration of 2.5 g l^{-1} (RT = 5, 4, 3 and 1 d; indicated on the right of the figure). DAPI staining (panel A and C) was compared to FISH performed with the general bacterial probe EUB338-Fam and the Deltaproteobacterial probe DELTA495a-Fam (panel B and D, respectively). The scale for all micrographs was 10 μm as indicated in Panel A.

The prevalence of SRB species within the BSR communities in these reactors were visualised using the Deltaproteobacterial probe, DELTA495a-Fam. As mentioned above, FISH with the EUB338-Fam probe emitted a similar intensity fluorescence signal compared to DAPI stain across higher RTs of 5, 4 and 3 d (dilution rates of 0.0083, 0.010 and 0.014 h^{-1} in the one litre CSTRs) in both reactors (2.5 and

5 g l^{-1} sulphate) (Figures 6.1 and 6.2, panels C and D). Lower fluorescence signals were however observed with the DELTA495a-Fam probe at reduced RTs compared to the DAPI signal for samples at these RTs, suggesting SRB were 'washed' from the reactor system at high dilution rates. This was observed at 1.0 d (dilution rate of 0.042 h^{-1}) for the reactors receiving 2.5 g l^{-1} and 5.0 g l^{-1} sulphate (Figures 6.1 and 6.2, panes C and D).

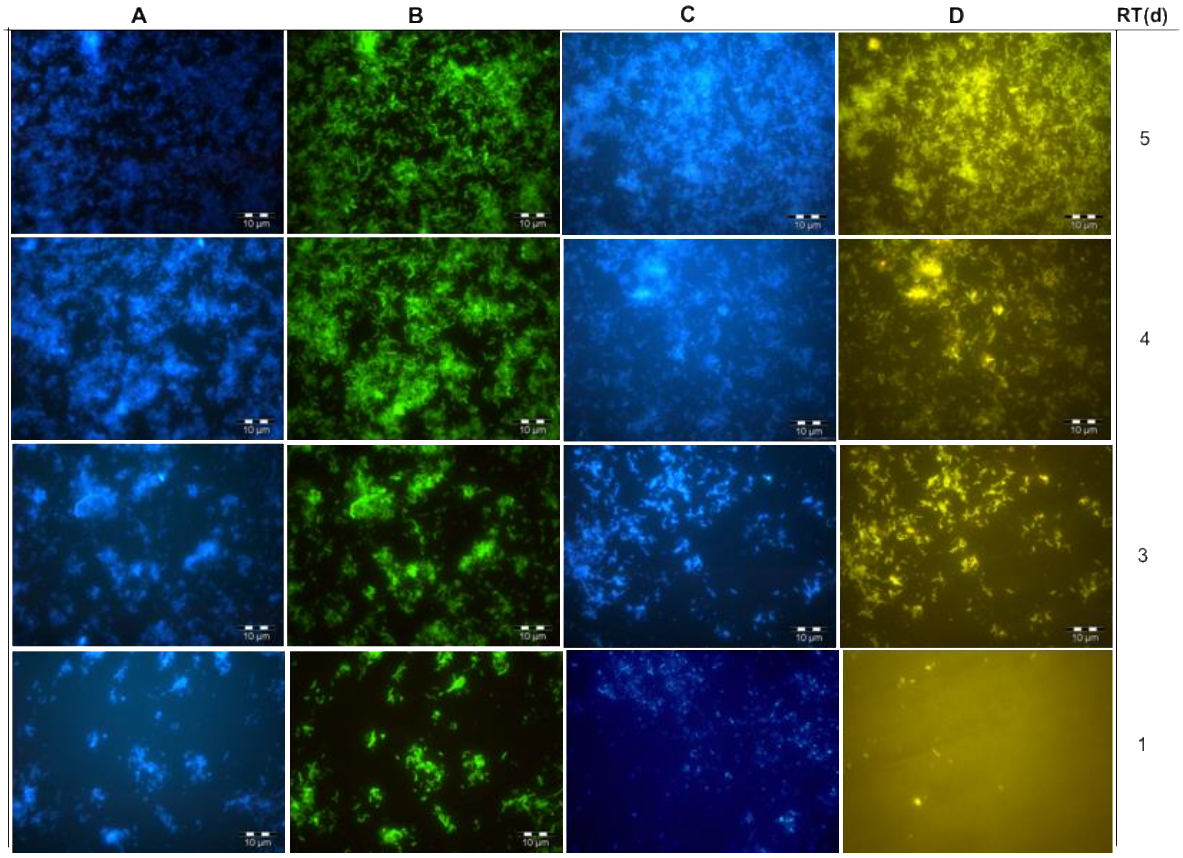


Figure 6.2. Epifluorescence micrographs of samples obtained from reactors receiving anaerobic digestate feed at a sulphate concentration of 5.0 g l^{-1} (RT = 5, 4, 3 and 1 d; indicated on the right of the figure). DAPI staining (panel A and C) was compared to FISH performed with the general bacterial probe EUB338-Fam and the Deltaproteobacterial probe DELTA495a-Fam (panel B and D, respectively). The scale for all micrographs was 10 μm.

In support of the FISH results shown here, *in silico* studies in Chapter 4, Section 4.3.2, revealed that the DELTA495a probe only hybridised to the 16S rRNA gene sequences homologous to those from SRB species. None of the non-SRB 16S rRNA gene sequences captured in the 16S rRNA gene library constructed from the inoculum community contained this probe sequence. These observations are corroborated by Oyekola (2008) who used FISH to demonstrate that the SRB community in the lactate fed CSTRs receiving feed sulphate concentration of 1.0 g l^{-1} declined as the RT was reduced from 5 to 0.5 d (dilution rates of 0.0083 to 0.083 h^{-1}). A similar decrease in the SRB numbers within the microbial

community was observed in the Oyekola (2008) study at a feed sulphate concentration of 2.5 g l^{-1} as the RT was reduced from 5 to 2 d. The low DAPI and DELTA495a-Fam signals observed at the lower residence times (Figures 6.1 and 6.2, panels A and B) suggested a loss of bacteria and SRB cells at these residence times.

FISH results suggest that the proportion of SRB species within the total bacterial population declined as the residence time was reduced (dilution rate increased). The decrease of SRBs in relation to bacteria were more pronounced for the higher sulphate concentration of 5.0 g l^{-1} compared to 2.5 g l^{-1}, which suggest that some SRB species were unable to proliferate and continue performing sulphate reduction at higher sulphate loading rates. This is also supported by the decrease of sulphate conversion observed at these residence times reported in Chapter 5, Section 5.3.2. The decrease in SRB species at high dilution rates and a sulphate concentration of 2.5 g l^{-1} is in agreement with findings in Chapter 5, Section 5.3.2.2, where bacterial biomass measured as dry weight (DW) was reduced from 1.488 g l^{-1} at a residence time of 5 d (0.0083 h^{-1}) to 0.955 g l^{-1} at a residence time of 1.0 d (dilution rate of 0.042 h^{-1}). At a feed sulphate concentration of 5.0 g l^{-1}, it was reported that bacterial dry mass was 3.161 g l^{-1} DW at a residence time of 5 d (dilution rate of 0.0083 h^{-1}) and was reduced to 0.715 g l^{-1} DW at a residence time of 1 d (dilution rate of 0.042 h^{-1}). This suggests that the high residence time of 5 d, feed sulphate concentration of 5.0 g l^{-1} supports a higher biomass concentration compared to sulphate concentration of 2.5 g l^{-1}. On the contrary, at the lower residence time of 1 d, feed sulphate concentration of 2.5 g l^{-1} supports a higher biomass concentration compared to sulphate concentration of 5.0 g l^{-1}. Total bacterial and SRB abundance were further validated quantitatively by qPCR in Section 6.3.1.2.

The prevalence of *Desulfomicrobium* species amongst the microbial communities in both reactors at 2.5 and 5.0 g l^{-1} sulphate was investigated using the DSM213-Fam probe. Comparison of the micrographs obtained from the DAPI stain and hybridisation with DSM213-Fam probe, showed high fluorescence levels across RTs of 5, 4 and 3 d (dilution rates of 0.0083, 0.010 and 0.014 h^{-1}) while lower fluorescence was observed at the lowest RT tested, 1 d (0.042 h^{-1}), in both reactors (Figures 6.3 and 6.4, panels A and B). This suggested that fewer *Desulfomicrobium* species could proliferate at a residence time of 1 d (dilution rate 0.042 h^{-1}), suggesting wash-out of these species from the reactors as the residence time was lowered.

The persistence of *Desulfovibrio* species with increased sulphate loading and dilution rates in anaerobic digestate fed reactors was evaluated using the DSV827 probe labelled with Fam. The results in Figures 6.3 and 6.4, panels C and D, indicated similar fluorescence levels at all RTs tested (5, 4, 3 and 1 d, dilution rates of 0.0083, 0.010, 0.014 and 0.042h^{-1}) in both reactors, 2.5 and 5.0 g l^{-1} sulphate. When comparing the hybridisation signal obtained with the DSM213-Fam probe, targeting *Desulfomicrobium*, in general a lower fluorescence signal was observed with the DSV827-Fam probe

for all samples. However, despite the low signal emitted by FISH targeting *Desulfovibrio* species at all RTs (5, 4, 3 and 1 d), the intensity of the hybridisation signal observed remained similar at all these RTs and did not decrease as was found for the *Desulfomicrobium* signal.

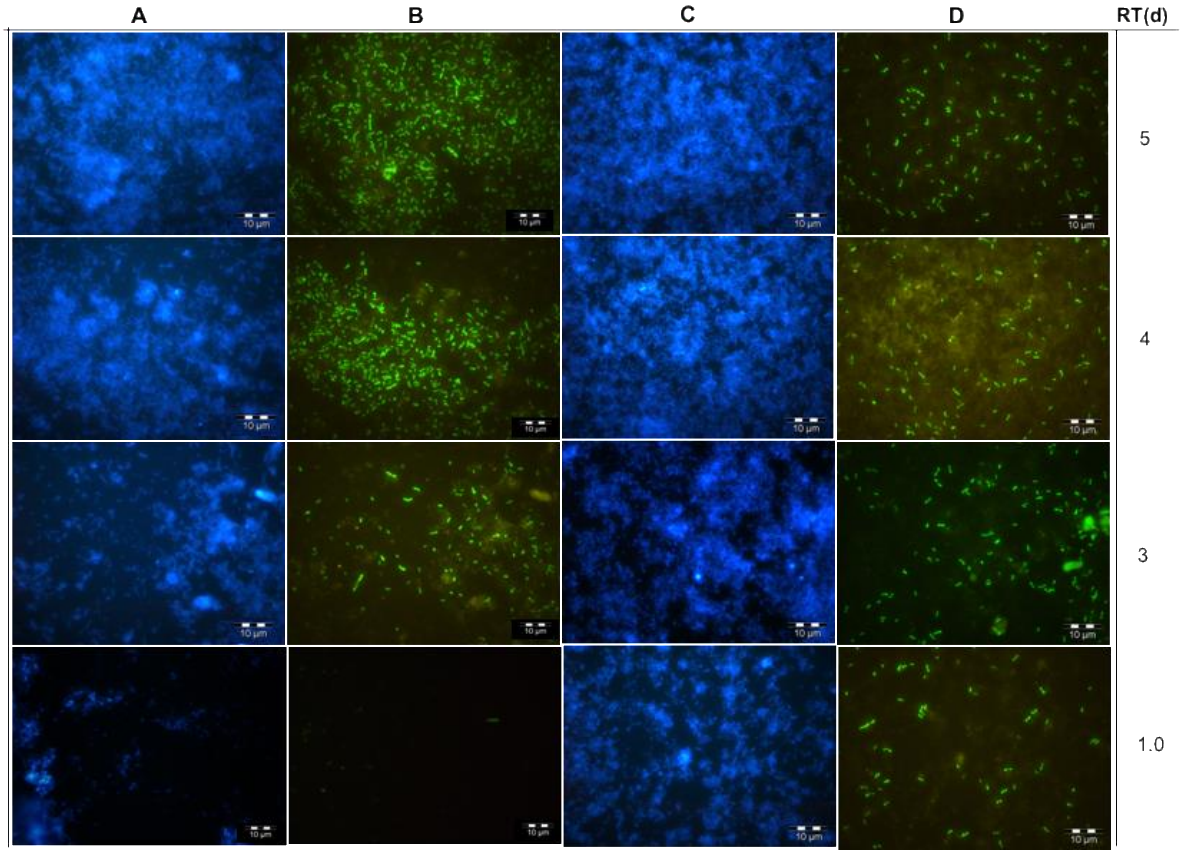


Figure 6.3. Epifluorescence micrographs of samples obtained from reactors receiving anaerobic digestate feed at a sulphate concentration of 2.5 g l^{-1}(RT = 5, 4, 3 and 1 d; indicated on the right of the figure). DAPI staining (panel A and C) was compared to FISH performed with the *Desulfomicrobium* genera probe DSM213-Fam and the *Desulfovibrio* genera probe DSV-Fam (panel B and D, respectively).

The results suggested that the *Desulfovibrio* species present in these reactors were more tolerant to increases in sulphate loading and dilution rate compared to cells belonging to *Desulfomicrobium* species. Icgen *et al.* (2007) also obtained a low signal with a *Desulfovibrionaceae* probe (SRB687) when FISH was performed on samples from a CSTR receiving acetate as a single carbon source and electron donor for BSR at a sulphate concentration of 2.5 g l^{-1}. This study and that by Icgen *et al.* (2007) suggest that *Desulfovibrio* species were not the abundant SRB at 2.5 g l^{-1} sulphate for the higher dilution rates tested.

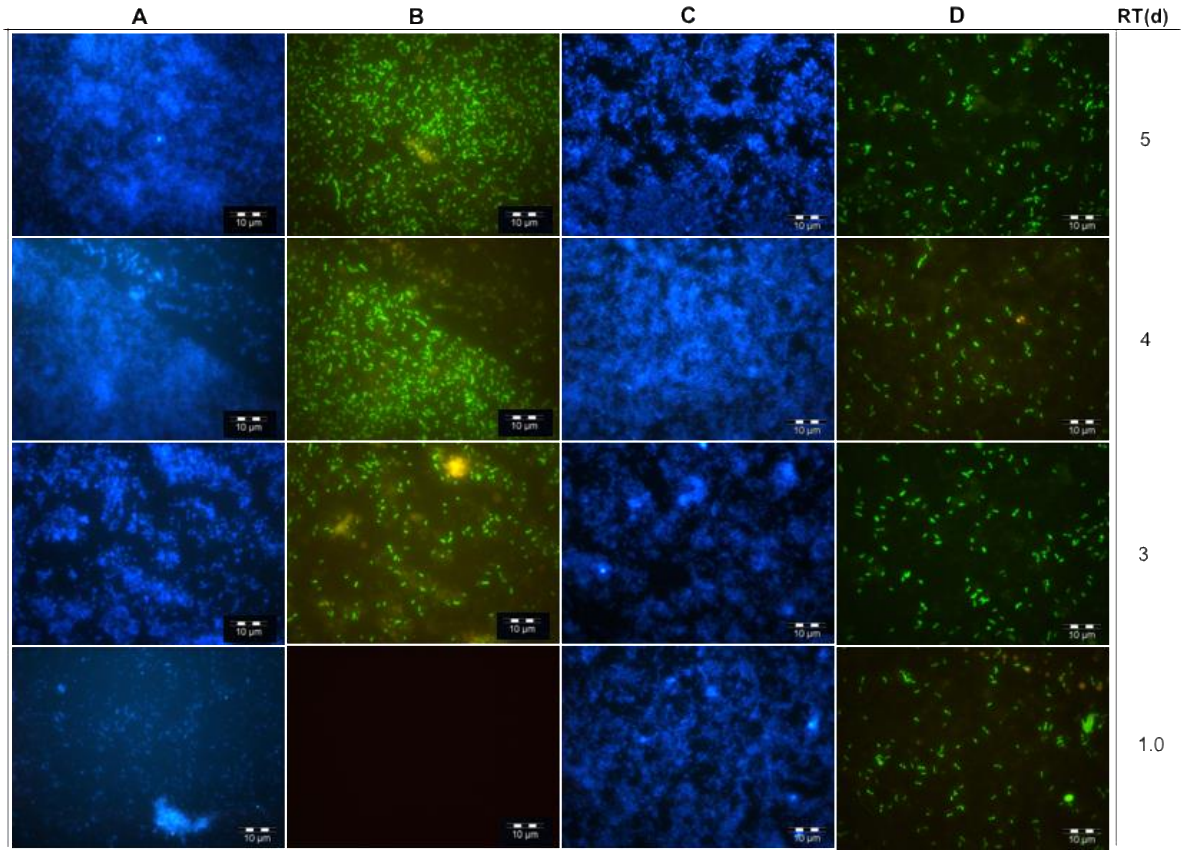


Figure 6.4. Epifluorescence micrographs of samples obtained from reactors receiving anaerobic digestate feed at a sulphate concentrations of 5.0 g l^{-1} (RT = 5, 4, 3 and 1 d; indicated on the right of the figure). DAPI staining (panel A and C) was compared to FISH performed with the *Desulfomicrobium* genera probe DSM213-Fam and the *Desulfovibrio* genera probe DSV-Fam (panel B and D, respectively).

A positive hybridisation signal was detected with the *Desulfobulbus* genus probe, SRB660-Fam, at RTs of 5 to 3 d (dilution rates of 0.0083 to 0.014 h^{-1}) at 2.5 and 5.0 g l^{-1} sulphate (Figure 6.5, panels C and D). Unlike the *Desulfovibrio* FISH results, the *Desulfobulbus* signal decreased as the residence time decreased and no visible signal was observed at a residence time of 1 d (dilution rate of 0.042 h^{-1}) at both sulphate concentrations. This suggested that *Desulfobulbus* species were not present in these reactors at low RTs as they were not able to proliferate at higher VSLRs and were washed from the reactors at high dilution rates. A quantitative assessment (qPCR) was performed on the different SRB community genera to complement the FISH results from this study.

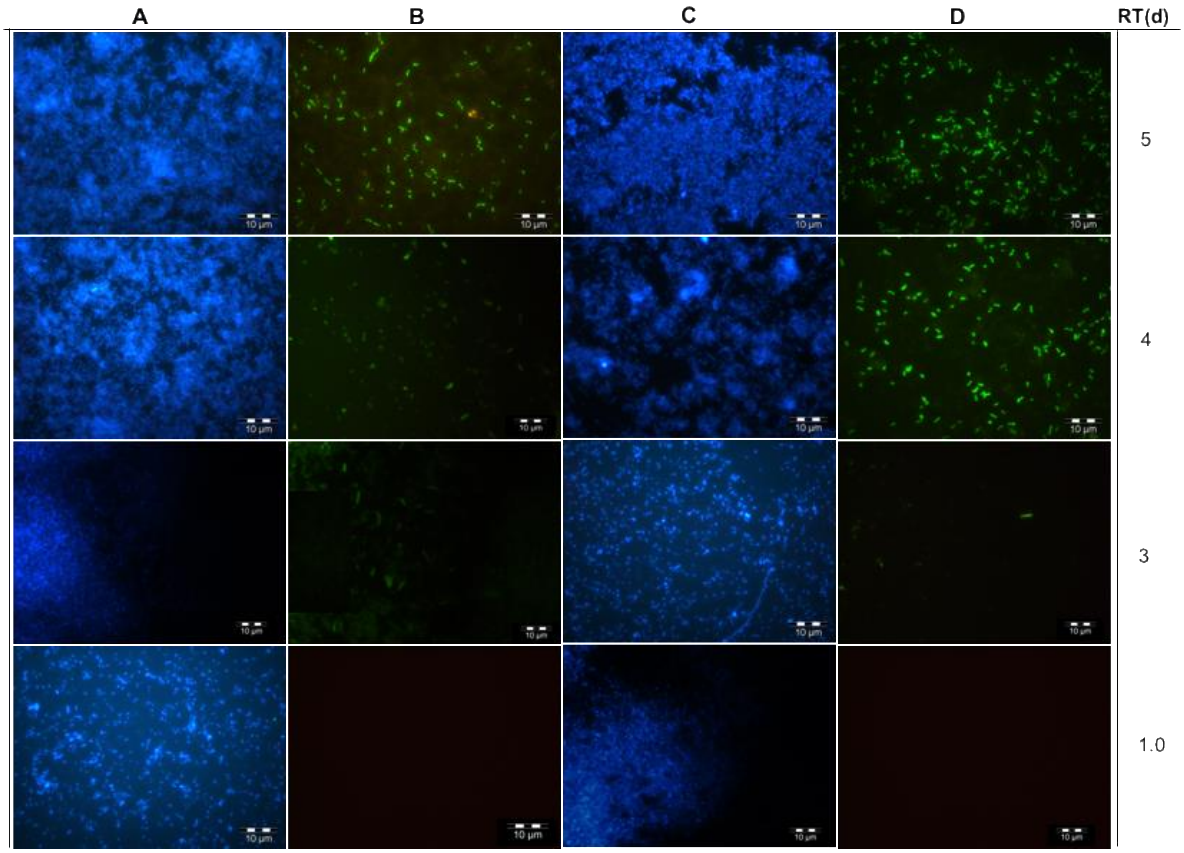


Figure 6.5. Epifluorescence micrographs of samples obtained from reactors receiving anaerobic digestate feed at a sulphate (S_o) concentrations of 2.5 (panel A and B) and 5.0 g l^{-1} (panel C and D) at residence times of 5, 4, 3 and 1 d; indicated on the right of the figure). DAPI staining (panel A and C) is compared to FISH performed with and the *Desulfobulbus* genera probe SRB660-Fam (panel B and D).

6.3.1.2 Quantitative PCR and 16S rRNA metagenomic analysis of microbial community

Quantitative PCR allows for assessment of changes in the abundance of species thus allowing us to study microbial community structure and dynamics in response to changes in VSLRs. qPCR results indicated that there was a higher proportion of total SRB species compared to total bacteria across the lower dilution rates of 0.0083 to 0.014 h^{-1} (RTs of 5 to 3 d) at both feed sulphate concentrations, 2.5 and 5.0 g l^{-1} (Figure 6.6A and B). The majority of SRB species captured belonged to the *Desulfomicrobium* genus. These results were in agreement FISH results and with observations from Figure 4.2 and Table 4.1 which showed that the most abundant 16S rRNA gene clones captured from the inoculum showed high homology to *Desulfomicrobium* species such as *Desulfomicrobium norvegicum*, *D. baculatum*, *D. escambiense* and *D. apsheronum*. The results in Figures 6.6A and B also suggest that the proportion of SRB compared to total bacterial copy numbers decreased as the dilution rates increased from 0.014 to 0.042 h^{-1}(RTs of 3 to 1 d), corroborating FISH results.

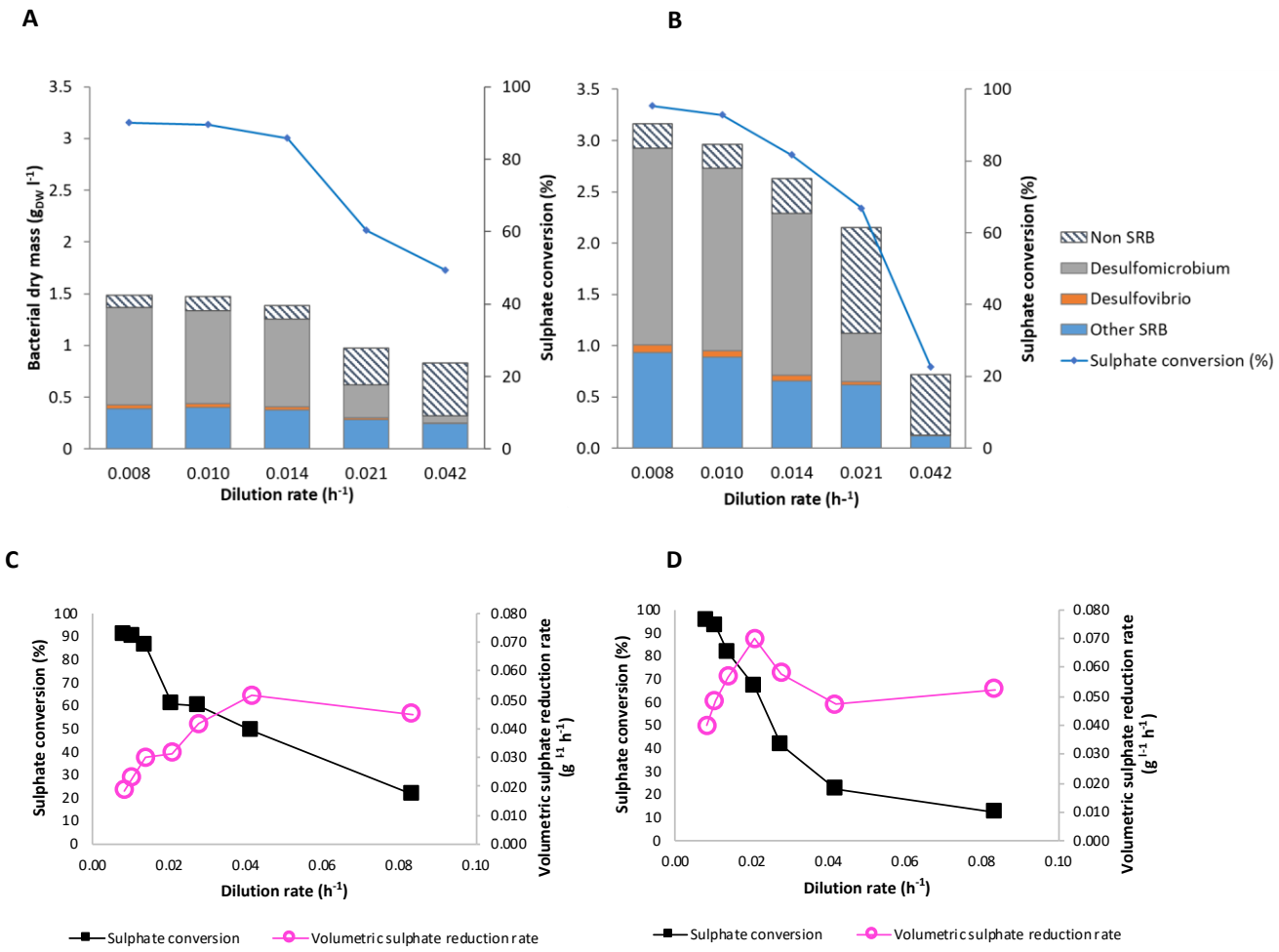


Figure 6.6. Graphical representation of the qPCR results obtained on steady state samples obtained from reactors receiving anaerobic digestate as a carbon source for biological sulphate reduction using total bacterial primers (TotalF and TotalR), *Desulfomicrobium* species primers (DSM442F and DSM632R) and *Desulfovibrio* species primers (DSV-III-312f[b] and DSV681R[b]). Results are represented as proportions relative the bacterial dry mass obtained per 1 ng DNA. It was assumed that that the copy numbers of the genes / cell and the mass of the cells of the various SRB species are the same. qPCR was performed with gDNA from SR CSTRs receiving feed sulphate concentrations of 2.5 g l^{-1} (A) and 5.0 g l^{-1} (B). The relationship between sulphate conversion and volumetric sulphate reduction rate across the dilution rate in the range 0.0083 and 0.083 h^{-1} [5 d to 0.5 d residence time (RT)] is shown for feed sulphate concentrations of 2.5 g l^{-1} (C) and 5.0 g l^{-1} (D). Note the inclusion of a dilution rate of 0.083 h^{-1} matching a RT of 0.5 d not included in the qPCR analyses shown in graphs A and B.

Although the decrease in the proportion of SRB to total bacteria correlated with the decrease in sulphate conversion at the lower residence times (higher dilution rates), the volumetric sulphate reduction rate (VSRR) still remained in the range of 0.019 to 0.051 g l^{-1} h^{-1} at a feed sulphate concentration of 2.5 g l^{-1} (Figure 6.6C) and in the range of 0.040 to 0.070 g l^{-1} h^{-1} at a feed sulphate concentration of 5.0 g l^{-1} (Figure 6.6D). This suggests that the reduced sulphate conversion efficiency observed with increased dilution rate, resulted from the inability of the microbial consortium to reach the required VSRR to

match the increased VSLR, rather than a substantial reduction in the VSRR. The proportion of SRB to total bacteria remained steady (91.1 ± 0.8%) across the dilution rates of 0.0083 to 0.014 h^{-1} (RT of 5 to 3 d) at a feed sulphate concentration of 2.5 g l^{-1}. The proportion of *Desulfomicrobium* and *Desulfovibrio* species also remained 61.9 ± 1.2 % and 2.3 ± 0.15 respectively, across these dilution rates (Figure 6.6A). Similar observations were made at a feed sulphate concentration of 5.0 g l^{-1} (Figure 6.6B). The proportion of SRB species compared to total bacteria within this reactor remained steady (90.6 ± 3.1%) across the dilution rates of 0.0083 to 0.014 h^{-1} (RTs of 5 to 3 d). As reported for a sulphate concentration of 2.5 g l^{-1}, *Desulfomicrobium* and *Desulfovibrio* species at these dilution rates also remained steady at 60.3 ± 0.49% and 2.3 ± 1.2% respectively (Figure 6.6B).

Increasing the dilution rate from 0.014 to 0.021 h^{-1} (RT of 3 to 2 d) resulted in a rapid decline of *Desulfomicrobium* species from 61 to 32.7% at a feed sulphate concentration of 2.5 g l^{-1} (Figure 6.6A) and from 60 to 22.1% at a feed sulphate concentration of 5.0 g l^{-1} (Figure 6.6B). A further decline in *Desulfomicrobium* species was observed as the RT was reduced to 1 d (dilution rates of 0.042 h^{-1}) for both reactors. This suggested that fewer *Desulfomicrobium* groups were present at the dilution rate of 0.042 h^{-1} as they probably have maximum specific growth rates (μ_{max}) < 0.042 h^{-1} or exhibited lower growth rates under the prevailing conditions within the reactor at that time and were therefore excluded from the reactors at these higher dilution rates (Chiu *et al*., 1972). Despite *Desulfovibrio* species being less abundant in either reactor, these microorganisms were able to proliferate at higher VSLRs of 0.104 and 0.208 g l^{-1} h^{-1} (Figure 6.6A and Figure 6.6B, respectively). Approximately 50% of the *Desulfovibrio* species present at the highest dilution rate testes (0.042 h^{-1}) remained in the reactors when the dilution rates were increased to 0.042 h^{-1} (RT of 1 d) suggesting that *Desulfovibrio* species have μ_{max} values higher than 0.042 h^{-1} (Figures 6.6A and B). These results are in agreement with observations made in Section 5.4.4 where microbial growth rates (μ) of 0.044 and 0.055 h^{-1} were determined for feed sulphate concentrations of 2.5 and 5.0 g l^{-1}, respectively. The total bacterial biomass that remained in the reactor with 2.5 g l^{-1} sulphate at the highest dilution rate tested (0.042 h^{-1}, RT of 1 d) was 0.761 g l^{-1}, which was 51.2% of the bacterial biomass concentration at the lowest dilution rate tested (0.0083 h^{-1}, RT of 5 d) (Figure 6.6A). In the reactor receiving 5.0 g l^{-1} sulphate, the total biomass concentration of 0.715 g l^{-1} was observed at 0.042 h^{-1} which was 22.6% of the total biomass determined at 0.0083 h^{-1} (Figure 6.6B).

These results suggest that the three SRB groups analysed using qPCR and FISH (*Desulfomicrobium, Desulfovibrio* and *Desulfobulbus*) played a role in sulphate reduction at the lower dilution rates (0.0083 to 0.014 h^{-1}, RTs of 5 to 3 d) when anaerobic digestate was utilised as electron donor for BSR. At higher dilution rates of 0.021 and 0.042 h^{-1} (and thus higher VSLRs) VFA oxidation and concomitant sulphate reduction was carried out mostly by "other SRB", captured by the *dsr AB* primer set, as fewer *Desulfomicrobium* and *Desulfobulbus* species remained in the reactors. Similarly to *Desulfomicrobium*

species, most *Desulfobulbus* species were washed out at dilution rates higher than 0.042 h^{-1}, which suggested that most *Desulfobulbus* members present had lower growth rates and could not proliferate at higher dilution rates and sulphate loadings. On the contrary, most *Desulfovibrio* members were able to tolerate high sulphate loadings, and had higher growth rates when anaerobic digestate (acetate, propionate and butyrate) was provided as an electron donor. Species for which growth rates are available in literature report different values which may be due to the different conditions such as electron donor, temperature and pH under which they were tested (Stams *et al*., 1984; O'Flaherty and Colleran, 1999; Suzuki *et al*., 2007) and therefore were not compared.

While lower hybridisation signals were observed with the *Desulfobulbus* genera probe SRB660-Fam at the highest dilution rate of 0.042 h^{-1} (RT of 1 d) (Figure 6.5), some propionate conversion to acetate was observed at feed sulphate concentrations of 2.5 and 5.0 g l^{-1} (21.7 and 56.3%, respectively) (Figure 5.6a and 5.7a). This suggested the presence of *Desulfobulbus* members which could oxidise the propionate present (Widdel and Pfennig, 1982; Stams *et al*., 1984; Widdel 1988; Gibson 1990a; Maillacheruvu *et al*., 1993) or other SRB groups within the unidentified "other SRB" that could utilise propionate in the presence of sulphate at this dilution rate. Two such microbial groups may be *Citrobacter* (Zhou *et al*., 2015) and *Firmicute species* (Wagner *et al*., 1998; Loy *et al*., 2004; Zverlov *et al*., 2005) which were identified within the inoculum sample (Table 4.1 and Figures 4.1A and B). *Citrobacter* species HCSR strain (Zhou *et al*., 2015) and *Desulfosporosinus* species from the phylum Firmicutes (Ramamoorthy *et al*., 2006; Vatsurina *et al*., 2008; Lee *et al*., 2009; Pester *et al*., 2010; Hausmann *et al*., 2016) can oxidise propionate in the presence of sulphate. In order to answer these questions, metagenomic analysis was carried.

16S rRNA metagenomic analysis of the BSR bacterial communities associated with the anaerobic digestate operated CSTR by 16S rRNA gene variable region was performed to validate the qPCR results and assist with the identification of the "other SRB" and non-SRB species. In this thesis, the metagenomic analysis was expected to assess the abundance of each operational taxonomic unit (OTU) and provide insight on which OTUs were associated with which electron donor. The proportion of SRB species was also assessed using the metagenomic data. The metagenomic data indicated the presence of a total of 14 SRB species within the reactors receiving anaerobic digestate as a carbon source for BSR. These were *Desulfomicrobium* species (*Desulfomicrobium hypogeium* and *Desulfomicrobium aestuarii*), *Desulfovibrio* species (*D. aminophilus, D. vulgaris, D. desulfuricans, D. intestinalis, D. oxamicus*, and *D. sulfodismutans*), *Desulfobulbus oligotrophicus, Desulfocurvus vexinensis, Desulfococcus biacutus, Desulfarculus baarsii, Desulfomonile tiedjei* and *Desulfobacca acetoxidans* (Figure 6.7 and Figure 6.8). *Desulfomicrobium hypogeium* (Krumholz *et al*., 1999) was the most abundant SRB species present at the lowest dilution rate tested of 0.0083 h^{-1} (RT of 5 d) in both reactors receiving feed sulphate of 2.5 and 5.0 g l^{-1} (Figures 6.7A and B). These contributed 81.2 and 78.6% to

the total SRB community in the reactors receiving feed sulphate concentration of 2.5 and 5.0 g l^{-1}, respectively. While the proportion

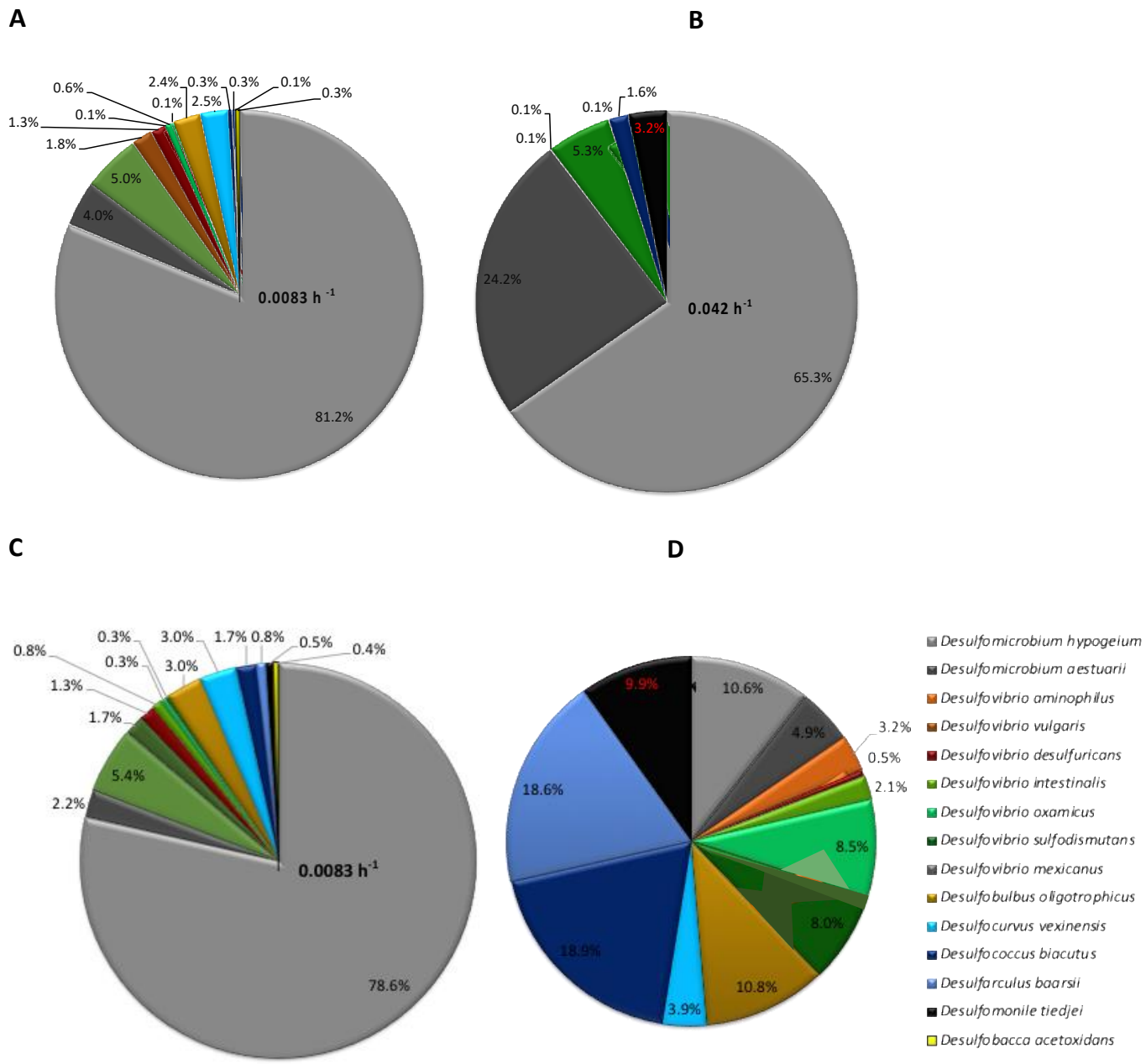


Figure 6.7. Metagenomic analysis of the sulphate reducing bacteria (SRB) structure at the species level and on steady state samples obtained from reactors receiving anaerobic digestate as a carbon source for biological sulphate reduction at feed sulphate concentration of 2.5 (A and B) and 5.0 g l^{-1} (C and D). The samples were obtained at lowest dilution rate tested (0.0083 h^{-1}, RT of 5 d) (A and C) and dilution rate of 0.042 h^{-1} (RT of 1 d) (B and D).

of *Desulfomicrobium aestuarii* compared to total SRB community was lower at this dilution rate (4.0% at a feed sulphate of 2.5 g l^{-1} and 2.2% at a feed sulphate of 5.0 g l^{-1}), the total proportions of *Desulfomicrobium* species in at feed sulphate of 2.5 and 5.0 g l^{-1} were 85.2 and 80.8% agreeing with

qPCR findings which indicated that *Desulfomicrobium* species were the most abundant SRB species at the lowest dilution rate of 0.0083 h^{-1} at both feed sulphate concentrations (Figure 6.6A and C). Assessment of the abundance of *Desulfovibrio* groups indicated that *Desulfovibrio aminophilus* (Baena *et al*., 1998) was the second most abundant SRB species at both feed sulphate concentrations with the proportion of 5.0 and 5.4% at feed sulphate of 2.5 and 5.0 g l^{-1}, respectively. The total proportion of all the *Desulfovibrio* species at this dilution rate, represented by *D. aminophilus, D. vulgaris, D. desulfuricans, D. intestinalis, D. oxamicus, D. sulfodismutans*, were similar with 9.0 ± 1.8 and 9.1 ± 1.8% in the reactors receiving 2.5 and 5.0 g l^{-1}, respectively (Figure 6.6A and C).

With increased volumetric sulphate loading rate (VSLR), mediated through feed sulphate concentration and dilutions rate, two things happened. Firstly, the structure of the SRB community changed. Secondly, the proportion of the total SRB community decreased (Figures 6.7 and 6.8). In the reactor receiving feed sulphate concentration of 2.5 g l^{-1}, increasing VSLR from 0.021 to 0.104 g l^{-1} h^{-1} (which corresponded to dilution rate of 0.0083 to 0.042 h^{-1}) resulted in the decrease in the proportion of *Desulfomicrobium hypogeium* from 81.2 to 65.3%. However, this resulted in the increase in the proportion of *Desulfomicrobium aestuarii* from 4.0 to 24.2 % (Figure 6.7 A and B). In the reactor receiving feed sulphate concentration of 5.0 g l^{-1}, increasing VSLR from 0.040 to 0.208 g l^{-1} h^{-1} (corresponding to dilution rate of 0.0083 to 0.042 h^{-1}) drastically decreased the proportion of *Desulfomicrobium hypogeium* from 78.6 to 10.6% but slightly increased the proportion of *Desulfomicrobium aestuarii* from 2.2 to 4.9 % (Figure 6.7 C and D). These observations suggested the following: (i) both *Desulfomicrobium hypogeium* and *Desulfomicrobium aestuarii* had higher enough growth rates in anaerobic digestate to survive the higher dilution rate of 0.042 h^{-1}, (ii) both of these species could tolerate high sulphate loadings of 0.208 g l^{-1} h^{-1} with anaerobic digestate and (iii) although these species can tolerate high sulphate loadings of 0.208 g l^{-1} h^{-1} when anaerobic digestate was provided at *Desulfomicrobium aestuarii* survives better at higher sulphate loading than *Desulfomicrobium hypogeium*.

Assessment of the *Desulfovibrio* species indicated that increasing the dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to VSLR of 0.021 to 0.104 g l^{-1} h^{-1} in the reactor receiving 2.5 g l^{-1} sulphate resulted in the complete washout of *D. vulgaris* (McInerney and Bryant, 1981), *D. intestinalis* (Fröhlich *et al.*, 1999) and *D. oxamicus* (Lopez-Cortes *et al.*, 2006). However, an increase in the proportion of *Desulfovibrio sulfodismutans* (Bak and Pfennig, 1987) from 0.02 to 5.3% was observed (Figures 6.7A and B). A similar observation was made at sulphate feed concentration of 5.0 g l^{-1} where all the other *Desulfovibrio* groups except *Desulfovibrio sulfodismutans* were washed out of the reactor when the dilution rate was increased from 0.0083 to 0.042h^{-1} which corresponded to the VSLR of 0.040 to 0.208 g l^{-1} h^{-1}). The proportion of *D. sulfodismutans* increased from 0.3 to 8.0% when the dilution rate was increased from 0.0083 to 0.042 h^{-1} (Figures 6.7C and D). These observations suggested that

amongst the *Desulfovibrio* groups observed in these reactors only *Desulfovibrio sulfodismutans* had high enough growth rates to survive the higher dilution rate of 0.042 h^{-1} and was the only *Desulfovibrio* species that can survive at the higher VSLR of 0.208 g l^{-1} h^{-1}. The complete washout of *D. vulgaris*, *D. intestinalis* and *D. oxamicus* was not surprising as none of these *Desulfovibrio* species have been reported to utilise neither acetate, propionate nor butyrate. No literature on *Desulfomicrobium hypogeium*, *Desulfomicrobium aestuarii*, *Desulfovibrio vulgaris*, *Desulfovibrio intestinalis* and *Desulfovibrio oxamicus* species' ability to utilise any of the VFAs in anaerobic digestate (acetate, propionate and butyrate) is currently available and therefore their role in these reactors remains unclear.

While the qPCR studies in this thesis could not elucidate "other SRB" communities present within these reactors, 16S rRNA metagenomic revealed these SRB groups to be *Desulfobulbus oligotrophicus, Desulfocurvus vexinensis, Desulfococcus biacutus, Desulfarculus baarsii, Desulfomonile tiedjei* and *Desulfobacca acetoxidans* (Figure 6.7, Figure 6.8 and Figure 6.9). The abundance of the incomplete propionate oxidiser, *Desulfobulbus oligotrophicus* OTU (El Houari *et al*., 2017) decreased as the dilution rate was increased from 0.0083 to 0.042 h^{-1} in both reactors. At feed sulphate concentration of 2.5 g l^{-1}, increasing the dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to VSLR of 0.021 to 0.104 g l^{-1} h^{-1}) resulted in the drastic decline of the abundance of the OTU assigned to *Desulfobulbus oligotrophicus* from 0.6 to 0.004% (Figure 6.8 and 6.9). On the contrary, at feed sulphate of 5.0 g l^{-1}, increasing the dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to VSLR 0.040 to 0.208 g l^{-1} h^{-1}) only decreased the abundance of *Desulfobulbus oligotrophicus* by two fold from 0.4 to 0.2% (Figure 6.8 and 6.9). These observations may suggest that *Desulfobulbus oligotrophicus* competed better for the available propionate within the anaerobic digestate at the higher VSLR of 0.208 g l^{-1} h^{-1} achieved by the higher sulphate concentration (5.0 g l^{-1}) than at the lower VSLR of 0.104 g l^{-1} h^{-1} and therefore remained in the reactors even at the highest dilution rate of 0.042 h^{-1} (RT 1 d) (Figure 6.8). Figure 7 indicates that the proportion of *Desulfobulbus oligotrophicus* compared to total SRB decreased from 0.6 to 0.1% when the dilution rate was increased from 0.0083 to 0.042 h^{-1} (corresponding to VSLR of 0.021 to 0.104 g l^{-1} h^{-1}) in the reactor receiving 2.5 g l^{-1} feed sulphate (Figure 6.7C). On the contrary, the proportion of this species increased from 3.0 to 10.8% when the dilution rate was increased from 0.0083 to 0.042 h^{-1} (corresponding to VSLR 0.040 to 0.208 g l^{-1} h^{-1}) at feed sulphate of 5.0 g l^{-1} (Figure 6.7D) supporting the above observations. Additionally, the decrease in species belonging to the *Desulfobulbus* genus observed was also supported by the observations made in FISH studies (Figure 6.5).

Reports by El Houari *et al*. (2017) demonstrated that *Desulfobulbus oligotrophicus* can oxidise propionate incompletely to acetate in the presence of sulphate, suggesting that when anaerobic digestate was supplied as a carbon source, propionate was being utilised for BSR. Although El Houari *et al*. (2017) reported lower growth rates with propionate (0.012 and 0.024 h^{-1}), the 16S rRNA metagenomics

data in this thesis suggests that the growth rates of *Desulfobulbus oligotrophicus* when anaerobic digestate the carbon source for BSR, the growth rates were as high as 0.042 h^{-1} (Figure 6.7).

Figure 6.8. Relative abundance of OTUs present in CSTRs receiving anaerobic digestate or lactate at different feed sulphate concentrations and residence times. The relative abundance of each OTU from the CSTR is shown by redscale. Each OTU is described by its species classification unless otherwise stated. OTUs classified with species containing known SRBs are in bold.

These differences could be due to the different experimental conditions used in the studies. For example, although the same temperature (35 °C) was used in this thesis and by El Houari *et al*. (2017), in this thesis pH of 8.0 ± 0.2 was maintain while El Houari *et al*. (2017) reported the growth rates at pH 7.6. Therefore, the differences in the pH conditions could have resulted in the different growth rates observed. Effects of different pH conditions on the growth rates of SRB have well been documented in literature (Omil et al., 1997; Lens *et al*., 1998; O'Flaherty, 1998; Cohen, 2006).

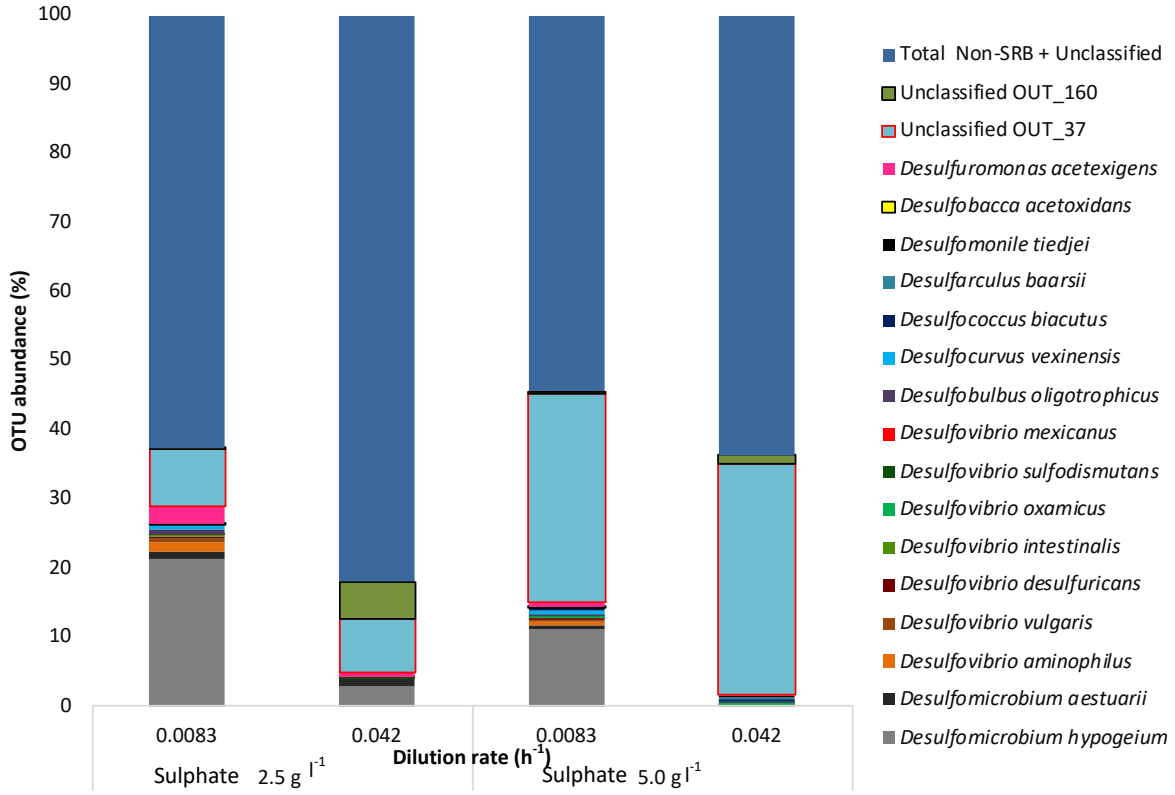


Figure 6.9. Relative abundance of OTUs present in CSTRs receiving anaerobic digestate at different dilution rates (0.0083 and 0.042 h^{-1}) and feed sulphate concentrations of 2.5 and 5.0 g l^{-1}. Each OTU is described by its species classification unless otherwise stated.

Despite being present in low abundance with OTU abundance of less than 0.1% at the lowest dilution rate tested (0.0083 h^{-1}, RT of 5 d), *Desulfococcus biacutus* OTU (Platen *et al*., 1990) and *Desulfomonile tiedjei* OTU (DeWeerd *et al*., 1990) were not washed out of the reactors when the dilution rate was increased to 0.024 h^{-1} (RT of 1 d) (Figure 6.7 and 6.9). This suggested that both SRB species had higher growth rates above 0.042 h^{-1} and could survive high sulphate loadings. The low abundance of *Desulfococcus biacutus* OTU (Figure 6.9) is in agreement with reports by Platen *et al*. (1990) who demonstrated that although *Desulfococcus biacutus* can grow on acetate, this growth is very slow and has a doubling time ranging between 19 of 24 d on acetate and sulphate as an electron acceptor. This

SRB grows better on acetone with a doubling time of 3.9 hours. *Desulfomonile tiedjei* cannot oxidise neither acetate, propionate nor butyrate and their presence in these reactors is not clear.

On the contrary, unlike *Desulfococcus biacutus*, the complete acetate oxidiser *Desulfobacca acetoxidans* (Oude Elferink *et al*. 1998b; Oude Elferink *et al*., 1999) was not able to withstand higher sulphate loading in either reactor and was washed out at the higher dilution rate of 0.042 h^{-1} (RT of 1 d) (Figure 6.7 and 6.9), suggesting that *Desulfobacca acetoxidans* had growth rates lower that 0.042 h^{-1} when anaerobic digestate was provided as a carbon source for BSR. However, Oude Elferink *et al*. (1998b) reported that when acetate served as a single carbon source for BSR, *Desulfobacca acetoxidans* exhibited a high μ_{max} ranging from 0.31 to 0.41 h^{-1} at 37 °C, suggesting that different growth conditions may have resulted in the washout of *Desulfobacca acetoxidans* at the dilution rate of 0.042 h^{-1} in this thesis. Acetate is oxidised completely to CO_2 via the carbon monoxide dehydrogenase pathway (Oude Elferink *et al*. 1998b; Oude Elferink *et al*., 1999).

The OTU abundance of *Desulfarculus baarsii* (Widdel, 1980; Kuever *et al*., 2005; Sun *et al*., 2010; Kuever, 2014) was 0.1% at the lowest dilution rate of 0.0083 h^{-1} (RT of 5) in both reactors and the OTU abundance of *Desulfocurvus vexinensis* was 0.7 and 0.4 in the reactors receiving feed sulphate of 2.5 and 5.0 g l^{-1}, respectively, at the same dilution rate (Figure 6.9). Increasing the dilution rate from 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d) resulted in the washout of both of these species in the reactor receiving feed sulphate of 2.5 g l^{-1} which corresponded to VSLR of 0.104 g l^{-1} h^{-1} (Figure 6.7A and B). However, in the reactor receiving feed sulphate concentration of 5.0 g l^{-1}, increasing the dilution rate from 0.0083 to 0.042 h^{-1} (VSLR of 0.042 to 0.208 g l^{-1} h^{-1}) resulted in the increase of *Desulfarculus baarsii* OTU abundance from 0.1 to 0.3% (Figure 6.9) and therefore dramatically increasing their proportion from 0.8 to 18.6% (Figure 6.7C and D). These observations suggested that *Desulfarculus baarsii* competed better for the substrate (anaerobic digestate) at higher VSLR of 0.208 g l^{-1} h^{-1} compared to lower VSLR of 0.104 g l^{-1} h^{-1}. Research by Widdel (1980), Kuever *et al*. (2005) and Kuever (2014) reported that *Desulfococcus biacutus* can oxidise acetate, propionate, butyrate and higher VFAs in the presence of sulphate the carbon monoxide dehydrogenase pathway. However, oxidation of acetate and propionate was observed to be very slow. Therefore, this suggested that in these reactors, *Desulfococcus biacutus* used butyrate as the main carbon source for BSR. Although increasing the VSLR from 0.042 to 0.208 g l^{-1} h^{-1}, corresponding to the dilution rate of 0.0083 to 0.042 h^{-1} in the reactor receiving feed sulphate concentration of 5.0 g l^{-1}, resulted in the decrease of *Desulfocurvus vexinensis* OTU from 0.4 to 0.1% (Figure 6.9) this resulted in a slight increase in the proportion of this SRB species from 3.0 to 3.9% (Figure 6.7C and D). However, *Desulfocurvus vexinensis* are not known to oxidise any of the components of anaerobic digestate. Therefore, their function, if any, in these reactors is not known.

While *Desulfomicrobium hypogeium* OTU was the most abundant OTU at the lowest dilution rate of 0.0083 h^{-1} (RT of 5 d) in some reactors (Figure 6.7, 6.8 and 6.9), neither *Desulfomicrobium hypogeium* nor *Desulfomicrobium aestuarii* is known to oxidise either VFA (acetate, propionate or butyrate) present in anaerobic digestate (Table 2.6 in Section 2.4.3). Similarly, *Desulfomonile tiedjei*, *Desulfocurvus vexinensis* and the identified *Desulfovibrio* species are not known to oxidise either VFA present in anaerobic digestate but were identified in the reactors receiving anaerobic digestate as an electron donor for BSR. In addition, some of these SRB stayed in these reactors even when the dilution rate was increased from 0.0083 to 0.042 h^{-1}. For example, *Desulfovibrio aminophilus* OTU (Baena *et al*., 1998), *Desulfovibrio desulfuricans* OTU (Okabe *et al*., 1992 and 1992a), *Desulfovibrio intestinalis* OTU (Fröhlich *et al*., 1999), *Desulfovibrio oxamicus* OTU (Lopez-Cortes *et al*., 2006) and *Desulfovibrio sulfodismutans* OTU (Bak and Pfennig, 1987) remained in the reactors even at the high dilution rate of 0.042 h^{-1} despite being present in low abundance. Only *Desulfovibrio vulgaris* OTU (McInerney and Bryant, 1981) and *Desulfovibrio mexicanus* OTU (Hernandez-Eugenio *et al*., 2000) were washed out of both reactors when the dilution rate was increased from 0.0083 to 0.042 h^{-1} (Figure 6.9). The function of *Desulfomonile tiedjei*, *Desulfocurvus vexinensis*, *Desulfomicrobium* and *Desulfovibrio* identified in these reactors remain unclear.

The decrease of *Desulfobulbus oligotrophicus* OTU from 0.6 to 0.004% at the dilution rates of 0.0083 and 0.042 h^{-1} respectively, indicated in Figure 6.9, coincided with the decline in propionate conversion from 100 to 21.7% reported in Chapter 5 (Sections 5.4.1. 2). Similarly, in the reactor receiving feed sulphate concentration of 5.0 g l^{-1}, the decline in the abundance of the *D. oligotrophicus* OTU from 0.4 to 0.2 % at the dilution rates of 0.0083 and 0.042 h^{-1} respectively, shown in Figure 6.9, coincided with the decline in propionate conversion from 100 to 56.3% reported in Chapter 5 (Sections 5.4.1. 3). These observations suggested that *D. oligotrophicus* as little as 0.004% could drive propionate oxidation and concomitant sulphate reduction when anaerobic digestate was provided as an electron donor for BSR. However, the fact that in this thesis, the decline in the abundance of the OTU of the propionate oxidiser *D. oligotrophicus* coincided with the decline in propionate conversion also suggests that prolific *D. oligotrophicus* numbers are important for better propionate oxidation and concomitant sulphate reduction efficiency. While *Desulfarculus baarsii* can oxidise propionate in the presence of sulphate, growth on propionate is very slow (Widdel, 1980; Kuever *et al.*, 2005; Kuever, 2014), suggesting that the oxidation of propionate and concomitant sulphate reduction observed in these reactors, reported in Chapter 5 (Sections 5.4.1. 2 and 5.4.1.3, respectively) was carried out by mainly by *D. oligotrophicus* and possibly to somewhat by *Desulfarculus baarsii.*

While both *Desulfarculus baarsii* and *Desulfococcus biacutus* can oxidise acetate in the presence of sulphate, and remained in these reactor even at the high VSLR of 0.208 g l^{-1} h^{-1} (Figures 6.7 and 6.9),

their growth on acetate is very slow (Widdel, 1980; Platen *et al*., 1990; Kuever *et al.*, 2005). This suggests that these SRB on their own are not effective for acetate oxidation and concomitant sulphate reduction.

Although *Desulfarculus baarsii* and *Desulfococcus biacutus* can oxidise acetate in the presence of sulphate, the two SRB were possibly growing too slowly to affect oxidation of acetate and concomitant sulphate reduction (Platen *et al*., 1990). On the contrary, *Desulfobacca acetoxidans* has a doubling time of 1.7-2.2 d on acetate and sulphate (Oude Elferink *et al*, 1999) suggesting that this SRB may have been responsible for the observed acetate oxidation and concomitant sulphate reduction. However, *Desulfobacca acetoxidans* were completely washed out at the higher dilution rate of 0.042 h^{-1} (RT of 1 d) in both reactors. Despite this, acetate utilisation was reported in both these reactors at this dilution rate in Chapter 5 (Sections 5.4.1.2 and 5.4.1.3). However, the stoichiometry of the BSR efficiency kinetics reported for these reactors (feed sulphate of 2.5 and 5.0 g l^{-1}, Sections 5.4.1.2 and 5.4.1.3) demonstrated that acetate oxidation and concomitant sulphate reduction in these reactors declined at the dilution rate of 0.042 h^{-1} (RT of 1 d). This suggested that at the dilution rate of 0.042 h^{-1}, acetate was being utilised by either "non-SRB" species or both SRB species and "non-SRB" species. One such non SRB species is *Desulfuromonas acetexigens* (Finster *et al.*, 1994). *Desulfuromonas acetexigens* can oxidise acetate in the presence of elemental sulphur using the citric acid pathway (Finster *et al*., 1994). Chapter 5 (Section 5.4.1.1) reported the visual observation of some white sulphur biofilms on the walls of the CSTRs which indicated that at least part of the sulphide was partially oxidised to elemental sulphur. At both feed sulphate concentrations, unlike *Desulfobacca acetoxidans*, *Desulfuromonas acetexigens* OTU remained in the reactors even at the higher dilution rate of 0.042 h^{-1} (Figures 6.8 and 6.9). *Desulfuromonas acetexigens* OTU was abundant at the lowest dilution rate of 0.0083 h^{-1} (RT of 5 d) with the abundance of 2.6 and 0.7% at feed sulphate of 2.5 and 5.0 g l^{-1} respectively and with the abundance of 0.6 and 0.04% at feed sulphate of 2.5 and 5.0 g l^{-1} respectively at the dilution rate of 0.042 h^{-1} (Figures 6.8 and 6.9). Grigoryan *et al*. (2008) also obtained elemental sulphur reducing *Desulfuromonas* species when denaturing gradient gel electrophoresis (DGGE) was performed on samples from a sulphate reducing bioreactor community receiving a mixture of acetate, propionate and butyrate as a carbon source and electron donor for BSR. Therefore this thesis hypothesise that *Desulfuromonas acetexigens* played a role in the utilisation of acetate at the dilution rate of 0.042 h^{-1}, and that the present elemental sulphur was the electron acceptor. Thus, suggesting that at the lowest dilution rates, *Desulfobacca acetoxidans* was responsible for the observed acetate oxidation and concomitant sulphate reduction. However, as these species were washed out of the reactors as the dilution rate was increased to 0.042 h^{-1} (RT of 1 d), there was competition for acetate the available acetate between SRB and *Desulfuromonas acetexigens*. Reduction of elemental sulphur is due to the constitution of the sulphur reductase EC 1.12.98.4-sulfhydrogenase, formerly known as EC 1.97.1.3-sulfur, observed in microorganisms such as *Desulfuromonas acetoxidans*, *Desulfovibrio* species (*D.*

gigas and *D. vulgaris*) and *Desulfomicrobium norvegicum* (Fauque, 1994; Fauque *et al*., 1994; Fauque and Barton, 2012; Barton *et al*., 2014). *Desulfuromonas* species have also been proposed to be involved in acetate fermentation metabolism in ways that is not yet understood (Goevert and Conrad, 2010; Callbeck *et al*., 2013).

Butyrate was also a major VFA present in anaerobic digestate. Oude Elferink *et al.* (1999) reported that *Desulfarculus baarsii* can oxidise butyrate in the presence of sulphate using the carbon monoxide dehydrogenase pathway. Although *Desulfarculus baarsii* OTU was the only known SRB OTU revealed in this thesis known to oxidise butyrate (Figure 6.9), there was no clear correlation between the OTU abundance and butyrate oxidation and concomitant sulphate reduction. For example, in the reactors receiving feed sulphate concentration of 2.5 g l^{-1}, the complete wash out of *Desulfarculus baarsii* OTU when the dilution rate was increased to 0.042 h^{-1} coincided with butyrate conversion of 16.5%. However, in the reactor receiving 5.0 g l^{-1} sulphate, increasing the dilution rate of 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d) resulted in the slight increase in the abundance of *Desulfarculus baarsii* OTU from 0.1 to 0.3% (Figure 6.9), which coincided with a decline in butyrate conversion from 100 to 8.7% (Figure 5.7a in Section 5.4.1.3). This suggested that some other microorganisms in these reactors may have partaken in the utilisation of the available butyrate. Some *Citrobacter* species and *Firmicutes* have been demonstrated to partake in the oxidation of butyrate (Wagner *et al*., 1998; Loy *et al*., 2004; Zverlov *et al*., 2005 Ramamoorthy *et al.*, 2006; Vatsurina *et al*., 2008; Lee *et al*., 2009; Pester *et al*., 2010; Zhou *et al.*, 2015; Hausmann *et al*., 2016). There were also unclassified OTUs in both reactors which their functions could not be identified. However, of the OTUs present at the relative abundance of ≥ 1% observed in Figure 6.8, none of these are known to oxidise butyrate.

These results show that when anaerobic digestate was provided as an electron donor for BSR, *Desulfobacca acetoxidans* (which specialises its growth on acetate), *Desulfarculus baarsii* (grows faster on butyrate than on acetate or propionate) and *Desulfobulbus oligotrophicus* (propionate oxidiser) were able to survive high VSLRs of up to 0.208 g l^{-1} h^{-1}. Therefore, this suggests that in BSR treatment processes with VSLRs of up to 0.208 g l^{-1} h^{-1} where anaerobic digestate is provided as an electron donor, a combination of these three SRB could be effective and thus resulting in high volumetric sulphate reduction rates (VSRRs) of up to 0.070 g l^{-1} h^{-1} which are reported in Chapter 5 (Section 5.4.1.3).

6.3.1.3 Microbial community structure and population dynamics of lactate operated BSR CSTRs

The quantitative assessment of changes in community structure and dynamics in response to changes in volumetric sulphate loading rate (VSLR), mediated through dilution rate and feed sulphate concentration, in the lactate (simple carbon source) fed reactors was carried out with qPCR and 16S rRNA metagenomics. The bacteria dry mass and sulphate conversion data were taken from Oyekola

(2008) and Oyekola *et al*. (2010; 2012). Similar to the reactors receiving anaerobic digestate, increasing VSLR changed SRB community structure and decreased the proportion of the total SRB within the mixed microbial community (Figures 6.9, 6.10 and 6.11). qPCR results indicated that *Desulfomicrobium* species were the most abundant group within these SRB communities (Figure 6.10). At all four feed sulphate concentrations (1.0, 2.5, 5.0 and 10.0 g l^{-1}) the proportion of SRB compared to total bacterial copy numbers declined with the increasing dilution rate (Figure 6.10). For the reactors receiving feed sulphate at 1.0 g l^{-1}, the proportions of *Desulfomicrobium* and *Desulfovibrio* species remained steady at the lower dilution rates of 0.0083 and 0.014 h^{-1} (RTs of 5 to 2 d) at 44.9 ± 4.9% and 1.3 ± 0.29%, respectively. The average sulphate conversion observed at these dilution rates was 86.4 ± 0.14% (Figure 6.9A). Increasing dilution rate to 0.028 h^{-1} (RT of 1.5 d) resulted in a pronounced decline of *Desulfomicrobium* species to 18.3 ± 0.81%, while the proportion of the *Desulfovibrio* species remained steady at 1.16 ± 0.06%. The pronounced decline of *Desulfomicrobium* species observed across the dilution rates of 0.028 and 0.042 h^{-1} (RTs of 1.5 and 1 d) did not affect sulphate conversion as it remained steady at 85.6 ± 0.35%. However, the decline of *Desulfovibrio* species from 1.1 ± 0.1% to 0.2 ± 0.06% observed when the dilution rate was increased from 0.042 to 0.083 h^{-1} (RTs of 1 to 0.5 d) resulted in a pronounced decline of sulphate conversion from 85.5 to 49.6% (Figure 6.10A). These observations were similar to observations made with anaerobic digestate, whereby the decrease of *Desulfovibrio* species was associated with a decline in sulphate conversion. Figures 6.10E shows that although the decrease in the proportion of SRB to total bacteria correlated with the decrease in sulphate conversion at higher dilution rates, the VSRR still remained in the range of 0.012 to 0.041 g l^{-1} h^{-1} at a feed sulphate concentration of 1.0 g l^{-1} and in the range of 0.032 to 0.090 g l^{-1} h^{-1} at a feed sulphate concentration of 10.0 g l^{-1}. This suggests that similar to when anaerobic digestate was used as carbon source and electron donor, the reduced sulphate conversion observed with the use of lactate as electron donor resulted from the inability of the VSSR of the microbial consortium to match the increased VSLR, rather than a reduction in VSRR.

These observations also suggested that despite being the less abundant species, like in the anaerobic digestate fed reactors, *Desulfovibrio* species played an important role in stabilising the sulphate reduction when lactate was used as a carbon source and electron donor for BSR. Similar observations were made in the lactate fed reactor receiving feed sulphate of 10.0 g l^{-1} (Figure 6.10D). Steady sulphate conversions (32.1 ± 7.1%) were correlated with steady proportions of *Desulfovibrio* species (1.5 ± 0.32%) across the dilution rates of 0.0083 to 0.042 h^{-1} (RTs of 5 to 1 d). The decline of *Desulfomicrobium* species from 21.2 ± 3.8% across the dilution rates of 0.0083 to 0.014 h^{-1} (RTs of 5 to 3 d) to 6.1 ± 0.66% at the dilution rate of 0.021 h^{-1} (RT of 2 d) did not result in a significant change in sulphate conversion. A further decline of *Desulfomicrobium* species to 0.43 ± 0.3% across the dilution rates of 0.028 to 0.042 h^{-1} (RTs of 1.5 to 1 d) also not result in a significant change in sulphate

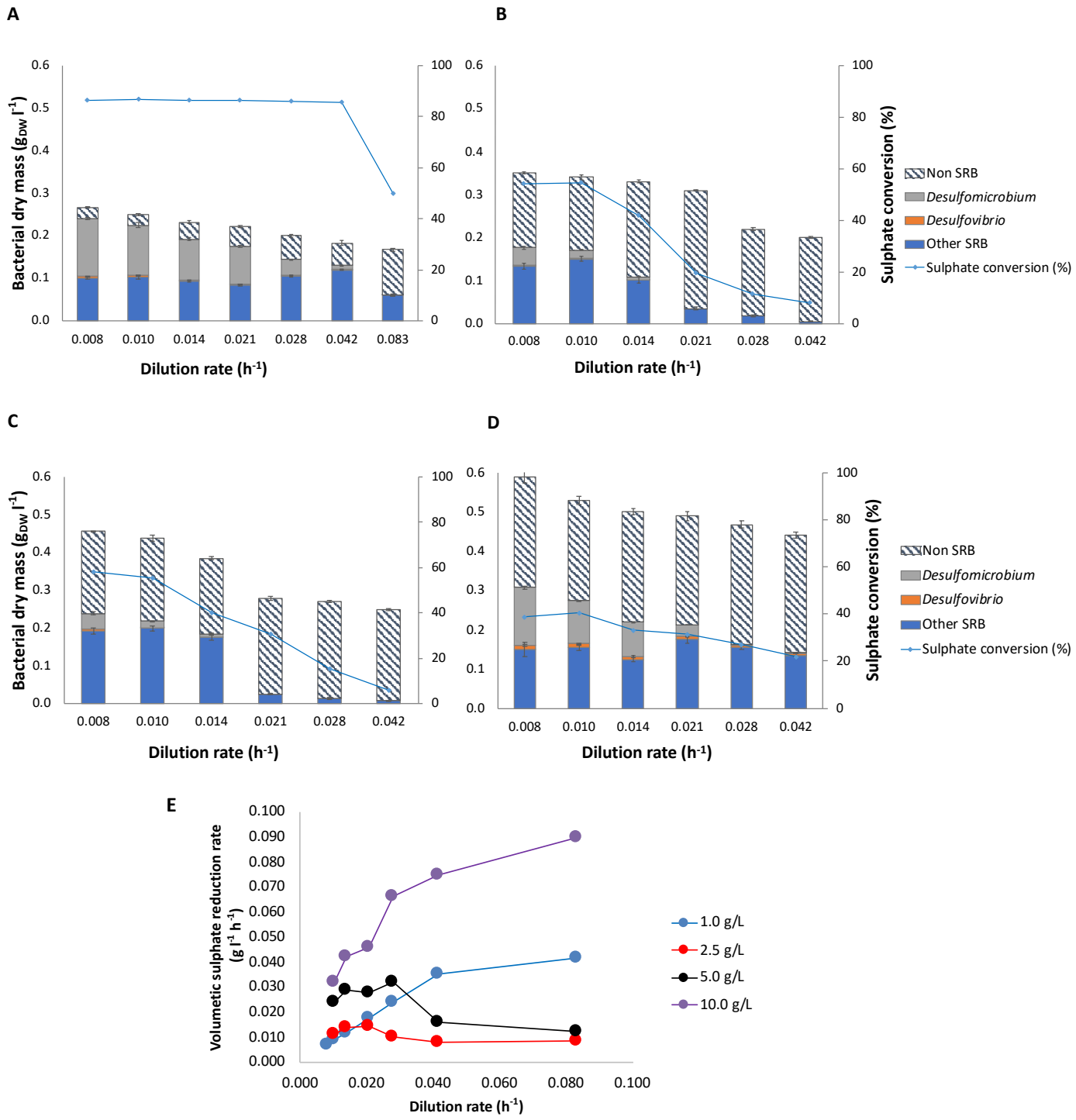


Figure 6.10. Graphical representation of the qPCR results obtained on steady state samples obtained from reactors lactate as a carbon source and electron donor for biological sulphate reduction using total bacterial primers (TotalF and TotalR), novel *Desulfomicrobium* species primers (DSM442F and DSM632R) and modified *Desulfovibrio* species primers (DSV-III-312f[b] and DSV681R[b]). The reactors received feed sulphate concentrations of 1.0 g l^{-1} (A), 2.5 g l^{-1} (B), 5.0 g l^{-1} (C) and 10.0 g l^{-1} (D). A comparison of VSRR obtained at these concentrations across the dilution rates of 0.0083 to 0.083 h^{-1} is given in (E). The bacterial dry mass, sulphate conversion and volumetric sulphate loading data used to generate this graph was obtained from Oyekola *et al.*, (2010). Conversion of the qPCR results to % of bacterial biomass assume that the copy numbers of the genes / cell and the mass of the cells of the various SRB is the same.

conversion (Figure 6.10D). These observations suggested that in the reactors receiving feed sulphate concentrations of 1.0 and 10.0 g l^{-1}, the present *Desulfovibrio* species had higher growth rates than *Desulfomicrobium* species. Also, these *Desulfovibrio* species could survive higher dilution rates as compared to *Desulfomicrobium* species. Oyekola *et al*. (2012) demonstrated that in these reactors (feed sulphate concentrations of 1.0 and 10.0 g l^{-1}), incomplete lactate oxidation and concomitant sulphate reduction were dominant in the reactors receiving suggesting that the presence of *Desulfomicrobium* and *Desulfovibrio* species was important for effective sulphate reduction.

A rapid decline in the proportion of *Desulfomicrobium* species was observed with the increase in dilution rates in the reactors receiving feed sulphate concentration of 2.5 and 5.0 g l^{-1} (Figure 6.9B and C). The proportion of *Desulfomicrobium* species in the reactor receiving feed sulphate at 2.5 g l^{-1} declined from 11.6 ± 0.85% at the lowest dilution rate tested of 0.0083 h^{-1} (RT of 5 d) and these species were completely washed out at the dilution rate of 0.021 h^{-1} (RT of 2 d) (Figure 6.9B). A similar observation was made for the reactor receiving feed sulphate concentration of 5.0 g l^{-1} whereby the *Desulfomicrobium* species declined from 8.9 ± 0.91% at the lowest dilution rate tested of 0.0083 h^{-1} (RT of 5 d) to being completely washed out dilution rate of 0.021 h^{-1} (RT of 2 d) (Figure 6.9C). The proportion of the *Desulfovibrio* species declined from 0.9 ± 0.06% at the dilution rate of 0.0083 h^{-1} (RT of 5 d) to 0.3 ± 0.15% at the dilution rate of 0.014 h^{-1} (RT of 3 d) in the reactor receiving feed sulphate of 2.5 g l^{-1}. Approximately 50% (0.5 ± 0.15%) of the *Desulfovibrio* species observed at the dilution rate tested of 0.0083 h^{-1} (RT of 5 d) were observed at the dilution rate of 0.010 h^{-1} (RT of 4 d) and no *Desulfovibrio* species were detected at the dilution rate of 0.014 h^{-1} (RT of 3 d) in the reactor receiving feed sulphate concentration of 5.0 g l^{-1} (Figure 6.10B and C). The decline in the *Desulfomicrobium*, *Desulfovibrio* and "other SRB" communities resulted in the decline in BSR (Figure 6.10B and C). The proportion of "non-SRB species" at in reactor with feed sulphate of 2.5 g l^{-1} increased from 55.4 ± 8.7% across the dilution rates of 0.0083 to 0.014 h^{-1} (RTs of 5 to 3 d) to 92.5 ± 4.0% across the dilution rates of 0.021 to 0.042 h^{-1} (RTs of 2 to 1 d). Similarly, the proportion of "non-SRB species" at in reactor with feed sulphate of 5.0 g l^{-1} increased from 50.7 ± 2.4% across the dilution rates of 0.0083 to 0.014 h^{-1} (RTs of 5 to 3 d) to 94.4 ± 3.0% across the dilution rates of 0.021 to 0.042 h^{-1} (RTs of 2 to 1 d). These observations are in agreement with studies by Oyekola *et al*. (2012) who demonstrated that in these reactors (feed sulphate of 2.5 and 5.0 g l^{-1}) the substantial decline in VSRRs and lactate oxidation at these dilution rates was associated with lactate fermentation, suggesting that *Desulfovibrio* and *Desulfomicrobium* were out competed by fermenters and could not thrive. The prevalence of *Desulfovibrio* and *Desulfomicrobium* species in these reactors is supported by the results from the bacterial 16S rRNA gene clone library of the inoculum which was obtained from a lactate fed CSTR receiving sulphate at 1.0 g l^{-1} at a RT of 5 d reported in Chapter 4 (Figure 4.2 and Table 4.1). In addition, Figure 6.10 indicates the presence of other SRB species (and non-SRB species) within the reactors that

the qPCR experiments in this thesis could not elucidate. Therefore, metagenomic analysis of the BSR bacterial communities associated with the lactate operated CSTR by 16S rRNA gene variable region was performed to validate the qPCR results and assist with the identification of the "unknown" bacteria (Figures 6.8, 6.11 and 6.12).

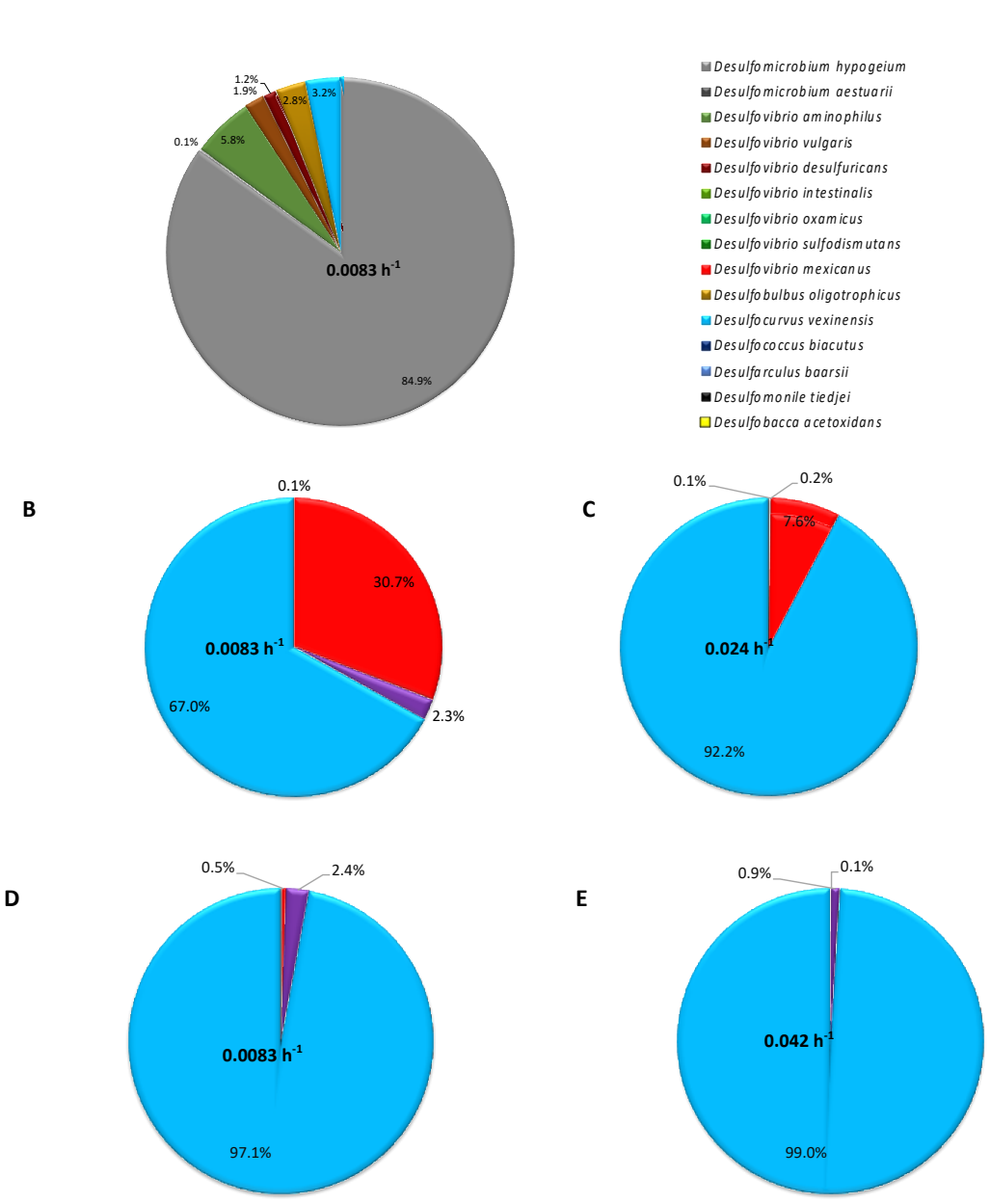


Figure 6.11. Metagenomic analysis of the SRB structure at the species level on steady state samples obtained from reactors operating on lactate as a carbon source for BSR at feed sulphate concentration of 1.0 g l^{-1} (A), 5.0 g l^{-1} (B and C) and 10.0 g l^{-1} (D and E). The samples were obtained at lowest dilution rate tested (0.0083 h^{-1}, RT of 5 d) (A, B and D) and dilution rate of 0.042 h^{-1} (RT of 1 d) (C and E).

The metagenomic data indicated the presence of 10 SRB species belonging to the *Desulfomicrobium* groups (*Desulfomicrobium hypogeium* and *Desulfomicrobium aestuarii*), *Desulfovibrio* groups (*D.*

aminophilus, D. vulgaris, D. desulfuricans, D. intestinalis, D. sulfodismutans and *D. mexicanus*), *Desulfobulbus* species (*D. oligotrophicus* and *Desulfocurvus* species (*D. vexinensis*) (Figure 6.11 and Figure 6.12). In the reactor receiving feed sulphate concentration of 1.0 g l^{-1} at a dilution rate of 0.0083 h^{-1} (RT of 5 d), which was also the inoculum used to initiate the reactors as described in Chapter 4 (Table 4.1 and Figure 4.2), *Desulfomicrobium hypogeium* OTU was the most abundant SRB species at 29.0% abundance (Figure 6.12). This accounted for 84.9% of the total SRB proportion in the reactor (Figure 6.11A). These results were in corroboration with observations made with qPCR in Figure 6.10A and Section 4.2 (Figure 4.2), where it was demonstrated the *Desulfomicrobium* species were the most abundant microbial group within the inoculum used to initiate the reactors in this thesis. Contrary to the observations made in this reactor, metagenomics data indicated that at the lowest dilution rate of 0.0083 h^{-1} (RT of 5 d), the incomplete lactate oxidiser, *Desulfocurvus vexinensis* OTU (Klouche *et al*., 2009), was the most abundant SRB species in the reactors receiving feed sulphate concentrations of 5.0 and 10.0 g l^{-1} with the abundance of 24.8 and 30.6%, respectively (Figures 6.8 and 6.12). *Desulfocurvus vexinensis* species accounted for 67.0% of the total SRB proportion at feed sulphate concentration of 5.0 g l^{-1} (Figure 6.11B) and 97.1% of the total SRB proportion at feed sulphate of 10.0 g l^{-1} (Figure 6.11D). Increasing dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to volumetric sulphate loading rate of 0.042 to 0.208 g l^{-1} h^{-1}) resulted in the decline of *Desulfocurvus vexinensis* OTU from 24.8 to 4.1% at feed sulphate concentration of 5.0 g l^{-1} (Figure 6.12). Despite the decline in the abundance of *Desulfocurvus vexinensis* at the higher dilution rate of 0.042 h^{-1} (VSLR of to 0.208 g l^{-1} h^{-1}), *Desulfocurvus vexinensis* still accounted for 92.0% of the total SRB proportion in this reactor (Figure 6.11C). Similarly, increasing dilution rate from 0.0083 to 0.042 h^{-1} (which corresponded to VSLR rate of 0.083 g l^{-1} h^{-1} to 0.42 g l^{-1} h^{-1}) resulted in the decline of *Desulfocurvus vexinensis* OTU from 30.6 to 12.9% at feed sulphate concentration of 10.0 g l^{-1} (Figure 6.12). In spite of this decline of its abundance at the dilution rate of 0.042 h^{-1} (VSLR of to 0.42 g l^{-1} h^{-1}), *Desulfocurvus vexinensis* still accounted for 99.0% of the total SRB proportion in this reactor (Figure 6.11E).

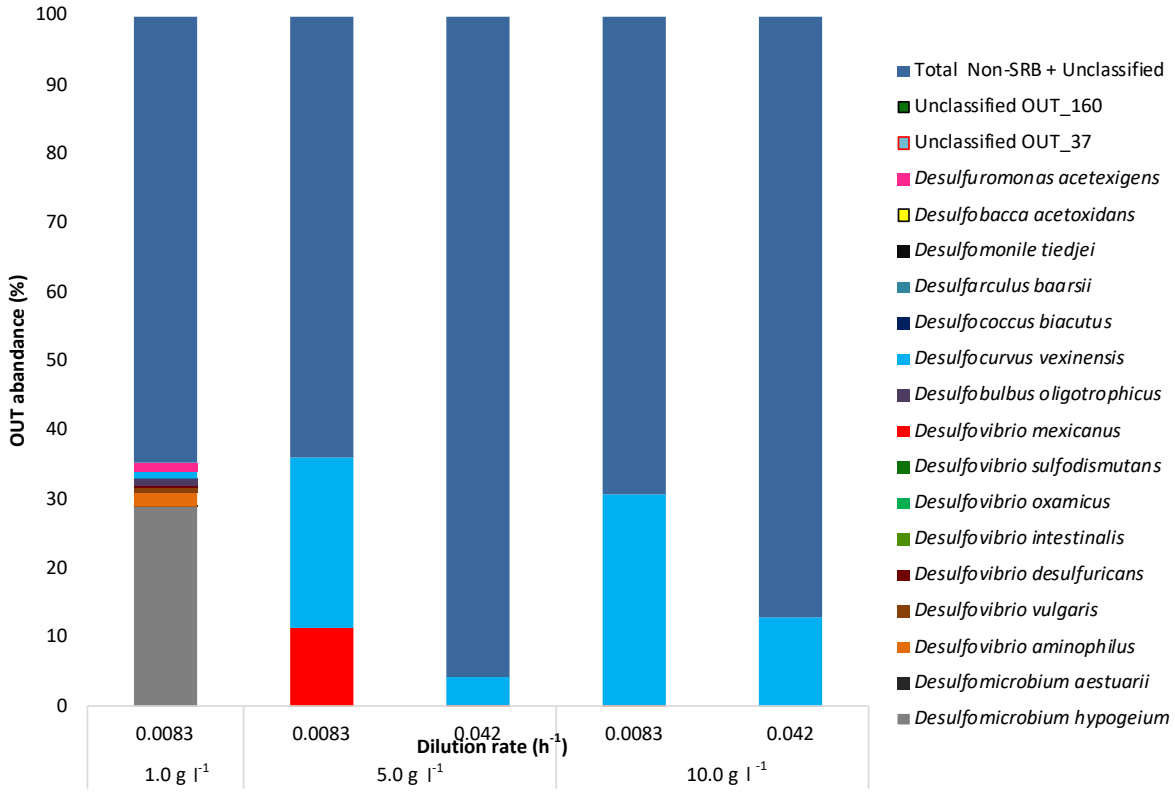


Figure 6.12. Relative abundance of OTUs present in CSTRs receiving lactate at different dilution rates and feed sulphate concentrations. Each OTU is described by its species classification unless otherwise stated.

These observations suggested three things: when lactate was provided as an electron donor for BSR (i) *Desulfocurvus vexinensis* had higher growth rates (> 0.042 g l^{-1} h^{-1}) than other SRB identified in this study, (ii) *Desulfocurvus vexinensis* could survive at high VSLR (>0.42 g l^{-1} h^{-1}) and (iii) *Desulfocurvus vexinensis* competed better for sulphate at higher loading than other classified microbial species identified in this study as this SRB was more abundant at higher feed sulphate concentration of 10.0 g l^{-1} compared to 1.0 and 5.0 g l^{-1} (Figures 6.8, 6.11 and 6.12). Klouche *et al*. (2009), reported that *Desulfocurvus vexinensis* exhibited high growth rates of on lactated with μ_{max} of 0.21 h^{-1} and a doubling time of 3.3 h. Oyekola *et al*. (2010) reported that high volumetric sulphate reduction rates (VSRRs) of up to 0.090 g l^{-1} h^{-1} were observed in the reactors receiving lactate as a carbon source for BSR. Therefore, these characteristics of *Desulfocurvus vexinensis* makes this SRB a good candidate for BSR treatment processes with VSLR of up to 0.42 g l^{-1} h^{-1}.

Another incomplete lactate oxidiser *Desulfobulbus oligotrophicus* OTU (El Houari *et al*., 2017) was observed at all three feed sulphate concentrations (1.0, 5.0 and 10.0 g l^{-1}) (Figures 6.8, 6.11 and 6.12). At the lowest dilution rate of 0.0083 h^{-1} (RT of 5 d), the abundance of *Desulfobulbus oligotrophicus* OTU was highest at feed sulphate concentration 1.0 g l^{-1} with the abundance of 1.0% (Figure 6.11A)

and similar at feed sulphate concentrations of 5.0 and 10.0 g l^{-1} with the abundance of 0.8% (Figure 6.11B and D, respectively). Increasing dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to volumetric sulphate loading rate of 0.042 to 0.208 g l^{-1} h^{-1}) resulted in total washout of the *Desulfobulbus oligotrophicus* OTU at feed sulphate concentration of 5.0 g l^{-1} (Figure 6.11C and Figure 6.12). At feed sulphate concentration of 10.0 g l^{-1}, increasing dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to VSLR of 0.083 to 0.42 g l^{-1} h^{-1}) resulted in the decline of *Desulfobulbus oligotrophicus* OTU from 0.8 to 0.1% (Figure 6.12), which resulted in the proportion of this SBR declining from 2.4 to 0.9% (Figures 6.11D and 6.11E). These observations suggested that (i) *Desulfobulbus oligotrophicus* had higher growth rates at higher sulphate loading and competed effected better for substrate at higher VSLR of 0.42 g l^{-1} h^{-1} than at 0.208 g l^{-1} h^{-1}. Observations by studies El Houari *et al*. (2017) reported that growth rates for *Desulfobulbus oligotrophicus* on lactate and sulphate were between 0.012 and 0.024 h^{-1} at 35°C and pH 7.6. The fact that the conditions in this thesis were 35°C and pH 8 ± 0.2, could have had an effect of the higher growth rates observed for the reactor receiving feed sulphate concentration of 10.0 g l^{-1} (Figure 6.11E), therefore making *Desulfobulbus oligotrophicus* a good candidate for BSR treatment processes with VSLR of up to 0.42 g l^{-1} h^{-1}.

Although incomplete lactate oxidiser *Desulfovibrio mexicanus* OTU which was originally isolated by from the upflow anaerobic sludge blanket (UASB) reactor treating cheese factory wastewater Hernandez-Eugenio *et al*. (2000) was not observed in the reactor with feed sulphate of 1.0 g l^{-1} at the dilution rate of 0.0083 h^{-1} (RT of 5 d) (Figures 6.8, 6.11A and 6.12), this SRB was the second most abundant SRB at 11.3% at feed sulphate concentrations of 5.0 g l^{-1} (Figure 6.12). This accounted for 30.7% of the total SRB proportion at at this dilution rate (Figure 6.11B). Increasing dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to VSLR of 0.042 to 0.208 g l^{-1} h^{-1}) resulted in drastic decline of *Desulfovibrio mexicanus* OTU from 11.3% to 0.3% (Figure 6.12) which accounted for the decline from the proportion of 30.7% to 7.6% (Figure 6.11B and 11C). However, in the reactor with feed sulphate of 10.0 g l^{-1}, *Desulfovibrio mexicanus* OTU only had the abundance of 0.2% (Figures 6.8 and 6.12). Increasing dilution rate from 0.0083 to 0.042 h^{-1} (corresponding to VSLR of 0.083 to 0.42 g l^{-1} h^{-1}) resulted in the complete washout of *Desulfovibrio mexicanus* (Figures 6.11D and 6.11E). These observations showed that although *Desulfovibrio mexicanus* dominated at the lower VSLR of 0.042 g l^{-1} h^{-1}, this SRB could not survive at the higher VSLR of 0.42 g l^{-1} h^{-1} and thus suggesting that when lactate is used as a carbon source for BSR, *Desulfovibrio mexicanus* is a good candidate for BSR treatment processes with lower VSLRs. Hernandez-Eugenio *et al*. (2000) demonstrated that the growth rates for of *Desulfovibrio mexicanus* ranged between 0.07 and 0.35 h^{-1} between 20 and 40°C and pH between 6.3 and 8.2 on lactate and sulphate.

One *Desulfomicrobium* OTU, *Desulfomicrobium aestuarii* OTU and two *Desulfovibrio* OTUs (*Desulfovibrio vulgaris* OTU and *Desulfovibrio desulfuricans* OTU) were observed at feed sulphate of

1.0 g l^{-1} but not at feed sulphate concentrations of 5.0 and 10.0 g l^{-1} (Figures 6.11 and 6.12). Extensive literature is available on the growth kinetics and growth rates of *Desulfovibrio desulfuricans* and studies have demonstrated that specific growth rates of *Desulfovibrio desulfuricans* range between 0.007 to 0.55 h^{-1}, which falls within the observations made with these reactors (Okabe and Characklis, 1992; Okabe *et al*., 1992; Reis *et al*., 1992; Okabe *et al*., 1995; Cooney *et al*., 1996; Nagpal *et al*., 2003). Ingvorsen and Jørgensen (1984) reported that the μ_{max} of 0.011 h^{-1} for *Desulfovibrio vulgaris* on lactate and sulphate. Dias *et al*. (2008) indicated that *Desulfomicrobium aestuarii* showed growth rate of 0.080 h^{-1} (with doubling time of 8.6 hours) at optimum growth conditions of 35°C and pH 7.2. The observations made in these reactors suggested that although all three SRB species can oxidise lactate in the presence of sulphate (McInerney and Bryant, 1981; Okabe *et al*., 1992a; Dias *et al*., 2008), the fact that they could not survive at higher sulphate loadings makes them unideal candidates for treating wastewaters containing sulphate concentrations of 5.0 or 10.0 g l^{-1} when lactate is provided as a carbon source for BSR.

Desulfovibrio aminophilus OTU (Baena *et al*., 1998) and another lactate oxidiser *Desulfovibrio sulfodismutans* OTU (Bak and Pfennig, 1987) were observed at feed sulphate concentrations of 1.0 and 5.0 g l^{-1}, they were not at feed sulphate of 10.0 g $^{-1}$. However, both SRB were observed at low abundance at feed sulphate of 5.0 g l^{-1} with *Desulfovibrio aminophilus* observed at the abundance of 0.004% and *Desulfovibrio sulfodismutans* OTU at 0.02% the dilution rate of 0.0083 h^{-1} (Figure 6.12). Increasing dilution rate to 0.042 h^{-1} (RT 1 d) resulted in the complete washout of both SRB in this reactor (Figure 6.11B and 6.11C). The abundance of 2.0 and 0.01% were observed for *Desulfovibrio aminophilus* OTU and *Desulfovibrio sulfodismutans* OTU respectively, at feed sulphate of 1.0 g l^{-1} (Figure 6.12). The complete washout of *Desulfovibrio sulfodismutans* OTU observed when dilution rate was increased from 0.0083 to 0.042 (corresponding to VSLR of 0.042 to 0.208 g l^{-1} h^{-1}) and the absence of this lactate oxidiser at higher sulphate concentrations suggests that it had lower growth rates and could not survive at the higher VSLR of 0.208 g l^{-1} h^{-1}. Therefore, it can be concluded that like *Desulfomicrobium aestuarii*, *Desulfovibrio vulgaris*, and *Desulfovibrio desulfuricans*, *Desulfovibrio sulfodismutans* may not be a good candidate for treating wastewaters containing sulphate concentrations of 10.0 g l^{-1} when lactate is provided as a carbon source for BSR. To date, there is no evidence that *Desulfovibrio aminophilus* can utilise lactate nor the products of its incomplete oxidation (acetate and propionate). Therefore, the presence of this SRB in these reactors remains unclear. The lactate oxidiser *Desulfovibrio intestinalis* OTU (Fröhlich *et al*., 1999) was only observed in the reactor receiving feed sulphate concentration of 5.0 g l^{-1} and not at feed sulphate of 1.0 or 10.0 g l^{-1}. Despite being present at low abundance (0.004%) at the lowest dilution rate of 0.0083 h^{-1} (corresponding to VSLR of 0.042 g l^{-1} h^{-1}) the increase in the dilution rate to 0.042 h^{-1} (corresponding to VSLR of 0.208 g l^{-1} h^{-1}) did not affect the abundance of this SRB as its abundance remained unchanged (0.004%) (Figure 6.12). These observations suggested that *Desulfovibrio intestinalis* had higher growth rates in the receiving feed

sulphate concentration of 5.0 g l^{-1} than in the reactors receiving sulphate concentrations of 1.0 and 10.g l^{-1}. *Desulfovibrio intestinalis* OTU was able to survive higher VSLR of 0.208 g l^{-1} h^{-1}. Fröhlich *et al*. (1999) reported the doubling time of 12.5 hours at 37°C for *Desulfovibrio intestinalis* species on lactate and sulphate. However, growth rate values were not reported for this SRB.

The lactate fermenters, *Anaerotignum propionicum* and *Anaerotignum aminivorans* (Ueki *et al*., 2017) and *Bacillus pseudofirmus* (Ma *et al*., 2012), were observed in the reactors receiving lactate and not in the reactors receiving anaerobic digestate. These microorganisms were also more prolific at the higher dilution rate of 0.042 h^{-1} (Rt of 1 d) when compared to the lower dilution rate of 0.0083 h^{-1} (RT of 5 d) as demonstrated by Figure 6.8 and Figure 6.12. This suggested that *Anaerotignum aminivorans* and *Bacillus pseudofirmus* did not only have high growth rates but also competed effectively for the present lactate at this dilution rate. The observations made in this thesis are supported by Oyekola *et al.* (2010) who demonstrated that although sulphate conversion was relatively low at feed sulphate concentrations of 2.5 and 5.0 g l^{-1} (8.0 and 6.0%, respectively) at the lower dilution rate of 0.042 h^{-1} (RT of 1 d), lactate conversion remained high at 100 and 64.2% at feed sulphate concentrations of 2.5 and 5.0 g l^{-1}, respectively, suggesting the presence of lactate fermenters which competed effectively for lactate with SRB. The lactate fermenters were characterised by a high μ_{max} of 0.3 h^{-1} while lactate oxidisers had a μ_{max} of 0.20 h^{-1} (Oyekola *et al*., 2012). The competition between lactate oxidisers and lactate fermenters is dependent on both lactate and sulphide concentrations. To date, the growth rates of the strict anaerobe *Anaerotignum aminivorans* on lactate has not been studied. Similarly, no specific growth rates for fermentation of lactate for *Anaerotignum propionicum*, formerly known as *Clostridium propionicum* (Cardon and Barker, 1946; Ueki *et al*., 2017; Oren *et al*., 2018) and *Petrimonas sulfuriphila* have been reported in the current literature.

The lactate fermenting and elemental sulphur reducing bacteria *Petrimonas sulfuriphila* (Grabowski *et al*., 2005) was observed in the reactor receiving feed sulphate of 1.0 g l^{-1} (Figure 6.8). This was not surprising as Oyekola (2008) observed sulphur biofilm on the walls of this reactor. Grabowski *et al*. (2005) demonstrated that elemental sulphur can stimulate growth of *Petrimonas sulfuriphila* thus supporting observations made in this thesis. The presence of elemental sulphur is the possible reason for the observations of *Desulfuromonas acetexigens* (Finster *et al.*, 1994), *Dethiosulfovibrio acidaminovorans* (Surkov *et al*., 2001), *Defluviitoga tunisiensis* (Hania *et al*., 2012) and *Petrimonas sulfuriphila* (Grabowski *et al*., 2005) and the strict anaerobe *Mesotoga prima* (Nesbø *et al*., 2014). *Desulfuromonas acetexigens* like *Petrimonas sulfuriphila* was observed at feed sulphate of 1.0 g l^{-1} but not at 5.0 and 10.0 g l^{-1}, suggesting that these organisms may have been inhibited by higher sulphate loadings.

The observations made here further demonstrated that increasing VSLR changed SRB community structure and decreased the proportion of the total SRB within the mixed microbial community within each reactor, which resulted in lower sulphate reduction rates (Oyekola *et al*., 2009; 2010; 2012). The results in Figures 6.8, 6.11 and 6.12 indicated that the reactor receiving feed sulphate concentration of 1.0 g l^{-1} and the inoculum used to initiate the reactors in this thesis, had a diverse SRB community with total of eight SRB species. Of these SRB species, six (*Desulfomicrobium aestuarii*, *Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans*, *Desulfovibrio sulfodismutans*, *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus*) were lactate oxidisers and thus allowing for reported sulphate conversion of 86.3% and VSRR of 0.0072 g l^{-1} h^{-1} (Oyekola *et al*., 2010; 2012). In the reactor with feed sulphate concentration of 5.0 g l^{-1}, a total of seven SRB species were observed and four of these SRB were lactate oxidisers. These were *Desulfovibrio sulfodismutans*, *Desulfocurvus vexinensis*, *Desulfobulbus oligotrophicus* and *Desulfovibrio mexicanus* and were possibly responsible for the sulphate conversion of 58.2% and VSRR of 0.024 g l^{-1} h^{-1} at the dilution rate of 0.0083 h^{-1}. However, only *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus* remained at the lower dilution rate of 0.042 h^{-1}which resulted in the sulphate conversion of 6.0% and VSRR of 0.012 g l^{-1} h^{-1}. Increasing the sulphate concentration to 10.0 g l^{-1}resulted in a total of four SRB species with only three SRB species (*Desulfocurvus vexinensis*, *Desulfobulbus oligotrophicus* and *Desulfovibrio mexicanus*) being lactate oxidisers. The corresponding sulphate conversions and VSRR were 38.8% and 0.032 g l^{-1} h^{-1}, respectively. Similar to feed sulphate concentration of 5.0 g l^{-1}, only *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus* remained at the lower dilution rate of 0.042 h^{-1}which resulted in the sulphate conversion of 21.7% and VSRR of 0.090 g l^{-1} h^{-1}.

Increasing VSLR changed SRB community structure and decreased the proportion of the total SRB within the mixed microbial community within each reactor. These SRB could utilise either lactate or the products of its incomplete oxidation. The results in Figure 6.8 and Figure 6.12 indicate that the reactor receiving feed sulphate concentration of 1.0 g l^{-1}, also the inoculum used to initiate the reactors in this thesis, had a more diverse SRB community that the other two reactors receiving lactate as an electron donor. In this reactor, the lactate oxidising SRB OTUs present were *Desulfomicrobium aestuarii* (0.04%), *Desulfovibrio vulgaris* (0.6%), *Desulfovibrio desulfuricans* (0.4%), *Desulfovibrio sulfodismutans* (0.01%), *Desulfocurvus vexinensis* (1.1%) and *Desulfobulbus oligotrophicus* (1.0%). Both *Desulfobulbus oligotrophicus* OTU and *Desulfocurvus vexinensis* OTU were present at feed sulphate concentrations of 2.5 and 5.0 g l^{-1}, with *Desulfocurvus vexinensis* OTU being the most abundant OTU at 24.8 and 30.6% respectively, at the lowest dilution rate of 0.0083 h^{-1}. These observations suggested that the more diverse the SRB community, the higher sulphate reducing potential (determined as sulphate conversion and VSRR) it has. In addition, the fact that *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus* had higher growth rates and were not washed out of the reactors at the higher dilution rate of 0.042 h^{-1} allowed to speculate that these two SRB played a crucial

role in the occurrence of the observed sulphate reduction in these reactors. Lastly, the survival of these two SRB species at high VSLRs of up to 0.42 g l^{-1} h^{-1} (corresponding to dilution rate of 0.042 h^{-1} at feed sulphate of 10.0 g l^{-1}) suggests that in BSR treatment processes with VSLRs of up to 0.42 g l^{-1} h^{-1} where lactate is provided as an electron donor, a combination of these two SRB species could be effective and thus resulting in high volumetric sulphate reduction rates (VSRRs) of up to 0.090 g l^{-1} h^{-1} reported by Oyekola *et al*., 2010. Oyekola *et al*. (2010) showed that propionate and acetate were the main products of lactate oxidation and fermentation in this reactor which suggested that *Desulfobulbus oligotrophicus* observed was not only oxidising lactate but propionate as well while acetate at feed sulphate concentration of 1.0 g l^{-1} was oxidised by *Desulfuromonas acetexigens* in the presence of elemental sulphur.

6.3.2 SRB community structure with different carbon sources and electron donors and its role in the success of BSR

SRB structure and dynamics in the CSTRs were influenced by both VSLRs, mediated through residence time and sulphate concentration, and carbon source provided. Two things remain clear. Firstly, the more diverse the SRB communities, the higher the sulphate reduction potential observed as sulphate conversion and VSRR. Secondly, the ability for the SRB species to tolerate high VSLRs was dependent on the carbon source provided. A total of 15 SRB species were identified within the CSTRs in this thesis. All 15 SRB species were observed in the reactors receiving the complex carbon source (anaerobic digestate) as an electron donor for BSR. However, only ten of these SRB species were observed in the reactors receiving the simple carbon source (lactate) (Table 6.1), supporting the hypothesis that the complex carbon source supported a more diverse group of SRBs compared to a single VFA source. Table 6.1 also indicates that the lactate oxidisers were the most diverse SRB species with a total of nine SRB species observed. The second most diverse SRB group was the acetate oxidisers with three SRB species *Desulfobacca acetoxidans*, *Desulfarculus baarsii* and *Desulfococcus biacutus*. However, the latter two SRB species are known to grow very slow on acetate (Platen *et al*., 1990; Sun *et al*., 2010) and were most likely did not contribute much to the sulphate reduction process. The butyrate oxidiser *Desulfarculus baarsii*, was observed only in the reactors receiving feed anaerobic digestate and not in the reactors supplemented with lactate.

Table 6.1. Comparison of SRB species observed with different anaerobic digestate and lactate and their effect on the sulphate reduction process

SRB	VFA oxidised				Carbon source in CSTR								
					Anaerobic digestate				Lactate				
					2.5 g l^{-1}		5.0 g l^{-1}		1.0 g l^{-1}	5.0 g l^{-1}		10.0 g l^{-1}	
	Ac	Pr	Bu	La	5 d RT	1 d RT	5 d RT	1 d RT	5 d RT	5 d RT	1 d RT	5 d RT	1 d RT
Desulfomicrobium hypogeium	-	-	-	-	Y	Y	Y	Y	Y	Y	Y	Y	Y
Desulfomicrobium aestuarii	-	-	-	+++	Y	Y	Y	Y	Y	-	-	-	
Desulfovibrio aminophilus	-	-	-	-	Y	Y	Y	Y	Y	Y	-	-	
Desulfovibrio vulgaris	-	-	-	+++	Y	-	Y	-	Y	-	-	-	
Desulfovibrio desulfuricans	-	-	-	+++	Y	Y	Y	Y	Y	-	-	-	
Desulfovibrio intestinalis	-	-	-	+++	Y	-	Y	Y	-	Y	Y	-	
Desulfovibrio oxamicus	-	-	-	+++	Y	-	Y	Y	-	-	-	-	
Desulfovibrio sulfodismutans	-	-	-	+++	Y	Y	Y	Y	Y	Y	-	-	
Desulfovibrio mexicanus	-	-	-	+++	Y	-	-	-	-	Y	Y	Y	
Desulfobulbus oligotrophicus	-	+++		+++	Y	Y	Y	Y	Y	Y	Y	Y	Y
Desulfocurvus vexinensis	-	-	-	+++	Y	-	Y	Y	Y	Y	Y	Y	Y
Desulfococcus biacutus	+	-	-	-	Y	Y	Y	Y	-	-	-	-	-
Desulfarculus baarsii	+	+	+++	-	Y	-	Y	Y	-	-	-	-	-
Desulfomonile tiedjei	-	-	-	-	Y	Y	Y	Y	-	-	-	-	-
Desulfobacca acetoxidans	+++	-	-	-	Y	-	Y	-	-	-	-	-	-
Total SRB number					15	8	14	12	8	7	5	4	3
Number of active VFA oxidisers					3	1	3	2	6	5	5	3	2
Sulphate conversion (%)					90.1	49.4	95.5	22.6	86.3	58.2	6.0	38.8	21.7
VSRR g l^{-1} h^{-1}					0.019	0.051	0.040	0.047	0.0072	0.024	0.012	0.032	0.090

KEY: Y= Yes, + = Very slow reaction, +++ = Fast reaction

Figures 6.8 and 6.9 indicated a high abundance of two unclassified OTUs (OTU_37 and OTU_160) which were only present in the reactors receiving anaerobic digestate and not in the reactors receiving lactate, with OTU_37 being the most abundant OTU (30.2 – 33.5%) at feed sulphate concentration of 5.0 g l^{-1}. Since these unclassified OTUs could only survive on anaerobic digestate and not on lactate, this suggested that these OTUs needed butyrate for growth. Despite being relatively diverse with five lactate oxidisers observed, the CSTR receiving lactate at feed sulphate of 5.0 g l^{-1} showed very low sulphate conversion of 6.0% and volumetric sulphate reduction rate of 0.012 g l^{-1} h^{-1}, at RT of 1 d (dilution rate of 0.042 h^{-1}). This was possibly due to the fact that there was a high abundance of lactate fermenters (*Anaerotignum propionicum*, *Anaerotignum aminivorans*) and *Bacillus pseudofirmus* which were competing effectively for lactate with lactate oxidisers. Lactate fermenters have been shown to have higher growth rates than lactate oxidisers (Oyekola *et al*., 2012). These observations are supported by the fact that in the reactors receiving anaerobic digestate where these lactate fermenters were not observed, sulphate conversion and VSRRs remained high even when the RT was decreased to 1 d (Table 6.1).

However, not all SRB observed in these CSTR were able to utilise the carbon source provided. For example, despite their inability to oxidise either acetate, propionate, butyrate or lactate, *Desulfomicrobium hypogeium* and *Desulfovibrio aminophilus* were observed with both carbon sources. *Desulfomicrobium hypogeium* was the most abundant species of all the SRB observed at the highest residence time of 5 d (dilution rate of 0.0083 h^{-1}) in the CSTRs receiving anaerobic digestate and CSTR receiving lactate at feed sulphate of 1.0 g l^{-1} (Figures 6.8, 6.9 and 6.12). In spite this, about 86.6% and 98.5% of the *Desulfomicrobium hypogeium* were washed out of the CSTRs receiving anaerobic digestate at feed sulphate of 2.5 and 5.0 g l^{-1}, respectively when the RT was decreased from 5 to 1 d (dilution rate of 0.0083 to 0.042 h^{-1}) which resulted from the abundance of the *Desulfomicrobium hypogeium* OTU from drastically decreasing from 21.5 to 2.9% at feed sulphate of 2.5 g l^{-1} (Figure 6.9) and from 11.3 to 0.2%[1] (Figure 6.12). *Desulfovibrio* species *Desulfovibrio desulfuricans* and *Desulfovibrio sulfodismutans* were more tolerant to changes in VSLR with anaerobic digestate but not with lactate. Studies by Icgen and Harrison (2006) demonstrated that when acetate served as a carbon source for BSR, exposure to different sulphide concentration caused shifts in population dynamics. However, although there is extensive literature on how sulphide affects the sulphate reduction kinetics (Okabe *et al*, 1992; Reis *et al*., 1992; O'Flaherty *et al*., 1998; Greben *et al*., 2004; Icgen and Harrison, 2006; Lopes *et al*., 2010; Oyekola *et al*., 2012; Ni *et al*., 2019; Fan *et al*., 2020) not much attention has been given to how sulphide affects different SRB species dynamics. Okabe *et al*. (1992) reported that 50% of *Desulfovibrio desulfuricans* species were inhibited by 0.5 g l^{-1} sulphide when lactate served as carbon sources and electron donors for BSR suggesting that the sulphide in the lactate reactors inhibited the growth both *Desulfovibrio desulfuricans* and *Desulfovibrio sulfodismutans*. However, sulphide sensitivity studies were not carried out in this thesis. The survival of *Desulfovibrio desulfuricans* and

Desulfovibrio sulfodismutans at higher VSLR with anaerobic digestate is supported by studies by Icgen and Harrison (2006) demonstrated that although *Desulfovibrio* species, the least dominant SRB in the reactor, were the most competitive SRB at sulphide loadings of 0.5, 0.75, 1.0 and 1.5 g l^{-1}. Table 6.1 also indicate that despite being present at lower abundance, *Desulfovibrio* species were the most common genera in these CSTRs. These observations are in agreement with repots by Das *et al*. (2013; 2015) who demonstrated that *Desulfovibrio* species were the most common SRB in a mixed microbial community receiving sweetmeat waste, which was a mixture of acetate, propionate, butyrate, lactate, ethanol and sucrose, as carbon source for BSR. Because some *Desulfovibrio* species can grow on sulphur as an alternative electron acceptor to sulphate this thus allow them to thrive in wastewater systems (Biebl and Pfennig, 1977; Barton *et al*., 2014). Okabe *et al.* (2003) suggested that *Desulfovibrio* species were crucial members of the hydrogen utilising bacteria in wastewater. Therefore, this explanation may suggest that despite being present al lower abundance, *Desulfovibrio* species may have played a crucial role in the success of the sulphate reduction process when lactate was supplied and therefore contributed to their success in the CSTRs. Hausmann *et al.* (2016) reported that low abundance *Desulfovibrio* species responded positively to sulphate in the presence of lactate.

The fact that the low abundance *Desulfovibrio* species may have played a crucial role the success of the sulphate reduction process would mean that the effectiveness VFA oxidation and concomitant sulphate reduction does not necessarily depend on the abundance of the SRB OTU, but on the presence of that OTU. This idea is further supported by the fact at the RT of 1 d, in the reactors receiving anaerobic digestion at feed sulphate concentration of 2.5 g l^{-}, *Desulfobulbus oligotrophicus* OTU (the only SRB identified as propionate oxidiser in Table 6.1) which constituted only 0.004% of the total OTUs (Figure 6.9) was able to drive propionate oxidation and concomitant sulphate reduction suggesting that its presence was more important than its abundance in the reactor. In Chapter 5, it was demonstrated that propionate conversion of 21.7% was recorded at this dilution rate (Figure 5.6a in Section 5.4.1.2). These observations are in agreement with studies by Pester *et al*. (2010) who reported that in peatlands, the sulphate reduction process was driven by the less abundant SRB species such as *Desulfosporosinus* which constituted only 0.006% the proportion of the total microbial communities. Studies by Saunders *et al.* (2015) and Hausmann *et al*. (2016) have also demonstrated that the core organisms may also be present in low numbers within the mixed microbial communities.

Minor oxygen leakages into the CSTRs during sampling were observed by Oyekola (2008) and this thesis (Section 5.4.1.1) and some species of *Desulfobulbus*, *Desulfomicrobium*, some *Desulfovibrio* species such as *Desulfovibrio desulfuricans* and some Firmicutes can tolerate some oxygen (Johnson *et al*., 1997; Eschemann *et al*., 1999; Nagpal *et al*., 2000; Sass *et al*., 2002a; Dolla e*t al*., 2006; Lobo *et al*., 2007; Sheoran *et al*., 2010; Galperin, 2013; El Houari *et al*., 2017). *Desulfovibrio desulfuricans* tolerates oxygen by aggregates formation and aerotaxis as discussed in Chapter 2 in Section 2.4.4.4.

(Eschemann *et al*., 1999; Dolla e*t al*., 2006) and has been shown to reduce oxygen by reoxidising sulphide to sulphate which can serve as a subsequent electron acceptor according to Reaction 6.1 and Reaction 6.2, and thus removing oxygen in bioreactors and creating an anoxic environment (Dannenberg *et al*., 1992; Fuseler *et al*., 1996; Sass *et al*., 2002b).

$$2HS^- + O_2 + 2H^+ \rightarrow 2S^0 + 2H_2O \qquad \text{Reaction 6.1}$$

$$4S^0 + 4H_2O \rightarrow 3HS^- + 5H^+ + SO_4^{2-} \qquad \text{Reaction 6.2}$$

The formation of polysulphides in the CSTRs were reported in Chapter 5 (Section 5.4.1.1) and in the reactors receiving lactate (Oyekola, 2008). Some *Desulfomicrobium* species such *Desulfomicrobium norvegicum* possess the tetraheme cytochrome c_3 enzyme which attacks (in the reduced form) the polysulphide chains of the colloidal S^0 leading to the collapse of the micelles (lipid molecules that arrange themselves in a spherical form in aqueous solutions as a response to the amphipathic nature of fatty acids) with the precipitation of S_8 molecules. When polysulphides are reduced, the sulphide produced leads to the opening up of the S_8 ring by a nucleophilic attack, resulting in the production of new molecules of polysulphides, which are in turn quickly reduced to sulphide by *Desulfomicrobium* species tetraheme cytochrome c_3 (Fauque *et al*., 1979; Cammack, *et al*., 1994; Barton *et al*., 2014). A study by Pereira *et al*. (1997) reported that the triheme cytochrome c_7 of *Desulfuromonas acetoxidans* and the [Fe] hydrogenase of *Desulfovibrio vulgaris* H, which were identified in the CSRTs in this thesis, played a role in the reduction of polysulphides. The activity of triheme cytochrome c_7 to completely reduce polysulphides was twice as high of the purified *Desulfomicrobium norvegicum* (Fauque, 1994, Barton *et al*., 2014). This suggests that *Desulfomicrobium*, *Desulfovibrio* and *Desulfuromonas* may have played a role in reduction of polysulphides in these reactors. Johnson *et al*. (1997) reported that *Desulfovibrio vulgaris* H can grow at oxygen concentrations of 0.02 to 0.04% (0.24 to 0.48 μM) and proposed that in the zones of transition from oxic to anoxic environment, this microorganism protects anoxic environments from intrusion of oxygen.

The sulphur biofilms observed in these reactors (with both carbon sources) may have been the possible reason for the stimulated growth of *Desulfuromonas acetexigens* (Finster *et al.*, 1994), *Dethiosulfovibrio acidaminovorans* (Surkov *et al*., 2001), *Defluviitoga tunisiensis* (Hania *et al*., 2012) and *Petrimonas sulfuriphila* (Grabowski *et al*., 2005) and the strict anaerobe *Mesotoga prima* (Nesbø *et al*., 2014). The thermophilic (25 to 55 °C) anaerobic *Lutaonella thermophila* (Arun *et al*., 2009), facultative anaerobe *Labilibacter marinus* (Lu *et al.*, 2017) and *Proteiniphilum acetatigenes* (Chen and Dong, 2005) all belonging to the phylum Bacteroidetes were found to be some of the most abundant species in the reactors (Figure 6.8) which was in agreement with studies which reported that Bacteroidetes is one of the most dominant phylum in sulphate reducing bioreactors and wastewater

treatment environments (Chen and Dong, 2005; Probst *et al*., 2013). *Desulfomonile tiedjei* which is known to reduce 3-chlorobenzoate to benzoate was observed in the reactors receiving anaerobic digestate as a carbon source for BSR but not in the reactors receiving lactate. The function of *Desulfomonile tiedjei* and other species in these reactors which cannot oxidise nor ferment the available VFAs remains unclear.

6.4 CONCLUSION

This chapter demonstrated that FISH, qPCR and 16S rRNA metagenomics can be used complementary to each other to report the diversity of microbial communities in responses to changes in VSLRs, mediated through dilution rate and feed sulphate concentration, in the reactors receiving a complex carbon source and electron donor. In this thesis, FISH confirmed the presence of different SRB groups within the reactors receiving anaerobic digestate as a carbon source for BSR. Performing FISH with simultaneous multicolour fluorophores such as Fam (maximum excitation wavelength and emission at 520 and 525 nm) and Cy3 (maximum excitation wavelength and emission at 550 and 570 nm) for EUB338-Fam (green colour) and DELTA495a-Cy3 (red colour) could be the preferred way to assess the change in the SRB community at different residence times. However, the DAPI and EUB338-Fam show similar staining and hybridisation patterns for these samples suggesting that the DAPI results are a reliable indication of total bacteria. FISH studies indicated that the predominance of *Desulfomicrobium*, *Desulfovibrio* and *Desulfobulbus* species decreased when the RT was decreased below 3 d (0.014 h^{-1}). qPCR confirmed the presence of different SRB groups (*Desulfomicrobium* and *Desulfovibrio*) and "non-SRB" groups across carbon sources and electron donors. Metagenomic data elucidated present SRB and non-SRB groups. Both metagenomic data and 16S rRNA metagenomics confirmed that *Desulfomicrobium* species were the most abundant SRB at the lowest dilution rate tested of 0.0083 h^{-1} (RT of 5 d). Metagenomic data indicated that the bioreactor fed with the complex carbon source (anaerobic digestate) had a more diverse SRB population than the bioreactor fed with the simple carbon source, with a total of 14 SRB species observed with anaerobic digestate and only 10 SRB species observed with lactate.

FISH, qPCR and 16S rRNA metagenomics indicated that as the VSLRs were changed across carbon sources, the community structure and microbial community dynamics changed. However, these changes were both carbon source dependent and VSLR dependent. When lactate was provided as an electron donor for BSR, the lactate oxidisers *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus* were able to survive high VSLRs of up to 0.42 g l^{-1} h^{-1} which suggests that in BSR treatment processes with VSLRs of up to 0.42 g l^{-1} h^{-1} where lactate is provided as an electron donor, one or two of these SRB species could be effective to treat sulphate contaminated wastewaters with VSLRs of up to 0.42 g l^{-1} h^{-1}. The acetate specialists, *Desulfobacca acetoxidans* and *Desulfarculus*

baarsii, which grow faster on butyrate than on acetate or propionate, and the propionate oxidiser *Desulfobulbus oligotrophicus*, were able to survive high VSLRs of up to 0.208 g l^{-1} h^{-1} suggesting that a combination of these three SRB species could be used in BSR treatment processes with VSLRs of up to 0.208 g l^{-1} h^{-1} where anaerobic digestate is provided as an electron donor. In addition, unlike in CSTRs receiving lactate as a carbon source, no lactate fermenters were observed with anaerobic digestate. The ability for anaerobic digestate to support a diverse SRB communities even at higher VSLRs is therefore attributed to the to the robustness of the reactors observed in **Chapter 5**.

CHAPTER 7
GENERAL CONCLUSIONS AND RECCOMMENDATIONS

7.1 INTRODUCTION

Mining and mineral beneficiation has been a key contributor towards world economies and industry, and a provider of primary energy needs such as electricity, key to supporting our modern day lifestyle. Further metals remain a critical component of the green economy. However, the mining of sulphidic minerals and coal can aggravate AMD generation, especially if extreme care is not taken with the long-term stabilisation of sulphidic mine wastes. AMD has detrimental effects on human, plant and animal life due to low acidity, high sulphate and metal loading. One approach for its treatment, especially the treatment of diffuse AMD generated remotely and needing ongoing treatment over extended time periods, is to use biological sulphate reduction.

In order to treat AMD and sulphate laden wastewaters by biological sulphate reduction, the availability, efficiency and cost-effectiveness of carbon sources and electron donors for sulphate reducing bacteria (SRB) and the kinetics of biological sulphate reduction (BSR) processes have been a subject of intensive research. With respect to the former, it is well recognised that the cost of electron donor itself and transport to the site as well as its efficiency of use is critical to the technoeconomic feasibility of BSR. A cyanobacterial species from the *Arthrospira* genus, commonly referred to as Spirulina, is readily cultivated in remote locations and a good substrate for anaerobic digestion (AD) due to its high content of chemical oxygen demand (COD). Subsequent to biogas formation, the effluent from AD contains a high residual COD content, primarily as volatile fatty acids (VFAs). The VFAs from AD (referred to in this thesis as anaerobic digestate) were found to be primarily acetate, propionate and butyrate. Owing to high VFA content of anaerobic digestate and the availability of *Arthrospira platensis* not limited to industrial activity of the region, the current study investigated the use of anaerobic digestate as a cost effective carbon source and electron donor for biological sulphate treatment in sulphate laden waters. Further, it focussed on developing tools for the rapid and routine analysis of the microbial community dynamics in BSR systems and their application across reactors operated on both anaerobic digestate and lactate across a range of dilution rates and feed sulphate concentrations to develop understanding of the interaction between the microbial consortium and the sulphate reduction efficiency. Such understanding is expected to be valuable in process optimisation.

The effect of volumetric sulphate loading rate (VSLR), controlled by dilution rate and feed sulphate concentration on the kinetics of BSR, was evaluated. The microbial communities mediating the BSR process were also evaluated. Three chemostats (one litre) operated with anaerobic digestate as a carbon source and electron donor ((35°C, pH 8 ± 0.2 and COD:SO_4^{2-} of 0.7 (g g^{-1})) at varying feed sulphate

concentrations and dilution rates was carried out and the BSR kinetics were monitored. Optimisation of molecular methods for the characterisation of microbial communities was carried out. Fluorescence *in situ* hybridisation (FISH) was used as a visual confirmation of the different microbial communities present within the inoculum and the reactors receiving anaerobic digestate as a carbon source and electron donor. Quantitative real-time PCR (qPCR) and 16S rRNA metagenomics were used to assess the microbial communities responsible for the observed kinetics within the reactors receiving anaerobic digestate (complex carbon source) and to compare the SRB communities within these reactors with the microbial communities within the reactors supplemented with lactate (simple carbon source). This chapter presents major findings in the study.

7.1.1 Optimisation of molecular toolkit for the characterisation of microbial communities in sulphidogenic CSTRs

Anaerobic organisms, particularly SRB, are difficult to isolate using traditional cultivation techniques. Molecular techniques such as amplified ribosomal DNA restriction analysis (ARDRA), fluorescent *in situ* hybridisation (FISH) and quantitative real-time PCR (qPCR) and 16S rRNA metagenomics which are based on the assessment the abundance of the 16S rRNA genes associated with specific species, offer an opportunity for the monitoring and analysis of the structure and dynamics of microbial communities. In this thesis, the utilisation of the clone library to characterise the microbial community comprising the inoculum used to initiate reactors receiving anaerobic digestate as a complex carbon source revealed a more diverse community than previously reported by Oyekola (2008). A subset of 96 recombinant bacterial clones were subjected to ARDRA. Of these, 49 clones were identified to have unique ribotypes on the basis of their *Hae*III and *Alu*I restriction profiles. The phylogenetic tree demonstrated that the 16S rRNA sequences, captured from the inoculum community, showed high similarity to well-known SRB species belonging to the *Desulfomicrobium, Desulfovibrio, Desulfuromonas, Desulfobulbus* and *Desulfocurvus* genera. Other "non-traditional SRB" species belonging to the *Firmicutes* and *Citrobacter* genus containing the *dissimilatory sulphite reductase* gene (*dsrAB*) within their genomes were also detected. A phylogenetic analysis revealed that the *Desulfomicrobium* group of SRB was the most diverse of the SRB groups represented in the bacterial community within the inoculum reactor. The optimisation of the FISH technique using general fluorescein labelled probes EUB338-Fam and DELTA495a-Fam allowed for the improvement of the technique for the qualitative characterisation of the eubacterial and *Deltaproteobacterial* communities respectively, taken from reactors receiving anaerobic digestate. Similarly, optimisation of FISH using the genus specific probes, DSM-Fam, DSV287-Fam and SRB660-Fam, allowed for the qualitative characterisation of *Desulfomicrobium, Desulfovibrio* and *Desulfobulbus* genera respectively, in these reactors.

A systematic approach was undertaken for the successful design of SRB genus primers for qPCR. *In silico* analysis of the 16S rRNA gene sequences captured from the clone library validated the specificity of the universal total bacterial primers (TotalF and TotalR), *Desulfovibrio* species primers (forward primer DSV-II-312f[b] and reverse primer DSV681R[b]) and the novel *Desulfomicrobium* species primers (forward primer DSM442F and reverse primer DSM632R) applied in this thesis. The total sulphate reducing potential of the mixed microbial community was captured by performing quantitative real-time PCR (qPCR) with *dissimilatory sulphite reductase* (*dsr*) specific primers (DSRp2060F and DSR4R). The results demonstrated that FISH and qPCR can be used in a manner complementary to each other to report the diversity of microbial communities in sulphate reducing CSTRs as a result of changing sulphate loading and residence time in the SRB reactors receiving complex or simple carbon source. These results are summarised in Section 7.3.

7.1.2 Evaluation of anaerobic digestate as an effective carbon source for BSR

Three chemostat reactors receiving anaerobic digestate as a carbon source and electron donor for BSR were supplemented with feed sulphate concentrations of 1.0, 2.5 or 5.0 g l^{-1}. The three reactors were operated across a range of dilution rates of 0.0083 to 0.083 h^{-1} (Residence times (RTs) of 5 to 0.5 d). Since VSLR is the product of dilution rate and feed sulphate concentration, volumetric sulphate reduction rates (VSRRs) were higher at equivalent dilution rates for higher feed sulphate concentration. A VSRR of up to 0.070 g l^{-1} h^{-1} and sulphate conversions as high as 95% were observed indicating that anaerobic digestate can serve as a carbon source and electron donor for BSR. Further simultaneous metabolism of the acetate, propionate and butyrate present in the digestate was observed. The kinetics observed when anaerobic digestate served as a carbon source and electron donor for BSR were well matched with the kinetics observed with lactate (Oyekola *et al*., 2010; 2012) ethanol (Hansford *et al*, 2007) and acetate (Moosa *et al*., 2002). Anaerobic digestate (a mixture of acetate, propionate and butyrate) was more robust and the reactors remained operational with active sulphate reduction at higher VSLRs than previously observed when acetate served as a single carbon source and electron donor for BSR. For the reactors receiving feed containing a sulphate concentration of 1.0 g l^{-1}, similar performances were observed between anaerobic digestate, lactate and ethanol. However, ethanol did not support high VSRRs owing to sulphide inhibition being experienced at lower sulphide concentrations (Erasmus *et al*., 2007). The VSRR demonstrated for acetate were lower at low feed sulphate concentration (Moosa *et al*., 2002). Higher VSRRs were observed with anaerobic digestate at feed containing sulphate concentrations of 2.5 and 5.0 g l^{-1}. The VSRR profiles exhibited at these concentrations were similar for all the four carbon sources and electron donors, reaching a maximum and declining as the VSLRs were increased beyond the metabolic capacity of the biomass present and partial biomass washout occurred. The bacterial dry mass concentrations obtained with anaerobic

digestate were higher than the ones obtained with lactate and ethanol, possibly due the presence of nitrogen and phosphate in the anaerobic digestate feed.

Based on the stoichiometry of sulphate reduction, simultaneous utilisation of acetate, propionate and butyrate was observed in the reactors receiving anaerobic digestate as a carbon source and electron donor for BSR. This was contrary to observations made with lactate (Oyekola *et al*., 2012), where lactate, and not its propionate nor acetate products, was preferentially used for oxidation reactions. Across the lower dilution rates of 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d), the experimental stoichiometry ratios of acetate, propionate and butyrate reactions, concurred largely with the theoretical ratios of acetate oxidation, incomplete propionate oxidation and incomplete butyrate oxidation and concomitant sulphate reduction in the reactor receiving sulphate concentration of 1.0 g l^{-1}. In the reactors supplemented with 2.5 or 5.0 g l^{-1} sulphate, these experimental stoichiometry ratios concurred largely with the theoretical ratios of acetate oxidation, incomplete propionate oxidation and incomplete butyrate oxidation and concomitant sulphate reduction across the lower dilution rates of 0.0083 to 0.028 h^{-1} (RTs of 5 to 1.5 d). All three reactors showed acetogenesis of the present VFAs at the highest dilution rate of 0.083 h^{-1} (RT of 5 to 0.5 d).

The growth parameters μ_{max} and K_S of the mixed SRB culture were demonstrated for each feed sulphate concentration using the steady-state data and the mathematical kinetic model described by Moosa *et al*. (2002), assuming sulphate as a limiting substrate. On selecting anaerobic digestate as a carbon source and electron donor for SRB in treating sulphate laden wastewaters, it is recommended that dilution rates used should be held below the maximum specific growth rate determined for the system to minimise biomass washout and maximise VSRR in the wastewaters rich in sulphate. Alternatively, biomass retention methods should be used, as reported by Hessler (2020) and Marais (2020).

7.1.3 The link between BSR reactor performance, community dynamics and diversity as a function of the carbon source and electron donor applied

FISH, qPCR and 16S rRNA metagenomics were successfully used in a manner complementary to each other to report the diversity of microbial communities as a result of changing sulphate concentration (2.5 and 5.0 g l^{-1}) and residence time (5 to 1 d) in the SRB reactors receiving complex (anaerobic digestate) or simple (lactate) carbon source. FISH confirmed the presence of *Desulfomicrobium, Desulfovibrio* and *Desulfobulbus* species within the CSTRs receiving anaerobic digestate. The results suggested that the proportion of the total SRB species within the total bacterial population and these three SRB genera (*Desulfomicrobium, Desulfovibrio* and *Desulfobulbus*) declined as the residence time was reduced (dilution rate increased) at sulphate concentrations 2.5 and 5.0 g l^{-1} in these reactors. qPCR confirmed the presence of the SRB groups *Desulfomicrobium* and *Desulfovibrio*, “other SRB” and

"non-SRB" groups within the reactors receiving anaerobic digestate or lactate. This supported the observations made from FISH suggesting that some SRB species could not survive as the VSLR was increased in these reactors. According to qPCR, the *Desulfomicrobium* group made up the highest proportion of the total SRB groups represented in the bacterial community at the lowest dilution rate tested (0.0083 h^{-1}, RT 5 d); increasing VSLR resulted in the substantive decrease of their proportion within these reactors.

In the reactors receiving anaerobic digestate as a carbon source for BSR, the proportion of *Desulfomicrobium* species remained steady at 61.9 ± 1.2 % across dilution rates of 0.0083 to 0.014 h^{-1} (RTs of 5 to 3 d) at feed sulphate concentration of 2.5 g l^{-1} and 60.3 ± 0.49% at feed sulphate concentration of 5.0 g l^{-1}. A small proportion of *Desulfomicrobium* species (6.9 ± 1.8%) remained in the reactor receiving sulphate at 2.5 g l^{-1} at the dilution rate of 0.042 h^{-1} while no *Desulfomicrobium* species remained at a feed sulphate of 5.0 g l^{-1} and D of 0.042 h^{-1} (RT of 1 d). Although *Desulfovibrio* species was present in lower proportion across similar dilution rates of 0.0083 to 0.014 h^{-1} (2.3 ± 0.15% and 2.2 ± 0.13% at 2.5 and 5.0 g l^{-1} sulphate respectively), some *Desulfovibrio* species remained in both reactors at the dilution rate of 0.042 h^{-1}. These observations suggested that the *Desulfovibrio* species in these reactors had higher growth rates (> 0.042 h^{-}) and could survive at higher sulphate loadings.
In the reactor receiving lactate as an electron donor for BSR supplemented with sulphate concentration of 1.0 g l^{-1}, the proportion of *Desulfomicrobium* species declined from 46.5 ± 4.5% at the dilution rate of 0.0083 h^{-1} to 0.3 ± 0.15% at the dilution rate of 0.083 h^{-1} (RT of 0.5 d). Similarly, the proportion of *Desulfomicrobium* species declined from 21.2 ± 3.8% at the dilution rate of 0.0083 h^{-1} to 0.2 ± 0.06% at the dilution rate of 0.042 h^{-1} (RT of 1 d) in the reactor receiving lactate as a carbon source and electron donor supplemented with sulphate concentration of 10.0 g l^{-1}. The proportion of *Desulfomicrobium* species was lower in the reactors receiving feed sulphate concentrations of 2.5 and 5.0 g l^{-1} (11.6 and 8.9%, respectively) at the dilution rate of 0.0083 h^{-1} (RT of 5d), and complete wash out of *Desulfomicrobium* species was observed at the dilution rate of 0.021 h^{-1} (RT of 2d) at these sulphate concentrations. Similar to observations made in the reactors receiving anaerobic digestate, despite being present in less proportion when compared to *Desulfomicrobium* genera, qPCR demonstrated that the *Desulfovibrio* groups were more resistant to changes in VSLRs in the reactors receiving lactate at feed sulphate concentration of 1.0 and 10 g l^{-1}. These results suggest that *Desulfovibrio* species might have played a critical role in the success of the lactate oxidation and concomitant sulphate reduction process; however there was out-competition by the fermenters, leading to *Desulfovibrio* not thriving.

16S rRNA metagenomics revealed that the reactors receiving the complex carbon source, anaerobic digestate, had a more diverse SRB community with a total of 14 SRB species observed at the lowest dilution rate of 0.0083 h^{-1} (RT of 5 d). These were *Desulfomicrobium* groups (*Desulfomicrobium*

hypogeium and *Desulfomicrobium aestuarii*) *Desulfovibrio* groups (*D. aminophilus, D. vulgaris, D. desulfuricans, D. intestinalis, D. oxamicus*, and *D. sulfodismutans*) *Desulfobulbus oligotrophicus, Desulfocurvus vexinensis, Desulfococcus biacutus, Desulfarculus baarsii, Desulfomonile tiedjei* and *Desulfobacca acetoxidans*. *Desulfomicrobium hypogeium* OTU was the most abundant at the lowest dilution rate of 0.0083 h^{-1} (RT of 1 d) in the reactors receiving anaerobic digestate (2.5 and 5.0 g l^{-1} feed sulphate) and in the reactor receiving lactate at feed sulphate concentration of 1.0 g l^{-1} (which was also the inoculum reactor used to initiate other reactors in this thesis). There was a high abundance of two unclassified operational taxonomic units (OTU_37 and OTU_160) which were only observed in the reactors receiving anaerobic digestate and not in the reactors receiving lactate, with OTU_37 being the most abundant OTU (30.2 – 33.5%) at feed sulphate concentration of 5.0 g l^{-1}.

Metagenomic data indicated that out of 14 SRB species, a total of five SRB species (*Desulfovibrio vulgaris, Desulfovibrio intestinalis, Desulfovibrio oxamicus, Desulfarculus baarsii* and the acetate specialist, *Desulfobacca acetoxidans*) were lost when the dilution rate was increased from 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d) in the reactor receiving anaerobic digestate at feed sulphate concentrations of 2.5 g l^{-1}. The washout of these SRB species corresponded with the decrease of sulphate conversion from 90.1 to 49.4% and bacterial dry mass from 1.488 to 0.761 g l^{-1} of dry weight, however, the VSRR remained in the range 0.040 to 0.050 g l^{-1} h^{-1}, and above some 60% of the maximum productivity measured at a feed of 5.0 g l^{-1} sulphate and equivalent to the maximum at 2.5 g l^{-1} sulphate. This confirms that the reduced sulphate conversion observed resulted from an inability of the microbial consortium to reach the increased VSLR, rather than substantial reduction in VSRR. Only two of these SRB species (*Desulfovibrio vulgaris* and *Desulfobacca acetoxidans*) were completely washed out of the reactor receiving feed sulphate of 5.0 g l^{-1} when the dilution rate was increased from 0.0083 to 0.042 h^{-1}. In this reactor, the complete washout of *Desulfovibrio vulgaris* and *Desulfobacca acetoxidans* corresponded with the decrease of sulphate conversion from 95.4 to 22.6% and bacterial dry mass from 3.161 to 0.715 g l^{-1}, but an increase in VSRR from 0.040 to 0.046 g l^{-1} h^{-1}, via a maximum of 0.068 g l^{-1} h^{-1}. This suggests an increase in the specific sulphate reduction activity with increasing dilution rate. Since the loss of both *Desulfovibrio vulgaris* and *Desulfobacca acetoxidans* corresponded with the decrease of sulphate conversion but only a small decrease in VSRR in both reactors it suggested that either these SRB species did not play an important role in the oxidation of anaerobic digestate and concomitant sulphate reduction or that they were not operating at maximum specific activity at lower dilution rates. However, only *Desulfobacca acetoxidans* can utilise the acetate component of anaerobic digestate, suggesting that the reduction in abundance of *Desulfobacca acetoxidans* in the reactors receiving anaerobic digestate must result in its increased specific activity to maintain the acetate-linked BSR rates.

Only a total of nine SRB species were observed at a dilution rate of 0.0083 h^{-1} (RT of 5 d) in the reactor receiving the simple carbon source, lactate, at feed sulphate concentration of 1.0 g l^{-1}. These SRB were shown to be *Desulfomicrobium hypogeium, Desulfomicrobium aestuarii, Desulfovibrio aminophilus, Desulfovibrio vulgaris, Desulfovibrio desulfuricans, Desulfovibrio sulfodismutans, Desulfovibrio mexicanus, Desulfobulbus oligotrophicus* and *Desulfocurvus vexinensis*. Lactate fermenters *Anaerotignum propionicum, Anaerotignum aminivorans* and *Bacillus pseudofirmus* were only observed in the reactors supplemented with lactate and not observed in the reactors receiving anaerobic digestate. An increase in sulphate concentration from 1.0 to 5.0 g l^{-1} or 10.0 g l^{-1} resulted in the inhibition of three SRB species (*Desulfomicrobium aestuarii, Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*). Therefore only six SRB species were observed at feed sulphate concentrations of 5.0 and 10.0 g l^{-1}. However, this increase in sulphate concentration from 1.0 to 5.0 g l^{-1} or 10.0 g l^{-1} resulted in the prolific abundance of *Desulfocurvus vexinensis* which suggested that this SRB species grew better at higher VSLRs.

In the reactors receiving feed sulphate concentration of 5.0 and 10.0 g l^{-1} and lactate, increasing the dilution rate from 0.0083 to 0.042 h^{-1} (RT of 5 to 1 d) resulted in the complete washout of the lactate oxidisers *Desulfovibrio sulfodismutans* and *Desulfovibrio mexicanus*, respectively. These observations correlated with the decrease of sulphate conversion from 58.2 to 6.0% and 38.8 to 21.7% at feed sulphate concentrations of 5.0 and 10.0 g l^{-1}, respectively, across the dilution rates of 0.0083 to 0.042 h^{-1}. The correlation of the decrease of sulphate conversion and the complete washout of *Desulfovibrio sulfodismutans* and *Desulfovibrio mexicanus* suggested the following: (i) both SRB could not survive higher sulphate loading, (ii) both SRB had lower growth rates on lactate than the competing lactate fermenters and (iii) *Desulfovibrio sulfodismutans* played an important role in the oxidation of lactate and concomitant sulphate reduction at feed sulphate concentration at feed sulphate of 5.0 g l^{-1} while *Desulfovibrio mexicanus* was important at feed sulphate concentration of 10.0 g l^{-1}. On the contrary, the lactate oxidisers *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus* had higher growth rates (greater than 0.042 h^{-1}) and were able to survive high sulphate loading. The lactate fermenters *Anaerotignum propionicum*, *Anaerotignum aminivorans* and *Bacillus pseudofirmus* were also more prolific at the higher dilution rate of 0.042 h^{-1} which suggested that they competed effectively for lactate at higher dilution rates.

In conclusion, these results suggest that SRB may not only have a preference for carbon source but also for sulphate loading. The acetate specialist *Desulfobacca acetoxidans* and the butyrate oxidiser *Desulfarculus baarsi* were observed only in reactors with anaerobic digestate and not in reactors supplemented with lactate, while the propionate oxidiser *Desulfobulbus oligotrophicus* was observed with both carbon sources. Therefore, it was assumed that in the reactors receiving the complex carbon source, oxidation of acetate, propionate and butyrate and their concomitant sulphate reduction were

performed by *Desulfobacca acetoxidans*, *Desulfobulbus oligotrophicus* and *Desulfarculus baarsii*, respectively. Since these three SRB were able to survive high VSLRs of up to 0.208 g l^{-1} h^{-1}, a combination of these three SRB could be effective and thus resulting in high volumetric sulphate reduction rates (VSRRs) of up to 0.070 g l^{-1} h^{-1}. In the reactors receiving lactate as a carbon, because the lactate oxidisers *Desulfocurvus vexinensis* and *Desulfobulbus oligotrophicus* have high growth rates and can survive high VSLRs of up to 0.42 g l^{-1} h^{-1}, these two SRB can be used to operate at high VSLRs of up to 0.42 g l^{-1} h^{-1} resulting in high volumetric sulphate reduction rates (VSRRs) of up to 0.090 g l^{-1} h^{-1} as demonstrated by Oyekola *et al*. (2010).

7.2 RECOMMENDATIONS

The following recommendations are made to aid in the further improvement in the application of anaerobic sulphate reduction processes in treating sulphate laden wastewaters

1. **The use of metagenomics to unpack "other SRB" and generate novel qPCR primers for the quantification of SRB**: The novel and modified genus specific qPCR primers in this thesis allowed for the quantification of total SRB groups, *Desulfomicrobium* and *Desulfovibrio* species, and the elucidation of the link between community dynamics and performance of SRB. However, species specific qPCR primers for these SRB and "other SRB" groups elucidated by 16S rRNA metagenomics could not be designed. Metagenomic sequence-based approaches can be used to elucidate the microbial diversity and identify their function. This information can then be used to generate both genus specific and species specific qPCR primers for SRB. In addition, metagenomic data across dilution rates can help identify transient organisms whose presence or absence can result in system failure (Saunders *et al*., 2016). This information can help to elevate the understanding of changes that occur in the microbial communities during anaerobic sulphate reduction treatment processes and inform bioreactor design, optimisation and operation of anaerobic sulphate reduction processes.

2. **The use of different dilution rates to operate reactors treating sulphate contaminated waters**: This thesis demonstrated that when anaerobic digestate was used as a carbon source for BSR, resultant VSRRs were affected by VSLRs. Therefore, in order to avoid acetogenesis of the VFAs present and ensure dominant sulphate reduction at feed sulphate concentration of 1.0 g l^{-1}, the reactors should be operated between 0.0083 to 0.042 h^{-1} (RTs of 5 to 1 d). At feed sulphate concentration of 2.5 or 5.0 g l^{-1}, the reactors can be operated between 0.0083 to 0.028 h^{-1} (RTs of 5 to 1.5 d) in order to ensure oxidation of the VFAs present and concomitant sulphate reduction are the dominant, processes thus avoiding the occurrence of acetogenesis.

3. **A comprehensive cost analysis comparing the cost of using anaerobic digestate as a carbon source and electron donor for BSR**: The cyanobacterial species *Arthrospira platensis* has to be grown, harvested and anaerobically digested for anaerobic digestate to be obtained. Therefore, the capital cost, operation and maintenance costs of obtaining anaerobic digestate should be taken into consideration.

4. **Comparative study between anaerobic digestate and synthetic VFAs (acetate, propionate and butyrate) as carbon sources for BSR**: In order to determine whether the higher biomass obtained was due to the presence of nitrogen contained in the biologically derived anaerobic digestate, it is recommended that a study with chemostat reactors operating on a synthetic mixture of acetate, propionate and butyrate under similar operating conditions used in this thesis is carried out

5. **Selection of certain SRB strains to construct a tailored mixed consortium**: The results suggest, and associated literature supports, that SRB do not only have a preference for VSLR but for carbon source. Therefore a tailored mixed consortium for each carbon source and sulphate loading treated may result in resilient microbial community and optimum treatment process

6. **Higher sulphate concentrations** should be examined to investigate the effect of higher VSLRs on the microbial community dynamics. Further, the potential to enhance the process at high VSLR by active biomass retention should be investigated.

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APPENDICES

1 Appendix A

Supplementary information for **Chapter 3**. This section describes media and buffer compositions.

1.1. Media and buffer composition

Phosphate buffered saline (PBS) (1 ×)

8 g of NaCl, 1.44 g of Na_2HPO4 , 0.24 g of KH_2PO4 and 0.2 g of KCl were dissolved in 800 ml. pH of solution was adjusted to 7.4. This was then made up to a final volume of 1 litre with (distilled water) dH_2O. The solution was sterilised by autoclaving.

TAE Buffer

242 g Tris-HCl, 57.1 ml of glacial acetic acid and 10 ml 0.5M EDTA were dissolved in 900 ml dH_2O. The solution was adjusted to pH8. This was then made up to a final volume of 1 litre with dH_2O. The solution was sterilised by autoclaving.

14% paraformaldehyde

33 ml of dH2O was heated to 60°C, 2 g of paraformaldehyde was added while stirring. 10 N NaOH was added drop wise until paraformaldehyde was completely dissolved. This was followed by the addition of 16.5 ml PBS buffer (3 ×). pH of solution was adjusted to 7.4. The solution was sterilised by filtering through a sterilised 0.45 μm membrane that could be autoclaved.

1.2. Solutions used for hydrogen sulphide assay

N,N-dimethyl-p-phenylene diamine dihydrochloride solution

2 g of N,N-dimethyl-p-phenylene diamine dihydrochloride was added to 267 ml concentrated hydrochloric acid (32 wt%). A volume of 233 ml of sterile dH_2O was added to give a final volume of 500 ml.

Ferric Chloride solution

Ferric chloride (8 g) was added to 267 ml concentrated hydrochloric acid (32 wt%) and made up to volume of 500 ml with sterile dH_2O.

Zinc acetate

10 g of zinc acetate was dissolved sterile dH_2O and made up to 1 litre.

1.3. Solutions for sulphate assay

Sodium Borate-Gluconate stock solution

16 g Sodium gluconate, 18g Boric acid and 25 g sodium tetraborate decahydrate were added to a beaker. A volume of 750 ml dH_2O was added followed by mixing with a magnetic stirrer bar on a magnetic stirrer. 250 ml of glycerol was added and mixed thoroughly. The solution was added to a 1 litre Schott bottle and autoclaved to prevent contamination.

Mobile phase solution (1 litre)

500 of dH_2O was added to a Schott bottle. This was followed by the addition of 20 ml sodium borate-gluconate stock solution, 20 ml 1-Butanol (HPLC grade), 120 ml Acetonitrile (HPLC grade) and 340 ml dH_2O to make a final volume of 1 litre.

1.4. Preparation of standard curves

Hydrogen sulphide standard curve was generated by diluting a 200 mg l^{-1} hydrogen sulphide standard solution to 1 m gl^{-1} made from sodium sulphide (Sigma-Aldrich). This was based on the fact that the ideal range for determining sulphide concentrations is 0-1 mg l^{-1}.

The sulphate standard curve was generated by running a set of sulphate standards at the beginning and end of each sample sequence during HPLC run

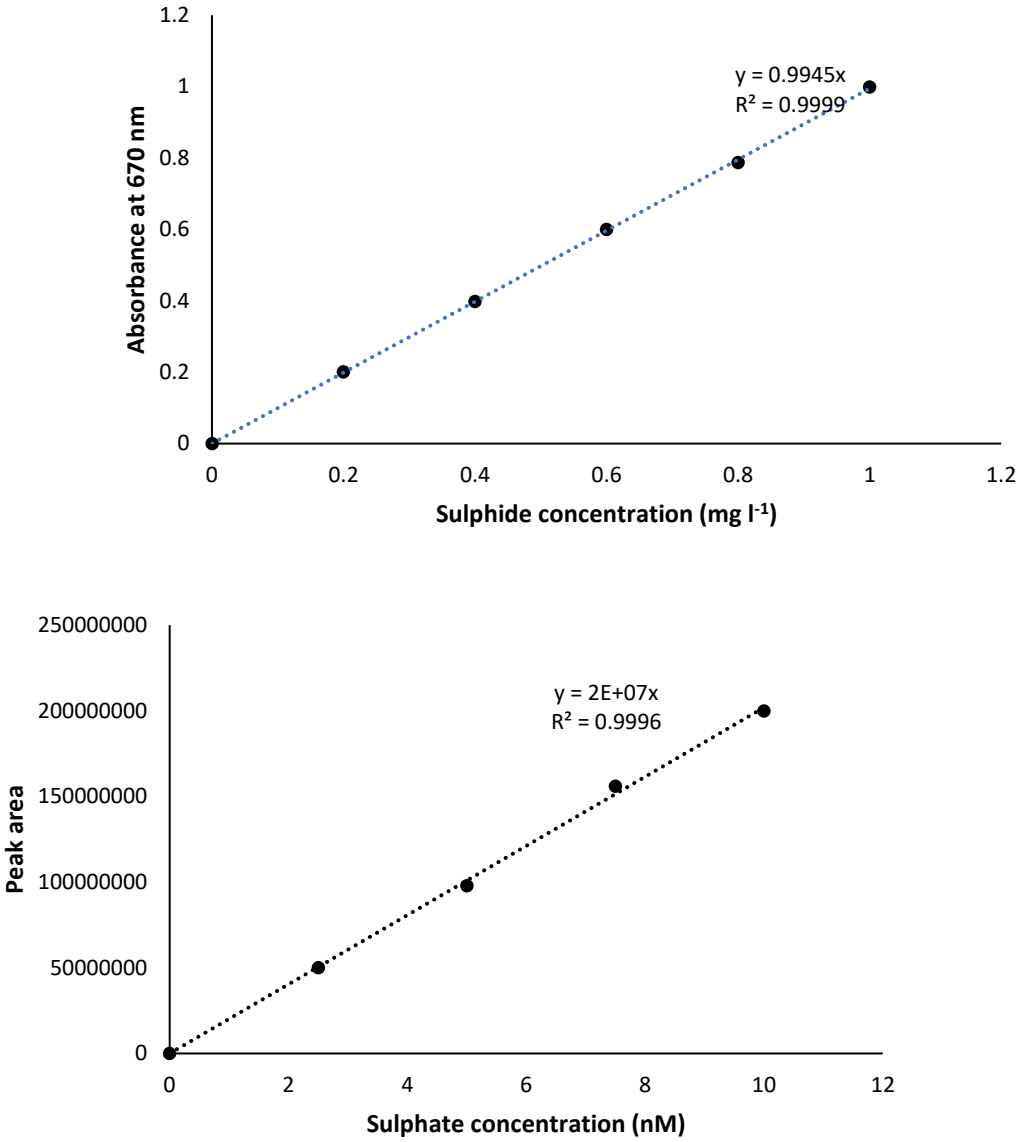


Figure A1. Standard curves of hydrogen sulphide (A) and sulphate (B)

The volatile fatty acids (VFAs) standard curves were generated by running VFA standards at the beginning and end of each sample sequence using HPLC showing peak area vs VFA concentration.

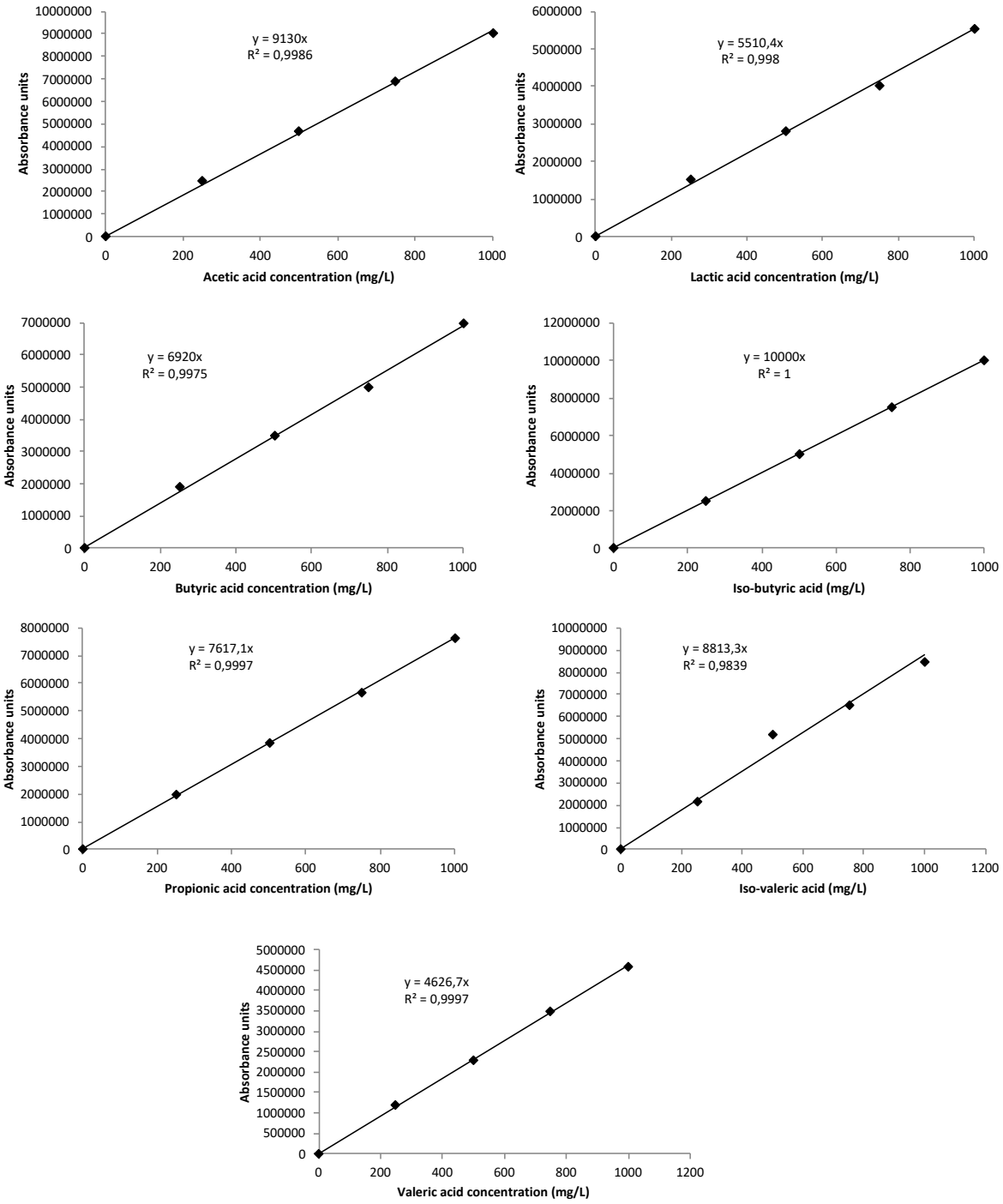


Figure A3. VFA standard curves for the concentration range 0 - 1000 mg/L

Each VFA compound elutes at a specific time provided the HPLC is operated as described in Table A.1 and Figure A.4a. Error analysis was performed with propionic acid as indicated in Figure A.4b

Table A.1: Elution times of all seven volatile fatty acid compounds

Compound	Retention time (min)
Lactic acid Acetic	13.10
Acetic acid	15.79
Propionic acid	18.67
Iso-butyric acid	20.97
Butyric acid	23.18
Iso-valeric acid	26.77
Valeric acid	34.03

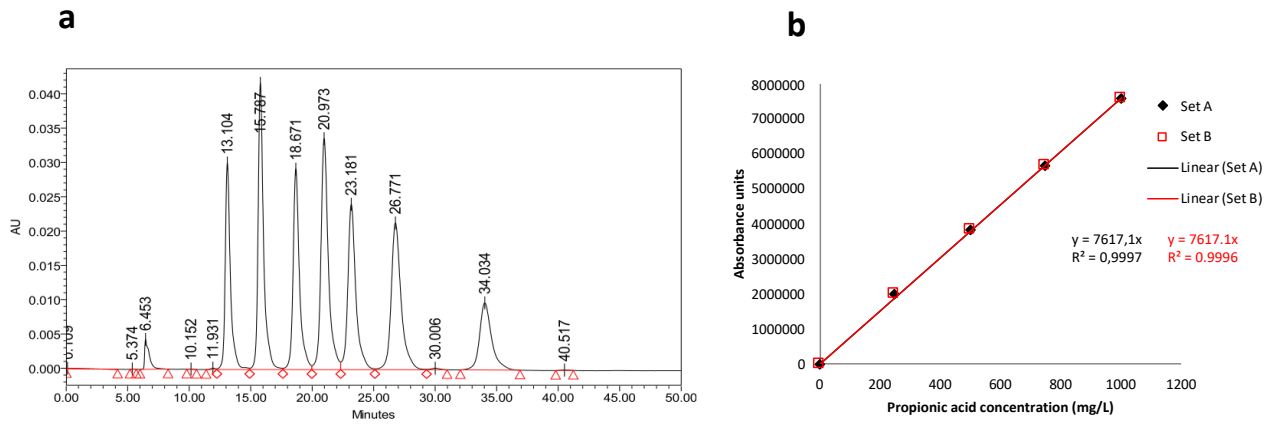
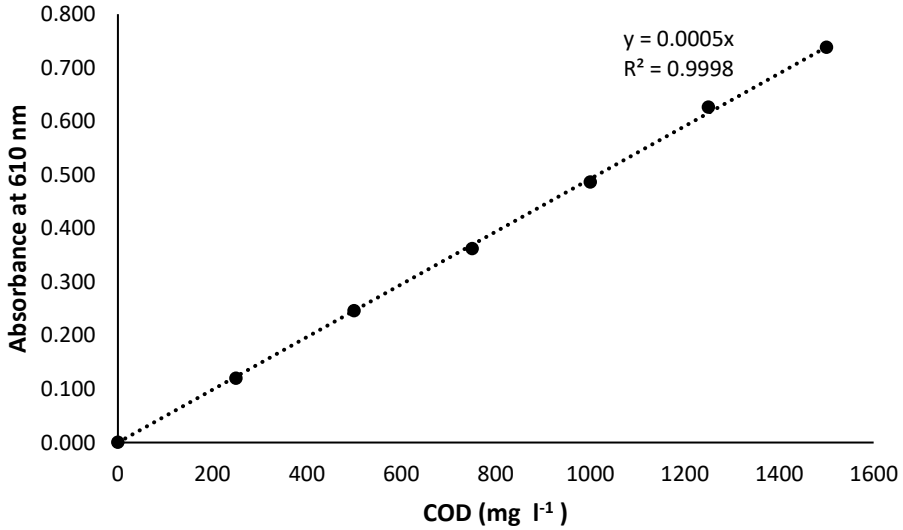


Figure A.4. High performance liquid chromatography chromatogram of volatile fatty acid compounds

A standard curve for the COD analysis was generated using potassium hydrogen phthalate. This compound is known to have a COD of 10000 mg COD per litre at a concentration of 8.5 g l^{-1}. Both high range and low range standard curves were prepared.

A



B

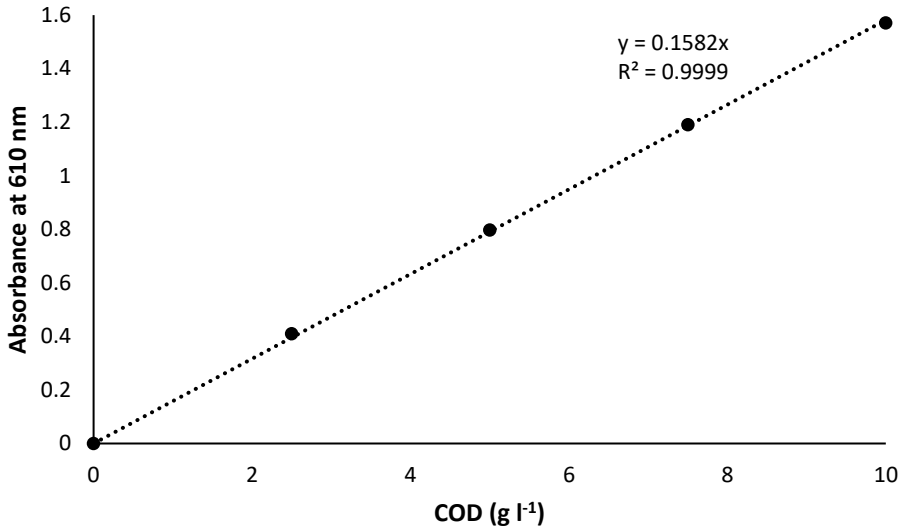


Figure A.5. Standard curve of low (A) and high range (B) COD

1.5. Calculation of alkalinity

Total alkalinity as mg l^{-1} $CaCO_3$ equivalents, $C = \frac{A \text{ X } N \text{ X } 50\,000}{\text{ml Sample}}$

Where A = ml standard acid used and N = normality of standard acid

Total alkalinity as mg l^{-1} $NaHCO_3$ equivalents, $D = C \text{ x } \frac{84}{50}$

Molar concentration of bicarbonate = Molar concentration of D – Molar concentration of sulphide estimated from analysis.

2 Appendix B

Supplementary information for **Chapter 4**. This section describes methods carried out in Chapter 4.

A **B**

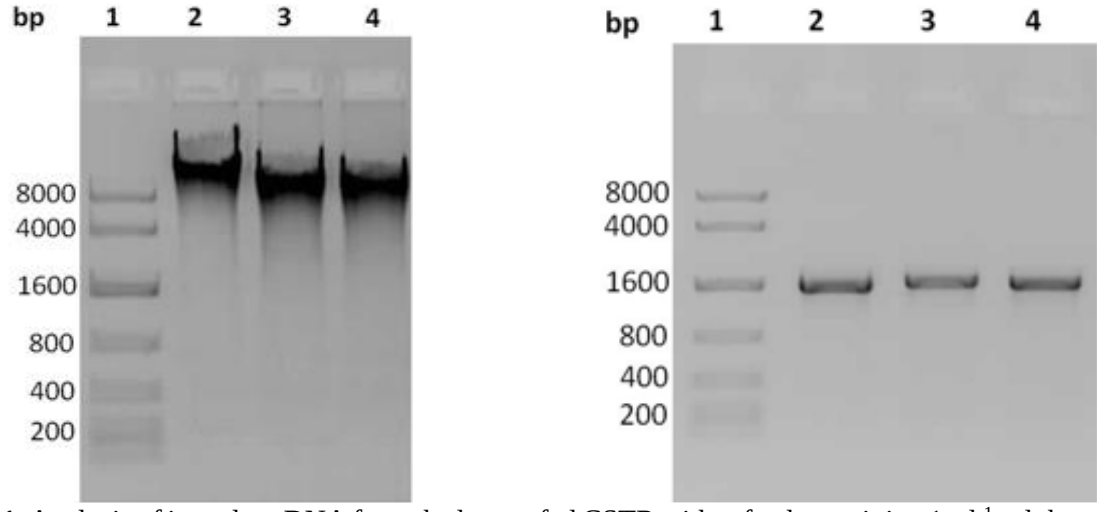


Figure B1. Analysis of inoculum DNA from the lactate fed CSTR with a feed containing 1 g l^{-1} sulphate at residence time of 5 days **A**. Genomic DNA extracted from the three samples taken on 3 different days separated on a 0.8% agarose gel in 1X TAE. Lanes 2-4: 25 ng DNA from samples 1-3 respectively. **B**. Gel electrophoresis analysis of the PCR fragments of the bacterial 16S rRNA from the lactate fed

CSTR receiving 1 g l^{-1} sulphate concentration at a residence time of 5 day. PCR amplification was carried out with the universal forward (27Fg) and reverse (1492Rg) PCR primers. Lanes 2-4: samples 1-3, respectively. On both gels, Lane 1 is 100 bp molecular weight marker (KAPA™ Express ladder, Kapa Biosystems, South Africa).

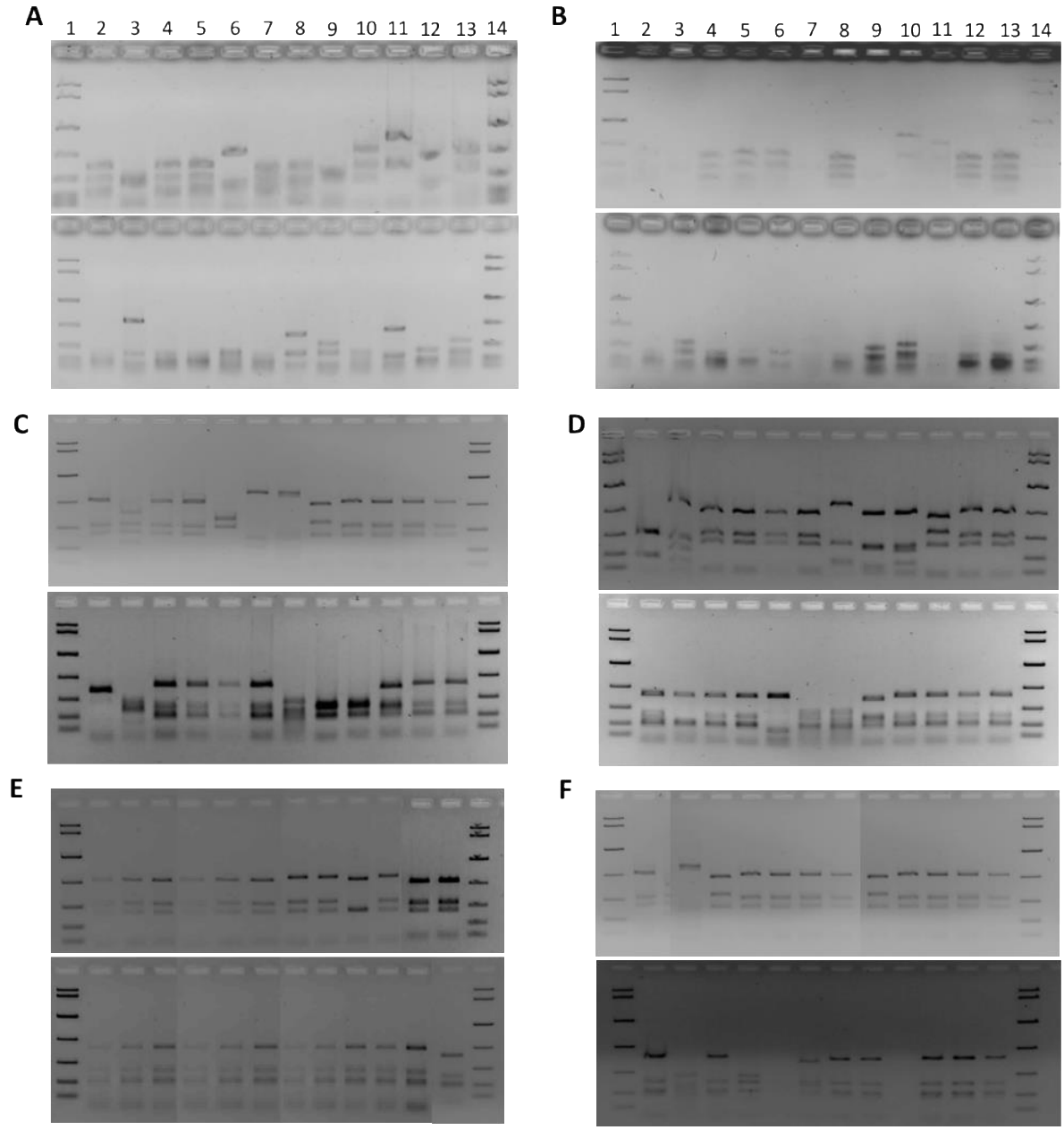


Figure B2. Restriction enzyme digestion analyses of PCR product from the inoculum reactor supplemented with lactate and 1.0 g l^{-1} sulphate at residence time of 5 days using *Hae*III (top) and *Alu*I (Bottom). Lane 1 and Lane 14: Molecular weight marker (KAPA™ Express ladder, Kapa Biosystems (all gels). Gel A: Lanes 2-13 contains LB25 to LB36; Gel B: Lanes 2-13 contains LB37 to LB48; Gel C: Lanes 2-13 contains LB49 to LB60; Gel D: Lanes 2-13 contains LB61 to LB72; Gel E: lanes 2-13 contains LB73 to LB84; and Gel F: Lanes 2-13 contains LB85 to LB96.

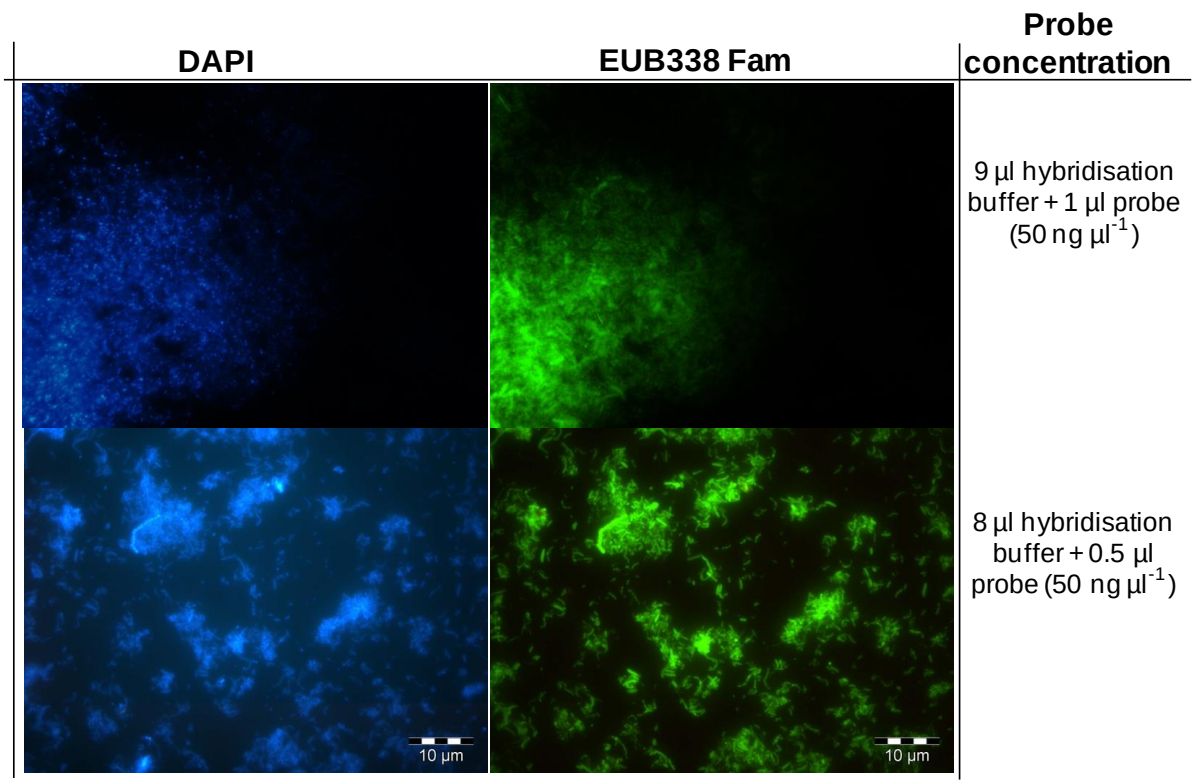


Figure B3. Epifluorescence micrographs showing optimisation of the probe concentration of samples obtained from reactors receiving anaerobic digestate feed at sulphate (S_o) concentrations of 5.0 g l^{-1}(RT = 5 d). DAPI staining on the left column is compared FISH performed with general bacteria probe EUB338-Fam at a series of formamide concentrations on the right column (same microscopic field). The hybridisation reaction was carried out at formamide concentration of 50%.

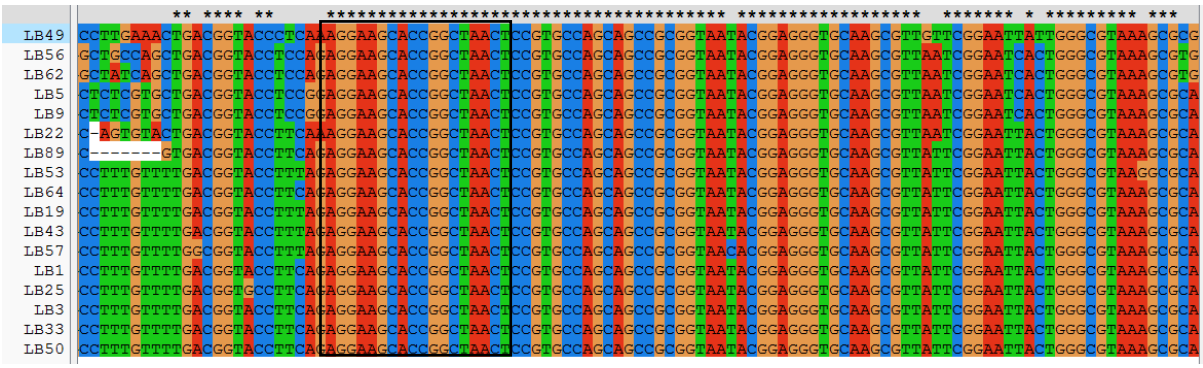


Figure B4: Deduced nucleotide sequence alignments of bacterial clones 16S rRNA sequences from inoculum reactor supplemented with lactate and 1.0 g l^{-1} sulphate with the DELTA495a probe sequence (**5'-**AGT TAG CCG GTG CTT CCT-**3')** which is shown in the black box. Alignments were performed in CLUSTALX.2. The bacterial clone 16S rRNA sequences of LB1 99% to uncultured *Desulfomicrobium* species, LB5 (99% to *Desulfovibrio aminophilus*), LB9 (99% to *Desulfovibrio aminophilus*), LB19 (99% to *Desulfomicrobium Norvegicum*), LB22 (99% *Desulfovibrio desulfuricans* and *Desulfovibrio multispirans*), LB25 (99% to *Desulfomicrobium Norvegicum*), LB33 (99% to *Desulfomicrobium Norvegicum*), LB43 (99% *Desulfomicrobium bacalatum*), LB49 (Uncultured *Desulfuromonas* species), LB50 (99% to *Desulfomicrobium Norvegicum*), LB53 (Uncultured *Desulfomicrobium* species), LB57 (99% to *Desulfomicrobium bacalatum*), LB62 (94% to *Desulfocurvus vexinensis*), LB64 (99% to *Desulfomicrobium escambiense*) and LB89 (93% to *Desulfomicrobium apsheronum*).

Figure B5. Nucleotide sequence alignments of the forward primer TotalF with 16S rRNA sequences of bacterial clones; LB36 (high similarity to *Sphaerochaeta globosa* LB44 (high similarity to Uncultured *Synergistete*s) and LB71 (high similarity to Uncultured *Chloroflexi* species) from the inoculum reactor receiving lactate at a sulphate concentration of 1 g l^{-1}. The mismatches are indicated as nucleotide bases.

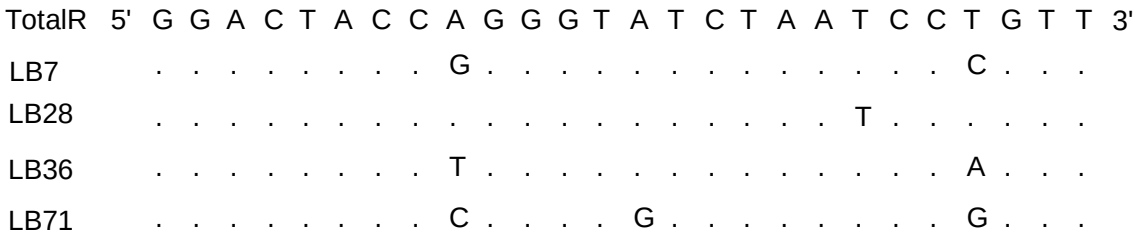


Figure B6. Nucleotide sequence alignments of the forward primer TotalF with 16S rRNA sequences of bacterial clones; LB28 (high similarity to *Mesotoga inferera*), LB36 (high similarity to *Sphaerochaeta globosa* LB44 (high similarity to Uncultured *Synergistete*s) and LB71 (high similarity to Uncultured *Chloroflexi* species) from the inoculum reactor receiving lactate at a sulphate concentration of 1 g l^{-1}. The mismatches are indicated as nucleotide bases.

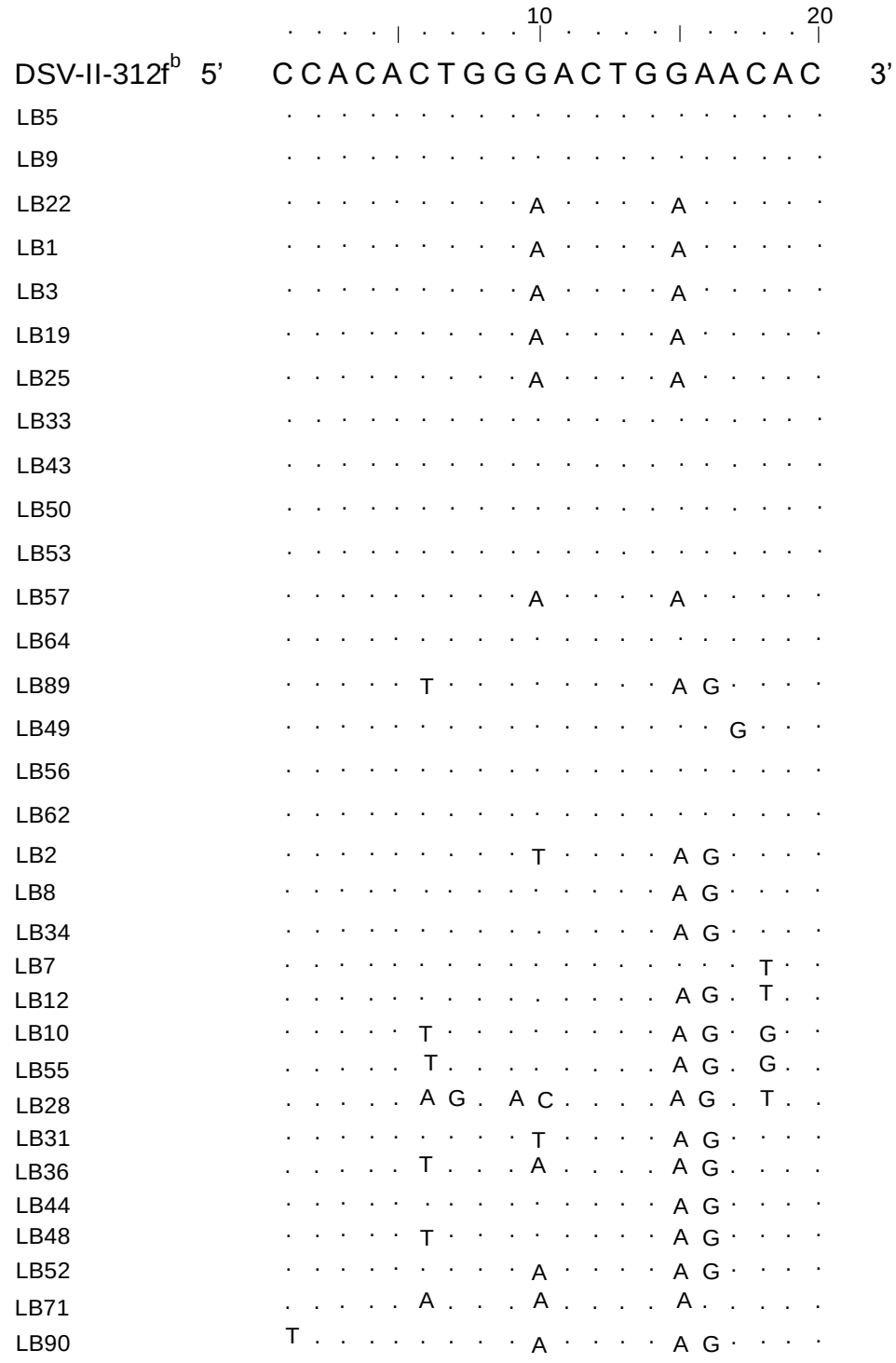


Figure B7. Nucleotide sequence alignments of the forward primer DSV-II-312f[a] with 16S rRNA sequences of bacterial clones that showed high similarity to *Desulfovibrio* species; LB5 (high similarity to *Desulfovibrio aminophilus*), LB9 (high similarity to *Desulfovibrio aminophilus* (high similarity to Uncultured *Synergistetes*) and LB22 (99% similarity to both *Desulfovibrio desulfuricans*, EU980606.1 and *Desulfovibrio multispirans* JX965382). The 16S rRNA sequences of other bacterial clones are indicated. The sample was obtained from the inoculum reactor receiving lactate at a sulphate concentration of 1 g l^{-1}. The mismatches are indicated as nucleotide bases.

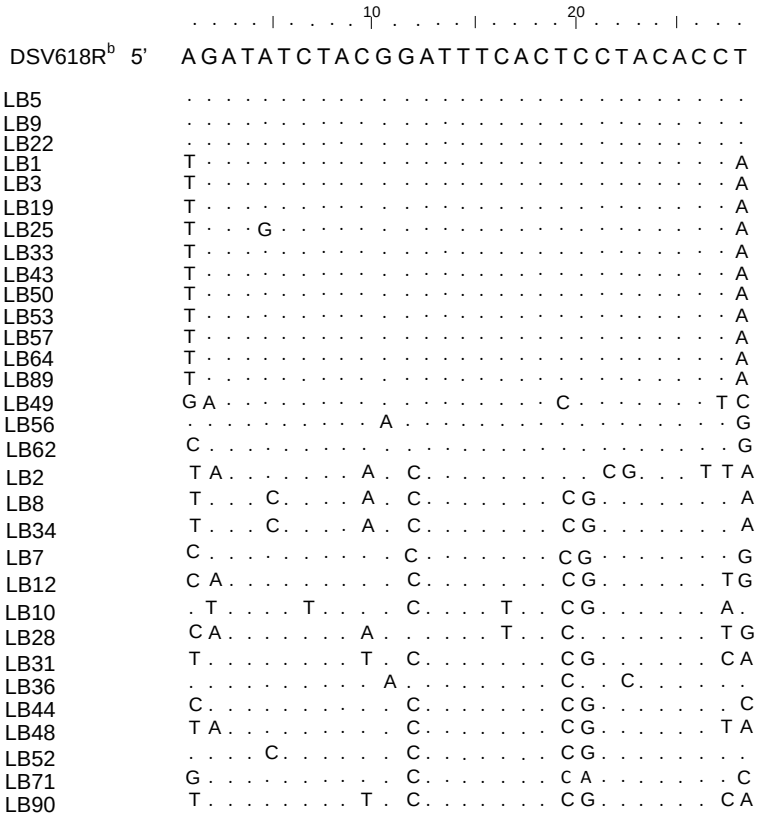


Figure B8 Nucleotide sequence alignments of the reverse primer DSV681R[b] with 16S rRNA sequences of bacterial clones that showed high similarity to *Desulfovibrio* species; LB5 (high similarity to *Desulfovibrio aminophilus*), LB9 (high similarity to *Desulfovibrio aminophilus* (high similarity to Uncultured *Synergistete*s) and LB22 (99% similarity to both *Desulfovibrio desulfuricans*, EU980606.1 and *Desulfovibrio multispirans* JX965382). The 16S rRNA sequences of other bacterial clones are indicated. The sample was obtained from the inoculum reactor receiving lactate at a sulphate concentration of 1 g l^{-1}. The mismatches are indicated as nucleotide bases.

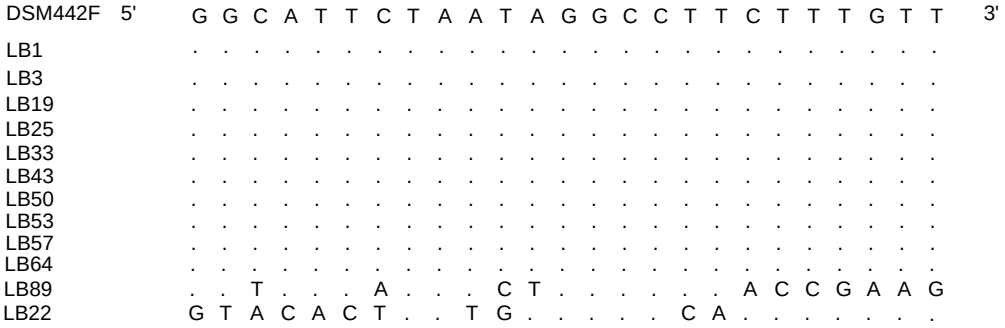


Figure B9. Nucleotide sequence alignments of the forward primer DSM442F with 16S rRNA sequences of bacterial clones that showed high similarity to *Desulfomicrobium* species; LB1 (high similarity to *Desulfovibrio aminophilus*), LB3 (high similarity to *Desulfovibrio aminophilus* (high similarity to Uncultured *Synergistete*s) and LB19 (99% similarity to both *Desulfovibrio desulfuricans*, EU980606.1 and *Desulfovibrio multispirans* JX965382), LB25 (high similarity to *Desulfovibrio aminophilus*), LB33 (high similarity to *Desulfovibrio aminophilus*), LB43 (high similarity to *Desulfovibrio aminophilus*), LB50 (high similarity to *Desulfovibrio aminophilus*), LB53 (high similarity to *Desulfovibrio aminophilus*), LB57 (high similarity to *Desulfovibrio aminophilus*), LB64 (high similarity to *Desulfovibrio aminophilus*) and LB89 (high similarity to *Desulfomicrobium apsheronum*). The 16S rRNA sequences of LB22 (99% similarity to both *Desulfovibrio desulfuricans*, EU980606.1 and *Desulfovibrio multispirans* JX965382 is indicated. The sample was obtained from the inoculum reactor receiving lactate at a sulphate concentration of 1 g l^{-1}. The mismatches are indicated as nucleotide bases.

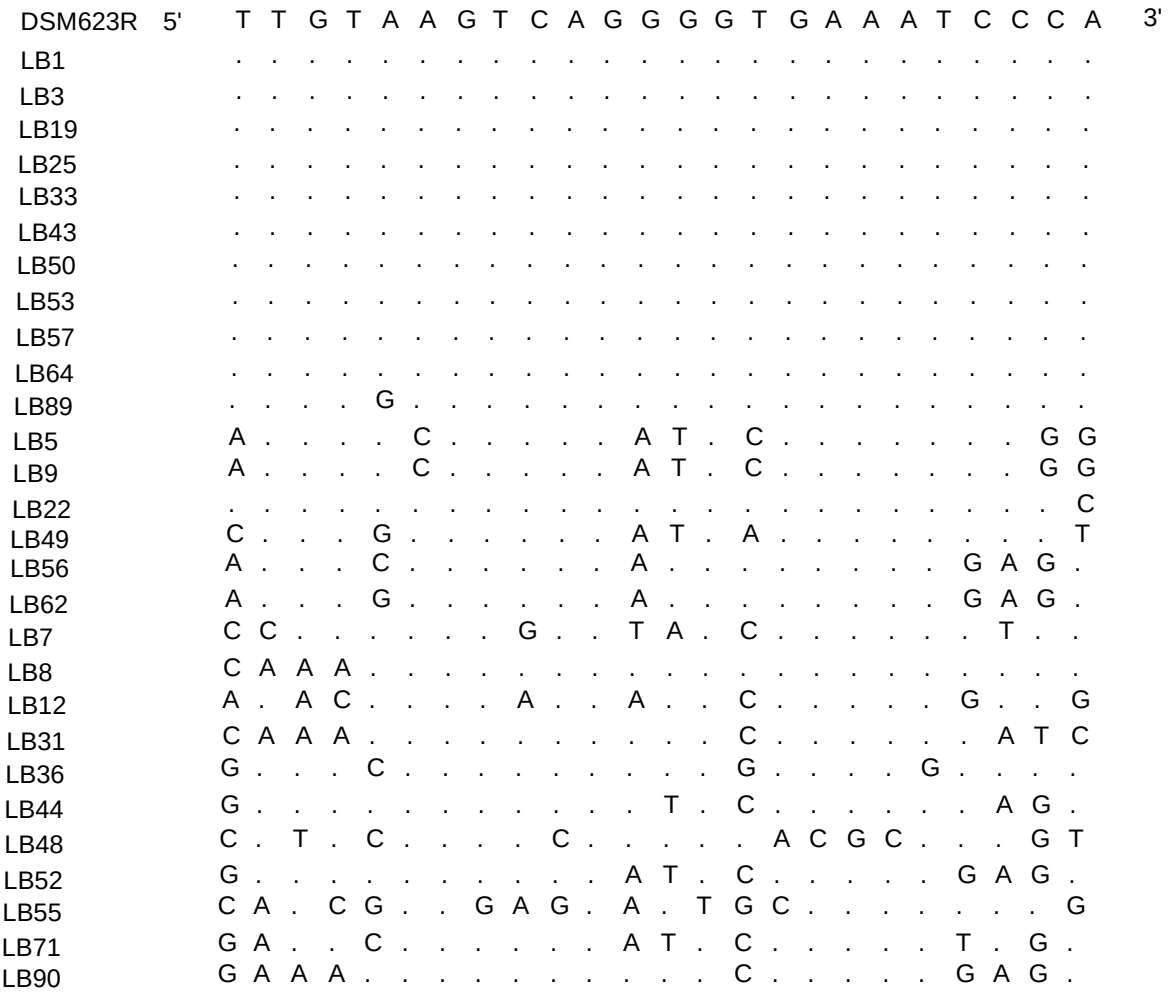


Figure B10. Nucleotide sequence alignments of the reverse primer DSM623R with 16S rRNA sequences of bacterial clones that showed high similarity to *Desulfomicrobium* species; (LB1, LB3, LB19, LB25, LB33, LB43, LB50, LB53, LB57, LB64 and LB89) but showed high variability to 16S rRNA sequences of other SRB that showed high similarity with [*Desulfovibrio* (LB5, LB9 and LB22)], Uncultured *Desulfuromonas* (LB49), *Desulfocurvus vexinensis* (LB62), *Desulfocurvus thunnarius* (LB56), uncultured *Synergistetes* species (LB7, LB12 and LB44), uncultured *Bacteriodetes* species (LB8 and LB31), *Mesotoga inferera* (LB28), *Sphaerochaeta globosa* strain Buddy (LB36), *Citrobacter freundii* (LB52), *Acholeplasma species* (LB55), uncultured *Chloroflexi s*pecies (LB71) and *Bacteriodetes* species (LB90). The mismatches are indicated as nucleotide bases.

3 Appendix C

Supplementary information for **Chapter 5**. This section gives raw data and calculations of experiments reported in Chapter 5.

Table C1. Comparison of assayed sulphide and expected sulphide (ES) conncentrations across the environmental conditions investigated for BSR in CSTRs.

RT (d)	Dilution rate (h^{-1})	Assayed sulphide (AS) expected sulphide (ES) concentrations (g l^{-1}) at different feed sulphate concentrations (S_0) S_0 (g l^{-1})								
		1.0			2.5			5.0		
		AS	ES	% Loss	AS	ES	% Loss	AS	ES	% Loss
0.5	0.083	0.071	0.172	41.3	nd	0.186		nd	0.215	
1	0.042	0.171	0.309	55.3	0.010	0.425	2.3	0.081	0.388	20.9
1.5	0.028	0.176	0.309	56.9	0.200	0.516	38.8	0.467	0.717	65.1
2	0.021	0.184	0.316	58.2	0.204	0.519	39.3	0.722	1.15	62.8
3	0.014	0.190	0.316	60.1	0.451	0.738	61.1	0.755	1.40	53.8
4	0.010	0.191	0.316	60.4	0.500	0.770	64.9	0.911	1.59	57.1
5	0.0083	0.191	0.316	60.4	0.511	0.774	66.0	0.985	1.64	60.1

Table C2. Steady-state data of sulphate conversion across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Sulphate conversion (%) at different feed sulphate concentrations (S_0) S_0 g l^{-1}		
		1.0	2.5	5.0
0.5	0.083	50.0	21.6	12.5
1.0	0.042	90.0	49.4	22.6
1.5	0.028	90.0	60.0	41.7
2.0	0.021	92.0	60.4	66.9
3.0	0.014	92.0	85.8	81.7
4.0	0.010	92.0	89.6	92.8
5.0	0.0083	92.0	90.1	95.4

Table C3. Steady-state data of propionate conversion across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Propionate conversion (%) at different feed sulphate concentrations (S_0) S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	7.5	24.2	5.8
1.0	0.042	100	21.7	56.3
1.5	0.028	100	100	100
2.0	0.021	100	100	100
3.0	0.014	100	100	100
4.0	0.010	100	100	100
5.0	0.0083	100	100	100

Table C4. Steady-state data of butyrate conversion across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Butyrate conversion (%) at different feed sulphate concentrations (S_0) S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	7.3	4.4	0.3
1.0	0.042	100	16.5	8.7
1.5	0.028	100	100	95.1
2.0	0.021	100	100	100
3.0	0.014	100	100	100
4.0	0.010	100	100	100
5.0	0.0083	100	100	100

Table C5. Steady-state data of volumetric sulphate reduction rate across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Volumetric sulphate reduction rate (g l^{-1} h^{-1}) at different feed sulphate concentrations (S_0) S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.042	0.045	0.052
1.0	0.042	0.038	0.051	0.047
1.5	0.028	0.025	0.042	0.058
2.0	0.021	0.019	0.031	0.070
3.0	0.014	0.013	0.030	0.057
4.0	0.010	0.010	0.023	0.048
5.0	0.0083	0.008	0.019	0.040

Table C6. Steady-state data of bacterial dry mass across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Bacterial dry mass (g_{DW} l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.160	0.160	0.211
1.0	0.042	0.596	0.761	0.715
1.5	0.028	0.597	0.955	1.285
2.0	0.021	0.600	0.974	2.151
3.0	0.014	0.626	1.386	2.616
4.0	0.010	0.627	1.472	2.963
5.0	0.0083	0.628	1.488	3.161

Table C7. Steady-state data of residual sulphate across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Residual sulphate concentration (g l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.50	1.96	4.37
1.0	0.042	0.10	1.27	3.87
1.5	0.028	0.10	1.00	2.91
2.0	0.021	0.08	0.99	1.65
3.0	0.014	0.08	0.35	0.91
4.0	0.010	0.08	0.26	0.36
5.0	0.0083	0.08	0.25	0.23

Table C8. Steady-state data of residual acetate across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Residual acetate concentration (g l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.134	0.975	1.99
1.0	0.042	0.311	0.711	2.03
1.5	0.028	0.306	1.11	2.47
2.0	0.021	0.301	1.11	2.03
3.0	0.014	0.317	0.902	1.88
4.0	0.010	0.329	0.843	1.88
5.0	0.0083	0.329	0.871	1.65

Table C9. Steady-state data of residual propionate across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Residual propionate concentration (g l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.110	0.220	0.292
1.0	0.042	0	0.220	0.169
1.5	0.028	0	0	0
2.0	0.021	0	0	0
3.0	0.014	0	0	0
4.0	0.010	0	0	0
5.0	0.0083	0	0	0

Table C10. Steady-state data of residual butyrate concentration across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Residual butyrate concentration (g l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.100	0.117	0.311
1.0	0.042	0	0.103	0.260
1.5	0.028	0	0	0.009
2.0	0.021	0	0	0
3.0	0.014	0	0	0
4.0	0.010	0	0	0
5.0	0.0083	0	0	0

Table C11. Steady-state data of bicarbonate concentration across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Bicarbonate concentration (g l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.113	0.213	0.213
1.0	0.042	0.296	0.331	0.231
1.5	0.028	0.312	0.781	0.810
2.0	0.021	0.322	0.800	0.880
3.0	0.014	0.342	0.834	0.934
4.0	0.010	0.351	0.861	1.16
5.0	0.0083	0.370	0.911	1.21

Table C12. Steady-state data of initial acetate concentration across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Initial acetate concentration (g l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.269	0.991	2.10
1.0	0.042	0.269	1.02	2.02
1.5	0.028	0.269	1.00	2.02
2.0	0.021	0.269	1.06	2.04
3.0	0.014	0.269	0.888	1.99
4.0	0.010	0.269	0.969	1.98
5.0	0.0083	0.269	0.811	2.02

Table C13. Steady-state data of initial propionate concentration across the experimental conditions investigated in this current study.

RT (d)	Dilution rate (h^{-1})	Initial propionate concentration (g l^{-1}) at different feed sulphate concentration (S_0)		
		(S_0)		
		1.0	**2.5**	**5.0**
0.5	0.083	0.119	0.290	0.310
1.0	0.042	0.119	0.281	0.388
1.5	0.028	0.119	0.321	0.548
2.0	0.021	0.119	0.246	0.595
3.0	0.014	0.119	0.333	0.391
4.0	0.010	0.119	0.308	0.361
5.0	0.0083	0.119	0.434	0.369

Table C14. Steady-state data of initial butyrate concentration across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Initial butyrate concentration (g l^{-1}) at different feed sulphate concentrations (S_0)		
		S_0 g l^{-1}		
		1.0	**2.5**	**5.0**
0.5	0.083	0.108	0.122	0.312
1.0	0.042	0.108	0.129	0.285
1.5	0.028	0.108	0.111	0.185
2.0	0.021	0.108	0.129	0.219
3.0	0.014	0.108	0.182	0.298
4.0	0.010	0.108	0.119	0.300
5.0	0.0083	0.108	0.120	0.322