Cape Peninsula University of Technology

Tannery Effluent Characterisation & Culture Enrichment for Enhancement of Biological Treatment

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Declaration

I, Emma Jane Horn, declare that the contents of this thesis represent my own unaided work, and that the thesis/dissertation has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed  **Date** **10/06/2020**

Abstract

Pollution of water resources has become a critical problem around the world. The tanning process, used in leather manufacturing, is water and chemical intensive and has developed a reputation as critically water polluting. The hides and skins are salted to preserve them. Sodium hydroxide and sodium sulphide are used in the initial phase of the tanning process to remove the hair. Therefore, tannery effluent is characterised by a high organic load, high salinity and high concentrations of sulphur species. Consequently, comprehensive wastewater treatment is required before the effluent can be discharged.

Tannery effluent has a high organic load, which makes it theoretically attractive for anaerobic digestion (AD) to achieve energy recovery as part of the treatment process. The high sulphur species load, in particular sulphide (HS^-), inhibits the methanogenic microbial species responsible for biogas generation. Therefore, in its raw form, the effluent is not a suitable substrate for AD. Pre-treatment, particularly to remove the HS^- components, would require organisms that are capable of withstanding the high salinity associated with the effluent. However, there is relatively little research on saline tolerant bacterial consortia capable of removing sulphur compounds.

The primary aims of this study were to collect and enrich microbial communities from saline, anaerobic environments and use them to test the hypothesis that tannery effluent can be pre-treated in a novel reactor system to manage the sulphur species, making the effluent more amenable to AD. Samples were collected from five marine and estuarine environments and enriched in batch reactors of increasing volumes, on lactate-supplemented artificial seawater. Their ability to reduce sulphate (SO_4^{2-}) was evaluated and promising candidates were selected for further tests.

Tannery effluent was obtained from a local tannery that processes ostrich skins and another that processes bovine and ovine hides. These were characterised in detail and used as substrates to evaluate biological sulphate reduction (BSR) and sulphide oxidation (SO), initially in batch reactors, and then in a hybrid linear flow channel reactor (HLFCR) under continuous operation at a 4-day hydraulic residence time (HRT). The HLCFR was designed to support simultaneous reduction of SO_4^{2-} in the bulk volume and partial oxidation of HS^- to elemental sulphur (S^0) within a floating sulphur biofilm (FSB).

A molecular biology approach was used to characterise and quantify the sulphate reducing communities in the environmental samples, tannery effluent, and reactor contents, thereby elucidating the relationship between microbial community structure and function and process performance. This was based on metagenomic sequencing and quantification of the dissimilatory sulphite reductase (dsr) gene.

The ostrich tannery effluent was characterised by low sulphide concentrations (< 5 mg/ℓ) and relatively low sulphate (average 650 mg/ℓ), so it did not require pre-treatment prior to AD. The bovine/ovine tannery effluent (BTE) exhibited a high salinity (electrical conductivity (EC) 32 mS/cm) and variable sulphide and sulphate concentrations, reaching almost 900 mg/ℓ and 4000 mg/ℓ, respectively. Preliminary AD tests using raw BTE confirmed the need for pre-treatment.

The performance of a HLFCR that was inoculated with enriched microbial consortia was compared with the performance of a non-inoculated reactor. The results demonstrated that both the

endogenous and exogenous microbial communities were capable of BSR and SO, and were able to maintain a FSB. The non-inoculated reactor was able to reach 99% reduction of SO_4^{2-} over 21 days of batch operation, while the inoculated reactor attained 80% sulphate reduction (SR) efficiency during batch operation.

Together, when operating both the reactors continuously in series for > 100 days, the system reached a maximum SR of 96.6% in the bulk liquid at a 4-day HRT and was able to achieve an average sulphate reduction rate (SRR) of 170 mg/ℓ.day (maximum of 444 mg/ℓ.day), with a maximum of 81% reduction of influent SO_4^{2-}. The SRR improved under continuous operation due to the near first order relationship of BSR to SO_4^{2-} concentration.

Both reactors were consistently capable of near-complete HS^- removal during stable operation. The effluent from the non-inoculated HLFCR had an average HS^- concentration of 8.4 mg/ℓ when operated alone, while the average HS^- concentration in the effluent from the system in series was 9.7 mg/ℓ when the reactors were operated in series.

The SRB communities in the enrichment consortia were dominated by the *Desulfovibrio* genus, in particular, the uncultured *Desulfovibrio.* sp. MCM B_508 (34.2-60.5% relative abundance (RA)), with contributions from a variety of other species. The BTE batches contained an endogenous SRB community dominated by an unidentified *Desulfovibrio* sp. (enrichment culture HCB4; 18-59% RA across the batches). The community structure was relatively diverse and varied across the five batches of bovine/ovine tannery effluent.

During continuous operation, the SRB community within the HLFCRs treating BTE changed significantly. The inocula were dominated by *Desulfovibrio* species, but continuous operation selected for *Desulfobacterium autotrophicum* and *Desulfomicrobium orale.* The metabolic versatility of *D. autotrophicum* is likely responsible for its swift emergence within the system.

This research provided a technical proof of concept for the process using the enriched SRB/SOB community to reduce SO_4^{2-} under saline conditions. Preliminary AD studies on the BTE treated in the HLFCRs showed substantial improvement in biogas generation relative to AD of raw and partially treated BTE. It was concluded that pre-treatment of BTE using a HLFCR inoculated with well acclimated microbial consortia, followed by AD is a feasible option for the simultaneous remediation and valorisation of tannery effluent.

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Abbreviations and Acronyms

Systematic Chemical Formulae (IUPAC)	
$Ca(OH)_2$	Calcium hydroxide
$BaCl_2$	Barium chloride
$CaSO_4$	Calcium sulphate
CH_4	Methane
C_6H_6O	Phenol
CO_2	Carbon dioxide
Cr^{3+}	Trivalent chromium
Cr^{6+}	Hexavalent chromium
$CrSO_4$	Chromium sulphate
$FeCl_3$	Ferric chloride
H_3BO_4	Boric acid
HCN	Hydrogen cyanide
HCO_3	Bicarbonate
H_2O_2	Hydrogen peroxide
H_2S, HS^-, S^{2-}	Sulphide
H_2SO_4	Sulphuric acid
KCl	Potassium chloride
MgO	Magnesium oxide
$MgSO_4$	Magnesium sulphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaHS	Sodium hydrosulphide
NaOH	Sodium hydroxide
Na_2S	Sodium sulphide
Na_2SO_4	Sodium sulphate
NH_3-N	Ammonia as nitrogen
NH_{4+}	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
PO_4^{2-}	Phosphate
$S_2O_3^{2-}$	Thiosulphate
$S_4O_6^-$	Tetrathionate
SO_3^{2-}	Sulphite
SO_4^{2-}	Sulphate

Abbreviations			
AAE	Acetic acid equivalents	MCC	Microcrystalline cellulose
AD	Anaerobic digestion	nMDS	Non-metric multidimensional scaling
AES	Atomic emission spectroscopy		
AFP	Advanced facultative pond	MPA	Methane-producing archaea
ALK	Alkalinity	MR DNA	Molecular Research
AOP	Advanced oxidation process	MS	Mass spectroscopy
ARD	Acid rock drainage	NCBI	National Centre for Biotechnology Information
ASBR	Anaerobic sequencing batch reactor		
ASP	Activated sludge process	NF	Nanofiltration
bcl	bacteriochlorophyll	NGS	Next Generation Sequencing
BMP	Bio-methane potential	NWA	National Water Act
BOD	Biochemical oxygen demand	OA	Old Airstrip culture
Bp	Base pairs	OD	Oxidation ditch
BSR	Biological sulphate reduction	OHPA	Obligate hydrogen-producing acetogens
BTE	Bovine/ovine tannery effluent		
CAF	Central Analytical Facility	OLR	Organic loading rate
COD	Chemical oxygen demand	OTE	Ostrich tannery effluent
CSTR	Continuous stirred tank reactor	OTU	Operational taxonomic units
DAF	Dissolved-air floatation	PSB	Purple sulphur bacteria
DNA	Deoxyribonucleic acid	PLV	Pull lock valve
DO	Dissolved oxygen	qPCR	Quantitative polymerase chain reaction
dsr	Dissimilatory sulphite reductase		
dsDNA	Double stranded DNA	RA	Relative abundance
DWS	Department of Water and Sanitation	RO	Reverse osmosis
EGSB	Expanded granular sludge bed	SBR	Sequencing batch bioreactor
EPS	Extracellular polymeric substances	SIMPER	Similarity percentage
EO	Electro oxidation	SO	Sulphide oxidation
ETP	Effluent treatment plant	SOB	Sulphide oxidising bacteria
FB	Fermentative bacteria	SR	Sulphate reduction
FF	Fixed film	SRB	Sulphate reducing bacteria
FSB	Floating sulphur biofilm	SRR	Sulphate reduction rate
GSB	Green/brown sulphur bacteria	TDS	Total dissolved solids
HAc	Homoacetogenic bacteria	TE	Trace element
HLFCR	Hybrid linear flow channel reactor	TKN	Total Kjeldahl nitrogen
HRT	Hydraulic retention time	TOC	Total organic carbon
IC_{50}	Half maximal inhibition concentration	TR	Trench reactor
IC	Ion chromatography	TSS	Total suspended solids
ICP	Inductively coupled plasma (ICP)	TVS	Total volatile solids
K_A	Kasouga culture grown on acetate	UASB	Up-flow anaerobic sludge blanket
K_L	Kasouga culture grown on lactate	VFA	Volatile fatty acid
KP	Kathy Park culture	VOA_t	Total volatile organic acid
LB	Luria-Bertani	WSP	Waste stabilisation pond
MBR	Membrane bioreactor	Z	Zandvlei culture

Glossary

Term	
Anaerobic digestion	A series of biological processes where microorganisms decompose biodegradable material, in the absence of oxygen, to produce biogas and digestate (Deublein and Steinhauser, 2008).
Biochemical oxygen demand (BOD)	The quantity of oxygen required by microorganisms to degrade organic materials and oxidisable inorganic matter in the substrate at standard conditions (Buljan and Král, 2011). The reaction time is limited to five days.
Chemical oxygen demand (COD)	The quantity of oxygen required for the total oxidation of the organic and inorganic oxidisable matter (Buljan and Král, 2011). A substrate's COD is always greater than its BOD.
Methanogenesis	The metabolic production of methane (CH_4) by microbes known as methanogens (Buljan and Král, 2011).
Sulphate reduction /sulphidogenesis	A form of anaerobic metabolism where sulphate (SO_4^{2-}) is used as a terminal electron acceptor with sulphide (H_2S, HS^- and S^{2-}, depending on pH) as a reaction product (Sabumon, 2016).
Dissimilatory and assimilatory SO_4^{2-} reduction	Dissimilatory SO_4^{2-} reduction occurs when the HS^- product is released to the environment, as opposed to assimilatory SO_4^{2-} reduction where HS^- is assimilated into various organic compounds in the microbial cells (Sabumon, 2016).
Sulphide oxidation	An aerobic process where sulphide (HS^-) is converted into elemental sulphur (S^0) by partial oxidation or into sulphate (SO_4^{2-}) by complete oxidation (Midha and Dey, 2008).
Total Kjeldahl nitrogen (TKN)	Both organic nitrogen (nitrogen contained in amino acids/proteinaceous compounds) and nitrogen present in ammonia (NH_3-N) and ammonium (NH_4^+) salts are included in TKN (APHA, 2012).
Total nitrogen	A measure of all the nitrogen present in effluent. Includes organically bound nitrogen, and Total Inorganic Nitrogen, which is made up of nitrogen present in nitrites (NO_2^--N), nitrates (NO_3^--N) and NH_3-N (APHA, 2012).

1 Introduction

1.1 Background and context

Tanning is one of the oldest raw material processing techniques in the world and is an important economic activity in many countries, regardless of their economic status (Sabumon, 2016). As long as animals have been slaughtered for food, humans have found use for their skins, or had to dispose of them. To use the hide or skin, it must first be cleaned, then washed in tanning agents and dried to preserve it. This process converts the hide into leather (Midha and Dey, 2008). While most hides are by-products of the meat and dairy industries, animals, like crocodiles, can be farmed specifically for their exotic skin (UN FAO, 2013). Leather is internationally traded and made into a large variety of durable products (Swartz *et al*., 2017).

The tanning process is chemically intensive and generates large quantities of highly turbid, saline and foul smelling effluent that is laden with toxic metal salts (Cr^{+3}), suspended solids, as well as organic and inorganic (NH_4-N, SO_4^{2-}, HS^-, chlorides) residues (Midha and Dey, 2008; Oyekola *et al*., 2018). The uncontrolled release of tannery wastewater contaminates the environment and can result in public health risks. Therefore, while tanneries address the problem of disposing of hides and skins they create another issue, with the challenge of treatment and disposal of their effluent as old as the tanning industry itself (Naturgerechte, 2002).

The variability of tanning processes, size of tannery, type of desired product and the intermittent processing of hides or skins means that pollution loads in the generated wastewater are not consistent at one site over time, let alone in different tanneries around the world (Buljan and Král, 2011; Goswami and Mazumder, 2013). Effective treatment of this complex wastewater is challenging and expensive, which often results in tanning firms paying fines for releasing non-compliant, partially treated effluent into the municipal sewer (Brink, 2018). Presently, tanneries operating in South Africa are obliged to comply with municipal effluent discharge standards and release their treated effluent into municipal sewers and dispose of solid wastes in sanitary landfills. Most South African tanneries pay fines, as they are not able to treat the complex wastewater to the discharge standards set (Brink, 2018). The National Waste Management Strategy strives to promote waste prevention, reuse, recycling and has energy recovery targets of reducing landfilling of biodegradable solid wastes by 50% by 2024, including through the use of AD (DST, 2014).

One of the major challenges facing the tanning industry is that the removal of hair from animal hides uses chemicals with high concentrations of sulphide (Swartz *et al*., 2017). While most of the sulphide in the slightly alkaline effluent is present as aqueous bisulphide (HS^-), hydrogen sulphide (H_2S), which is corrosive, highly toxic and has a distinctive malodour at concentrations of only 1 ppm, can occur at high concentrations in more acidic process water (Midha and Dey, 2008). Current treatment technologies, such as balancing and aerobic treatment, may not effectively eliminate HS^- and can increase the sulphate (SO_4^{2-}) load of the wastewater (Oyekola *et al.*, 2018). Therefore, there is the potential for the development of an alternative, supplementary treatment to remove HS^-.

Tannery effluent contains a high organic matter load, which also needs to be reduced prior to discharge. Sulphide removal would increase the potential of using anaerobic digestion (AD) to lower the organic matter content, as opposed to the more commonly used aerobic treatment. During the AD process, organic matter is converted into methane-rich biogas, so is potentially a method for

energy recovery from tannery wastewater. Therefore, AD could be preferable to an aerobic treatment, like the conventional activated sludge process (ASP), as significantly less sludge is generated, energy and nutrient requirements are low, and relatively high organic loading rates (OLR) and short retention times can be employed, whilst producing a renewable energy (Appels *et al*., 2008; Welz *et al.*, 2018). However, while AD is an attractive option to reduce chemical oxygen demand (COD) and sludge volume, methanogens are strongly inhibited by HS^-. Methanogens are the microorganisms responsible for producing methane (CH_4) from organic material. Similarly problematic, high HS^- and SO_4^{2-} concentrations in AD reactors can lead to competition between sulphate reducing bacteria (SRB) and methanogens (Midha and Dey, 2008). Therefore, the development of a supplementary treatment that effectively reduces SO_4^{2-} and HS^- in the effluent could increase the potential to use AD for COD reduction and the generation of renewable energy.

The overall aim of the research was to contribute to the development of a pre-treatment process that reduces the sulphur species load, improves effluent quality, manages odour problems, recovers elemental sulphur and transforms the effluent into a substrate that is more amenable to AD.

1.2 Scope and limitations (and key issues effecting the study)

The following indicate the scope of the research:

- Acquisition of enrichment cultures from coastal and estuarine areas of the Western Cape and Eastern Cape
- Technical proof of concept of using these enriched cultures for biological SO_4^{2-} reduction (BSR) and HS^- oxidation (SO), illustrating the feasibility of the physiological process at laboratory scale
- Collection and characterisation of ostrich tannery effluent (OTE) and raw bovine/ovine tannery effluent (BTE) with a conductivity that ranged between 3-42 mS/cm
- Operation and monitoring of reactors under a mesophilic temperature range
- Investigation of BSR and partial SO with the OTE and BTE in a hybrid linear flow reactor under continuous operation
- Molecular characterisation of microbial communities in selected samples and preliminary investigation into potential structure/function relationships

The following are boundaries for what were not covered by the research:

- Detailed kinetics of BSR and SO
- Study of the scalability and economic viability of the process
- Identification of where and how the reactor could be integrated into current treatment systems
- Data on effluent discharged from respective tanneries' treatment plants
- Transformation of other sulphur species, aside from HS^- and SO_4^{2-}
- Comprehensive anaerobic digestion tests and a focus on organic compound degradation

1.3 Statement of the research problem

The tanning of hides and skins involves processes and chemicals that produce an effluent that is high in COD. The variation in these processes and water consumption, as well as the intermittent processing of hides or skins means that the pollution load in the wastewater is not consistent in composition at one site over time, let alone in different tanneries around the world (Buljan and Král, 2011). Effective treatment of tannery wastewater is challenging, expensive and different in each situation. Consequently, in many cases, current technologies applied to treat tannery wastewater in South Africa are inadequate. If this sub-standard wastewater is discharged, not only is there a threat to the environment, there is also an economic threat to the industry due to the heavy fines incurred from the Department of Water and Sanitation (DWS) and/or local municipalities for non-compliance (Swartz *et al.*, 2017).

Tannery effluent is a complex wastewater. While there is the potential for treatment by AD, the presence of sulphur species in the wastewater presents a major challenge to this process. Conventional treatment methods have drawbacks of high chemical, energy and disposal costs (Midha and Dey, 2008). Therefore, there is a need for robust, halotolerant cultures capable of reducing the sulphur species load in tannery effluent. These cultures need to be sourced, enriched and adapted to utilising typical OTE and BTE as a substrate for BSR and partial SO, and then applied to its treatment.

2 Literature Review

2.1 Tanning and leather finishing processes

The primary purpose of tanning and leather finishing processes is to produce durable material for upholstery, shoe uppers and accessories (Swartz *et al*., 2017). A range of raw material types are processed in tannery operations – namely, bovine, ovine and exotic hides, using a range of operational stages, commonly including wet blue tanning, retanning and leather finishing (Swartz *et al*., 2017). These distinctions can be found in Table 1.

Table 1: Range of raw materials, processes and products in the tanning industry (adapted from Swartz et al., 2017)

Hides/skins	Processes	Products
• Bovine (cattle) • Ovine (sheep) • Exotic (ostrich, crocodile, game)	• Wet blue tanning (chrome) • Wet white tanning • Vegetable tanning • Leather finishing	• Automotive upholstery • Furniture upholstery • Shoe uppers & clothing accessories • Hair-on hides • Exotic skin products

Generally, leather manufacturing can be divided into four parts, the beam house operations, or pre-tanning, the tanning and dye house processes, and the finishing steps. Vegetable tanning does not require as many steps as wet blue tanning after the vegetable tanning stage is complete. A firm that performs the complete process, as opposed to just wet blue tanning or vegetable tanning, is said to be a full-house tannery (Swartz *et al*., 2017). A diagram of the wet blue tanning process is shown in Figure 1 and includes inputs, by-products and wastes.

2.1.1 *Beam house operations*

In the beam house, where 80% of the biochemical oxygen demand (BOD) load is produced, the first step is to remove blood and dirt by washing (Lens et al., 1998a; Sabumon, 2016). The hides are then soaked in water to soften and remove salts. Fatty tissue can then be removed during the fleshing stage, after which, liming is performed to remove hair and epidermis with an alkaline medium of HS^- and lime. Liming also swells the hides to improve the penetration of tanning agents later on. The effluent from this stage contains a high concentration of sodium sulphide (Na_2S), lime and organic matter (Midha and Dey, 2008). The hides then undergo deliming and bating, where they are neutralised with acid ammonium (NH_4^+) salts and treated with enzymes to remove hair remnants and degrade proteins, creating a clean, smooth and soft hide surface (Swartz *et al*., 2017). This accounts for a major part of the NH_4^+ load in the effluent (Midha and Dey, 2008).

2.1.2 *Tanyard section*

Before tanning can take place, the hide or skin is first degreased to prevent the non-uniform penetration of tan or dye (Swartz *et al.*, 2017). Thereafter, pickling is usually performed to prepare the hides or skins for tanning. In this chemical process, acid (predominantly sulphuric acid, H_2SO_4) is added

to allow for better penetration of the tanning chemicals and salt is added to prevent swelling under the acidic conditions (Swartz *et al.*, 2017).

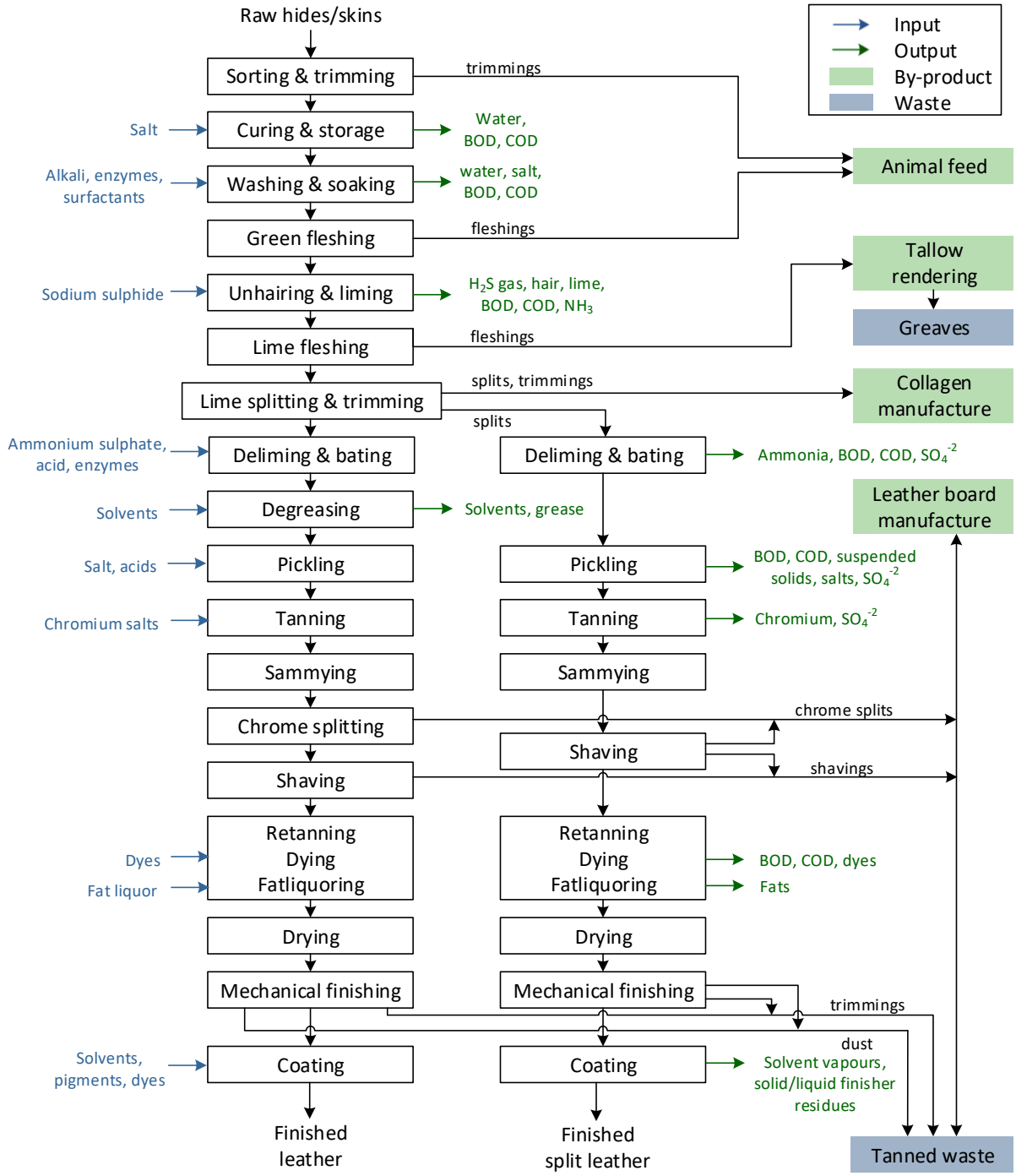

Figure 1: Wet blue tanning process and its processes, by-products and wastes (adapted from Midha and Dey, 2008; Swartz et al., 2017)

BOD – biochemical oxygen demand; COD – chemical oxygen demand.
Note: inputs and outputs are the same for leather and split leather processes that are repeated.

Finally, tanning takes place. During the tanning process, about 300 kg of chemicals are introduced per ton of hides (Midha and Dey, 2008). The tanning agents generally used are alums, syntans,

formaldehyde, glutaraldehyde and heavy oils. Based on the agents used, the tanning operations will be classed as chrome tanning or vegetable tanning (Midha and Dey, 2008).

Vegetable tanning is typically performed in a series of vats using natural organic substances, while chrome tanning is achieved under more alkaline conditions using trivalent chromium (Cr^{3+}) salts. Chrome tanning is the most prevalent leather tanning method in the world (Buljan and Kral, 2011). The Cr^{3+} ion has been found to have limited toxicity and the limits for its disposal have been relaxed in the EU and the US (Swartz *et al*., 2017). However, chrome or wet blue processing is still the most polluting of all tanning processes due to the partial conversion of Cr^{3+} to highly toxic hexavalent chromium (Cr^{6+}). However, the resulting wet blue product is stable and an internationally traded commodity. Therefore, the consistent properties wet blue processing imparts to the leather are highly desirable, making it a preferable, if polluting, process (Swartz *et al*., 2017).

The more alkaline conditions ensure binding of tanning chemicals to the hide (Swartz *et al.*, 2017). After tanning, a final wash is performed to remove any unbound chemicals from the hide. The tanned leather is then piled down, wrung (or sammyed) and graded for thickness and quality (Midha and Dey, 2008; Sharma and Sanghi, 2012). This product is now ready to be sold or dyed to specification.

2.1.3 *Dye house operations*

In the dye house, neutralisation and retanning are first performed, where the pH is raised to allow penetration of chemicals and to give the leather properties of flexibility, fullness and the desired texture (Swartz *et al*., 2017). Dyeing gives the leather the preferred colour by combining either powder or liquid dyes with the leather to form an insoluble compound (Lens *et al.*, 1998a; Sharma and Sanghi, 2012). Fatliquoring introduces an oil emulsion to the skin before it is dried, to replace the natural oils that were lost during beam house and tanyard operations, making it soft and flexible (Sharma and Sanghi, 2012). The leather is then wrung, set out and dried (Sharma and Sanghi, 2012).

2.1.4 *Finishing operations*

The finishing process refers to all steps carried out after drying the leather and is the final stage in the tanning process. This is begun by covering the grain surface with a chemical compound and brushing it (Sharma and Sanghi, 2012). Buffing, plating and embossing are performed to achieve a softer and aesthetically pleasing final product (Midha and Dey, 2008). Depending on the desired product, various waxes, pigments, glazes and oils are added to appeal to buyers (Sharma and Sanghi, 2012).

While there are a variety of useful by-products generated during the tanning process, attention needs to be paid to the potential toxicity of much of the waste.

2.2 Wastewater composition

The processing of hides or skins into leather generates a complex, highly turbid and foul smelling wastewater (Midha and Dey, 2008). Tannery effluent is commonly highly saline and has many compounds that can harm the environment and have a detrimental effect on biological sewage treatment operations if this effluent is released directly into the municipal waste stream (Swartz *et al.*, 2017). A summary of the inputs and outputs through a full-house tannery is shown in Figure 2.

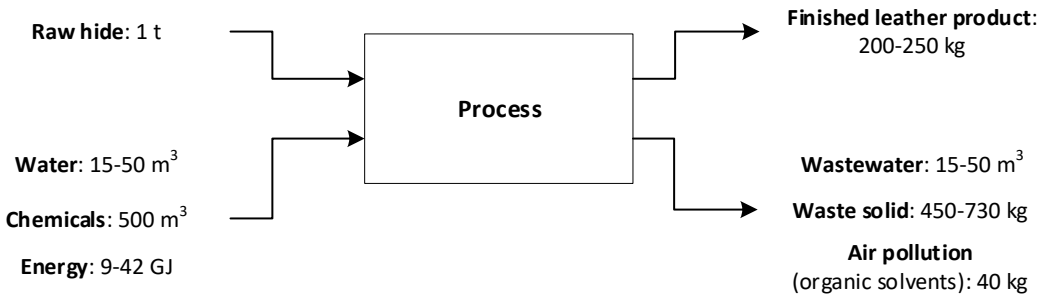

Figure 2: Overall inputs and outputs of a full-house tannery (adapted from Sharma and Sanghi, 2012)

A large amount of wastewater (~32.5 m^3) is generated for each ton of raw hide processed (Figure 2). The effluents produced by tanneries have high organic loads and elevated concentrations of dissolved (TDS) and suspended solids (TSS) (Swartz *et al.*, 2017). Other major components of the effluent include HS^-, SO_4^{2-}, chromium salts, nitrogen compounds, surfactants and large quantities of solid waste and suspended solids, like animal hair and trimmings (Midha and Dey, 2008). A summary of typical solid waste loads is shown in Table 2 per ton of raw hide. Table 3 shows typical contaminant concentration ranges in the wastewater, as well as typical pollution loads in the effluents per ton of raw hide.

Table 2: Typical solid waste loads generated in tanneries per ton of raw hide (adapted from Swartz et al., 2017)

Solid load	Mass per ton of raw hide (kg/t)
Raw trimmings	120
Fleshings	150
Wet blue trimmings & shavings	100
Buffing	2
Finished leather trimmings	32

Table 3: Mean composition of tannery wastewater, based on average effluent of 45 m^3/t raw hide (adapted from Midha and Dey, 2008; Sabumon, 2016; Swartz et al., 2017)

Contaminant	Contaminant load	
	(mg/ℓ)	(kg/t)
BOD	210-4 300	100
COD	180-27 000	242
TSS	925-36 000	150
Cr	3-350	5
HS^-	1-500	10
SO_4^{2-}	1156-2444	81
Cl^-	1 500-2 800	10
TKN	90-630	12-18
NH_3-N	17-380	5
Fats & oils	49-620	-
Phenolic compounds	0.4-100	-

COD – chemical oxygen demand; BOD – biochemical oxygen demand; Cr – Chromium; HS^- - total aqueous sulphides; Cl^- – chloride; NH_3-N – ammonia as nitrogen; SO_4^{2-} – sulphate; t – Ton

Some tanneries in South Africa combine all the effluent streams. However, keeping the beam house and tanyard streams separate is preferable for treatment purposes (Swartz *et al.*, 2017).

The various components in the effluent can negatively affect humans, agriculture and livestock. In the worst case, they can cause severe ailments and death to tannery workers (Midha and Dey, 2008).

A high COD and BOD in water can disrupt the natural balance of available oxygen in the water body, causing eutrophication and consequent oxygen deficiency that can kill aquatic life. Similarly, high organic nitrogen, NH_4^+ and nitrate (NO_3^-) concentrations in water can cause accelerated microbial activity and contribute to eutrophication (Yu, 2012).

Ammonia (NH_3), Cr^{6+} and HS^- are acutely toxic, causing bacterial inhibition due to an increase in maintenance energy requirement and enzyme reaction inhibition (Wittmann *et al.*, 1995); carcinogenic and teratogenic effects (Seiler *et al*., 1994); and cell metabolism interference (Parkin and Owen, 1986; Wiemann et al., 1998), respectively.

There are waste and effluent discharge regulations, under the National Water Act (NWA), specifying the quality of wastewater released into each municipality's sewer and the environment. These cover pH, temperature, COD, suspended solids and metals (Swartz *et al.*, 2017). The penalties associated with the releasing wastewater laden with HS^- and Cr^{3+} into the environment are particularly problematic to tanneries (Swartz *et al.*, 2017). Therefore, the removal of HS^- and Cr^{3+} from wastewater is required.

2.2.1 *Source of pollutants in tannery wastewater*

An overall view of how much each individual tannery operation can contribute to the load of each contaminant is shown in Table 4 and salinity and HS^-, specifically, are discussed further.

Table 4: Contribution of individual tannery operations to total pollution loads (adapted from Sabumon, 2016)

Operation /Process	WW (m^3/T)	Pollution load (kg/t raw hide) employing conventional technology								
		SS	**COD**	**BOD**	**Cr**	**HS^-**	**NH_3-N**	**TKN**	**Cl^-**	**SO_4^{2-}**
Soaking	7-9	11-17	22-33	7-11	-	-	0.1-0.2	1-2	85-113	1-2
Liming	9-15	53-97	79-122	28-45	-	3.9-8.7	0.4-0.5	6-8	5-15	1-2
Deliming*	7-11	8-12	13-20	5-9	-	0.1-0.3	2.6-3.9	3-5	2-4	10-26
Tanning	3-5	5-10	7-11	2-4	2-5	-	0.6-0.9	0.6-0.9	40-60	30-55
Post tanning	7-13	6-11	24-40	8-15	1-2	-	0.3-0.5	1-2	5-10	10-25
Finishing	1-3	0-2	0-5	0-2	-	-	-	-	-	-
Total	34-56	83-149	145-231	50-86	3-7	4-9	4-6	12-18	137-202	52-110

*/Bating

2.2.2 *Source of salinity in tannery wastewater*

The primary source of salinity in tannery processes comes from the initial hide and skin preservation technique. Fresh hides and skins are salt-cured to prevent decomposition immediately after they are stripped at the abattoir. This salt (sodium chloride: NaCl) has to be removed by soaking at the tannery before processing can begin. This requires a lot of water and constitutes the first source of effluent in the tanning process (Lefebvre *et al*., 2005). Generally, this soak water provides 60% of the effluent

salinity with the remainder from the acid salts used to suppress pelt swelling (Buljan and Král, 2012; Swartz *et al*., 2017; UNIDO, 1991).

Hides can be dry-salted or wet-salted. Instead of undergoing salt-curing, raw hides and skins can be transported chilled, green or chemically cured. In this case, the salinity of tannery effluent decreases considerably. However, only in rare cases are hides and skins not salted in South Africa (Swartz, *et al.*, 2017).

In situations where tanneries are located next to and integrated with an abattoir, the green skins do not need to be preserved before they enter the beam house operations (Brink, 2018). Hence, salt concentrations in this wastewater can be approximately 20-50% of the concentrations seen in typical effluents (Brink, 2018).

2.2.3 *Source of sulphide in tannery wastewater*

The main source of HS^- in tannery wastewater is from the beam house operations (Midha and Dey, 2008). Sulphides are used in the de-hairing and liming stage of the tanning process to chemically remove hair and swell the skins, so that lime fleshing can take place to mechanically remove fats and flesh from the inside of the hide or skin (Swartz *et al*., 2017). Sulphides such as Na_2S and sodium hydrosulphide (NaHS) are used to relax the structure of the hide or skin and swell the hides. This can result in HS^- concentrations of between 10-5000 mg/ℓ (Midha and Dey, 2008; Swartz *et al.*, 2017).

Reducing the amount of sulphides used has negative implications on the quality of the leather product. Leather quality is prized above all else, so the toxic effluent has to be dealt with afterwards (Brink, 2018). During the effluent treatment process, SO_4^{2-} used in a range of processes such as liming, deliming, bating, pickling and chrome tanning may be converted to HS^-, further increasing the concentration of HS^- (Li, 2005; Swartz *et al*., 2017; Zhao *et al*., 2011).

2.3 Tannery effluent treatment

Wastewater treatment is a process where wastewater quality is improved before it is released into the environment or reused. The primary objective of wastewater treatment is to reduce or remove pollutants, including organic matter, nutrients, solids and heavy metals (Buljan and Král, 2011).

The tanning process is complex, so the effluent composition can vary substantially. Not all effluent treatment plants (ETP) are similar, as they need to be tailored to the requirements of the site and the tanning processes employed (Buljan and Král, 2011).

2.3.1 *Overview*

In South Africa, most tannery wastewater requires three or four stages of treatment to meet the stringent discharge standards set by the Department of Water and Sanitation (DWS), local or regional municipal structures, for discharge into the municipal waste stream or the environment (Sabumon, 2016; Swartz *et al.*, 2017). These include preliminary, primary and secondary treatment, but if the quality is still unsatisfactory, tertiary stage treatment may be required. The separate stages are required due to the complex composition of the effluent. Further, the classification of processes occurring in each of the stages are often inconsistent across literature (Buljan and Král, 2011). For

example, preliminary treatment is often considered a part of primary treatment (Sabumon, 2016). For the purposes of this study, they will be considered separate treatment stages.

Tanneries that perform wet blue retanning and leather finishing mostly use only preliminary and primary treatment processes, while full-house tanneries also use secondary treatment (Swartz *et al.*, 2017). In the case of a tannery that releases their effluent into a public sewerage system, the tannery often does not include tertiary treatment in their ETP (Di Iaconi *et al*., 2003). Lastly, some tanneries in South Africa combine all effluent streams from the beam house and tanyard, as well as with soaking wastewater and other general effluents. As there are particular constituents in each that are not in the others, it is preferable to segregate these streams and pre-treat them separately (Swartz *et al*., 2017). This separation and independent treatment of each effluent stream reduces treatment and sludge disposal costs and reduces possible safety risks (Buljan and Král, 2011). The mixing of these streams causes the production of toxic H_2S gas as the pH becomes less alkaline, which is the most frequent cause of death in tannery accidents (Buljan and Král, 2011).

A typical full-house tannery ETP for segregated beam house and tanyard streams, utilising a SO tank and biological aeration tank, is shown in the flow diagram in Figure 3.

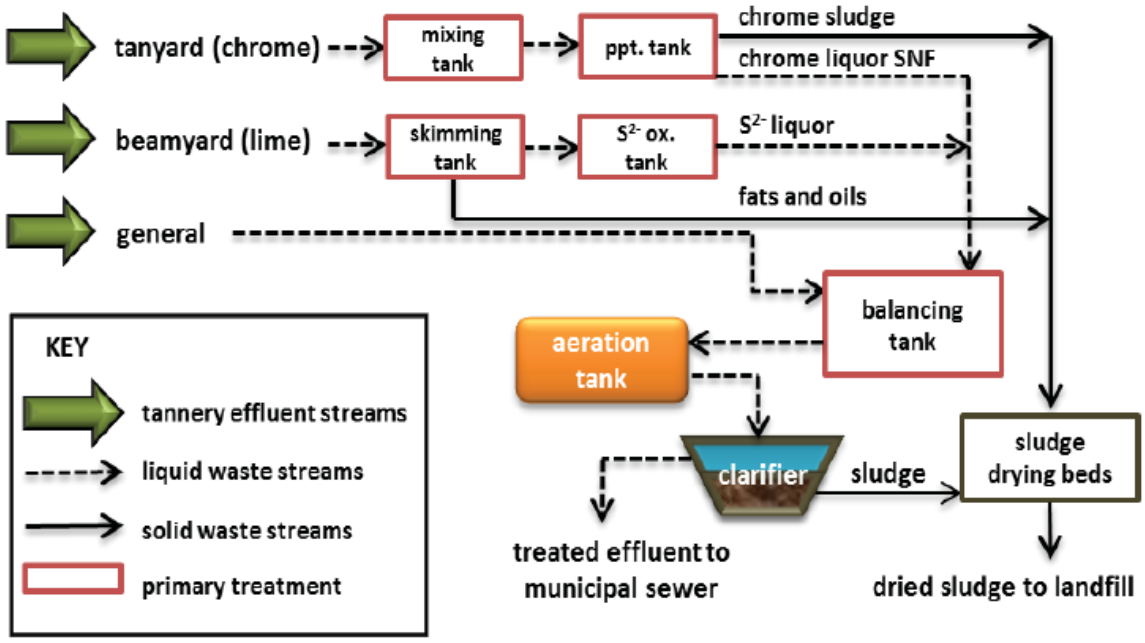

Figure 3: Full-house tannery conventional primary, secondary and tertiary effluent treatment process for segregated streams (Swartz et al., 2017)

2.3.2 *Preliminary treatment*

The objective of preliminary treatment is to remove coarse material from the beam house effluent that can clog pipes, pumps and sewer lines (Buljan and Král, 2011). Secondary to this is to produce a more homogenous stream that can be treated consistently in subsequent stages (Buljan and Král, 2011). To accomplish this, mechanical screens, grit removal apparatus and equalisation tanks are employed (Swartz *et al.*, 2017).

First, the effluent flows through a rough bar screen that removes larger solids. Preliminary settling usually follows this and is a simple, non-aerated grit removal chamber which can separate up to 30% of the COD present in the form of organic solids (Sahasranaman and Emmanuel, 2001; Swartz *et al.*,

2017; UNIDO, 1991). Self-cleaning screens remove most of the remaining larger solids, including hairs, sand and grease, resulting in the removal of 30-40% of the suspended solids from the raw waste (Swartz *et al.*, 2017; UNIDO, 1991).

In preparation for the subsequent physicochemical treatment, it is very important to keep all particulates in suspension (Buljan and Král, 2011). An equalisation and homogenisation tank with a stirrer is used to accomplish this. At this point, HS^- can be oxidised by using mixing-aeration devices in the equalisation tank, such that the HS^- undergoes catalytic oxidation (Buljan and Král, 2011).

2.3.3 *Primary treatment*

The objective of primary effluent treatment is to improve and accelerate the settling of suspended solids, specifically, fine and colloidal matter (Buljan and Král, 2011). In addition, the removal of chromium and HS^- from the effluent are key processes that occur during primary treatment (Swartz *et al.*, 2017). As the removal and recycling of chromium has been integral to the tanning process for centuries, this part of the effluent treatment has been well refined. By contrast, the HS^- removal process continues to present challenges and innovation opportunities, if it is included at all.

2.3.3.1 Coagulation and flocculation

Coagulation and flocculation are utilised to separate suspended solids from the effluent. This process is characterised by an optimum pH, so the addition of chemicals to correct the pH is often required (Swartz *et al*., 2017).

The negative charges on the surface of the finely dispersed colloids are destabilised through the addition of a coagulant, which neutralises the forces that keep them apart (Buljan and Král, 2011). As a result, the particles collide to form larger particles, known as flocs. However, care needs to be taken to not overdose the coagulant, which can cause a complete charge reversal and the colloid complex to restabilise (Buljan and Král, 2011).

Following this, flocculation occurs where bridges form between flocs to bind particles into clumps (Buljan and Král, 2011). The developing agglomerate particles are fragile and require slow and gentle mixing of the flocculating agent into the effluent. As with the coagulating agent, caution must be exercised to not overdose the polymer as this will cause settling or clarification complications (Buljan and Král, 2011).

2.3.3.2 Primary sedimentation

These large, flocculated particles are usually removed from the effluent by sedimentation, filtration, straining or floatation (Buljan and Král, 2011). Flocculation also makes the aggregates less gelatinous and as a result, easier to dewater (Buljan and Král, 2011). Fats, waxes, mineral oils and floating non-fatty materials that were not separated during screening or grit removal are also removed here.

The suspended solids are separated, most commonly, by circular settling tanks called clarifiers, which have continuous grease removal on top and sludge removal at the bottom (Buljan and Král, 2011). Circular tanks allow for easier recirculation than rectangular tanks, and in cases where there is space shortage, solids can be separated by floatation, usually using a dissolved-air floatation (DAF) system (Buljan and Král, 2011).

Clarified effluent is collected over the edge of the clarifier's effluent weir and most commonly, continues through to the HS^- treatment next. Sludge disposal will be discussed after the rest of the treatment stages in Section 2.3.6.

2.3.3.3 Sulphide removal

In South Africa, the chromium-rich tanyard stream is typically not mixed with this HS^--rich beam house effluent (Swartz *et al*., 2017). In this case, the pH of the stream is kept high until the HS^- is treated (Naturgerechte, 2002).

Globally, many tanneries do not target the detoxification of HS^- in the effluent (Valeika *et al*., 2006). There are three main reasons for this oversight (Valeika *et al*., 2006):

- National discharge standards are insufficiently strict with respect to HS^-,
- Some HS^- removal methods are too expensive, and
- Some methods are very complicated

However, the toxicity, corrosion and odour problems associated with the presence of HS^- are irrefutable and removal methods have received attention over the years and continue to be developed.

Conventionally, HS^- is removed through stripping, precipitation or oxidation, either by adding hydrogen peroxide (H_2O_2) to the beam house effluent or through the use of an oxidation catalyst (Swartz *et al.*, 2017; Valeika *et al.*, 2006). These treatment methods and others will be discussed further in Section 2.5.3.

After HS^- removal treatment, the stream is discharged into a balancing tank.

2.3.3.4 Chromium removal

Conventionally, the chromium-rich tanyard effluent stream undergoes precipitation with an alkaline reagent. Magnesium oxide (MgO) is typically used as it produces a denser sludge (Naturgerechte, 2002; Swartz *et al*., 2017). Once the sludge is dewatered, the filter cake can be re-acidified with H_2SO_4 to generate chromium sulphate ($CrSO_4$) (Swartz *et al*., 2017). This can be recycled into the tanning process or dried on sludge-drying beds. The supernatant is relatively chromium-free and is then discharged into the effluent balancing tank with the other effluent streams (Swartz *et al.*, 2017).

It is once again preferable to have segregation of HS^-- and chromium-rich flows (Swartz *et al.*, 2017). Beyond H_2S gas problems, not only can this avoid dilution of highly polluted streams, this also improves the efficiency of the ETP (Naturgerechte, 2002; Swartz *et al*., 2017).

2.3.4 *Secondary treatment*

The objective of the secondary treatment stage is to further reduce the amount of organic matter (expressed as BOD and COD) and other residual nutrients so that the effluent meets the discharge standards into municipal sewers or surface waters (Buljan and Král, 2011). Following treatment, the remaining suspended and colloidal solids are flocculated and adsorbed from the effluent (Buljan and Král, 2011).

The majority of secondary treatment processes make use of biological reactors, although chemical oxidation and advanced oxidation processes (AOPs) may be used. The latter are more often applied in tertiary treatment.

2.3.4.1 Biological treatment

Biological treatment processes aim to replicate natural phenomena under controlled conditions, to greatly accelerate the rate of the reactions. In practice, the biodegradability of the pollutant largely determines the efficiency of the treatment (Buljan and Král, 2011).

The major types of biological treatment processes include aerobic, anaerobic and facultative (aerobic-anaerobic) treatment. The aerobic methods for tannery effluent treatment include activated sludge processes (ASPs), the oxidation ditch (OD), sequencing batch reactor (SBR), wetlands and stabilisation ponds (Yusif *et al*., 2016). Common anaerobic processes include the anaerobic digester (AD), anaerobic filter (AF), and up flow anaerobic sludge blanket (UASB) (Ahmed and Lan, 2012; Lin and Chuang, 1994; Yusif *et al*., 2016).

While this variety exists, in practice aerobic systems are almost exclusively used in the tanning industry, except in countries with a hot climate and large amounts of available land, where facultative lagoons may be used (Buljan and Král, 2011). This is largely due to the characteristics of tannery effluent, primarily the HS^- and SO_4^{2-} present that results in the release of H_2S gas when the effluent is anaerobically digested (Buljan and Král, 2011; Swartz *et al*., 2017). Therefore, anaerobic treatment is used predominantly in sludge digestion, as it reduces the volume of sludge at lower costs to other methods (Buljan and Král, 2011; Swartz *et al.*, 2017). AD will be discussed in more detail in Section 2.4.

For aerobic treatment, the most widely used process is the conventional ASP with extended aeration. In this process, microorganisms are stimulated to convert harmful, oxygen-demanding organic compounds into low-energy, stable compounds. Treatment is efficient, with BOD_5 removals of 90-97% observed for tannery effluent (Buljan and Král, 2011; Haydar *et al.*, 2016). The qualitative biochemical reaction is summarised in Equation 1 (Buljan and Král, 2011):

$$inert\ matter + organic\ matter + O_2 + nutrients + microbes \rightarrow new\ microbes + CO_2 + H_2O + additional\ inert\ matter \quad (1)$$

Generally, this biological treatment stage is the most complex step of the ETP, requiring the highest capital investment and operational costs, as well as considerable operational skills and experience (Buljan and Kral, 2011). The ASP coupled with physicochemical methods is capable of reducing BOD_5 to below 10 mg/l and sludge volume by 20% (Buljan and Král, 2011; Swartz *et al*., 2017).

Operational issues that can arise include sludge expansion and rising and excessive foaming. Therefore, experienced operators are required on site (Yusif *et al.*, 2016).

In some cases, TKN, ammonia as nitrogen (NH_3-N) and oxidised nitrogen compounds also need to be removed as a part of secondary treatment. Discharge of effluent laden with nitrogen compounds promotes eutrophication in receiving water bodies. Conventional nitrogen-removal wastewater treatment systems include both aerobic nitrification and anaerobic denitrification steps (Sabumon,

2016). This can be done either in two separate units or by altering aeration and no aeration in the same unit.

Following biological treatment, clarifiers and sand filter beds separate solids from the effluent and dewater the sludge. The sludge undergoes further digestion and is sun-dried in drying beds before disposal to landfills. Some tanneries use belt filter presses, but drying beds are an inexpensive option. Medium to large tanneries usually discharge the remaining liquid effluent into municipal sewers (Swartz *et al.*, 2017).

2.3.5 *Tertiary treatment*

In certain cases, despite undergoing the steps described above, the quality of the final effluent does not satisfy the stipulated discharge standards (Buljan and Král, 2011). This tertiary polishing step may be particularly necessary if the effluent is going to be discharged into superficial water bodies as recalcitrant organic compounds can result in a COD (~500 mg/ℓ) that is still too high for discharge due to the risk of eutrophication (Buljan and Král, 2011; Di Iaconi *et al*., 2003). Other issues of disagreeable colour and the presence of specific organic pollutants can make tertiary treatment necessary (Buljan and Král, 2011).

In these circumstances, additional, more sophisticated and relatively expensive treatments need to be implemented to meet specific effluent quality requirements (Swartz *et al.*, 2017). The treatments include high efficiency carbon filtration, membrane technologies like ultrafiltration (UF) and reverse osmosis (RO), as well as ion exchange, elecrodialysis, high-rate evaporation and biological treatment with biomass or in polishing lagoons and reed beds (Buljan and Král, 2012; Swartz *et al.*, 2017).

2.3.6 *Sludge dewatering, handling and disposal*

The objective of sludge dewatering is twofold, to reduce the volume and weight of the material that needs to be transported, as well as to attain the dry matter content that landfills require. Much of the sludge that is first collected is in the form of a slurry, with a dry-solid content of just 2-4%. Generally, this slurry first undergoes thickening in sludge thickeners, then either mechanical dewatering in filter presses, belt-filter presses or decanters/centrifuges, or natural drying in sludge-drying beds. Apart from capital cost, power and chemical requirements, the achievable dry matter content of the sludge is a key consideration for equipment selection (Buljan and Král, 2011).

2.3.7 *Critical assessment of treatment*

A summary of the various treatment systems commonly used in the tanning industry, illustrating their function and benefit to treatment is shown in Table 5.

In South Africa, the two most common secondary treatment technologies are the ASP system with extended aeration like the OD and anaerobic ponds (Swartz *et al*., 2017). Apart from these, SBRs and membrane bioreactors (MBRs) have also been introduced for tannery effluent treatment in other parts of the world. Newer techniques, such as the granular activated carbon MBR and AOPs may become more popular in the future with further investigation (Swartz *et al*., 2017). A comparison of the major advantages and disadvantages of these popular secondary treatment processes is shown in Table 6.

Table 5: Summary of tannery effluent treatment systems most widely used in the tanning industry (adapted from Swartz et al., 2017)

Treatment Stage	Function	Benefits
Preliminary treatment		
Screening	Removes large particles of suspended solids	Reduces suspended solids in the effluent
Grit removal chamber	Separates organic solids	Can separate up to 30% of the COD present in the form of organic solids
Primary treatment		
Lime pre-settling	Removes a large amount of suspended solids – pulped hair and undissolved lime	Improves HS^- oxidation and lowers catalyst requirements
Sulphide oxidation	Reduces HS^- content	Minimises odour and improves biological processes
Chromium precipitation	Removes Cr from effluent	Allows effluent and sludge to meet discharge and disposal limits
Secondary treatment		
Activated sludge	Breaks down soluble and suspended organic matter, as well as others	Reduces pollution and discharge tariffs
Addition of phosphates	Improves breakdown of organics and increases process efficiency by ~30%	More efficiently reduces pollutants and improves sludge digestion
Anaerobic digestion	Breaks down soluble and suspended organic matter	Allows the formation of biogas and lowers sludge volume
Secondary settling	Removes biomass from effluent, allowing reinoculation of fresh effluent with biomass in sludge	Enables minimal suspended solids and COD/BOD in effluent discharged
Chemical precipitation	Precipitates suspended solids	Reduces suspended solids and COD/BOD
Tertiary treatment		
Ultrafiltration	Removes fine particles of suspended solids and soluble fats	Preparation for reverse osmosis
Reverse osmosis	Removes total dissolved inorganic solids and salt (NaCl)	Removes TDS and salt, lowering discharge tariffs
Sludge dewatering		
Drying beds	Dewaters sludge by evaporation and drainage	Reduces potential odour nuisance and landfill transport costs
Centrifuges	Separates water from solids	Reduces potential odour nuisance and landfill transport costs

While biological treatment of tannery effluent with the ASP is regarded as the standard for organic carbon and nitrogen removal, this technology may be ineffective in some situations due to the wastewater's particular characteristics (Goswami and Mazumder, 2013). These characteristics could be the presence of excess concentrations of biological inhibitors, including chromium, HS^-, chlorides, etc. (Goswami and Mazumder, 2013).

Table 6: Advantages and disadvantages of secondary treatment processes used in the treatment of tanning wastewater (adapted from Swartz et al., 2017)

Treatment	Advantages	Disadvantages	References
ASP with extended aeration	• Widely used in South Africa • Moderate capital cost • Can achieve high organic matter removal rates (COD: 60-98%; BOD_5: of 90-97%) • Continuous operation • Lab scale • Can be paired with chromium removal • Can be paired with nitrification and denitrification	• Reliant on floc formation • Occurrence of bulking and foaming • High operating costs of extended aeration for satisfactory BOD/COD removal • Larger footprint than: membrane bioreactor, SBR, UASB • Secondary settler requires separate clarifier • Unstable when variable hydraulic and pollutant loading	Mandal *et al*. (2010) Goswami and Mazumder (2013) Haydar *et al*. (2016) Buljan and Král (2011)
Membrane bioreactor (MBR)	• Recoverable products • Water can be reused • No reliance on floc formation • Small footprint • Adjusts well to variable hydraulic and pollutant loading • No separate clarifier required	• Highest capital and operating costs • Membranes prone to fouling due to effluent's high fat content • Requires skilled operation	Durai *et al.* (2011) Faouzi *et al*. (2013) Jafarinejad (2016)
Sequencing batch bioreactor (SBR)	• Decreased bulking compared to CASP • Small footprint • Adjusts well to variable hydraulic and pollutant loading • No separate clarifier required • COD removal efficiency of 80-95% • Treatment cycles can be adjusted to attain complete N removal, more effectively than CASP	• Reliant on floc formation • Higher capital and operating costs than CASP • Higher energy requirements than CASP • Batch operation	Singh and Srivastava (2011) Patil *et al*. (2013) Goswami and Mazumder (2013) Jafarinejad (2016)
Anaerobic ponds	• Inexpensive capital and operating costs • Low energy requirements • Low sludge production • Economic energy recovery	• Water cannot be reused • Leakage and emissions are threat to environment • Difficult to desludge • Malodorous	Buljan and Král (2011) Burton *et al*. (2009) Goswami and Mazumder (2013)
Up-flow anaerobic sludge blanket reactor (UASB)	• Favourable for high strength wastewater • Low sludge production • Biogas can be utilised for energy • Economic energy recovery	• Unstable when variable hydraulic and pollutant loading • Methanogens are sensitive to HS^- • Long start-up periods required after shutdown	Rajeshwari *et al*. (2000) Goswami and Mazumder (2013) Tamilchelvan and Mohan (2012)

2.3.8 *Compliance with discharge levels*

Most municipalities or local authorities have prescribed standards for the quality of wastewater released to the sewer covering broad (pH, COD, TSS) and specific (e.g. total Cr, SO_4^{2-}) parameters. South Africa has relatively stringent discharge quality requirements and examples from municipalities with tanning operations are shown in Table 7. Industries must comply and pay tariffs according to their effluent volume and specific pollutants that remain. As a result of these water quality standards, most tanneries in South Africa employ full treatment, from preliminary through to secondary, and tertiary only in special circumstances (Swartz *et al*., 2017).

Table 7: Effluent quality requirements regulated for tannery effluent (adapted from Swartz, et al., 2017)

Region	**pH**	**COD** (mg/ℓ)	**Phosphate** (mg/ℓ P)	**TSS** (mg/ℓ)	**Conductivity** (mS/m)	**SO_4^{2-}** (mg/ℓ)	**Total Cr** (mg/ℓ)	**Cl^-** (mg/ℓ)
City of Cape Town	5.5-12	5 000	25	1 000	500	1 500	10	1 500
Nelson Mandela Bay	6-12	10 000	-	1 000	500	1 500	20	1 000
Mossel Bay	6-11	3 000	-	1 000	500	500	10	1 000

2.3.9 *Challenges facing tannery effluent treatment*

The high organic load is one of the properties of tannery wastewater that needs to be addressed by any comprehensive tannery effluent treatment plant. While some of this load is easily removed, the presence of recalcitrant organic material requires further treatment and therefore, further investment (Buljan and Král, 2011).

The presence of SO_4^{2-} and HS^- complicates treatment due to toxicity and corrosivity of sulphide. Aqueous HS^- is present in the effluent as a result of dehairing chemicals and may form H_2S gas during secondary treatment as the mixing of streams causes the effluent to become less alkaline (Swartz *et al*., 2017). This H_2S slows the removal of COD (Sabumon, 2016).

While aerobic treatment can convert HS^- to SO_4^{2-}, removing the malodour; SO_4^{2-} can be rapidly metabolised by anaerobic bacteria to HS^- once more if the effluent remains stagnant (UNIDO, 2016). Any successful treatment needs to address the removal of these substances, which can be costly.

High salinity can further complicate treatment by affecting oxygen uptake in aerobic processes. Upon discharge, high salinity negatively affects receiving water bodies or low-flow municipal sewerage systems (Buljan and Král, 2011; Swartz *et al*., 2017).

Certain process routes result in the formation of large volumes of sludge. This sludge needs to go through a dewatering process and eventually ends up in landfill (Buljan and Král, 2011). High sludge

volumes result in additional processing and transport costs. Therefore, minimising the volume of sludge formed is a priority. Anaerobic digestion (AD) is a well-known method of reducing sludge volume (Swartz *et al*., 2017).

In full-house tanneries, primary treatment is typically followed by aerobic or anaerobic biological treatment to reduce the COD and BOD. Effluents with a high concentration of organics are potential energy sources, through AD of the wastewater (Rajeshwari *et al.*, 2000). However, the presence of sulphides may inhibit methane-producing archaea (MPA) and the presence of SO_4^{2-} may cause substrate competition, posing a bottleneck to AD (Hulshoff Pol et al., 1998; Vazifehkhoran et al., 2018).

2.4 Anaerobic digestion

The overall process of AD (Figure 4) results in the organic load being mineralised into sludge and biogas, which mainly consists of methane (CH_4) and carbon dioxide (CO_2), with some impurities. Anaerobic digestion is a complex process consisting of several phases. Different groups of microbes carry out degradation processes including hydrolysis, acidogenesis, acetogenesis and methanogenesis, which are partly dependent on one another's products as well as different environmental conditions (Deublein and Steinhauser, 2008).

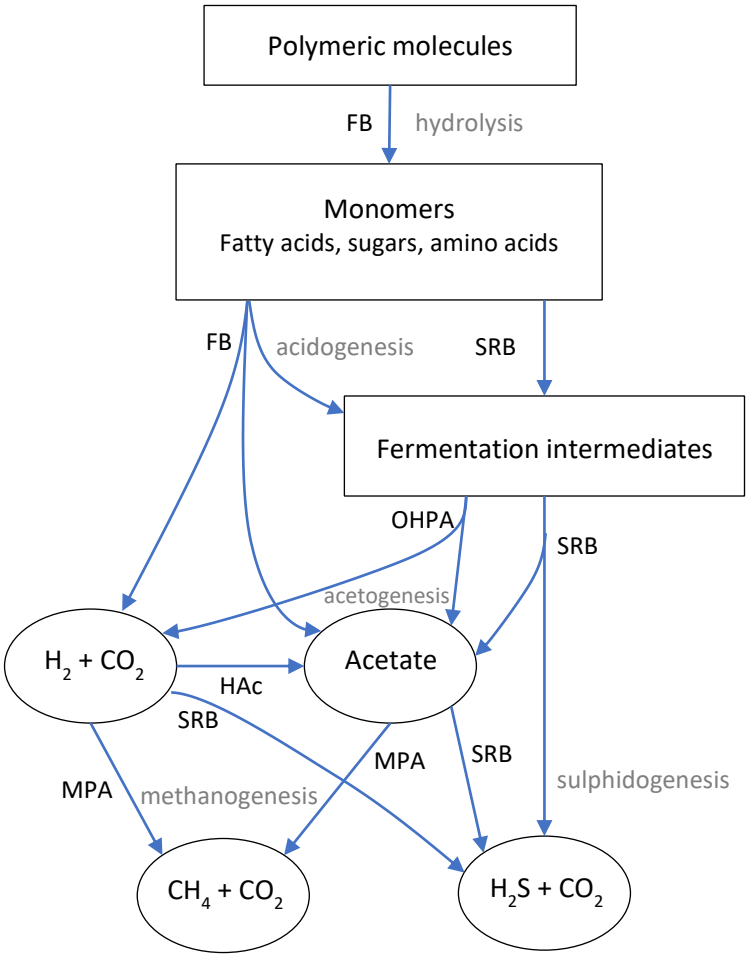

Figure 4: Organic compound degradation pathways under both methanogenic and sulphidogenic conditions (adapted from Colleran et al., 1995)

FB – fermentative bacteria; OHPA – obligate hydrogen-producing acetogens; HAc – homoacetogenic bacteria; MPA – methane-producing archaea; SRB – sulphate reducing bacteria.

Figure 4 shows the pathways of organic compound degradation required to sustain methanogenesis and sulphidogenesis. In the first stage (hydrolysis), extracellular enzymes from fermentative bacteria (FB) solubilise polymeric organic matter, such as starches, cellulose, proteins, lipids and fats into water-soluble monomers (Boshoff *et al.*, 2004). Proteins are hydrolysed into soluble organics and amino acids while lipids are split into long- and thereafter, short-chain fatty acids (Boshoff *et al*., 2004). Hydrolysis is a relatively slow process and can be the rate limiting step of the overall AD process when using a solid waste substrate (Bajpai, 2017).

Acidogenic microorganisms convert these soluble organic products into higher organic acids, alcohols, H_2 and CO_2 during the acidogenesis stage. Of these, the higher organic acid substrates are then metabolised by acetogenic microorganisms to produce acetate (the primary organic substrate for methanogensis), carbonic acid and alcohols, as well as H_2 and CO_2 (Bajpai, 2017; Deublein and Steinhauser, 2008).

Methanogenesis is a critical step of AD, where the organic pollution load (COD and BOD) is significantly reduced. The preceding steps merely converted the organic material from one form to another, with relatively limited complete oxidation to CO_2 (Bajpai, 2017). Methanogenic archaea are a diverse group of organisms, but dominant genera include *Methanobacterium, Methanospirillum* and *Methanosarcina* (Deublein and Steinhauser, 2008). Some, like *Methanospirillum hungatii, which* utilises formate as a carbon source, are substrate specific, while others like *Methanosarcina* are able to grow on acetate, methanol and methylamines. Some species can convert H_2 and CO_2 directly to CH_4 (Deublein and Steinhauser, 2008).

Sulphate reducing bacteria (SRB) cannot hydrolyse polymeric carbon sources so rely on the hydrolysis products of the FB (Burton *et al*., 2009; Hansen, 1993). Similarly, SRB cannot effectively compete with fast-growing FB in terms of monomer degradation in anaerobic environments (Widdel, 1988). However, SRB have a higher affinity (lower K_m) for H_2 and volatile fatty acids (VFAs) than methanogenic archaea (Isa *et al.*, 1986b; Rinzema and Lettinga, 1988). When considered alongside yield coefficient data, SRB could effectively outcompete methanogens at limiting substrate levels, depending on process parameters. Moreover, from a thermodynamic point of view, the reduction of SO_4^{2-} is slightly more energetically favourable than the reduction of bicarbonate (Isa *et al.*, 1986b). Therefore, it is more likely that SRB will be involved in the final and second-last steps of mineralisation if BSR and methanogenesis occur simultaneously (Colleran et al., 1995; O'Flaherty et al., 1998).

2.4.1 *Common anaerobic digestion treatment techniques*

Anaerobic ponds are a common treatment method in developing countries and are capable of a high degree of organic matter reduction. However, these ponds take up a lot of space and suffer from odour generation. Anaerobic contact, UASB reactors, and AF reactors are common alternatives that allow for high organic loading rates (OLR) from 5-40 kg COD/m^3 day. High rate anaerobic treatment systems, such as UASB and fixed bed reactors, are less favoured for tannery effluents due to the high fat, oil and suspended matters in the wastewater that lower the efficiency and performance of the treatment system. Therefore, a pre-treatment to remove these substances would be required. While high rate systems handle higher organic concentrations, the anaerobic contact reactor is more suitable as it is not constrained by the presence of a high fat content and the lack of granule formation (which can be problematic with UASB reactors). However, it is energy intensive (Rajeshwari *et al*., 2000).

In the last decades, the anaerobic sequencing batch reactor (ASBR) process has been developed. The process operates in a cyclic batch mode of four distinct phases per cycle: filling, reacting, settling and release (Zupančič and Jemec, 2010). The ASBR process has several advantages compared to conventional digestion systems (Lee *et al.*, 2001; Shizas and Bagley, 2002; Wang *et al.*, 2009; Zhang *et al.*, 2000; Zupančič *et al*., 2007; Zupančič and Jemec, 2010):

- Better organic matter removal
- Higher biogas production
- Possible elimination of equalisation tanks and secondary clarifiers
- Relatively simple operations

Despite the high performance of anaerobic treatment, comparatively lower costs and wide use in other industries, the tanning industry has not integrated the process into conventional treatment due to the release of toxic, corrosive and flammable H_2S as the reaction progresses (Buljan and Král, 2011). Hence, for this to be a favoured treatment, the formation of H_2S gas needs to be adequately reduced.

2.4.2 *Potential to treat tannery effluent*

Tannery effluent contains substantial amounts of organic matter so its conversion into biogas holds potential to benefit the tanning industry from an effluent treatment and renewable energy perspective. However, AD reactors are sensitive to parameters such as effluent composition and the presence of toxic compounds, such as phenol and HS^- (Rajeshwari *et al*., 2000).

Microbial consortia require a variety of nutrients and extremely low concentrations of certain ions (e.g. metal co-factors) to support the functional metabolic processes required for effective AD (Rajeshwari *et al*., 2000). Therefore, it is important to characterise the effluent so that macro- and/or micro-nutrients can be added to support AD, if required.

The potential of utilising AD to treat tannery effluent can be better understood by considering inhibitors of the process and the concentrations at which they become inhibitory.

2.4.3 *Inhibitors of anaerobic digestion*

The feasibility of AD technology can be compromised when the microbial processes are inhibited, resulting in a decrease in overall process efficiency. Common disturbances include mixing problems and difficulty in maintaining the correct balance between acid-forming and CH_4-producing microbial communities (Burton *et al.*, 2009). Pertinently, several compounds have been shown to be inhibitory to AD. For example, methanogens are strict anaerobes and are susceptible to even small quantities of oxygen (Bajpai, 2017). Once the exclusion of oxygen is addressed, chromium, sodium and HS^- are most commonly thought to present problems.

2.4.3.1 Sodium chloride

Tannery wastewaters typically contain light metal cations. Moderate amounts of these ions are required for growth, although excessive amounts are inhibitory. Tannery effluent contains sodium chloride (NaCl) concentrations of between 2,500-12,500 mg/ℓ and Jackson-Moss *et al.* (1989) found that a NaCl concentration of up to 17,000 mg/ℓ had no inhibitory effects on AD. Literature values vary widely due to qualitative and quantitative differences in the microbial species and their degree of

acclimation, but half maximal inhibitory concentrations (IC_{50}) of Na range between 5.6-53 g/ℓ, depending on adaptation period, substrate and reactor configuration (Burton *et al*., 2009; Chen *et al*., 2008; Omil *et al*., 1995). Therefore, Na levels in tannery effluent are unlikely to be unsuitable to an acclimatised anaerobic digesting inoculum. However, the history of the sludge and the specific antagonistic and synergistic effects on the microbes can result in reduced performance.

When using a sludge-bed reactor, NaCl concentrations of > 13-15 g/ℓ have been found to hinder granulate development and therefore, should be avoided (Deublein and Steinhauser, 2008). Omil *et al*. (1995) reported that 50% inhibition of methanogens may occur at 6-40 g/ℓ of Na. Approximately 180 kg of Cl^- are generated per ton of raw wet salted hide and while excessive amounts inhibit the growth of plants, bacteria and fish, the concentrations in tannery effluent are typically not excessive (UNIDO, 2016).

2.4.3.2 Chromium and other heavy metals

Heavy metals are of particular concern because, unlike many other toxic substances, they are not biodegradable and can accumulate to toxic concentrations (Sterritt and Lester, 1980). Heavy metal toxicity is attributed to the disruption of enzyme structure and function by binding with groups on protein molecules or replacing other metals in enzyme prosthetic groups (Vallee and Ulmer, 1972). Those identified to have the greatest toxic effect to AD are chromium, iron, cobalt, nickel and cadmium (Abdel-Shafy and Mansour, 2014; Lin, 1992; Mekonnen *et al*., 2017; Thanh *et al*., 2016; Zayed and Winter, 2000). Inhibitory metal concentrations vary widely in literature because of differences in microbial community structures, temperature, synergistic and antagonistic effects, carbon source, sludge characteristics and reactor configurations (Altaş, 2009; Chen *et al*., 2008; Oleszkiewicz and Sharma, 1990).

While Altaş (2009) states that a 50% reduction in the cumulative CH_4 production can occur from 27-3000 mg/ℓ of chromium, Deublein and Steinhauser (2008) report that CH_4 production has still been reported to be high at chromium concentrations of 268 mg/ℓ (Zupančič and Jemec, 2010). Required trace element concentrations for various metals and their inhibition and toxicity threshold concentrations are shown in Table 8.

2.4.3.3 Fatty acids and amino acids

Organic acids may be present in both undissociated and dissociated forms in the substrate and are utilised in methanation. Undissociated acids have greater potential to be inhibitory as they can easily cross cell membranes and denature cell proteins. If the concentration of an organic acid becomes too high, acidification can occur. A decrease in pH intensifies this inhibitory effect as organic acids are more likely to be undissociated. Below neutral pH, the inhibiting threshold for acetic acid is 1000 mg/ℓ. For undissociated iso-butyric acid or iso-valeric acid, the inhibiting threshold can be as low as 50 mg/ℓ for non-adapted cultures (Deublein and Steinhauser, 2008).

2.4.3.4 Ammonium and ammonia

The speciation of ammonia depends on the pH of the wastewater. Ammonia (NH_3) is a weak base and the ammonium (NH_4^+) ion is a weak acid (pK_a = 9.25). Therefore, as the pH increases the equilibrium shifts toward ammonia. The NH_4^+ is practically harmless and can only become inhibitory at NH_4^+-N > 1,500-10,000 mg/ℓ. Ammonia, on the other hand, has an inhibiting threshold of 80 mg/ℓ and

becomes toxic at 150 mg/ℓ. Additionally, it has been found that NH_4-N concentrations of above 1000 mg/ℓ can restrict granulate development in a sludge-bed reactor (Deublein and Steinhauser, 2008).

Table 8: Inhibition and toxicity concentrations of various metals in solution (adapted from Deublein and Steinhauser, 2008; Mudrack and Kunst, 1994; Sahm, 1981)

Substance	Minimum amount required as trace element (mg/ℓ)	Parameter affected	Concentration at which inhibition begins (mg/ℓ)		Toxicity for adapted MO (mg/ℓ)
			Free ions*	Carbonate*	
Cr	0.005-50	-	28-300	530	500
Fe	1-10	-	N/A	1750	N/A
Ni	0.005-0.5	-	10-300	N/A	30-1000
Cu	Essentially with acetogenic MO	-	5-300	170	170-300
Zn	Essentially with acetogenic MO	-	3-400	160	250-600
Cd	N/A	-	70-600	180	20-600
Pb	0.2-200	-	8-340	N/A	340
Na	N/A	pH - Wert	5 000-30 000	N/A	60 000
K	N/A	Osmosis of CH_4 formers	2500-5 000	N/A	N/A
Ca	N/A	Long-chain fatty acids	2 500-7 000	N/A	N/A
Mg	Essentially with acetogenic MO	Fatty acids	1 000-2 400	N/A	N/A
Co	0.06	-	N/A	N/A	N/A
Mo	0.05	-	N/A	N/A	N/A
Se	0.008	-	N/A	N/A	N/A
Mn	0.005-50	-	1 500	N/A	N/A
HCN	0.0	-	5-30	N/A	N/A
C_6H_6O	Inhibiting until microorganisms are adapted, then all is completely degraded.				

*The level of inhibition depends on whether the metals are present as ions or carbonates.
N/A – not applicable; MO – microorganisms.

2.4.3.5 Sulphide

High concentrations of sulphide are toxic to many microbes. Multiple publications have quantified the inhibiting effect of sulphide in anaerobic wastewater treatment (Hulshoff Pol *et al*., 1998; Wiemann *et al*., 1998). However, results for the inhibitory concentration differ widely. There are a number of factors that have been postulated to be responsible (Wiemann *et al*., 1998):

- Two inhibiting effects of HS^- or BSR on AD are known and the extent of each depends on the experimental system:
    1. Direct toxicity of H_2S
    2. Precipitation of trace elements by HS^- (Loka Bharathi et al., 1990)
- Direct toxicity exclusively correlates with the amount of undissociated sulphide (H_2S), because only uncharged molecules can pass through the cell membrane without an active transport

mechanism (Boshoff *et al.*, 2004; Hulshoff Pol *et al.*, 1998). H_2S impedes cellular activity by denaturing the microbes' native proteins, obstructing coenzyme HS^- linkages and interfering with the assimilatory HS^- metabolism, as well as intracellular pH (Burton *et al.*, 2009; Hulshoff Pol *et al.*, 1998; Parkin and Owen, 1986). Due to the degree of dissociation being mostly dependent on the pH, this needs to be known when H_2S toxicity is quantified. For speciation of sulphide at varying pH, see Figure 6, Section 2.5.3.

- The extent of inhibition is incumbent on the type of substrate and the functional microbial consortia that are present.

Therefore, it can be seen that the accumulation of sulphide can cause severe inhibition of the process (Hulshoff Pol *et al.*, 1998). Fermentative bacteria are less susceptible to direct H_2S toxicity than acetogenic bacteria, while methanogens are the most susceptible (Maillacheruvu *et al.*, 1993; McCartney and Oleszkiewicz, 1991; Oyekola, 2008). Literature shows considerable discrepancy with respect to exact levels of H_2S and HS^- that cause inhibition to the various trophic groups, in part due to information on pH rarely being included (Chen *et al.*, 2008). The published IC_{50} values range between 50-250 mg H_2S/ℓ for methanogens, with higher IC_{50} values for lower pH values (Burton *et al.*, 2009; Chen *et al.*, 2008; McCartney and Oleszkiewicz, 1993). While the lower end of the aforementioned range has been found to be true for acetoclastic methanogens (acetophilic) with their complete inhibition occurring at free H_2S concentrations of 200 mg/ℓ (Kroiss and Wabnegg, 1983).

Methanogens are inhibited to a significantly greater extent than SRB above a pH of 7.8, while the degree of inhibition below a pH of 7.0 is very similar for both groups (Koster *et al.*, 1986).

On the other hand, microorganisms can adapt to sulphide, where some acetoclastic and hydrogenotrophic methanogens are only significantly inhibited at concentrations greater than 1000 mg/ℓ free H_2S (Deublein and Steinhauser, 2008; Isa *et al.*, 1986a). In terms of total dissolved HS^-, inhibitory effects have been recorded for a range as wide as 150-1100 mg/ℓ (Omil *et al.*, 1995). Reactor type and configuration can have an impact on the susceptibility to sulphide inhibition. For example, H_2S toxicity is experienced at lower concentrations in suspended growth systems than in anaerobic filters (Maillacheruvu *et al.*, 1993; Parkin *et al.*, 1991).

Due to toxic and corrosive H_2S gas being the product of BSR, avoiding BSR altogether could be considered most advantageous for AD processes. Specific inhibitors such as a SO_4^{2-} analog, transition elements or antibiotics to selectively suppress BSR have not been found for full-scale anaerobic reactors. Therefore, BSR cannot be prevented in practice and as a result, ideally should take place prior to methanogenesis with subsequent/combined removal of the sulphide product to avoid the possibility of substrate competition and sulphide inhibition (Hao et al., 2014; Hulshoff Pol et al., 1998).

If up-front removal of SO_4^{2-} is not an option, organic matter removal in the presence of BSR is possible, whereby both the SRB and the methanogens will digest the organic matter (Hulshoff Pol *et al.*, 1998). The extent of competition between methanogens and SRB has been observed to be related to the initial microbial concentration of each and the COD:SO_4^{2-} ratio (Burton *et al.*, 2009). Sulphate reducing bacteria dominate at ratios below 1.7 and methanogens above 2.7, with active competition occurring in between (Burton *et al.*, 2009). However, AD has been shown to proceed successfully for a COD:SO_4^{2-} ratio exceeding 10 (O'Flaherty et al., 1998; Rinzema and Lettinga, 1988; Sabumon, 2016). Therefore, provided the initial conditions are favourable, methanogens can outcompete SRB and AD can be

successful. On the other hand, if the initial conditions are unfavourable, the risk of HS^- inhibition increases and pre-treatment of the SO_4^{2-} and HS^- is necessary.

2.5 Sulphur species removal

Tannery effluent contains HS^- and SO_4^{2-} in concentrations as high as 500 mg/ℓ and 2444 mg/ℓ, respectively (Section 2.2). Sulphur species are problematic in an anaerobic environment and reduce the potential of AD. The following section discusses the various forms of sulphur, the intricacies of the reduction of SO_4^{2-} and oxidation of HS^-, and the technologies available for their removal from tannery wastewater.

2.5.1 *Sulphur cycle*

Sulphur is an example of an element whose state and transformation in nature are critically dependent on microbial activities. Sulphur plays an important role as a structural element, redox centre (electron carrier), carbon carrier, and in the generation and conservation of biochemical energy (Klotz *et al.*, 2011). This is due to its chemical properties, and principally, its range of stable redox states which are included in Table 9.

Table 9: Oxidation states of sulphur in common compounds (Steudel, 2000)

Compound	**Formula**	**Oxidation state**
Sulphide & other organic sulphhydryl groups	H_2S	-2
Disulphane	H_2S_2	-1
Elemental sulphur	S	0
Thiosulphate	$S_2O_3^{2-}$	+2
Dithionite	$S_2O_4^{2-}$	+3
Sulphite	SO_3^{2-}	+4
Dithionate	$S_2O_6^{2-}$	+5
Sulphate	SO_4^{2-}	+6

Hence, sulphur is constantly being transformed from one oxidation state to another. The microbial sulphur cycle is shown in Figure 5.

Bisulphide (HS^-) is formed by various microbial processes: the respiration of SO_4^{2-} and partially oxidised sulphur species, known as dissimilatory sulphur compound reduction; disproportionation reactions; and during the decomposition of sulphur-containing amino acids in organic material, known as desulfurylation.

Sulphur-oxidising prokaryotes partially oxidise sulphide to intermediate sulphur species, such as elemental sulphur, sulphite (SO_3^{2-}) and thiosulphate ($S_2O_3^{2-}$), or completely oxidise it to SO_4^{2-}. Through the recycle of reduced sulphur compounds back into their oxidised form, anaerobic microorganisms are able to use them as electron acceptors (Lenk, 2011).

The green path shown in Figure 5 demonstrates what commonly occurs in the microbial reduction of SO_4^{2-} and oxidation of HS^-, as will be discussed in Sections 2.5.2 and 2.5.3.

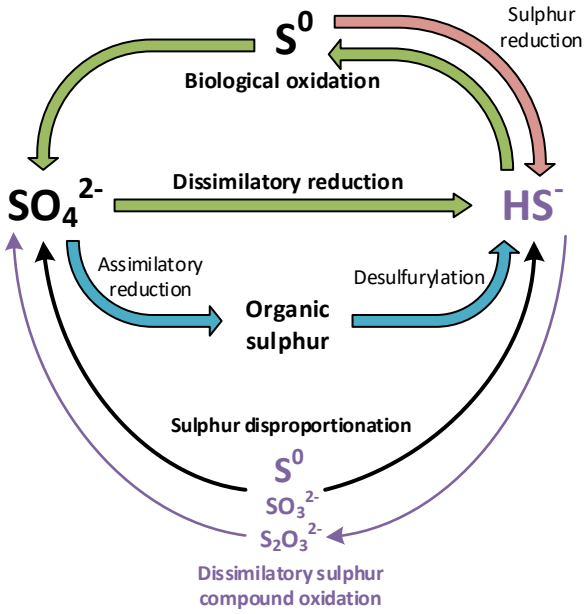

Figure 5: The microbial sulphur cycle (adapted from Brüser et al., 2000; Lenk, 2011)

2.5.2 *Sulphate reduction*

Sulphate itself is chemically inert, non-volatile and non-toxic (Hulshoff Pol *et al.*, 1998). Although, high concentrations of SO_4^{2-} can cause an imbalance in the sulphur cycle (Figure 5) and increased salinity can be a problem for agriculture, as well as the cost of treatment to potable quality.

2.5.2.1 Dissimilatory sulphate reduction

Under anaerobic conditions, SO_4^{2-} is used as a terminal electron acceptor in the degradation of organic matter for bioenergetic purposes, similarly to the way oxygen (O_2) is used under aerobic conditions, except with the expulsion of sulphides instead of CO_2 (Sabumon, 2016). This process is called dissimilatory sulphate reduction or sulphidogenesis, where HS^- is not assimilated into the bacteria.

The bacteria that mediate this process are known as sulphate reducing bacteria (SRB) (Sabumon, 2016). Hence, the problem that arises from the anaerobic treatment of SO_4^{2-} containing effluent is the resultant production of toxic HS^-. When effluent laden with SO_4^{2-} is discharged into the environment this can result in the formation of acid water and malodour due to the H_2SO_4 and HS^- that can form (UNIDO, 2016).

2.5.2.2 Suitable habitats for sulphate reducing bacteria

Sulphate reducing bacteria are found in anoxic sediments, oil deposits, deep sea hydrothermal vents, acid mine water and hypersaline lakes, a wide range of environments are suitable for the growth of SRB (Fauque, 1995). Dissimilatory SO_4^{2-} reduction or methanogenesis are the major degradation processes in anaerobic environments, with methanogenesis dominating in SO_4^{2-}-poor environments and SRBs in SO_4^{2-}-rich habitats, often in freshwater and marine environments, respectively (Fauque, 1995; Widdel, 1988). Sulphate reducing bacteria are able to survive temperatures ranging from 0˚C to at least 100˚C, pH values spanning 3-9.8 and salinities from freshwater to hypersaline conditions (Mackenzie, 2005). However, generally, the sulphate reduction rate (SRR) is inversely proportional to salinity (Kerkar and Loka Bharathi, 2007; Sørensen *et al.*, 2004).

Most of the halophilic SRBs isolated are marine or slightly halophilic with an optimum salinity range between 1-4% NaCl (Fauque, 1995; Ollivier *et al*., 1994). For reference, seawater typically has a salinity of 3.5%. Moderately halophilic species have an average optimum salinity of 6-10% NaCl (Caumette *et al.*, 1991). Further, in spite of their obligatory anaerobic metabolism, SRB can be found in aerobic habitats (Dworkin et al., 2006; Wieringa et al., 2000). These bacteria are highly adaptable and therefore, pervasive.

2.5.2.3 Substrates utilised by sulphate reducing bacteria

Many different organic substrates can be used by SRB as electron donors, including H_2, formate, acetate, propionate and higher fatty acids, branched fatty acids, lactate, methanol, ethanol and higher alcohols, fumarate, malate and aromatic compounds. The rate of BSR is considerably effected by the electron donor used, and therefore reported hydraulic retention times (HRTs) have a range of 1-480 hours (Hao *et al*., 2014).

While acetate is a common electron donor, an increasing concentration can inhibit SRBs. A study found that SRB growth was inhibited by 50% at a concentration of about 54 mg/ℓ of undissociated acetic acid (Reis *et al*., 1990). Lactate and propionate are the most favourable substrates when considering biomass yield, energy release, production of alkalinity and heterotrophic biological sulphur conversion (Das *et al.*, 2013; Liamleam and Annachhatre, 2007; Van Kuijk and Stams, 1995). However, as the various species of SRBs demand different substrates for their metabolisms, a mixture of electron donors is favourable for SRB growth (Hao *et al.*, 2013; Kleikemper *et al*., 2002; Waybrant *et al*., 1998).

To date, there has been no correlation found between substrate and SRB genus, where species within a single genus are capable of using different substrates (Hao *et al*., 2014). The most commonly found SRB genera when treating wastewater laden with SO_4^{2-} in bioreactors are incomplete organic oxidisers *Desulfovibrio*, *Desulfobulbus* and *Desulfomicrobium* and, the only dominant complete organics oxidiser, *Desulfobacter* (Hao *et al.*, 2014).

Beyond the reduction of SO_4^{2-}, the reduction of other oxidised sulphur electron acceptors, SO_3^{2-} and $S_2O_3^{2-}$, is also very common amongst SRB (Visser, 1995). NO_3^-, nitrite (NO_2^-) and other compounds are also suitable as electron acceptors for some SRB (Hao *et al*., 2014).

Therefore, while SRBs use inorganic molecules as their electron acceptor or energy source, they use organic molecules as electron donors and carbon sources, making them chemoorganotrophic heterotrophs (Atlas, 1997).

Equations 2 and 3 show the typical overall conversion of SO_4^{2-} by SRBs using each acetate and lactate, respectively, as carbon sources (Oude Elferink, 1998; Thauer *et al.*, 1977):

$$SO_4^{2-} + acetate^- = HS^- + 2HCO_3^- \qquad (2)$$

$$(\Delta G° = -47.6 \frac{kJ}{mol\ SO_4^{2-}})$$

$$^1/_2\, SO_4^{2-} + lactate^- = {}^1/_2\, HS^- + acetate + HCO_3^- + {}^1/_2\, H^+ \qquad (3)$$

$$(\Delta G° = -80.0 \frac{kJ}{mol\ SO_4^{2-}})$$

Theoretically, when organic material is oxidised through BSR, one molecule of SO_4^{2-} can accept 8 electrons. One molecule of oxygen can accept only 4 electrons, so the electron accepting capacity of 2 moles of O_2 equates to that of 1 mole of SO_4^{2-}. This equals 0.67 g of O_2 per gram of SO_4^{2-} (Choi and Rim, 1991; Lens et al., 1998a). Consequently, wastewater streams with a COD/SO_4^{2-} ratio of 0.67 have enough available SO_4^{2-} to remove the organic matter present completely via BSR. It follows that when the COD/SO_4^{2-} ratio in an effluent is lower than 0.67, there is insufficient organic matter to reduce all the SO_4^{2-} present and extra substrate would be required to remove all the SO_4^{2-}. On the other hand, if the ratio is greater than 0.67, all the organic material can only be removed if another reaction, such as methanogenesis, is also carried out (Lens *et al.*, 1998a).

2.5.2.4 Competition with other species

In an anaerobic environment, for an effluent containing both SO_4^{2-} and abundant organic matter, the organics are likely to be removed via both BSR and AD processes (i.e. there may be competition for the organic matter substrates they share) (Section 2.5.2.2). There are a multitude of factors that affect this competition, including the composition of the feed, the concentration of substrates and SO_4^{2-}, the pH value within the reactor, presence of inhibitors, as well as the type of seed sludge, its populations and their degree of acclimation (Chen *et al.*, 2008; Lens *et al.*, 1998a).

From a thermodynamic and kinetic perspective, acetoclastic SRB (ASRB) (acetate-utilising SRB) have an advantage over acetoclastic methanogens, whereby ASRB gain more energy from the consumption of acetate and have higher growth rates than methanogens (Lens *et al.*, 1998a). This is especially so at low acetate concentrations as ASRB have a higher affinity (lower K_m) and therefore a lower threshold value for acetate than methanogens, which allows ASRB to maintain acetate at concentrations lower than methanogens are able to utilise (Colleran et al., 1995; Isa et al., 1986b; Rinzema and Lettinga, 1988; Sabumon, 2016). However, it has been concluded that acetoclastic methanogens can often predominate in AD systems because acetate, as well as hydrogen, is a key intermediate through which organic matter is channelled and therefore, is often at concentrations above acetoclastic methanogens' threshold value. In addition, SRB have a lower affinity for acetate than they do for other substrates (Colleran and Pender, 2002; Sabumon, 2016).

Despite the extensive research over the past four decades, literature data on the topic is still contradictory, owing to the various factors at play and the variety of conditions employed. Therefore, practical guidelines to direct the outcome of the competition between methanogens and SRB have yet to be clarified (Chen *et al.*, 2008; Lens *et al*., 1998a).

There are indications that ASRB have a lower affinity for SO_4^{2-} in comparison to hydrogenotrophic SRB (HSRB) and therefore, HSRB outcompete the ARSB for available SO_4^{2-} and leave acetate in the effluent (Lens *et al*., 1998b; Nedwell and Reynolds, 1996; Omil *et al*., 1996). This is an advantage for the sequential AD of this effluent where methanogens use acetate as their primary substrate. Conversely, this is a drawback when there is no subsequent AD process stage and then acetate-scavenging processes need to be integrated into the ETP to lower the residual COD in the effluent (Lens and Hulshoff Pol, 2000). Nitrification or staged reactors which allow the development of chiefly acetotrophic biomass in the later stages are commonly suggested (Lens *et al.*, 1998b).

2.5.2.5 *Sulphide inhibition of biological sulphate reduction*

Relatively little attention has been paid to the inhibition of SRBs by H_2S in comparison to its effect on methanogenesis, nevertheless, van Houten *et al*. (1997, 1994) reported that SRB growth becomes inhibited at 250 mg/ℓ H_2S and that growth was possible up to 450 mg/ℓ. On the other hand, Hao *et al*. (2014) found that the activity of SRB is inhibited when H_2S concentrations become higher than 60-70 mg/ℓ. Complete inhibition was observed for a free H_2S concentration of 547 mg/ℓ for a *Desulfovibrio* sp. culture growing on lactate and SO_4^{2-} at a pH of ca. 6.5 (Reis *et al*., 1992). Brahmacharimayum *et al*. (2019) have listed various toxicity levels of HS^- for a variety of cultures and reactor types, ranging 34-2767 mg/ℓ. It has been confirmed that even when completely inhibited by sulphide, the microbes recover from this toxicity entirely once it is removed from the reactor (Okabe *et al*., 1995; van Houten *et al*., 1997, 1994). Once again, adaptation or acclimation to high sulphide concentrations will be crucial to effective treatment of sulphur species laden wastewater.

Therefore, serious problems occur when SO_4^{2-}-rich wastewaters, such as tannery effluents, are treated anaerobically. However, when used together with a biological HS^- removal step, AD can also serve as a method to remove SO_4^{2-} and other oxidised sulphur compounds from effluents, in addition to the subsequent more amenable generation of biogas (Hulshoff Pol *et al*., 1998).

2.5.3 *Sulphide oxidation*

Sulphide itself presents a number of problems when it is present in anaerobic reactor systems, increasing the complexity of biodegradation routes considerably (Hulshoff Pol *et al.*, 1998):

- Corrosion
- Potential toxicity
- Malodour
- Reduced CH_4 production
- Poor biogas quality, requiring the removal of H_2S
- Accumulation of inert material (such as metal sulphides) in the sludge

2.5.3.1 *Sulphide speciation*

Hydrogen sulphide is corrosive, toxic and highly malodourous and therefore, directly threatens the environment. The toxicity of sulphide is pH dependent as only the unionised form can pass through cell membranes, meaning that free H_2S gas is more toxic than other sulphide species. Hydrogen sulphide dissociates in water according to Equations 4 and 5 (Garrels *et al*., 1965):

$$H_2S \leftrightarrow H^+ + HS^-; (K_1 = 1.0 \times 10^{-7}) \quad (4)$$

$$HS^- \leftrightarrow H^+ + S^{-2}; (K_2 = 1.0 \times 10^{-14}) \quad (5)$$

When the pH is around 8-9, practically all dissolved sulphide is present as the bisulphide ion (HS^-), an ionised form (Sabumon, 2016). In wastewater, the concentration of the sulphide ion (S^{2-}) is typically negligible as the pH is rarely above 9 (Cadena and Peters, 1988; Sabumon, 2016). At neutral pH, as is typical of AD systems, between 20-50% of the dissolved sulphide is present as undissociated H_2S (Sabumon, 2016). Hence, small variations in the pH between 6-8 significantly affect the free H_2S concentration (Figure 6) (Hulshoff Pol *et al.*, 1998).

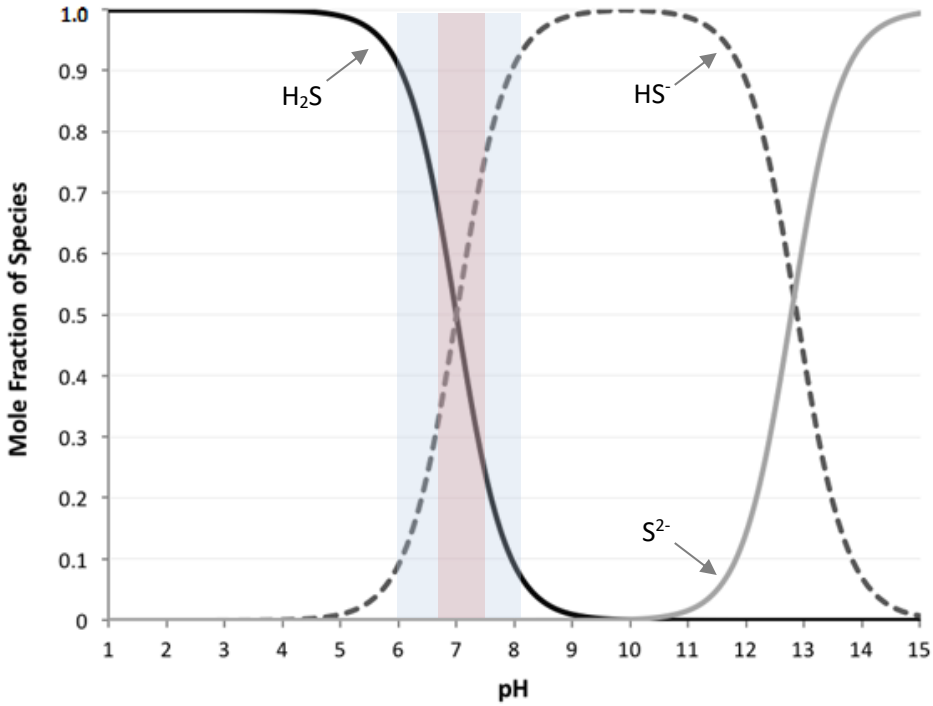

Figure 6: Distribution of sulphide forms for different pH values (adapted from Applied Analytics, 2020; Lens et al., 1998a).
Light shade = anaerobic digestion range; darker shade = methanogenic digestion optimal range.

Only the gaseous form of H_2S is malodourous, while HS^- and S^{2-} can only exist in solution and do not contribute to odours (Robert and McVay, 2011). In a bioreactor, H_2S will be distributed over the gas and liquid phase following this relationship governed by Henry's Law, given in Equation 6 (Hulshoff Pol *et al*., 1998):

$$[H_2S]_l = \propto \cdot [H_2S]_g \ (mol/m^3) \qquad (6)$$

Where $\propto$ is a dimensionless distribution coefficient. In the liquid phase, the total dissolved sulphide is present as both the unionised and the ionised form, depending on the pH and the temperature (Hulshoff Pol *et al*., 1998). The absorption coefficient $\propto$ of H_2S is about 1.99 at 30˚C, meaning that the concentration of H_2S in the liquid phase is almost twice that of H_2S in the gas phase at 30˚C (Lens *et al*., 1998a).

2.5.3.2 Aerobic sulphide oxidation

Apart from popular conventional methods such as the use of stripping and precipitation to remove HS^- from wastewater, both aerobic and anaerobic biological oxidation to elemental sulphur (S^0) have been investigated. In an aerobic HS^- removal system, the following overall biological reaction given by Equations 7 and 8 takes place (Lens *et al*., 1998a; Midha and Dey, 2008; Qaisar *et al*., 2007):

$$2\,HS^- + O_2 \rightarrow 2\,S^0 \downarrow + 2\,OH^- \qquad (\Delta G° = -129.5 \frac{kJ}{mol\ HS^-}) \qquad (7)$$

$$S^0 + H_2O + \frac{3}{2}\,O_2 \rightarrow SO_4^{2-} + 2\,H^+ \qquad (\Delta G° = -592.9 \frac{kJ}{mol\ S^0}) \qquad (8)$$

As can be seen in the reactions, the degree of oxidation, to either S^0 or to SO_4^{2-}, is dependent on the amount of oxygen in the system. The production of S^0 requires four times less O_2 than the full oxidation

to SO_4^{2-}. When the system is under O_2 limited conditions, S^0 is the primary end product of SO, on the other hand, SO_4^{2-} is produced when HS^- is limiting (Janssen *et al*., 1997, 1995). Comparing the Gibbs free energy (ΔG°) of Equation 7 to the sum of Equations 7 and 8, it is most energetically favourable for sulphide oxidising bacteria (SOB) to fully oxidise HS^- to SO_4^{2-} instead of partially to S^0 via just Equation 7.

The equilibrium of sulphur species at each pH and redox potential is shown in Figure 7. The shaded shape on the figure illustrates the small window of opportunity for the occurrence of partial oxidation of HS^- to S^0. In reality, the formation of S^0 is even less likely due to most microbial processes occurring closer to neutral pH. This highlights the requirement for fine control of O_2 availability and redox potential. In an active system, tight process control is required.

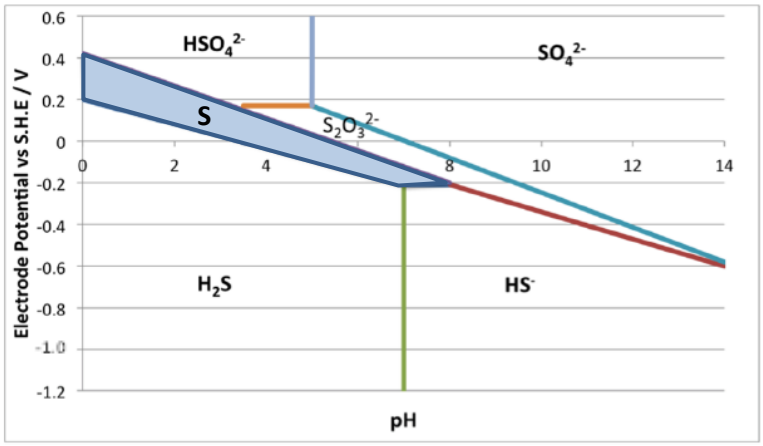

Figure 7: Potential-pH diagram for sulphur-water system at 298.15 K (adapted from Mooruth, 2017)

2.5.3.3 Colourless sulphur bacteria

Colourless SOB are predominantly bacteria but also exist in both archaea and eubacteria domains, and are commonly referred to as SOBs. Colourless sulphur oxidising bacteria are diverse in genera: *Achromatium, Acidianus., Acidothiobacillus, Beggiatoa, Leptospirillum, Macromonus, Sulfolobus, Thermothrix, Thiobacterium, Thiodendron, Thiomicrospira, Thiothrix* and *Thiovulum.* Members of these genera require different pH and temperature conditions for growth (Alesia, 2015). The oxidation process may use O_2, NO_3, manganese (IV) or iron (III) as terminal electron acceptors (Alesia, 2015). Some of the aforementioned organisms are also capable of denitrification (Ito *et al.*, 2004; Nielsen *et al*., 2000).

The colourless SOB can be either aerobic or anaerobic, with anaerobic bacteria using H_2 or iron (III) as electron acceptors (Alesia, 2015). Aerobic chemotrophic microorganisms can use inorganic carbon (CO_2) as a carbon source, and oxidise reduced inorganic compounds (such as HS^-) for chemical energy (Atlas, 1997). The simple nutritional requirements and the high H_2S tolerance of chemotrophic SOB favours their use in biotechnological SO technologies (Midha and Dey, 2008).

Several microbes have been studied for their ability to remove H_2S. Of these, *Thiobacillus* species and the heterotroph *Pseudomonas putida* are the most commonly utilised and extensively studied (Midha and Dey, 2008; Nelson and Hagen, 1995). The genus *Thiobacillus* includes nearly all non-photosynthetic, rod-shaped chemoautotrophs that perform sulphur compound oxidation and CO_2 fixation (Nelson and Hagen, 1995).

2.5.3.4 Photosynthetic sulphur bacteria

Some anaerobic, phototrophic microorganisms use CO_2 as a carbon source and light for energy (Midha and Dey, 2008). Purple sulphur bacteria (PSB) are bacteria that oxidise various inorganic reduced sulphur compounds, such as HS^-, polysulphides, $S_2O_3^-$, tetrathionate ($S_4O_6^-$), S^0 or SO_3^- for energy and are predominantly living by anoxygenic photosynthesis (Canfield *et al*., 2005; Megonigal *et al*., 2003). While most are obligate anaerobes, some species are capable of tolerating low concentrations of oxygen and can grow chemolithoautotrophically in the absence of light, using oxygen to oxidise HS^- (Pfennig, 1989). The PSB group is split into the *Chromatiaceae* and the *Ectothiohodospiraceae* families.

Green/brown sulphur bacteria (GSB) consist of only the *Chlorobiaceae* family and are obligate anaerobic phototrophs that use a range of electron donors (HS^-, S^0, $S_2O_3^-$ or H_2) for anoxygenic photosynthesis (Camacho, 2009). Both PSB and GSB produce S^0 as an intermediate product of SO (van Gemerden, 1986).

Under limiting light and CO_2 conditions, S^0 is the major product of HS^- oxidation (Equation 9) (Larsen, 1952). While, in the presence of abundant light and CO_2, the ultimate product of HS^- oxidation for both of these groups is the complete oxidation to SO_4^{2-} after always first forming SO_3^{2-} (Equation 10) (Alesia, 2015; Larsen, 1952; van Gemerden, 1986).

$$CO_2 + 2H_2S \rightarrow CH_2O + H_2O + 2S^0 \quad (9)$$

$$2CO_2 + H_2S + 2H_2O \rightarrow 2(CH_2O) + H_2SO_4 \quad (10)$$

Of these groups, the *Chromatiaceae* family store their produced S^0 internally in sulphur granules while the others deposit the produced S^0 extracellularly (van Gemerden, 1986). In most cases, internally stored S^0 granules make separation of cells and S^0, and therefore S^0 reclamation, impossible (Kleinjan *et al*., 2003).

If S^0 recovery is required, Kleinjan *et al.* (2003) recommends the use of only microbes that excrete S^0. Johnson (2000) found that the GSB, *Chlorobium limicola*, when used in SO bioreactors could achieve a 90% conversion of HS^- to S^0. While highly successful, the amount of light required by phototrophs on the scale necessary for industry effluent treatment results in high operating costs and restricts their use (Midha and Dey, 2008).

2.5.4 *Technologies available for sulphate and sulphide removal from wastewater*

A diverse range of technologies have been developed to treat SO_4^{2-} and HS^- in industrial wastewaters. Some have been applied specifically to tannery effluent. In these cases, the most appropriate technique depends on the particular tannery, the volume of effluent and the concentration of the types of contaminants (Lens *et al.*, 1998a).

In practice, the reduction of SO_4^{2-} cannot be prevented, especially when using AD for the removal of organic matter. The selective inhibition of SRB by using SO_4^{2-} analogs like molybdate, as well as transition metals and antibiotics has been unsuccessful at full scale (Hao et al., 2014; Lens et al., 1998a). The aim of the treatment, whether the removal of organic matter, SO_4^{2-}, HS^- or all three, determines the treatment strategy. Wastewaters need to have a COD/ SO_4^{2-} ratio of at least 0.67 to remove all SO_4^{2-} by using SRB (Brahmacharimayum et al., 2019; Choi and Rim, 1991; Lens et al., 1998a). The resultant production of HS^- necessitates the coupling of a SO removal step to the BSR step; this

can be performed in two separate reactors or combined into one reactor, as discussed in Section 2.5.4.5.

Treatment methods can essentially be divided into active and passive treatment types and further into biotic and abiotic treatment methods.

2.5.4.1 Active treatment

Active treatment typically involves a reactor system that requires constant energy input, such as the use of agitation or active aeration, and skilled operators. Similarly, active treatment systems often require continual addition of chemicals, which can become costly with the voluminous quantities of effluent to be treated (Mooruth, 2013).

2.5.4.1.1 Active physicochemical treatment

Several chemical and physicochemical treatment techniques can be applied for the removal of SO_4^{2-} from wastewater. Implementation of these end of pipe treatments typically first require exhaustive conventional primary and secondary treatment of the effluent, after which membrane processes like RO, electrodialysis and nanofiltration (NF) can be carried out (Galiana-Aleixandre *et al.*, 2011; Lens *et al.*, 1998a; Suthanthararajan *et al.*, 2004). Despite the extensive pre-treatment, severe fouling of the membrane may occur and post-treatment of the wastewater is required, which, together with the initial high capital cost, makes these techniques expensive (Galiana-Aleixandre *et al.*, 2011; Lens *et al.*, 1998a).

In this type of effluent, ion exchange is not economically viable due to the high concentration of various other ions and the high flow rate required to treat the large volumes of effluent (Galiana-Aleixandre *et al.*, 2011). Biological SO_4^{2-} removal is a cost-effective alternative for high-strength wastewaters like tannery effluent (Lens et al., 1998a; Sabumon, 2016). This will be discussed further in Section 2.5.4.1.2.

In South Africa, the chromium-rich tanyard stream is not usually mixed with the HS^--rich beam house effluent, with the latter kept at a high pH until the HS^- is treated (Naturgerechte, 2002; Swartz *et al.*, 2017). A number of physicochemical methods, such as direct air stripping, chemical precipitation and oxidation, are utilised to achieve the removal of HS^- (Midha and Dey, 2008). Air stripping is best carried out on effluents with a low concentration of HS^-, due to high energy costs, which is not common in the case of tannery wastewater. The gas phase carrying the removed HS^- requires an effective and consequently, a costly gas purification system that is capable of the recovery of sulphur (Berardino, 2009; Verink, 1988). The S^0 recovery unit often includes a stripper column, absorber column, regeneration unit and sulphur separator (Midha and Dey, 2008). Further, stripping methods require close regulation of the pH to minimise the release of CO_2 from the effluent, the concentration of which can vary from 2% (pH 8) to 30% (pH 7), thereby disturbing alkalinity equilibria and eventually resulting in reactor instability (Lens *et al.*, 1998a). Integrating an UASB with a stripper has resulted in a HS^- removal efficiency of 65-95% for a HS^- load of 1-10 kg/day (Suthanthararajan *et al.*, 2004).

Many metals, including iron, zinc and copper, can precipitate out the HS^- as insoluble metal sulphides. While simple, inexpensive and effective, this creates a lot of sludge that needs to be separated from the effluent, dried and disposed of (Midha and Dey, 2008; Valeika *et al.*, 2006).

The various oxidation processes include aeration, chlorination, ozonation, catalytic chemical oxidation or partial biological oxidation. The oxidation method selected depends on its cost and level of toxicity it may impart to the wastewater (Valeika *et al*., 2006). In the acidic range, oxidation of H_2S is incomplete and results in the formation of elemental sulphur, provided excessive doses of oxidant are not used (see Figure 7) (Cadena and Peters, 1988). In the basic range, full oxidation of HS^- to SO_4^{2-} occurs. In practice, one of the most common ways of oxidising the H_2S in the beam house effluent is by adding H_2O_2 (Valeika *et al*., 2006). The H_2S is oxidised by the H_2O_2 to elemental sulphur as in Equation 11 if the pH is below 7.5 (Midha and Dey, 2008):

$$8\,H_2S + 8\,H_2O_2 \rightarrow S_8 + 16\,H_2O \qquad (11)$$

When 2.4 g of H_2O_2 is used per gram of free sulphide in sewerage wastewater, the reaction goes to 97% completion in 30 minutes (Cadena and Peters, 1988). This is a relatively short amount of time for such a successful reaction. However, the high chemical costs of this method make it unattractive for large scale ETPs (Valeika *et al.*, 2006). Similarly, aeration is time intensive and demands energy dissipation for circulation of the wastewater (Valeika *et al.*, 2006). Oxidation with air using a manganese catalyst is an acceptable and highly effective method in practice, allowing removal of HS^- with relatively inexpensive materials, yet the process is time consuming and expensive and the catalysts may contribute to the pollution load in the wastewater after treatment (Midha and Dey, 2008; Valeika *et al.*, 2006).

2.5.4.1.2 Active biological treatment

Biological SO_4^{2-} and HS^- removal have the potential to be economically viable alternatives to other, costly, active chemical processes. Microbes may also partially oxidise HS^- to S^0, which allows for sulphur recovery. As S^0 is insoluble, it can be harvested and used as a feedstock in chemical, fertiliser and materials manufacturing industries if not recycled back into the tanning process (Midha and Dey, 2008). Tannery effluent has a high organic load relative to other wastewaters and without it, there would likely be prohibitively high costs incurred to purchase substrate for the biological removal of sulphur species (Mooruth, 2013). Additionally, due to the high organic load, there may be competition between SRB and methanogens under anaerobic conditions, and the removal efficiencies of SO_4^{2-} and COD will depend on the biodegradability of the COD and the operating conditions (Galiana-Aleixandre *et al.*, 2011).

Several microbes have been studied for their ability to remove SO_4^{2-} and HS^- as mentioned in Sections 2.5.2 and 2.5.3. However, there is relatively little published literature of this being performed under saline conditions, in tannery wastewater, or beyond laboratory scale (Giordano *et al.*, 2015; Hao *et al*., 2014; Omil *et al.*, 1995; Zhao *et al*., 2011).

Various reactor types that have been used for BSR are shown in Table 10. Several factors can affect the performance of BSR bioreactors, as shown in Table 11, most effecting microbial growth and activity, and selection of species.

Boshoff *et al.* (2004) evaluated a pilot scale waste stabilisation pond (WSP) system for the treatment of tannery effluent and acid rock drainage (ARD). They compared an UASB, a CSTR and a trench reactor (TR) for their ability to treat ARD by first assessing whether tannery effluent can be used as a carbon source for BSR. The UASB reactor subsequently became a CSTR when stirred. The TR is a semi-passive

BSR treatment technique and will be discussed in Section 2.5.4.3. All three reactors achieved 60-80% SO_4^{2-} removal for a SO_4^{2-} feed concentration of up to 1800 mg/ℓ. The UASB achieved an 80% SO_4^{2-} removal efficiency with SO_4^{2-} and COD removal rates of 600 and 500 mg/ℓ.day, respectively. The CSTR investigated obtained the lowest SO_4^{2-} removal rate of 250 mg/ℓ.day and therefore, the lowest COD removal rate of 200-600 mg/ℓ.day.

Table 10: Summary of reactor types and sulphate removal efficiencies in anaerobic digestion of sulphate-rich wastewaters (adapted from Lens et al., 1998a)

Reactor type	**Influent**			**SO_4^{2-} removal**		**Reference**
	Type	COD (g/ℓ)	SO_4^{2-} (g/ℓ)	% SO_4^{2-} reduced	% COD by SRB	
CSTR	Molasses	40.9	4.2-5.1	38-71	3-8	Hilton and Archer (1988)
UASB	Acetate	1.5-2.1	0.7-3.4	70	50-90	Visser *et al.* (1993)
EGSB	Acetate/propionate/butyrate	0.5-2.5	1.2-4.6	27-68	59-97	Omil *et al.* (1996)
USSB	Acetate/propionate/butyrate	0.5-6	1-12	35	67-81	Lens *et al.* (1998b)
MUSB	Acetate	0.2-0.4	0.1-0.2	40-80	100	Arora *et a*l. (1995)
FSB	Cane juice silage	26	1.5	95	4	Callander and Barford (1983)
AF	Citric acid	25.8	3.4	93	18	Colleran *et al.* (1994)
BER	Glucose	0.4	0.7-3	35-55	n.d.	Watanabe *et al.* (1997)
Hybrid	Landfill leachate	19.6-42	5.9	>90	n.d.	Nedwell and Reynolds (1996)
CAD	Sea food	10-60	0.6-2.7	96	3-12	Omil *et al.* (1995)

CSTR – continuously stirred tank reactor; UASB – up-flow anaerobic sludge bed reactor; EGSB – expanded granular sludge bed reactor; USSB – up-flow staged sludge bed reactor; MUSB – microaerophilic granular sludge bed reactor; FSB – flocculant sludge bed reactor; AF – anaerobic filter; BER – Bio-electro reactor; Hybrid – hybrid reactor; CAD – central activity digester; n.d. – no data.

For comparison, Zhao *et al*. (2011) also used an UASB reactor, but inoculated it with *Citrobacter freundii* CZ1001, a SRB strain isolated and enriched from anaerobic activated sludge. They achieved a SO_4^{2-} removal efficiency of 90% from tannery effluent with an initial SO_4^{2-} concentration of 1069 mg/ℓ, which was 12% higher than when the treatment was carried out without augmentation with the isolated SRB strain.

Several aerobic bioreactor systems have been employed at laboratory scale for partial SO. These are commonly: the three phase fluidised bed bioreactor, a biofilter system and a reverse fluidised loop reactor. Each of these systems achieved a H_2S removal efficiency of 94-100% from synthetic medium, with these efficiencies dropping with increasing flow rates (Midha and Dey, 2008). The biofilter system saw the HS^- removal efficiency drop about 18% when the flow rate doubled from 93 ℓ/h and the reverse fluidised loop reactor had a 10% drop in HS^- removal efficiency when the HS^- loading rate was increased by 50% from 19 kg/m^3.d (Midha and Dey, 2008). However, these systems used synthetic HS^- solutions and not tannery effluent, which is a far more complex medium to treat.

Once again, various anaerobic SO bioreactor types have been tested at laboratory scale: the continuous stirred tank reactor (CSTR), the up flow fixed film (FF) reactor and the plug flow FF reactor (Midha and Dey, 2008). In a FF CSTR, the sulphur recovery rate ranged 95-99% for a HS^- loading rate of 94-286 mg/ℓ.h (Midha and Dey, 2008). However, the major problems with using phototrophs on a large scale is the requirement of a strong light source and the necessity of keeping the system

anaerobic (Midha and Dey, 2008). While both aerobic and anaerobic biological HS^- removal treatments are possible, aerobic partial oxidation of HS^- is favoured for large scale operations (Midha and Dey, 2008).

*Table 11: Common components affecting the performance of sulphate reducing bioreactors (adapted from Hao et al., 2014)**

Component	Effect(s)	Condition(s) preferred
SO_4^{2-} concentration	Affects SRB growth, activity and microbial activity; May be out-competed at low concentration; High concentration inhibits SRB activity	Typically a COD/SO_4^{2-} value = 0.7-1.5, depending on carbon source; Optimal for COD removal = 0.6-1.2; for SO_4^{2-} removal = 2.4-4.8
Trace element	Fe, Cu, Zn, Co, Mo, Ni are needed for electron transport, redox-active metalloenzymes and composition of some protein and enzymes; High Mo level inhibits SRB metabolism	High levels of Fe in culture media in order to compensate for amount precipitated by HS^-; †Mo > 2 mM completely inhibits SRB
Metal concentration	Elevated heavy metal concentration can reduce or terminate SRB activity	Desired concentration and the order of decreasing toxicity (mg/ℓ): Cu < 4, Cd < 11, Ni < 13, Zn <16.5, Cr < 35, Pb < 80
NO_3^- concentration	NO_2^- is a strong SRB growth and activity inhibitor	†Impact extent: 70 mM NO_3^- inhibits growth significantly; Long-term 0.25-0.33 mM injection inhibits number and activity
pH	Effect the growth and activity; Influence the SRB species diversity and competition with methanogens; Effects dissolved H_2S quantity	pH range for SRB: 5.5-10
Salinity	Influence SRB species present; Generally SRR is inversely proportional to salinity	Optimum salinity range: 6-12%
H_2S concentration	High H_2S direct and reversible toxicity effect on SRB, and inhibit the activity	Nitrogen purging; †Decrease in activity when H_2S is higher than 60-70 mg/ℓ

**See Hao et al. (2014) for associated references*
†Negative impact limitation

Table 12 summarises the advantages and disadvantages of various HS^- removal treatments used on segregated effluent, i.e. just on beam house effluent.

Table 12: Advantages and disadvantages of different sulphide removal techniques of segregated effluent

Method	Advantages	Disadvantages	References
Aeration	• Short contact time for low HS^- concentrations	• Some H_2S loss directly to atmosphere • High HS^- concentrations require long contact times and large reactor volumes • Under pressure, O_2 added to water renders it extremely corrosive, reducing equipment effectiveness • Formation of SO_4^{2-}, $S_2O_3^{2-}$ and combined with residual HS^- are difficult to separate and thus, expensive to treat	Midha and Dey (2008) Robert and McVay (2011)
Catalytic chemical oxidation (with air)	• Short contact time • Low capital cost • Easy operation • Possibility of treating highly variable loads • Residual HS^- concentration of 2 mg/ℓ	• Time consuming • High operating costs • Large footprint • Formation of SO_4^{2-}, $S_2O_3^{2-}$ and combined with residual HS^- are difficult to separate	Giordano *et al.* (2015) Naturgerechte (2002) Midha and Dey (2008)
Chlorination	• Simple process • Short contact time • Highly effective	• High chemical costs • Hazardous chlorinated organic chemicals formed • Formation of SO_4^{2-}, $S_2O_3^{2-}$ and combined with residual HS^- are difficult to separate • Oxidised S products can be reduced again by SRBs	Midha and Dey (2008) Robert and McVay (2011)
Direct air stripping (Claus Process)	• Removal from gas streams • Suitable for use in combination with anaerobic digestion without disturbance, by applying a low volumetric flux of stripping gas • Capable of reducing a 100 mg/ℓ H_2S concentration to 30 mg/ℓ	• High energy requirements • High chemical and disposal costs • Stripping gas requires purification • Environmental problems • Suitable for low concentrations of HS^- • Requires careful regulation of pH to minimise CO_2 release	Midha and Dey (2008) Verink (1988) Wiemann *et al.* (1998)

Electro oxidation (EO)	• Presence of high concentration of dissolved solids, especially chlorides, makes effluent particularly amenable to EO • High efficiency • Simple equipment • Easy operation • Low retention time	• Effectiveness strongly dependent on effluent composition, electrode materials, batch/continuous operation, and treatment conditions • High energy costs • Potential of chlorinated organics formation • Electrode fouling may occur	Yusif *et al*. (2016) Anglada *et al.* (2009)
Metal precipitation	• Quick and simple process • Effective at maintaining low HS^- in an AD reactor	• High chemical costs, although typically more economical than chlorination • High disposal costs due to large amount of sludge • Precipitates all essential trace metals required for methanogens • Pipeline and valve clogging	Hellinger and Trommer (1991) Hulshoff Pol *et al*. (1998) Naturgerechte (2002) Robert and McVay (2011) Sabumon (2016) Lens *et al.* (1998a)
Biological – aerobic /chemotrophic	• Highly cost effective • Very high removal efficiencies • Safe process operation with flexible conditions • Robust (stable) process operation • Simple nutritional requirements • Higher HS^- tolerance • Oxidises to recoverable S^0 • Low sludge production for disposal • Culture often selectable from native tannery sludge	• Requires careful control of O_2 else results in production of SO_4^{2-} • Local corrosion • Pipeline and valve clogging • Inconsistent complete oxidation to S^0	Janssen *et al.* (2001) Midha and Dey (2008) Giordano *et al*. (2015) Hao *et al.* (2014) Buisman *et al*. (1990) Lee and Sublette (1993) Wiemann *et al*. (1998)
Biological – anaerobic /phototrophic	• Highly cost effective • Commonly, HS^- removal efficiency of greater than 90% • Safe process operation with flexible conditions • Robust (stable) process operation • Low sludge production for disposal	• Difficult anaerobic set up on large scale • Requirement of strong light source • Internal storage of S^0 is common • Inconsistent complete oxidation to S^0	Janssen *et al.* (2001) Midha and Dey (2008) Wiemann *et al.* (1998)

2.5.4.2 Passive treatment

Passive treatment techniques do not require frequent human intervention or maintenance and are able to function for many years (> 5 years) without electrical power and few supplements. Generally, passive systems employ natural construction materials, including soils, clays and plant residues and promote natural vegetation growth, which filters the wastewater. Further, systems typically use gravity for water flow instead of pumping. While there are numerous passive treatment system configurations in industry, there are two types that have been well researched and exclusively used for BSR are anaerobic wetlands and packed bed reactors (Mooruth, 2013).

The amount of space required limits the application of reed beds, lagoons and other alternative options to smaller tannery plants (Buljan and Král, 2012). The AFP system mentioned in Section 2.4.1, although intended for biogas production, can select out the BSR and partial SO pathway, making this a feasible integrated passive sulphur species removal treatment for effluents with large amounts of SO_4^{2-} or a low COD/SO_4^{2-} ratio (Tadesse *et al.*, 2003a). Tadesse *et al*. (2003a) recorded a BSR efficiency of 90% as the SRBs out-competed the MPA. Much of the sulphide generated was present as HS^- and was converted to recoverable S^0 by pink photosynthetic sulphur bacteria present in extreme abundance in the upper, illuminated layer of the AFP. These included members of the genera *Thiocystis, Rhodobacter, Rhodospirillum* and *Rhodopseudomonas* (Tadesse *et al.*, 2003b).

2.5.4.3 Semi-passive treatment

There are many treatment systems which are not active, but cannot accurately be described as completely passive, due to the requirement of continuous or periodic input of chemicals, energy and materials (Higgins *et al.*, 2017). These hybrid systems are termed “semi-passive”. These systems may require active management to maintain conditions and processes (Martin *et al*., 2010).

Boshoff *et al*. (2004) built a pilot scale WSP system on site at a tannery where, for an initial SO_4^{2-} concentration of up to 1800 mg/ℓ in the influent, the TR achieved a 72% SO_4^{2-} removal efficiency on average, for a SO_4^{2-} removal rate of 400-500 mg/ℓ.day. This equated to the TR reaching a COD removal rate of 500 mg/ℓ.day.

An example of a semi-passive treatment technique is the linear flow channel reactor, where there is no stirring or aeration required and relies on the principle of very laminar flow to achieve partial HS^- oxidation, S^0 biofilm formation and specific zones of oxygenation. This reactor is a novel concept and will be discussed in detail in Section 2.5.4.6.

2.5.4.4 Performance comparison

The performance of a variety of BSR bioreactors are given in Table 13 below for active, passive, continuous and batch reactors utilising a mixed microbial culture. The performance of the system is heavily reliant on the system parameters, including substrate, SO_4^{2-} loading rates, pH, redox potential, temperature and HRT, and have been given for comparison (Mooruth, 2013).

Table 13: Performance of SO_4^{2-} reducing bioreactors treating SO_4^{2-} laden wastewaters

Reactor type	T (˚C)	pH	Substrate	Feed SO_4 (g/ℓ.day)	HRT (days)	SRR (g/ℓ.day)	SR (%)	COD:SO_4	COD removal (%)	Reference
CSTR	35	7.5-8.0	Acetate	0.95	ND	0.768	80-90	ND	ND	Moosa *et al*. (2002)
CSTR	Amb	7.6-8.0	Tannery effluent	≤1.8	4	0.25	60-80	0.1	75	Boshoff *et al*. (2004)
UASB	Amb	7.4-7.9	Tannery effluent	≤1.8	4	0.6	80	0.56	95	Boshoff *et al.* (2004)
UASB	30	7-8	Acetate, propionate, butyrate	1.195	-	0.805	68	0.5	7-41	Omil *et al.* (1996)
SBBR	25	5.9-6.5	Butanol	0.48	2	0.743	99	3.67	32	Sarti and Zaiat (2011)
CSTR	35	8	Lactate	1-10	0.5-5	0.36-2.2	86	1.2	99	Oyekola *et al.* (2010)
ABR	35	7.2	Molasses	0.18-0.5	1	ND	96.8	6	>85	Vossoughi *et al.* (2003)
AnSBBR	30	7.2-8.4	Sodium lactate	1.6	1	ND	82	3.75	78	Mohan *et al*. (2005)
EGSB	30	ND	Lactate	1	0.75	ND	81.5	3	ND	Xu *et al*. (2012)
UHR	30	6.5-8.0	Sucrose	1.9-2.85	1	2.92	>90	1.3	>90	Sabumon (2008)
DPBR	Amb	6-9	Acetate, grass, wood chips, dry sewage sludge	2	1.75	0.903	79	ND	ND	Mooruth (2013)
DF-PBR†	25	ND	Wood chips, manure, primary sewage sludge	1.09	ND	0.013	1.8	ND	ND	Molwantwa *et al*. (2010)
UF-PBR†	25	6.4-7.4	Leaf compost, sawdust	0.243	ND	0.12	31.6	ND	7.5	Waybrant *et al.* (2002)
Batch†	22	8	Wood chips, leaf compost, poultry manure	3.39*	-	0.06	>95	ND	-	Zagury *et al.* (2006)
Batch	31	7.5	Sucrose	1.9*	-	ND	>90	0.7	>85	Sabumon (2008)
Batch	35	ND	Molasses	0.742*	-	ND	97.4	1.6	ND	Wang *et al*. (2008)

*initial SO_4^{2-} concentration

†passive

ABR – anaerobic baffled reactor; Amb – ambient temperature; AnSBBR – anaerobic sequential batch biofilm reactor; DF-PBR – down-flow packed bed reactor; DPBR – degrading packed bed reactor; EGSB - expanded granular sludge bed; LFCR – linear flow channel reactor; ND – no data; SBBR – sequential batch biofilm reactor; BSR – biological sulphate reduction; SRR – sulphate reduction rate; UASB – Up-flow anaerobic sludge blanket reactor; UF-PBR – up-flow packed bed reactor; UHR – up-flow hybrid reactor.

2.5.4.5 Strategies to integrate sulphur species removal and biogas production

Certain wastewaters contain both high concentrations of sulphur species and excess COD, so even effective sulphur species removal leaves an effluent with an elevated residual COD. The residual COD needs to be reduced to meet discharge specifications, but may also be a potential substrate for renewable energy generation by AD. Depending on the nature of effluent and the amount of sulphide generated, a sulphide removal step may be required to prevent inhibition of methanogens.

Three techniques for HS^- removal have been found to be suitable for integration into anaerobic wastewater treatment processes: precipitation, stripping and biological oxidation. The integration of stripping can be done without disturbance to the anaerobic treatment process by applying a low volumetric flux of the gas required for stripping. Stripping does not create sludge and requires no supplementary materials (Wiemann *et al.*, 1998).

Biological oxidation of HS^- is somewhat unreliable and does not always guarantee effective oxidation to elemental sulphur, even though it also requires few supplementary materials and is most cost effective (Wiemann *et al*., 1998).

Therefore, the efficacy and suitability of a treatment technique depends on the system in place and technical and economic considerations.

As stripping is more effective on wastewaters with a lower HS^- concentration, as discussed in Section 2.5.4.1.1, pairing the use of a stripper with a recycle of HS^--free effluent to dilute the wastewater and therefore, lower the reactor's influent HS^- and SO_4^{2-} concentration is a feasible option (Figure 8A & B) (Lens *et al*., 1998a). Similarly, the dissolved H_2S concentration can be significantly reduced by operating the reactor at a pH greater than 7.5 (Figure 6) (Rinzema and Lettinga, 1988).

While precipitation has been proven to be effective for maintaining low HS^- concentrations in anaerobic reactors, the high chemical costs, potential clogging of pipes and the accumulated metal sulphide sludge within the reactors make this solution unfeasible for large-scale tannery effluent treatment plants (Lens *et al.*, 1998a). However, if this technique were to be used, all three processes of AD, BSR and HS^- removal could happen in the same reactor (Figure 8C).

If sulphur compound loads are low and H_2S concentrations will not be toxic to methanogens, methanogenesis and BSR can occur first and a HS^- removal step can follow (Figure 8D). If this is not the case, the production of HS^- can be separated from methanogenesis by having two-stage AD, where pre-acidification is combined with BSR in the first stage and methanogenesis occurs in the second (Figure 8E) (Lens *et al.*, 1998a). The HS^- can be removed from within the first stage or between the two steps. It has been shown that acidogenesis can proceed successfully without being adversely affected by competition with SRBs or H_2S toxicity (Lens *et al*., 1998a; Wiemann *et al*., 1998). However, the complete reduction of SO_4^{2-} in the first stage needs to be assured to obtain a clean biogas stream in the methanogensis stage and results have shown that only up to 80-95% is converted to HS^- (Lens *et al*., 1998a). Therefore, H_2S removal from the biogas may be required.

Alternatively, if the wastewater stream has sufficient substrate for BSR without fermentation and acidogenesis occurring first, the BSR and HS^- removal can occur together or separately before any of the AD process stages (Figure 8F). While the majority of integrated SO_4^{2-} removal experiments consist

of two reactors, a separate one for SO_4^{2-} reduction and one for HS^- oxidising, it would be ideal for SRB and SOB to work in one reactor (Alesia, 2015).

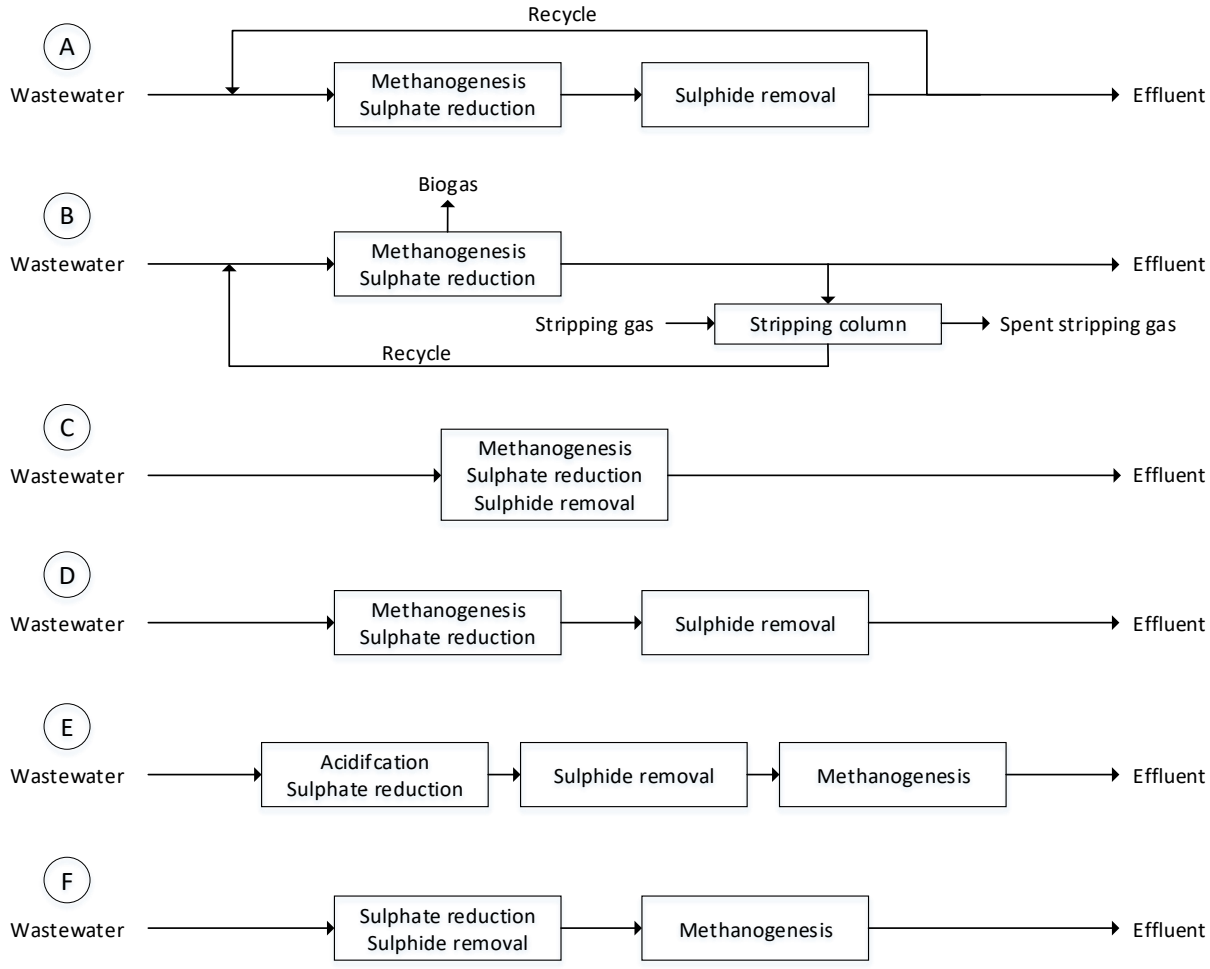

Figure 8: Process configurations for achieving sulphate reduction, sulphide removal and methanogenesis (adapted from Lens et al., 1998a)

2.5.4.6 Hybrid linear flow channel reactor

The hybrid linear flow channel reactor (HLFCR) is a new and novel semi-passive reactor design for simultaneous BSR and partial SO, as in the process route shown in Figure 8F. As this reactor is semi-passive, it is a more affordable treatment technique when compared with conventional active treatments. The LFCR was first utilised exclusively for partial SO as part of an acid rock drainage (ARD) treatment system (Mooruth, 2013). More recently, Marais *et al.* (2020) demonstrated that BSR and partial SO could occur in a single, hybrid reactor, which positively impacts process kinetics and could reduce equipment costs and footprint.

The LFCR configuration encourages the formation of a floating biofilm at the air-liquid interface and partial SO occurs within the biofilm. The biofilm floats on top of the bulk liquid, making recovery easier than systems that rely on the poor settling of S^0 particles (Janssen *et al.*, 1996). The biofilm restricts O_2 mass transfer and creates a microenvironment with the correct pH and redox potential to favour partial SO, rather than complete oxidation to SO_4^{2-}, with deposition of elemental sulphur within the biofilm matrix. These particular conditions required for the conversion of HS^- to S^0 can be seen in the

Pourbaix diagram in Figure 7. This S^0 and organic carbon mixture of the FSB can be easily harvested and used as a feedstock (Midha and Dey, 2008; Mooruth, 2013). The consumption of oxygen within the biofilm keeps the bulk liquid anaerobic for BSR.

In this hybrid reactor, the anaerobic and aerobic zones are spatially separated, albeit with a close interaction (Figure 9) (Lens and Hulshoff Pol, 2000). Thus, a sulphur cycle at the micro-scale is isolated.

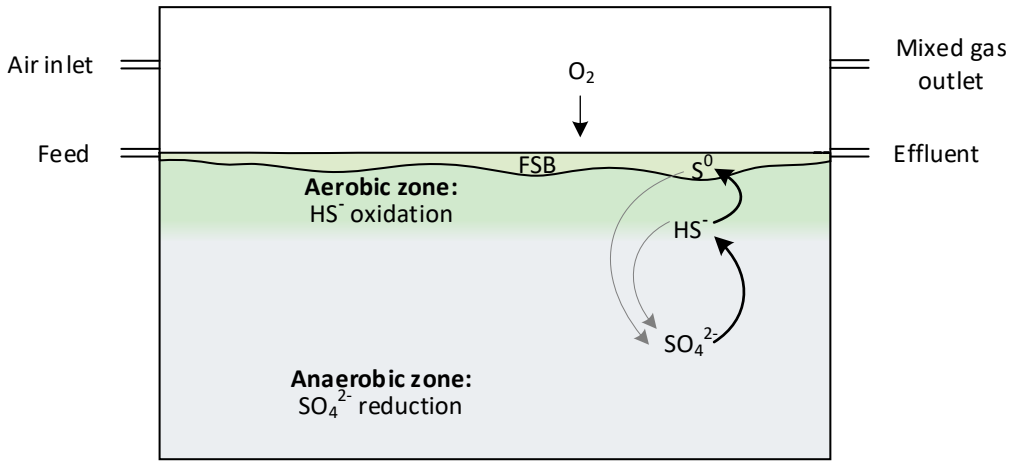

Figure 9: Sulphate reduction and sulphide oxidation zones of the HLFCR bulk liquid

Specifically, it is the hydrodynamics of the HLFCR that allows successful BSR and partial SO to harvestable S^0. A low flow rate and lack of turbulent mixing causes strictly laminar flow, with a Reynolds number (Re) of $0 < Re < 307$ (Mooruth, 2013). The requirement for laminar flow in a circular tube is *Re < 2300*. This extreme laminar flow within the HLFCR creates vertical stratification and the lack of turbulence is conducive to the formation of the FSB, which is crucial to the partial oxidation of HS^-.

While the LFCR has been conceptually described by Molwantwa (2008) and Molwantwa *et al.* (2009, 2007), fundamental knowledge on the hydrodynamics, optimal residence time, organic loading rate, sulphur speciation and improving S^0 recovery has only been receiving attention more recently (Mooruth, 2013). Demonstration of successful proof of concept of the hybrid system was even more recent (Marais *et al.*, 2020).

While the intention, with the presence of the FSB, is to allow anaerobic BSR and aerobic partial SO to S^0, some complete SO may occur when there is excess O_2 present, producing SO_4^{2-} once more (Figure 9). Similarly, a fraction of the S^0 that has been formed can be oxidised to SO_4^{2-}. Hence, the control of O_2 mass transfer by the biofilm is essential.

2.5.4.6.1 Biofilm structure and function

Costerton *et al*. (1995) defined a biofilm as "matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces." More broadly, they are a natural accumulation of microorganisms at an interface (Gjaltema, 1996). Generally, biofilms attach to a solid substrate that provides anchor points for stability and encourages proliferation (Gjaltema, 1996). Biofilms may also form on a liquid surface, as is the case with the HLFCR. The air-liquid interface of a water body allows easy access to gaseous O_2 as well as nutrients from within the liquid phase and is therefore an ideal site for biofilm development and aerobic organism colonisation (Spiers *et al*., 2003). These biofilms

are able to stay at the surface due to the hydrophobic nature of the organisms (and produced compounds) rather than their own buoyancy (Spiers *et al.*, 2003).

Biofilms have a characteristic internal structure. It has been concluded that the biofilm is not a random formation of bacteria but instead a complex arrangement that maximises the influx of nutrients. Biofilms consist of highly complex heterogenous films that incorporate channels, layers, voids and cell clusters (Mooruth, 2013).

The microbes in a microcolony produce extracellular polymeric substances (EPS) made up of polysaccharides, proteins and nucleic acids, which allows attachment to a substrate or to other microcolonies, as well as forming the framework for the biofilm (Costerton *et al.*, 1995; Stoodley *et al.*, 2002). The EPS supports the structure of the biofilm and provides resistance to environmental stresses.

One of the most important fundamental characteristics of bacterial biofilms is their capacity for focused and cooperative proliferation. This involves anything from a particular cell lying dormant until conditions improve, to stress responses and protection from predators like amoebae (Costerton, 2000; Jefferson, 2004). Generally, the microbial community lives in a state of symbiosis whereby the metabolic end-products of one population are utilised as a resource by another species, resulting in an intricate metabolic cooperative that produces highly complex and heterogeneous biofilms (Costerton, 2000; Lappin-Scott and Costerton, 1995). These relationships are contained in microcolonies, where its own matrix and the metabolic activity of its component cells controls its internal environment, attaining a measure of homeostasis (Costerton *et al.*, 1995). A single microcolony is the basic unit of biofilm growth and it follows that several microcolonies combine together to form the biofilm (Costerton *et al.*, 1995). Therefore, biofilms can be considered a micro-ecosystem of various microbial species and microcolonies working together in synergy for the general well-being of the whole bacterial community, resulting in a community that is greater than the sum of its parts.

Mooruth (2013) proposed a conceptual model for biofilm formation in the LFCR (Figure 10). In the absence of the biofilm unimpeded oxygen mass transfer occurs over the interface, resulting in partial sulphide oxidation in the bulk liquid and the formation of a colloidal sulphur layer (Step 1 of Figure 10). Biofilm formation is initiated by the formation of an EPS strand network at the air-liquid interface during the first 12 hours of FSB growth. This provides the scaffolding to support the attached microbes and deposited sulphur.

Step 2 in Figure 10 shows day 2-3 of FSB growth, where a thick yellow/cream S^0 layer has been deposited within the FSB. Other inorganic precipitates, depending on the media composition (e.g. magnesium sulphate ($MgSO_4$) and calcium sulphate ($CaSO_4$)) may form by evaporative crystallisation. The SOB, S^0 deposits and metal-HS^- precipitates reside in the newly formed biofilm layer shown in blue. This structural matrix is enlarged in the image and, supported by the EPS strand network, provides the framework for the next biofilm layer (Mooruth, 2013).

Some organic carbon is required to support the heterotrophs responsible for the EPS formation. Mooruth (2013) determined that the minimum amount of acetate required for optimal FSB development and growth was 0.1 g/ℓ. In the absence of the organic carbon complete FSB formation

did not occur and when acetate was limiting the FSB was not structurally robust enough to withstand the weight of S^0 deposits and the biofilm collapsed prematurely.

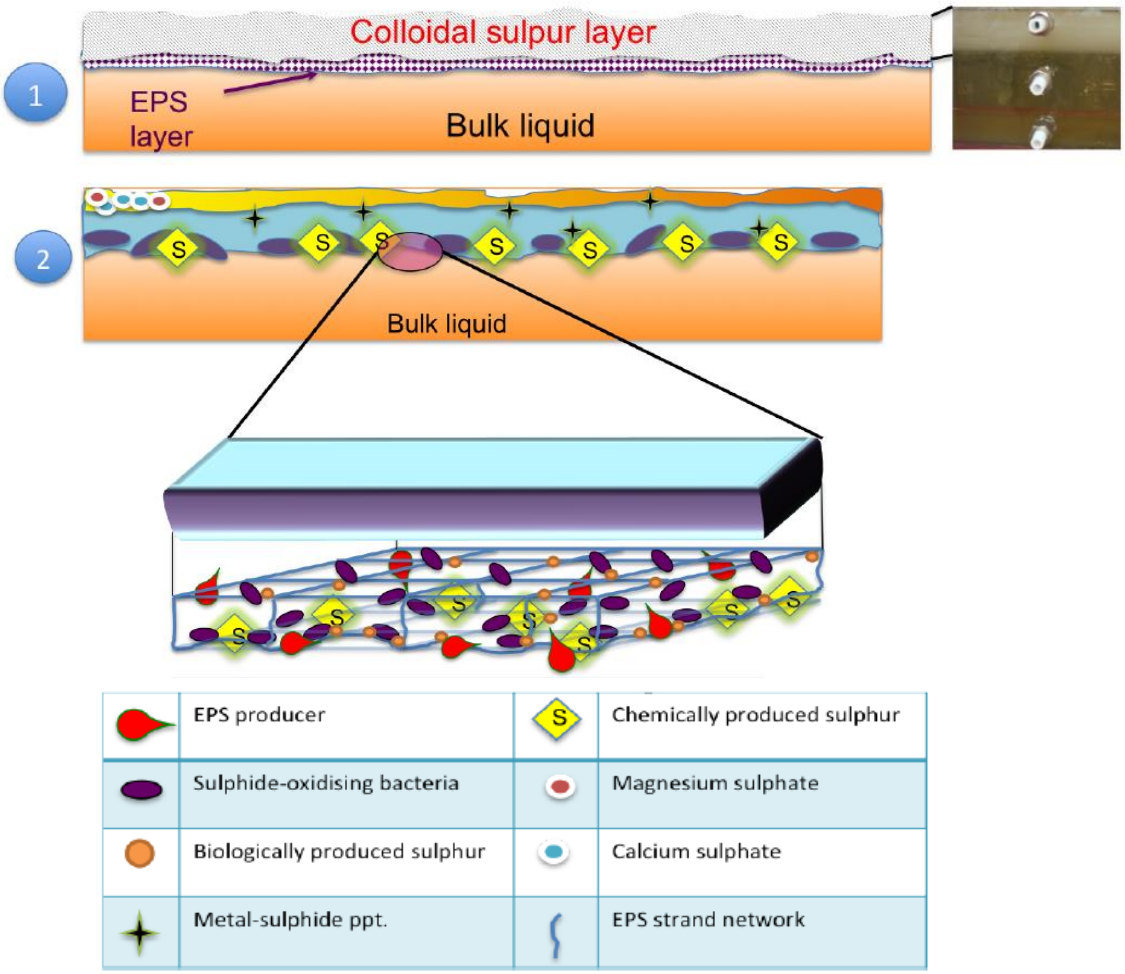

Figure 10: The structure of a floating sulphur biofilm at the air-liquid interface (Mooruth, 2013)

2.5.4.6.2 Sulphide oxidation performance as a linear flow channel reactor

Mooruth (2013) conducted a detailed study on the performance of the LFCR for the sole purpose of sulphide oxidation. Sulphate reduction occurred in a packed bed reactor and effluent from the reactor was fed into the LFCR. As a control he performed an abiotic SO study and found that the primary products produced were $S_2O_3^{2-}$, polysulphides (S_n^{2-}, where $n > 3$) and a small amount of colloidal sulphur. As seen in Figure 6, the pH plays a critical role in product speciation. The maximum HS^- removal in the abiotic linear flow channel reactor was 24-32%.

The primary products in the biologically catalysed system were S^0 and SO_4^{2-}, often with more than half of the converted HS^- becoming S^0. This paired with the significantly higher conversion of HS^- (65-96%) than in the abiotic system, shows that biological SO reactions dominate in the channel reactor, instead of chemical conversion. Mooruth (2013) found that the optimum residence time for his system was 1-2 days, requiring the FSB be harvested every 3-4 residence times and a minimum organic loading rate of 0.1 acetate/ℓ. Thereby, obtaining the average maximum SO rate of 3.0-4.7 mmol/ℓ.day and a S^0 yield of between 75-92%. The SO rate was highest soon after FSB formation was complete and thereafter decreased with time as oxygen mass transfer slowed, reaching a minimum after 4 residence

times. Therefore, the system became oxygen limited and was unable to reach a 100% HS^- removal efficiency (Mooruth, 2013).

While Mooruth's work was limited to evaluating the LFCR as a SO unit, the underlying mechanisms of biofilm formation, structure and performance are likely to be similar in the hybrid reactor system.

2.6 Community structure analysis

Many biological treatment processes rely on the contributions of many different microbial populations within a complex, mixed community. These processes were typically developed and refined based on iterative modifications and observation, while the microbial communities were viewed as a 'black-box' (Hessler, 2020). These mixed microbial cultures typically prove to be more effective than pure cultures, likely due to the expanded metabolic capabilities across multiple microorganisms and the synergism between these microorganisms (Hays *et al*., 2015). A meaningful understanding of the community structure increases the potential to understand structure-function relationships, track changes in dominance as operating conditions change, and provides valuable information to assess robustness of the community.

Basic assays to determine cell numbers, such as direct cell counting or gravimetric analyses are almost impossible for environments like tannery effluent, with high levels of suspended solids and biomass flocs. At best, these assays provide an indication of biomass concentration, but no information on diversity and dominance.

Prior to the development of the polymerase chain reaction (PCR) technology there were no effective techniques to characterise mixed microbial communities, given that only a tiny fraction of species within a given habitat can be cultured *in vitro* (Amann *et al*., 1995).

The PCR technique allows for the exponential amplification of a fragment of DNA between a set of forward and reverse primers, short pieces of single stranded DNA that anneal to complementary sequence on the target DNA. Functionally, the PCR reaction cycles through a series of short duration temperature steps that cause the double stranded DNA to separate into single strands (denaturation, 94-96˚C), the primers to bind to the specific sequences (annealing, around 68˚C), the polymerase enzyme to facilitate addition of new nucleotides (elongation, around 72˚C). Each cycle doubles the number of amplified fragments, so 30 cycles would result in x^{30} amplicons, where x is the number of copies of the target sequence added as template. The 16S ribosomal RNA gene (rRNA) is often targeted for community structure analysis as it contains a hypervariable region flanked by highly conserved regions. Primers targeting the conserved regions allow almost all bacteria or archaea to be covered, while differences in the sequence of the hypervariable region allows discrimination between groups, often to a species level.

The first metagenomic based studies involved PCR amplification from metagenomic DNA and cloning followed by functional screening (Schrenk *et al*., 1998). Metagenomics has since been defined as the direct genetic analysis of the total metagenomic DNA representing all microorganisms in a given environmental sample (Thomas *et al*., 2012). This approach allowed a shift from culture-based methods to molecular techniques, generally targeting the 16S rRNA gene as a phylogenetic marker gene. Post-amplification analytical techniques included constructing clone libraries, terminal

restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and quantitative real-time PCR (Kim *et al*., 2013; Nocker *et al.*, 2007).

The phylogenetic diversity of SRB meant that 16S rRNA sequencing was not always a suitable approach for the evaluation of SRB diversity in environmental samples, so the techniques mentioned above were often applied to genes, including *dsrAB*, specifically involved in BSR (Klein *et al.*, 2001; Wagner *et al*., 1998; Zverlov *et al.*, 2005) and *apr* (Meyer and Kuever, 2007).

Investigations into the microbial communities responsible for a range of water treatment processes have been ongoing for some time. The goals of these studies have remained similar over the years, but the techniques used have advanced considerably (Hessler, 2020).

The microbial communities of several BSR reactors fed with various electron donors were evaluated by Dar *et al.* (2007) using the PCR amplification of 16S rRNA and *dsrAB* genes, followed by DGGE and analysis of clone libraries. This study was able to conclude that the SRB which became dominant in these reactors, originating from the same inoculum, were selected for by the supplied electron donors, with *Desulfobulbus*, *Desulfovibrio* and *Desulfobacca* species appearing in ethanol- fed reactors and *Desulfosarcina* and *Desulfoarculus* species occurring in reactors fed a mixture of organic compounds. The same approach was employed by Dar *et al.* (2008) to monitor the competition between SRB, fermentative microorganisms and methanogens in lactate- supplemented bioreactors. Burns *et al.* (2012) used a similar method to characterise the microbial communities before, during and after entering a passive BSR reactor treating coal ARD. A single SRB could be identified, a *Desulfobacca* species, and led the authors to suggest that the bioreactor be modified in order to stimulate the growth of additional SRB (Hessler, 2020).

The use of next generation sequencing (NGS) has become more common and has facilitated the shift from molecular techniques described above to high-throughput DNA sequencing for the rapid and high-resolution evaluation of multiple microbial communities simultaneously (Hessler, 2020). Illumina amplicon sequencing was employed to assess changes in BSR community structure in response to changes in electron donor (Marais, under review). Marais used 16S rRNA gene amplicon sequencing to assess the microbial communities of several LFCRs operated under a range of operating conditions. This study found the microbial communities, including SRBs, to be strongly influenced by the electron donor, the influent SO_4^{2-} concentration and differences in the conditions experienced by sessile cells in the biofilm and planktonic cells in the bulk liquid.

Hessler (2020) noted that these studies illustrate the capacity of metagenomics, using various molecular, DNA sequencing, and bioinformatic techniques to resolve complex microbial communities and understand key microorganisms from a process perspective, leading to a better understanding of the fundamental mechanisms within these processes. This can, in turn, inform how these systems should be designed and operated for improved performance and system robustness.

3 Objectives of the Research

3.1 Aims

The aim of the research is to characterise OTE and BTE to determine their pollutant loads, to enrich cultures from saline environments that are capable of partial sulphide oxidation (SO) and biological sulphate reduction (BSR) and to evaluate the proof of concept of using the hybrid linear flow channel reactor (HLFCR) as a pre-treatment reactor.

3.2 Objectives

The objectives of the research are to:

- Characterise tannery effluent from active operations, specifically with respect to sulphur species, organic load and salinity
- Culture potential halophilic microbial consortia from natural anaerobic environments in the laboratory
- Develop a robust consortium as an inoculum for BSR and SO, through enrichment and operation in the HLFCR system using artificial seawater system
- Culture the bacteria under anaerobic conditions, using tannery effluent, to evaluate their suitability for tannery effluent treatment
- Use molecular biology techniques to characterise the microbial consortia present in enrichment consortia and effluents and investigate if and how the community structure changes during tannery effluent treatment
- Pre-treat OTE and BTE in a continuous HLFCR system to ascertain the success of the treatment system for tannery effluent

3.3 Hypothesis

The pre-treatment of tannery effluent, using acclimated, enriched microbial cultures in a HLFCR, to remove sulphur species by BSR and partial SO, will make it more amenable to subsequent anaerobic digestion (AD).

3.4 Research questions

1. What is the concentration of HS^- and SO_4^{2-} in the OTE and BTE?
2. What level of organic matter and salinity do each of the tannery effluents have?
3. Are the enrichment cultures likely to produce HS^- at concentrations that become inhibitory?
4. How much does the COD decrease during pre-treatment?
    a. Will there still be sufficient COD after pre-treatment to make AD viable?
5. What is the maximum rate of BSR and SO and how does this compare to conventional processes?
6. What are the critical species/groups of microorganisms performing BSR and partial SO?

4 Research Design and Methodology

4.1 Microbial consortia isolation and enrichment

4.1.1 *Description of sites*

Samples were collected from suitable anaerobic water bodies and sediments of varying salinities in coastal and estuarine areas of South Africa. Namely, a Muizenberg bog, Zandvlei in Muizenberg, Kasouga River Estuary and the Knysna Estuary, at Kathy Park and the Old Airstrip, as shown in Figure 11.

Figure 11: Sampling sites. A) Muizenberg bog. B) Zandvlei. C) Kathy Park. D) Old Airstrip. E) Kasouga River Estuary

4.1.2 *Method of collection*

Samples contained seawater, fresh water, sediment and if available, plant matter. Samples were collected in 500 mℓ glass jars, except for the Kasouga River Estuary sample, which was collected in a 400 mℓ plastic jar, and as close as possible to departure time for the Knysna and Kasouga samples for transport back to Cape Town.

All sample containers were tightly sealed and kept at ambient temperature during transport and on arrival at the laboratory. Samples were enriched in batch reactors within 3 days of arrival, except for the Muizenberg bog sample, which was enriched 84 days after collection, following the carbon source selection tests (Section 4.1.4).

4.1.3 *Artificial seawater*

The composition of the artificial seawater used throughout the tests is shown in Table 14. The artificial seawater contained 2.71 g/ℓ SO_4^{2-} and had an electrical conductivity (EC) of ~43 mS/cm.

Table 14: Artificial seawater composition

Salt	Concentration (g/ℓ)
NaCl	23.9
Na_2SO_4	4.01
KCl	0.67
$NaHCO_3$	0.2
H_3BO_4	0.03

4.1.4 *Carbon source selection in artificial seawater batch tests*

Anaerobic batch tests were performed to determine the preferred carbon source for the microbial consortia (carbon source selection test, Table 15). The sample, carbon source and artificial seawater (final volume 20 mℓ) were added into McCartney bottles and sealed. These were evaluated to measure HS^- concentration increases over time. The bottles were maintained at ambient temperature for 32 days and sampled every 5-8 days. The consortia were then enriched using the most appropriate carbon source in sealed 250 mℓ Duran Schott bottles (initial enrichment consortia, Table 15), with final volume 270 mℓ. These were used as inocula for the experiments described later in this chapter.

Table 15: Composition of each carbon source solution and initial batch reactor

Component	Concentration (g/ 100 mℓ)	Reactor composition (mℓ)	
		Carbon source selection test	Initial enrichment consortia
Carbon source solution		1	12.5
Glycerol	4.25		
Sodium acetate	7.5		
Sodium lactate*	12.15		
Molasses	5.85		
Sample		4	>125
Artificial seawater		15	<132.5
Total		20	270

*60% sodium lactate solution (Merck (Darmstadt, Germany); Cat no.: L1375-500ML), stored at 5˚C

4.2 Tannery effluent

4.2.1 *The tanneries*

Tannery effluent was procured from an ostrich tannery and a bovine tannery in South Africa. The ostrich tannery, Southern Cape Ostrich Tannery (SCOT), processes ostrich skins and produces ostrich leather for the exotic leather market. When fully operating, the tannery's current capacity is 6 250 skins per month. This tannery is situated next door to an abattoir, so the skins do not need not be preserved with salt and the resulting effluent has roughly a quarter of the salinity of a usual tannery.

The bovine tannery, Cape Produce, is one of the largest hides and skin tanneries in the country and supplies various countries with wet blue tanned hides and wet or dry salted hides. They process 100 000 bovine hides and 250 000 ovine skins per month (Cape Produce Company, 2018).

4.2.2 *Effluent collection, storage, and characterisation*

Six weekly composite batches of OTE were taken from the effluent balancing tank, prior to secondary treatment in an oxidation ditch (OD) (Figure 3), over the months of March to August 2018. The batches of OTE were couriered to the laboratory on the day of sampling. Upon arrival, samples were taken for chemical analyses on the solid and liquid fractions. Effluents were characterised to determine sulphur species, organic load, anions, cations, metals and physical properties, as in Table 23. These were performed within 48 h of arrival.

The remainder of each batch was frozen at -18˚C. All six batches were later defrosted and thoroughly blended to ensure consistent feed to each experimental study, after which the blended OTE was refrozen until required. Two additional batches of OTE were obtained later, one of which was taken from a high HS^- stream (Figure 3) and the other from the effluent balancing tank, as before.

Five batches of BTE from two different streams, one a blend of "strong" and "weak" effluent streams (raw BTE) and the second after the oxidation stage of the process (partially treated BTE), were obtained over the months of April to June 2019. These were characterised and as per the OTE batches. For reasons of confidentiality, the exact BTE treatment processes and sampling locations cannot be divulged.

In addition to the physical and chemical analyses, DNA extractions were performed on selected samples.

4.3 Reactors

4.3.1 *Scale-up of enrichment consortia*

The original enrichment consortia (Section 4.1.4) were used to inoculate batch reactors to scale up the enrichment consortia. These were performed at ambient temperature in sealed Duran Schott bottles of 500 mℓ, 1 ℓ and 2 ℓ, each with final volumes of 530 mℓ, 1050 mℓ and 2100 mℓ, respectively, to minimise headspace.

The batch enrichment reactors were assessed for their ability to reduce SO_4^{2-}. The reactors' performance was monitored by measuring the pH, conductivity, redox potential and HS^- and SO_4^{2-} concentrations for 341-420 days from date of first inoculation. Decreasing SO_4^{2-} concentrations in combination with increasing HS^- concentrations indicated an active BSR consortium.

Each of the original batch enrichment reactors were progressively scaled up from 250 mℓ to 1 ℓ and some 5 ℓ. Sub-culturing or scale up was performed when the SO_4^{2-} was depleted, the HS^- concentration was above 400 mg/ℓ or the sulphate reduction rate (SRR) slowed substantially. Enrichment cultures were scaled up by transferring half of their volume into the next size of Schott bottle, adding carbon source solution to the original and scaled-up reactors (2.55 g/ℓ lactate or 2.70 g/ℓ acetate), and topping up with artificial seawater (Table 14). For scale up to 1 ℓ reactors the inocula were made up using half of the original 250 mℓ and 500 mℓ enrichment cultures, where possible, or else 300 mℓ of the 500 mℓ consortia.

4.3.2 *Batch anaerobic digestion reactors*

The assessment of the suitability of raw and pre-treated tannery effluent as a substrate for anaerobic digestion was performed using 1 ℓ batch reactors. The reactors consisted of a 1 ℓ Schott bottle fitted with a modified lid to allow sampling of the liquid fraction and collection of any biogas generated (Figure 12). The lid contained three ports, two of which contained stainless steel tubing that ended in the headspace while the third tube penetrated about half way into the liquid fraction. A 10 mℓ syringe connected to the third port was used to sample the liquid fraction. Biogas was collected in a Supel™ inert foil pull lock valve (PLV) gas sampling bag connected to one of the headspace ports. The second headspace port was used as a vent when the reactor was flushed with nitrogen at the start of an experiment, then sealed for the duration.

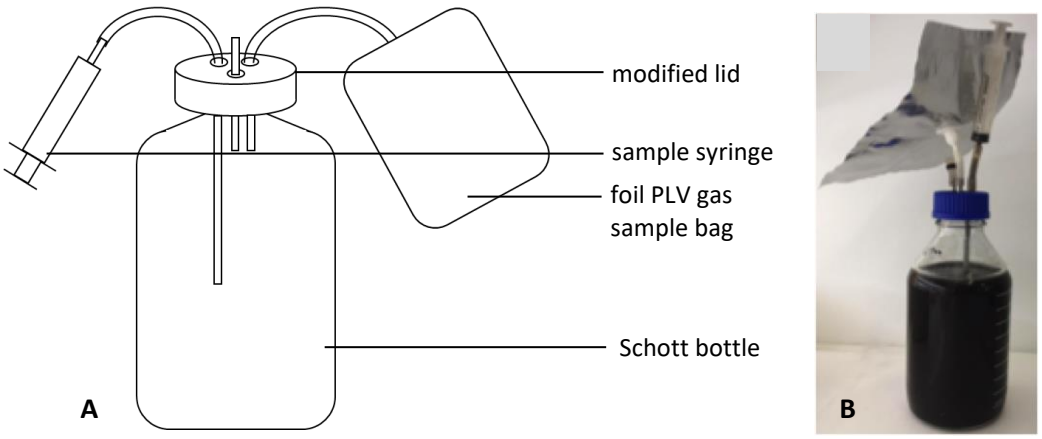

Figure 12: Diagram (A) and example (B) of anaerobic digestion reactors

4.3.3 *Hybrid linear flow channel reactor (HLFCR)*

The continuous pre-treatment experiments were performed using a HLFCR similar to that previously used in experiments to treat mining-impacted water (Marais *et al.*, 2020). A schematic diagram of the plexi-glass reactor is shown in Figure 13.

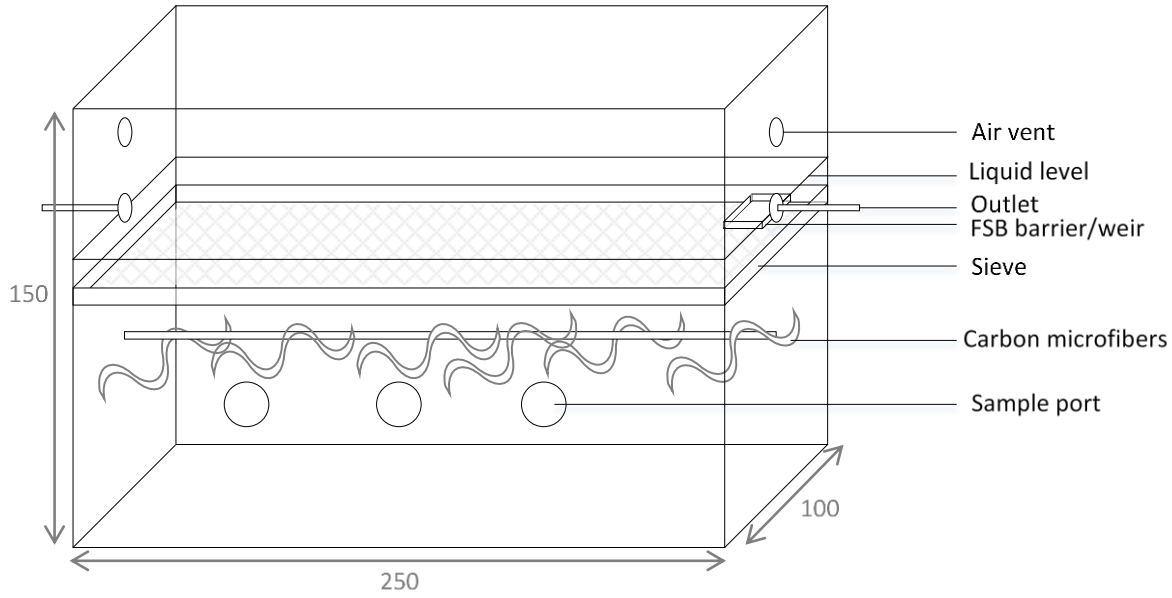

Figure 13: Simplified diagram of a hybrid linear flow channel reactor (channel). Dimensions in mm.

Six threaded pillars were embedded in the reactor, at the corners and midway along the long side, allowing a lid to be fitted. The lid was tightened using wingnuts and a silicon gasket ensured an airtight seal. The reactor contained carbon microfibers, held between two aluminium plates, suspended in the bulk liquid as a substrate for microbial attachment. The front of the reactor contained three sampling ports at approximately half the height of the bulk liquid. Each port was sealed with a rubber septum allowing sampling using a syringe fitted with a 70 mm hypodermic needle. A plastic mesh screen, held in place by wire supports, lay just below liquid surface and could be lifted to harvest the biofilm. A rectangular weir was fitted to the outlet port to prevent washout of the biofilm. Two additional ports, located midway between the feed/effluent port and the lid, allowed air flow and could be opened or closed depending on O_2 requirements.

4.4 Analytical methods

4.4.1 *pH, electrical conductivity and oxidation-reduction potential*

The pH, EC and redox potential were determined using a Eutech PC2700 multiparameter bench meter with standard EC and redox probes. An XS (Carpi, Italy) Sensor 2-pore S7 pH probe with a 6 mm tip allowed the measurement of pH in small volume samples. Total dissolved solids (TDS) were calculated from the EC, using a conversion factor of 0.67.

4.4.2 *Total solids and total volatile solids*

The total solids (TS) and total volatile solids (TVS) concentrations were measured following the loss on ignition standard gravimetric methods (APHA, 2012), using an oven at 105˚C and a furnace at 550˚C, respectively.

4.4.3 *Spectrophotometric analyses*

Spectrophotometric methods were employed to quantify HS^-, SO_4^{2-}, NH_3, COD and protein concentrations in the effluent samples as well as during experimental runs. Full assay methods and reagent formulations are provided in Appendix A: Analytical methods.

4.4.3.1 Sulphide assay

Aqueous sulphide was quantified using the N,N-dimethyl-p-phenylenediamine (DMPD) method (APHA, 2012). The sample volume was added to 200 µℓ zinc acetate solution and diluted to 5 mℓ with deoxygenated deionised water, after which 500 mℓ of the DMPD and ferric chloride ($FeCl_3$) reagents were added. The absorbance of the well-mixed solution was measured at a wavelength of 670 nm against a blank containing deionised water and the reagents.

4.4.3.2 Sulphate assay

Sulphate was measured using the barium chloride ($BaCl_2$) method (APHA, 2012). The sample volume, typically 100 µℓ, was diluted to 5 mℓ and 500 µℓ conditioning reagent, followed by 10 µℓ of $BaCl_2$ solution were added. A saturated $BaCl_2$ solution (0.3 g $BaCl_2$ in 1 mℓ water), was prepared each time a set of analyses was performed and used instead of powdered $BaCl_2$. This was shown to provide more accurate results. The mixture was vortexed, to ensure complete suspension of the barium sulphate particles, and the absorbance measured at a wavelength of 420 nm.

4.4.3.3 Ammonia assay

Total ammonia nitrogen (TAN) (NH_3-N) was determined using the Merck Spectroquant® test kit as per the manufacturer's instructions. Separate kits were available for low and high concentration ranges (see Appendix A: Analytical methods for concentration ranges and applicable volumes of sample and reagents) The absorbance was measured at a wavelength of 690 nm.

4.4.3.4 Chemical oxygen demand (COD)

The COD assays were performed using the Merck test reagents appropriate for low (10-150 mg/ℓ) and high (500 mg/ℓ - 10 g/ℓ) concentration ranges, according to the manufacturer's instructions (see Appendix A: Analytical methods). The absorbance of the solution after digestion at 150˚C for 2 hours was read at a wavelength of 610 nm.

4.4.3.5 *Protein*

Protein concentration was determined using the method of Bradford (1976). A volume of 1 mℓ of Bradford reagent, at room temperature, was added to 1 mℓ of well-mixed, filtered sample. The absorbance was measured at a wavelength of 595 nm.

4.4.4 *Anions*

Anion concentrations (Cl^-, F^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) were determined by ion chromatography (IC) on a Thermo Scientific (Waltham, USA) DIONEX ICS-1600 system with an IonPac AG16 anion column using a 10 µl injection loop and a conductivity detector with suppression. A 22 mM NaOH solution was used as the mobile phase at a flow rate of 1 mℓ/min. Data were analysed using the Chromeleon software package.

4.4.5 *Metals*

Transition metal (Al, Cd, Cr, Co, Cu, Fe, Ni, Pb and Zn) and other cation (Ca, K, Mg, Na) concentrations were quantified by inductively coupled plasma (ICP) atomic emission spectroscopy (AES) using a Thermo ICap 6200 ICP-AES instrument or by ICP-mass spectrometry (MS) using an Agilent (Santa Clara, USA) 7900 ICP-MS instrument, for trace concentrations. Analyses were performed by the Central Analytical Facility (CAF) at Stellenbosch University.

4.4.6 *Additional analyses performed on the tannery effluent samples*

Project partners at CPUT characterised effluent samples using a Merck Spectroquant Pharo® Spectrophotometer together with Merck cell tests or kits for COD (cat no: 14555), BOD_5 (cat no: 00687), total volatile organic acids (VOA_t) as acetic acid equivalents (AAE) (cat no: 01763), total organic carbon (TOC) (cat no: 14879), total alkalinity (ALK) as calcium carbonate ($CaCO_3$) (cat no: 101758) and total phosphate (TP) as phosphorous (PO_4^{2-}-P) (cat no: 14729) according to the manufacturers' instructions.

4.4.7 *Molecular methods*

Molecular biology techniques were applied to quantify *dsr*B gene abundance by quantitative polymerase chain reaction (qPCR) and SRB community structure by next generation sequencing (NGS).

4.4.7.1 *Deoxyribonucleic acid extraction*

Total metagenomic DNA was extracted from bulk liquid (16 mℓ) and solids (0.25 g FSB) samples using a Qiagen (Hilden, Germany) PowerLyzer Ultraclean Microbial DNA isolation kit (Cat No. 12224-50) and Qiagen PowerLyzer PowerSoil DNA isolation kit (Cat No. 12855-50) respectively, according to manufacturers' instructions.

Each extraction was performed in duplicate and the DNA concentration determined using a Jenway Genova (Bibby Scientific, Staffordshire, United Kingdom) NanoDrop spectrophotometer. Equimolar amounts of each replicate were combined for amplicon sequencing.

4.4.7.2 *Quantitative polymerase chain reaction*

The relative abundance of SRB and SOB in the reactors was investigated through quantitative polymerase chain reaction (qPCR), targeting the *dsr* gene, using a CF×96 Real-Time PCR Detection system (Bio-Rad, Hercules, USA). The qPCR assays were carried out in a total volume of 20 µℓ, consisting of 10 µℓ 2x SsoAdvanced universal SYBR Green Supermix (Bio-Rad), 0.6 µℓ of each primer

(Section 4.4.7.3), 3.8 µℓ PCR-grade polymerase-free water and 5 µℓ template DNA (adjusted to 5 ng/µℓ).

After loading, the qPCR tray was vortexed for one minute and then centrifuged for two minutes. The qPCR was run under thermal cycling conditions which consisted of an initial denaturation at 94˚C for 15 min followed by 39 cycles of denaturation (94˚C for 30 sec), annealing (60˚C for 20 sec) and elongation (72˚C for 30 sec), and then a fluorescent read after 10 sec at 95˚C and 5 sec at 72˚C. Following amplification, product purity was assessed by viewing melt peak curves determined across a range of temperatures (70-95˚C).

4.4.7.2.1 Cloning

Plasmids were generated from the PCR product of full-length *dsrAB* genes, purified and cloned as documented by Kabangzou (MSc thesis) and ligated into the pGEM®-T and pGEM®-T Easy Vector (Promega, Madison, USA) according to the manufacturer's instructions. Plasmids were transformed into *Escherichia coli* JM109 high efficiency competent cells (Promega). After overnight incubation, white colonies were randomly selected from the Luria-Bertani (LB) agar plates and used to inoculate 5 mℓ LB broth containing 5 µℓ ampicillin (100 mg/mℓ). The cultures were placed in a shaking incubator at 37˚C at ~160 rpm for 16 hours. The High Pure plasmid isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to isolate the plasmids according to the manufacturer's instructions. Positive clones, containing the *dsr* amplicons, were verified by PCR amplification using the same conditions and primers as mentioned in Section 4.4.7.2 and 4.4.7.3, respectively. These clones were visualised on a 1.5% agarose gel electrophoresis stained with Pronasafe or ethidium bromide, after purification with the NucleoSpin kit (Machery-Nagel, Duren, Germany). Cloned amplicons were sequenced at Inqaba™ Biotechnical laboratories (Pretoria, South Africa) with the relevant primers. The sequences were aligned using DNA Baser Assembler software, Chromas (Technelysium, Brisbane, Australia) and compared using BLASTn against a curated NCBI database.

Cloned template DNA was used to generate the standard curve for the calculation of copy numbers.

4.4.7.2.2 Standard curve

Quantification was based on a standard curve generated using genes amplified from plasmids containing full-length *dsrAB* amplicons and a 10-fold dilution series, in triplicate, from 2.5 ng/µℓ to 2.5 x 10^{-6} ng/µℓ for the first run and to 2.5 x10^{-7} ng for the repeated run. The total copy number was calculated using the quantification cycle values, as in Equation 12.

$$copy\ numbers = \frac{amount \times 6.022 \times 10^{23}}{length\ of\ DNA \times 10^{9} \times 660} \qquad (12)$$

Where, 6.022×10^{23} represents Avagadro's number;

660 is the average molecular weight of double stranded DNA (dsDNA);

Length of DNA = 350 base pairs (bp); and

1×10^{9} is the conversion factor for ng

4.4.7.3 Next generation sequencing and statistical analyses

Next generation sequencing (NGS) was performed for 22 samples using the Illumina (San Diego, USA) NovaSeq 6000 workflow as per the manufacturer's guidelines at Molecular Research (MR DNA) (Shallowater, USA). Targeted sequencing using primer sets for the gene encoding for dissimilatory

sulphite reductase (*dsr* gene) as the phylogenetic marker was accomplished. The primer pair used for the *dsr* gene was, with the forward primer being barcoded:

- dsr2061F 5'-CAACATCGTYCAYACCCAGGG-3' (Geets *et al*., 2006)
- dsr4R 5'-GTGTAGCAGTTACCGCA-3' (Wagner *et al*., 1998)

An initial denaturation step at 95 °C for 5 min was followed by 30 cycles of denaturation (95 °C for 30 sec), annealing (53 °C for 40 sec), and extension (72 °C for 1 min) using the HotStarTaq Plus Master Mix kit (Qiagen). The PCR products were quality checked by visualization in 2% agarose gel. Aliquots of samples containing equimolar amounts of DNA were pooled and purified using calibrated Ampure XP beads (Beckman Coulter, Brea, USA) and used to prepare the DNA library according to the Illumina TruSeq protocol. Amplicon sequencing was performed using an Illumina NovaSeq instrument according to the manufacturer's instructions.

The data were analysed using the MR DNA analysis pipeline: The sequences were joined and the barcodes removed. Then, the sequences with < 150 bp and/or ambiguous base calls were removed, and the sequences were denoised. Operational taxonomic units (OTUs) were then generated and chimeras were removed using UCHIME (Edgar *et al.*, 2011). The OTUs were defined by clustering at 3% divergence using UCLUST (Edgar, 2010). The OTUs were then taxonomically classified using BLASTn against a curated National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

Relative abundance data matrices were saved as Microsoft Excel files and analysed with the software, Primer 7 (Primer-E Ltd, UK). All data was square root transformed and then a Bray-Curtis similarity matrix was constructed. A cluster plot (group average linkages) and non-metric multidimensional scaling (nMDS) plot based on sample abundance of operational taxonomic units was made and subsequently overlaid. Similarity percentage (SIMPER) analyses were also performed on the transformed data.

4.5 Research approach and experimental methodology

This section provides a broad overview of the approach adopted, followed by details on the specific sets of laboratory experiments. The research approach is summarised in Figure 14.

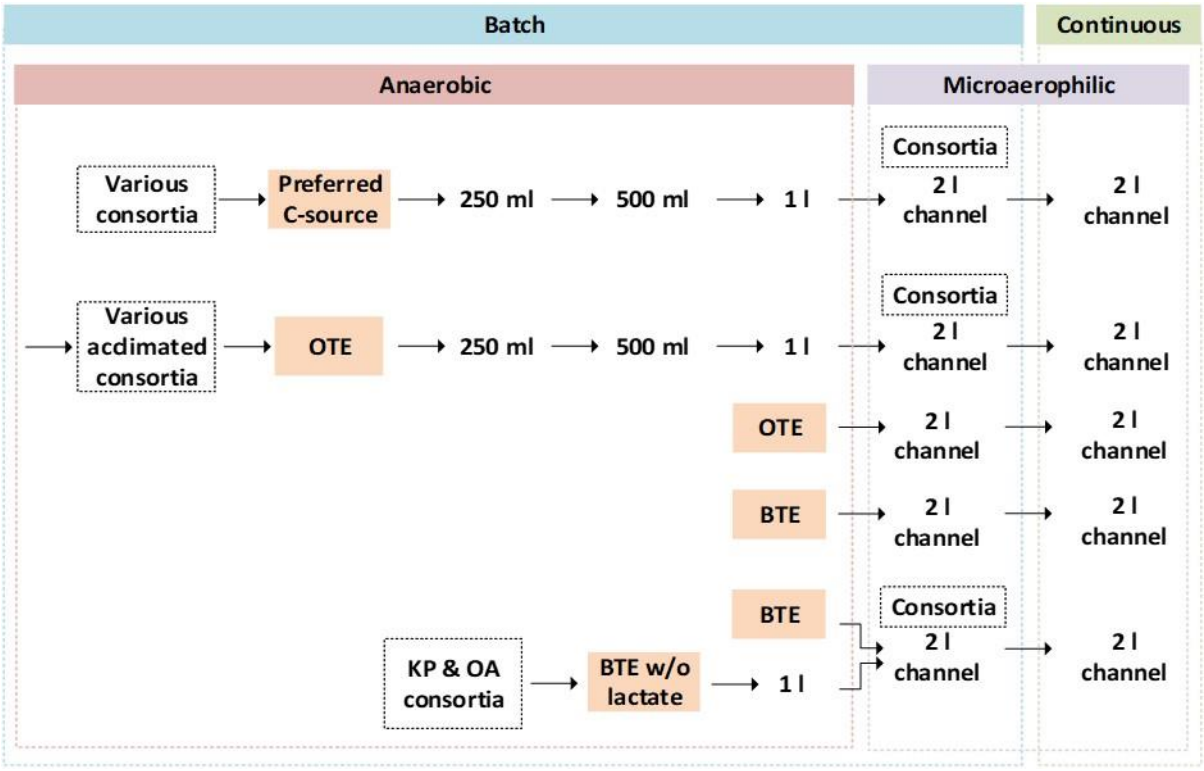

Figure 14: Overview of the research approach

4.5.1 *Batch anaerobic digestion tests on raw and pre-treated BTE*

A key hypothesis for this research was that the BTE was not suitable as a substrate for AD without pre-treatment to reduce the sulphur species concentrations. To evaluate this, batch AD tests were performed using the BTE as received, alongside a series of controls.

Each reactor was inoculated with 200 mℓ of active AD sludge from an industrial scale facility processing abattoir effluent with the remaining 793 mℓ consisting of either of the BTE or diluted BTE (Table 16). Positive controls containing either acetate or microcrystalline cellulose (MCC) were included (3 g COD/ℓ). Each reactor was supplemented with 6 mℓ of macro nutrient solution (170 g/ℓ NH_4Cl, 8 g/ℓ $CaCl_2.2H_2O$ and 9 g/ℓ $MgSO_4.7H_2O$) and 0.6 ml trace element (TE) solution (2 g/ℓ $FeCl_3.4H_2O$, 2 g/ℓ $CoCl_2.6H_2O$, 0.5 g/ℓ $MnCl_2.4H_2O$, 30 mg/ℓ $CuCl_2.2H_2O$, 50 mg/ℓ $ZnCl_2$, 50 mg/ℓ HBO_3, 90 mg/ℓ $(NH_4)_6Mo_7O_2.4H_2O$, 100 mg/ℓ $Na_2SeO_3.5H_2O$, 50 mg/ℓ $NiCl_2.6H_2O$, 1 g/ℓ EDTA, and 1 mℓ/ℓ HCl 36% (Hobo *et al.*, 2019)).

The reactors were then sparged with N_2 and immediately sealed. The reactors were leak tested to ensure all gas produced was collected in the foil PLV bags. The reactors were placed on a shaker at 120 rpm in an incubator at 38˚C.

The reactors were sampled periodically by withdrawing a 5 mℓ volume using the syringe. The sample was immediately tested for pH, before a 2 mℓ fraction was centrifuged at 14,000 rpm for 5 min and the supernatant filtered through a 0.45 µm filter before being used to measure soluble COD and ammonia. Biogas composition was measured using a Geotech BIOGAS 5000 portable gas analyser and

the volume determined by displacement, using an inverted, graduated 1 ℓ measuring cylinder. The cylinder was filled with acidified water to prevent CO_2 dissolution and the volume was adjusted to account for the "dead volume" in the tubing between the analyser and the cylinder.

Table 16: Composition of batch AD reactors testing untreated BTE

Reactor	Sludge (mℓ)	BTE (mℓ)	Macro (mℓ)	TE (mℓ)	Water (mℓ)	Substrate
Pos control 1	200	-	6	0.6	793	3.8 g Na acetate
Pos control 2	200	-	6	0.6	793	3 g MCC
BTE 1 A & B*	200	793	6	0.6	-	
BTE 2 A & B	200	793	6	0.6	-	3.8 g Na acetate
BTE 3 A & B	200	397	6	0.6	396	

*A: raw BTE and B: partially treated BTE

A second set of AD reactor tests was performed using treated BTE (Section 4.6.3), following the same protocol as before. Two positive control reactors were set up, the first in water and the second in ASW, with an EC of 26.3 mS/cm. Both were provided with 3.8 g sodium acetate (3 g COD/ℓ) as substrate. Tests were performed using full-strength treated BTE, as well as treated BTE diluted 50% with water to reduce the EC and concentration of potential inhibitors. A third experimental reactor was set up using the diluted treated BTE supplemented with sodium acetate (3.8 g/ℓ). The reactors were sampled as described above.

4.5.2 *Acclimation of enriched seawater consortia to tannery effluents in batch reactors*

4.5.2.1 Ostrich tannery effluent

Active enrichment cultures growing in lactate or acetate supplemented artificial seawater with HS^- concentrations of > 250 mg/ℓ, were evaluated for their ability to grow in OTE, with a lower salinity (7.5 mS/cm in comparison to ~43 mS/cm for artificial seawater). Initially, separate 250 mℓ reactors were inoculated with enrichment cultures from each location and topped up with well-mixed OTE (50% vol./vol.).

Each consortium was assessed for its ability to use OTE as a carbon source and electron donor for the reduction of SO_4^{2-} by monitoring pH, conductivity, redox potential and HS^-, SO_4^{2-} and COD concentrations. Active cultures were scaled up by increasing batch reactor volumes (Figure 14), as previously described (Section 4.3.1). If necessary, Na_2SO_4 was added to provide the consortia with a similar initial SO_4^{2-} concentration as the artificial seawater batch tests (~2000 mg/ℓ) (Section 4.1.3). The reactors were operated at ambient temperature for up to 293 days, from initial inoculation. Active cultures were used to inoculate the HLFCR.

4.5.2.2 Bovine tannery effluent

The BTE had a conductivity that was very similar to that of seawater (32 mS/cm) so the consortia did not need to be re-acclimated in terms of salinity. However, the BTE may have contained other potentially inhibitory substances to which the microbial communities needed to adapt. In addition, the suitability of the organic matter within the BTE as a substrate for SRB was tested by assessing whether supplementation with lactate, a more readily available carbon source, would be beneficial.

The KP and OA consortia were used to inoculate the raw BTE and partially treated BTE separately in duplicate 1 ℓ reactors, with and without lactate supplementation (Table 17; Figure 14). These batch reactors were designated by:

(i) the geographical location where the consortia was obtained (KP: Kathy Park or OA: Old Airstrip),
(ii) which type of BTE the consortia were grown on (R: raw BTE, PT: partially treated BTE), and
(iii) whether the consortia were supplemented with lactate.

For example, $OA_{R,Lac}$ = Old Airstrip consortia grown on raw BTE and supplemented with lactate.

Table 17: Formula for BTE batch tests with and without lactate supplementation

Component	Volume (mℓ)
Original enrichment*	40
KP_{2L} or OA_{5L}	250
Raw or partially treated BTE	710/660
Lactate solution, if added	50

*Refers to the original 250 mℓ Kathy Park or Old Airstrip consortium

The reactors were maintained for 127 days at ambient temperature. They were monitored in terms of pH, conductivity, redox potential and HS^-, SO_4^{2-} and COD concentrations. If any of the reactors depleted the SO_4^{2-}, 600 mℓ was decanted into a 5 ℓ container and stored and the reactor refilled with the appropriate BTE. On day 101, OA_R, $OA_{R,Lac}$, KP_R and $KP_{R,Lac}$ were all sub-cultured and topped up with partially treated BTE, to reduce the HS^- concentrations and provide more SO_4^{2-} for reduction.

4.6 Continuous operation in the hybrid linear flow channel reactor

The hybrid linear flow channel reactors are interchangeably referred to as either “HLFCRs” or “channels” in this thesis.

4.6.1 *Hybrid linear flow channel reactors operated on artificial seawater*

The initial tests using the HLFCR, to assess the suitability of the reactor, were performed using a feed consisting of artificial seawater (2.71 g/ℓ SO_4^{2-}) with lactate (2.55 g/ℓ) as the substrate.

The reactors were inoculated with a consortium made up of a blend from the separate batch enrichment cultures, detailed in Table 18. Enrichment cultures with high HS^- concentrations were preferred. The channel was supplemented with lactate and topped up with artificial seawater (Table 14).

Table 18: Ratio of each enrichment consortia in inoculum of each channel grown on lactate and volume of artificial seawater topped up with

Run	Ratio of consortia in inoculum					Seawater (mℓ)
	Z	K_L	K_A	KP	OA	
1	1	1	0.8	0.2	0.2	50
2	0.27	0.7	0.5	1	1	265
3	0.7	0.7	0.27	1	1	165

At the start of each run the reactor was operated in batch mode until a FSB formed at the air-liquid interface in the channel. If the FSB did not form spontaneously the reactor was seeded with dried FSB powder to stimulate the formation of biofilm. Once continuous operation started, feed was pumped into the channel through the feed port using an 8-head variable speed peristaltic pump. The volumetric flow rate was calculated from the desired hydraulic retention time (HRT) and reactor volume according to Equation 13.

$$\dot{V} = \frac{V}{\tau} \qquad (13)$$

During continuous operation, the channel was operated at a HRT of 4 days, unless otherwise stated. Two runs were performed for 56 and 70 days, respectively, at ambient temperature. Samples (5 mℓ) were drawn from the bulk liquid through the sample ports using a syringe and hypodermic needle. The pH, redox potential and HS^- concentrations were measured immediately. The remaining sample was centrifuged and filtered and used to measure COD and SO_4^{2-}.

During continuous operation, if the biofilm was disrupted, washed out or harvested, the vent holes were temporarily closed and the channel was run in batch mode until the FSB re-formed.

Between each run, the channel was washed out, scrubbed, dried and then prepared for the next run.

4.6.2 *Hybrid linear flow channel reactors operated on ostrich tannery effluent*

Two HLFCR runs were performed using blended OTE as a substrate, following the same protocol as for artificial seawater (Section 4.6.1). For the first run, the HLFCR was inoculated with enriched consortia acclimated to OTE (Section 4.5.2.1) and the second was filled with the 7th batch of OTE (high HS^- effluent), that had a greater likelihood of containing an adapted endogenous community capable of BSR and SO (Figure 14). The ratio of consortia used in the inoculum of the first channel is shown in Table 19, run 1. The channels were operated at ambient temperature for 56 and 130 days respectively. The channels were sampled as previously described and monitored for pH, conductivity, redox potential and HS^-, SO_4^{2-} and COD concentrations.

Table 19: Ratio of each enrichment consortia in inoculated HLFCR grown on OTE and volume of tannery effluent required to fill the reactor

Run	Ratio of consortia in inoculum					Tannery effluent (mℓ)
	Z	K_L	K_A	KP	OA	
1_{OTE}	1	0.11	0.44	0.11	0.11	100

4.6.3 *Hybrid linear flow channel reactors operated on bovine/ovine tannery effluent*

The HLFCRs, operated using BTE as the substrate, were set up and monitored using the same protocol as for the artificial seawater and OTE reactors. Where there were significant changes to the protocol, these are detailed in the subsequent sections.

4.6.3.1 Single HLFCR without inoculation

There was a strong suspicion that the BTE contained an active endogenous SRB community. The first channel was set up to assess the activity of the endogenous community, so was initially filled with the first batch of raw BTE.

The reactor was maintained in batch mode for six days, until a thick biofilm completely covered the reactor surface. Continuous operation was started at a 1-day HRT, but it quickly became apparent that the feed rate was too high as the residual SO_4^{2-} concentration rapidly increased. The reactor was switched back to batch mode until almost all the SO_4^{2-} in the bulk liquid had been reduced (day 31). The reactor was switched back to continuous mode at a 2-day HRT for one cycle, after which the feed rate was further reduced to achieve a 4-day HRT, which was maintained for the remained of the experiment (104 days).

Unlike the OTE samples, which were all received well before the channel reactor experiments started, so a blended feed could be used, the BTE samples were received as the channel experiments progressed. Due to the large variation in composition it was necessary to blend the raw and partially treated BTE, sometimes mixing different batches (e.g. raw batch 3 with partially treated batch 2) to produce a feed with a relatively consistent pH, SO_4^{2-} and HS^- concentration. The specific BTE batches used to make the blended feed as the experiment progressed are highlighted on the relevant graphs in the results chapter (Section 7.3.4).

The reactor was sampled from the front and back sample ports 3 times per week during batch mode and 5-7 times per week during continuous operation. The feed composition was measured at the start of each HRT. At each sampling time, when available, the effluent composition was also measured. The effluent sample was obtained from the effluent pipe during the first phase of operation and directly from the weir, using a syringe, once the second reactor had been connected in series. The FSB was periodically disrupted by physically breaking it up and allowing the fragments to settle on the harvesting screen, or harvested, where the screen was removed from the reactor and the accumulated FSB recovered. The FSB was dried at 37˚C for 3 days to determine dry mass and a sample of the dried FSB was used for elemental analysis to determine C, N, H and S content.

4.6.3.2 Channel 2 – inoculated with enrichment cultures and operated in series

The data obtained from the first channel showed that BSR, biofilm formation and partial SO were possible in a HLFCR populated with the endogenous microbial community. The residual SO_4^{2-} concentration in the effluent from channel 1 was still relatively high, suggesting two reactors in series could further improve performance. As a consequence, a second, identical HLFCR was set up, this time inoculated with a mixture of enrichment cultures adapted to BTE and raw tannery effluent. The composition of the inoculum is summarised in Table 20.

Table 20: Ratio of enrichment consortia and volume of tannery effluent used to inoculate the second HLFCR

Run	Ratio of consortia in inoculum					Tannery effluent (mℓ)
	Z	K_L	K_A	KP	OA	
2_{BTE}	0.56	0	0	1	1	1082

The second channel was started after the first channel had been running for 42 days. It was initially operated in batch mode until 75% of the SO_4^{2-} present was reduced. The channels were then connected in series, with the second channel receiving effluent from the first as its feed. The reactors were connected on day 64 of operation of channel 1. At this stage channel 1 was operating at a 4-day HRT so the overall HRT across the reactor pair system was effectively 8 days.

The second channel was sampled at the same time as the first, using the same protocol.

4.6.3.3 Sampling for microbial community structure analysis

To assess whether continuous operation in the HLFCRs resulted in changes in microbial community structure, the channel reactors were sampled each time a complete FSB formed after a change in influent raw BTE batch and once pseudo-steady state had been achieved. For the bulk liquid, a 16 mℓ sample was taken, while for the FSB, DNA was extracted from 0.25 g of solid material. These samples were analysed alongside previous samples taken from each batch of raw BTE (2-4 mℓ sample) and the enrichment cultures (16 mℓ sample) prior to inoculation of the reactors.

5 Consortia isolation, enrichment, characterisation & pre-treatment proof of concept

5.1 Introduction

The majority of literature describing BSR and SO focuses on solutions with relatively low salinity, particularly where the technology is applied to wastewater. Tannery effluent is typically characterised by high salinity, so the selection of microbial communities from saline environments will be preferable to trying to adapt laboratory cultures grown on defined, low salinity media.

The purpose of the work described in this chapter was to source bacteria capable of BSR and SO, to enrich them with a suitable carbon source and use them to inoculate a HLFCR system and evaluate this pre-treatment as a proof of concept.

5.2 Research approach

The various parts of the study are related to each other as shown in Figure 15. The beginning of each results chapter will contain the same figure, with the part of the study under discussion bordered in red.

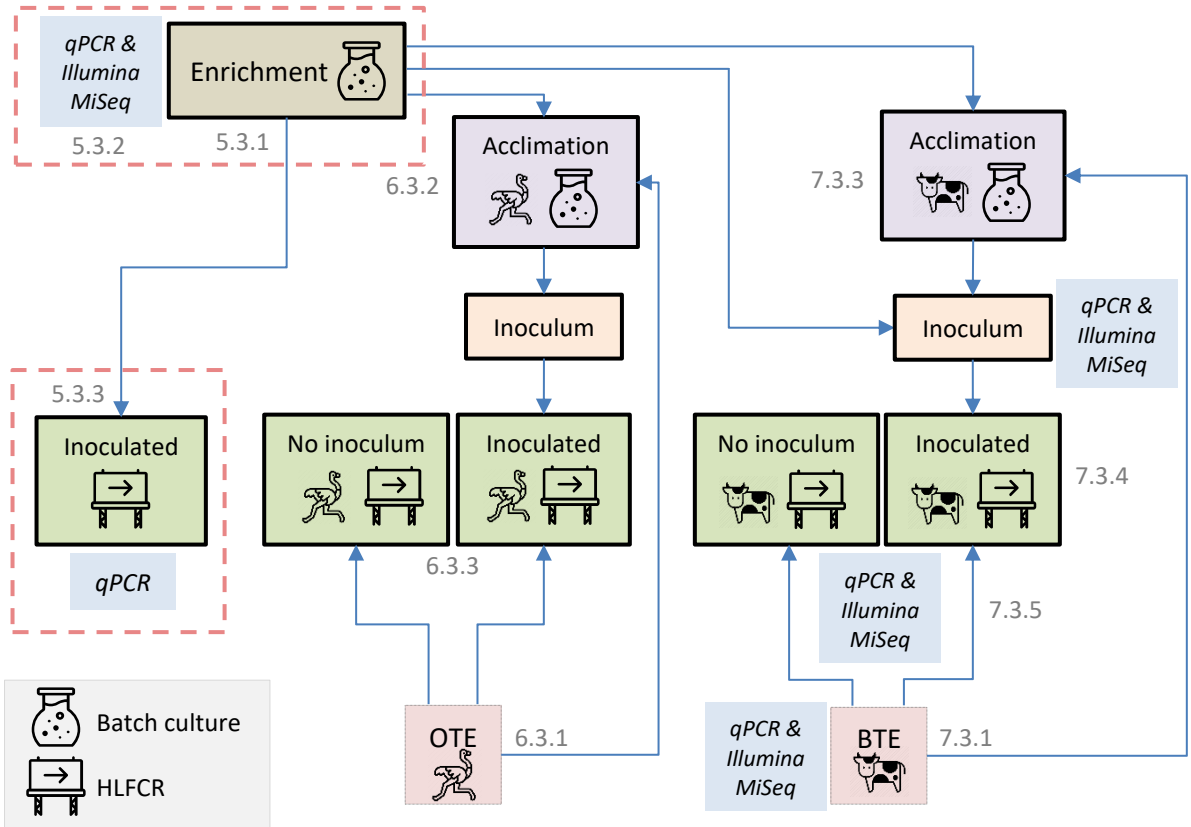

Figure 15: Flow diagram depicting the relationship of the batch enrichment cultures and inoculated HLFCRs to rest of the study
(Step 1: Batch enrichment of consortia and HLFCR proof of concept)

The first objective was to isolate consortia capable of BSR from marine environments and enrich them in anaerobic batch reactors with a suitable carbon source. Those that showed potential were sub-

cultured in increasing volumes and maintained for subsequent tests. The enrichment cultures were designated by:

(i) the geographical location where the sample was obtained (M: Muizenberg, Z: Zandvlei, K: Kasouga, KP: Kathy Park, OA: Old Airstrip),
(ii) for the Kasouga consortia, the specific substrate used to maintain the culture (A: acetate, L: lactate). The absence of a subscript prefix label indicates the culture from that location was only grown on lactate, and
(iii) volume of the batch reactor.

For example, Z_{1000} = Zandvlei consortium grown on lactate in a 1 ℓ reactor or K_{A500} = Kasouga consortium grown on acetate in a 500 mℓ reactor. The original enrichment consortium from each geographical area was the only reactor of its size and therefore, it was labelled with only the geographical area.

The most active consortia were used to inoculate HLFCRs to assess whether BSR and SO could take place in artificial seawater with the mixed consortia grown on the suitable substrate. Where possible, the HLFCRs were run continuously, with regular monitoring of pH, redox potential, HS^- and SO_4^{2-}. Floating sulphur biofilm formation was evaluated visually.

Metagenomic DNA was extracted from each of the enrichment consortia after at least seven months of maintenance, as well as from the HLFCRs at specific times. The DNA from all samples was used to determine *dsr*B gene copy numbers by qPCR, while the enrichment consortia extracts were used for NGS sequencing to evaluate their sulphur species metabolising community structure.

5.3 Results and discussion

5.3.1 *Isolation and batch enrichment of seawater consortia*

The consortia were sourced from semi-stagnant saline or estuarine water. The distinctive odour of rotten eggs (H_2S) was indicative of active BSR.

The samples had similar physical and chemical properties (Table 21). All samples had a pH close to neutral and high salinity, with conductivities ranging between 35-49 mS/cm, similar to artificial seawater (~43 mS/cm; Section 4.1.3). The SO_4^{2-} concentration of the Zandvlei, Kathy Park (KP) and Old Airstrip (OA) samples were close to that of artificial seawater (2710 mg/ℓ), while the Kasouga River Estuary and the Muizenberg bog samples contained 42% and 61% of the SO_4^{2-} present in the artificial seawater. For the most part, the HS^- present was negligible (<2.7 mg/ℓ), however, even this small amount confirmed the presence of SRBs.

Table 21: Characteristics of samples from the different sites

Property	Muizenberg	Kasouga	Zandvlei	Kathy Park	Old Airstrip
pH	7.88	7.24	7.24	7.31	6.55
Conductivity (mS/cm)	39.4	35.4	42.1	43	48.5
HS^- (mg/ℓ)	2.67	0.06	0.00	0.59	0.30
SO_4^{2-} (mg/ℓ)	1141	1650	2150	2295	2300

All samples were similar in appearance, containing murky liquid, plant matter and sediment (Figure 16A). The Kasouga River Estuary sample was visibly pink-purple, suggesting the presence of purple sulphur bacteria (PSB) (Figure 16B). Purple sulphur bacteria are bacteria that oxidise various inorganic reduced sulphur compounds, such as HS^-, polysulphides, $S_2O_3^-$, $S_4O_6^-$, S^0 or SO_3^- for energy and are predominantly living by anoxygenic photosynthesis (Canfield *et al*., 2005; Megonigal *et al*., 2003). While most PSB are obligate anaerobes, some species are capable of tolerating low concentrations of oxygen and can grow chemolithoautotrophically in the absence of light, using oxygen to oxidise HS^- (Pfennig, 1989). The PSB contain a single photosystem, bacteriochlorophyll a (bcl *a*) (Dworkin *et al*., 2006). They produce carotenoid accessory pigments, which impart a purple colour and widen the spectrum of light utilised (Dworkin *et al*., 2006).

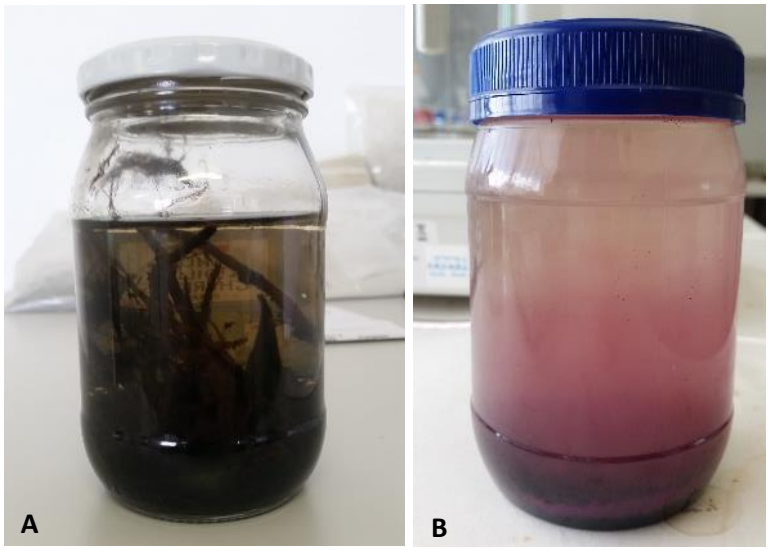

Figure 16: Photographs of environmental samples. A) Muizenberg bog, B) Kasouga River

5.3.1.1 Selection of a suitable carbon source

The Muizenberg bog sample was the first sample collected and was screened under anaerobic conditions, using all four potential carbon sources. The results are shown in Figure 17.

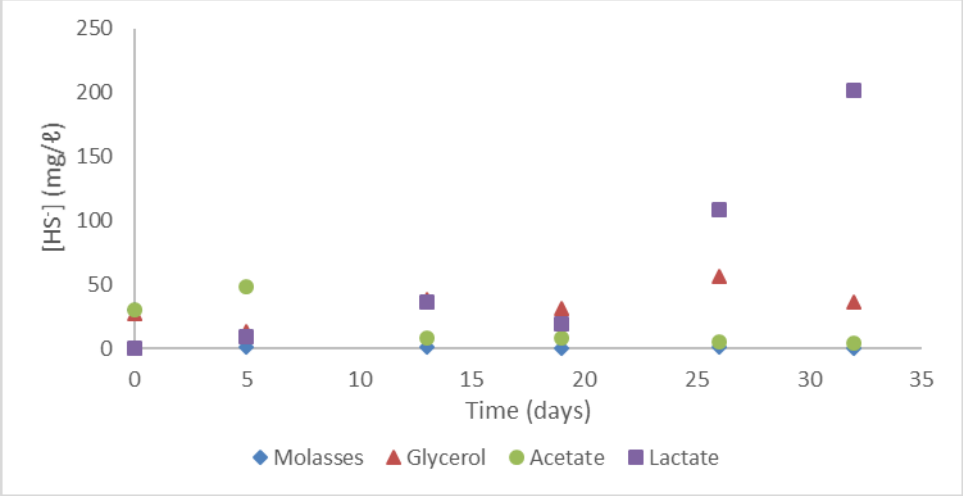

Figure 17: Change in sulphide over time for the Muizenberg consortia growing on different carbon sources

The consortium grown on acetate ($M_{acetate}$) showed signs of BSR activity over the first five days, but declined to almost zero by day 13. The $M_{glycerol}$ and $M_{lactate}$ cultures showed similar levels of activity until day 18 (38 and 36 mg/ℓ HS^- on day 18). Thereafter $M_{lactate}$ became far more active, achieving at least 45 mg SO_4^{2-}/ℓ.day between days 26-32 and reaching a HS^- concentration of 202 mg/ℓ, while $M_{glycerol}$ became inactive. The culture showed no BSR on molasses throughout the experiment.

Therefore, lactate showed the most potential as a carbon source for this halophilic BSR consortium and was used in subsequent tests. Lactate is a popular carbon source for laboratory studies as it supports a wide spectrum of SRB, so encourages a diverse community (Hao *et al*., 2014). However, it is seldom used at scale due to availability and its high cost. Glycerol and molasses were excluded from subsequent tests. In contrast to these findings, Zouch *et al*. (2017) found that acetate outperformed lactate in the long term (> 13 days) for enrichment of marine cultures grown on Na_2SO_4.

5.3.1.2 Batch anaerobic enrichment of environmental samples with lactate or acetate supplementation

Data were acquired for each environmental sample for at least 300 days. The intention was to assess activity and enrich the cultures, not to collect detailed performance data, so only some of the data are presented to illustrate trends and general procedures followed.

Acetate has been used as the sole carbon source for certain SRBs (Brahmacharimayum *et al*., 2019) and has been found to improve SO and FSB formation (Mooruth, 2013). It was employed as a carbon source and compared to lactate in a pair of tests on the Kasouga River Estuary consortium.

The Muizenberg sample showed relatively low activity during the extended monitoring so was not selected as an inoculum for the tannery effluent tests.

5.3.1.2.1 Zandvlei culture enrichment

The Zandvlei culture showed almost immediate BSR activity on lactate, achieving an aqueous sulphide concentration of 400 mg/ℓ after 1 week. After sub-culturing, the 250 mℓ consortium (Z) achieved almost complete sulphate reduction (99.6%) and a maximum HS- concentration of 616 mg/ℓ on day 21 (Figure 18).

All sub-cultures from the initial consortium showed good activity. For the first 50 days of operation, the pH of all Zandvlei cultures remained between pH 7.2 and pH 7.6, while the redox potential remained below -300 mV, after an initial value of -100 mV.

After the initial 50 days, the pH averaged around pH 7.0 for all reactors until day 225, before gradually decreasing to an average pH of 6.8. At this pH a greater proportion of the sulphide would be in its unionised toxic form of H_2S (Figure 6). This could explain why the activity of the consortia decreased significantly after day 225. The average SRR of Z between day 50 and 225 was 52 mg/ℓ.day, in comparison to 20 mg/ℓ.day from day 225-420. It is postulated that H_2S inhibition may have played a role in slowing the activity of the consortia.

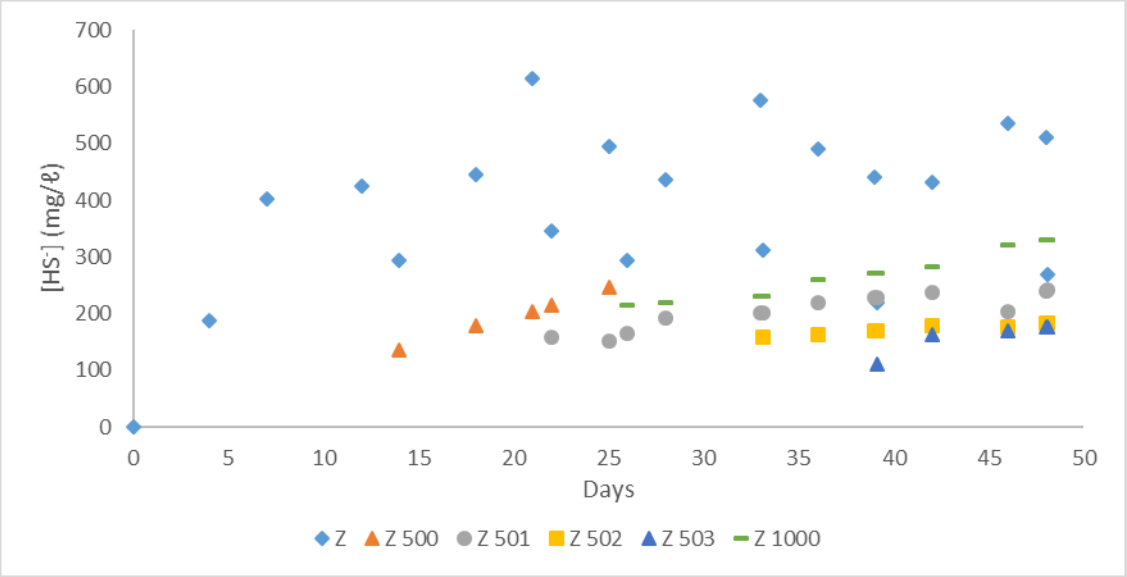

Figure 18: Change in HS^- concentration over time of the Zandvlei enrichment consortia. Z = original 250 mℓ enrichment consortia reactor; Z_{500} = first 500 mℓ enrichment consortia reactor; Z_{501} = second 500 mℓ enrichment consortia reactor; Z_{502} = third 500 mℓ enrichment consortia reactor; Z_{503} = fourth 500 mℓ enrichment consortia reactor; Z_{1000} = 1 ℓ enrichment consortia reactor.

5.3.1.2.2 Kasouga River Estuary culture enrichment supplemented with lactate or acetate

The Kasouga River Estuary sample was enriched using both lactate and acetate, in separate reactors. The original 250 mℓ culture growing on acetate (K_A) depleted most of the SO_4^{2-} (145 mg/ℓ SO_4^{2-} remaining from an initial concentration of 1982 mg/ℓ) after 25 days (92% SO_4^{2-} conversion), while the original on lactate (K_L) reached a SO_4^{2-} conversion of 89% after 36 days.

However, activity of all but one of the acetate-supplemented consortia started levelling off after 56 days and became much slower after 105 days. The exception was K_{A500}, which maintained an average SRR of 29 mg/ℓ.day for its last 48 days of operation, after which it was used to inoculate channel reactors.

The reactors fed with lactate generally outperformed those fed with acetate, with the exception of K_{A500}, in terms of mean SRR, although performance was similar for the 1 ℓ reactors. As with the Z reactors, the average SRR decreased as the experiment progressed with the mean values consistently < 20 mg/ℓ.day.

5.3.1.2.3 Knysna estuary culture enrichment

The reactors containing consortia sourced from the Knysna estuary achieved the highest maximum SRRs of 400 mg/ℓ.day and 500 mg/ℓ.day in the initial 250 mℓ reactors for the Old Airstrip (OA) and Kathy Park (KP) cultures respectively. The average rates did decrease through successive sub-culturing and scale up events, but remained among the highest of all enrichments. These consortia performed well in 2 ℓ and 5 ℓ reactors, with maximum SRRs of 38 and 26 mg/ℓ.day, respectively. The change in HS^- concentration for KP is shown in Figure 19 and illustrates that the culture maintained activity through regular sub-culturing (KP and KP_{500}) reactors and scale up (KP_{1000} and KP_{2000}).

These consortia appeared to be efficient SO_4^{2-} reducers, with the original KP culture reducing all the SO_4^{2-} present at the start (1830 mg/ℓ) in less than 29 days. After the first round of sub-culturing the

consortium reduced 87% of the added SO_4^{2-} over a 10-day period, equating to a SRR of 131 mg/ℓ.day. The OA culture achieved similar rates (data not shown).

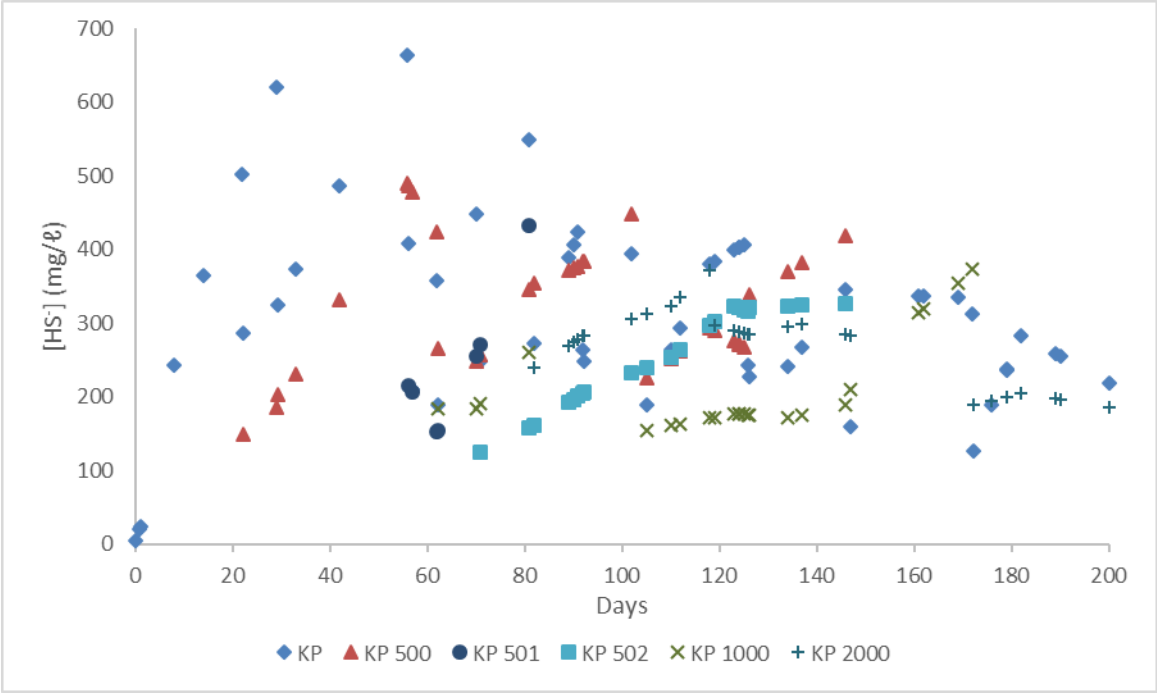

Figure 19: Change in HS^- concentration over time for each Kathy Park enrichment consortium

KP = original 250 mℓ enrichment consortia reactor; KP_{500} = 500 mℓ enrichment consortia reactor; KP_{1000} = 1 ℓ enrichment consortia reactor.

The redox potential for these reactors had initial values of around -130 mV, but quickly decreased to -300 mV and were maintained in a range between -295 and -411 mV, suitable for anaerobic BSR (Figure 7).

Reactor KP had an average pH > 7.0 until around day 100, which is when the activity of all the reactors started slowing. Meanwhile, reactor OA had an average pH > 7.0 until around day 180, which also aligned with a decrease in BSR activity. However, HS^- concentrations of ~500 mg/l were maintained in OA, which helped to keep the pH higher.

5.3.1.2.4 Overall performance of enrichment consortia

The batch cultures from all the environments followed a similar performance trend, with high initial SRRs over the first 103-147 days, followed by a steady decrease over the next 250-300 days. Several factors may limit SRR, such as the availability of nutrients (primarily N and P), insufficient amounts of labile organic matter, inadequate retention times and substrate limitations (Waybrant *et al*., 2002). As no N and P salts were added to the artificial seawater (Table 14), it is more likely that nutrient deficiency contributed to the slowing in activity, rather than retention time or organic carbon limitation. In addition, the reactors showed a relatively consistent trend of decreasing pH as the experiments progressed, which would have shifted sulphide speciation toward aqueous H_2S. Most lactate utilising SRB perform incomplete oxidation, resulting in the accumulation of acetate, which could have driven the decrease in pH value.

In summary, environmental samples were obtained from six locations and enriched in batch cultures on lactate and acetate. All six were capable of BSR and showed tolerance to high sulphide

concentrations. As time progressed, some became less active in terms of BSR. Overall, the Zandvlei, Kathy Park and Old Airstrip consortia were found to be the most effective at BSR, with reactors reaching high HS^- concentrations very quickly in comparison to the others.

5.3.2 *Characterisation of the enrichment cultures*

The most successful enrichment cultures were used to inoculate the HLFCRs on artificial seawater and later, the batch and continuous reactors on the tannery effluents. These were used for the molecular analysis to quantify *dsr*B gene abundance and characterise the SO_4^{2-} reducing community. The results are detailed below.

5.3.2.1 Variations in gene copy numbers

Samples for DNA extraction for qPCR quantification of the *dsr*B gene were taken after the cultures had been maintained for over six months in the laboratory, prior to inoculation of the second BTE channel (Section 7.3.4.2). The results and other relevant parameters are provided in Table 22.

Table 22: Sulphate reduction rates, dsrB gene abundance and their correlation for metagenomic DNA samples of original enrichment consortia

Sample	**DNA concentration** ng/µℓ	**Run day of sample** Days	***dsr*B gene abundance** Copies/ng	**SD** Copies/ng
K_L	43.2	222	1.48E+06	72,464
K_A	50.4	222	2.48E+05	13,542
Z	59.9	334	6.98E+05	11,774
KP	23.8	295/341*	1.26E+06	35,256
OA	24.4	295/341*	1.30E+06	15,247

*composite sample from two days

The concentration of DNA extracted from Kasouga and Zandvlei samples were substantially higher than for the Kathy Park and Old Airfield. It is possible that the biomass concentration could have been lower in the KP and OA cultures, due to the age of the cultures, although this does not explain the high value obtained for the Zandvlei culture. However, at the time of the final sampling of the KP and OA cultures, minimal BSR had occurred over the previous 18 days (producing < 2 mg HS^-), while the Zandvlei culture exhibited BSR that produced 23 mg HS^- at the time of the sample.

At the time of sampling, the K_L culture had a relatively high SRR, while K_A had a substantially lower activity, which decreased further after sampling.

The four cultures maintained on lactate showed a relatively consistent relationship between *dsr*B gene abundance and performance. The K_L and Z cultures had similar SRRs around the time of sampling. The specific *dsr*B gene concentration for Z was ~50% of that of K_L, but the total DNA concentration extracted from Z was ~50% higher than K_L. This suggests that there may be more non-SRB that persisted in the Z culture. As for KP and OA, both consortia were still active, requiring sub-culturing every 50 days and maintaining SRRs of 26-32 mg/ℓ.day, consistent with the relatively high *dsr*B gene concentration.

The K_A culture had a significantly lower *dsr*B gene concentration, which was consistent with the relatively low SRR, despite the extracted DNA concentration being high. This suggests that there could

be more non-SRBs in the enrichment and a broader diversity of anaerobes. There are fewer SRB species capable of utilising acetate than lactate.

It is theoretically possible to calculate cell number from qPCR data, but this requires knowledge of the copy number of the gene in all species present and confidence that the amplification efficiency is high. This is not possible in this case, so values are indicative only.

The quality of the data obtained, of the melt curves and standard curve, is discussed in Section 7.3.5.1.1.

5.3.2.2 Variations between consortia communities as a result of spatial and substrate differences

Samples were collected across a range of ca. 900 km along the Western and Eastern Cape coastline of South Africa. As expected, there was a diverse community of sulphur species metabolising (based on *dsr*B gene sequence) bacteria within each of these environmental samples. However, there was also a significant amount of similarity in community structure between the samples (56.2% across all samples, using SIMPER), demonstrated by the spatial distribution of points representing the enriched consortia in the non-metric multidimensional scaling (nMDS) plot overlaid with cluster analyses in Figure 20.

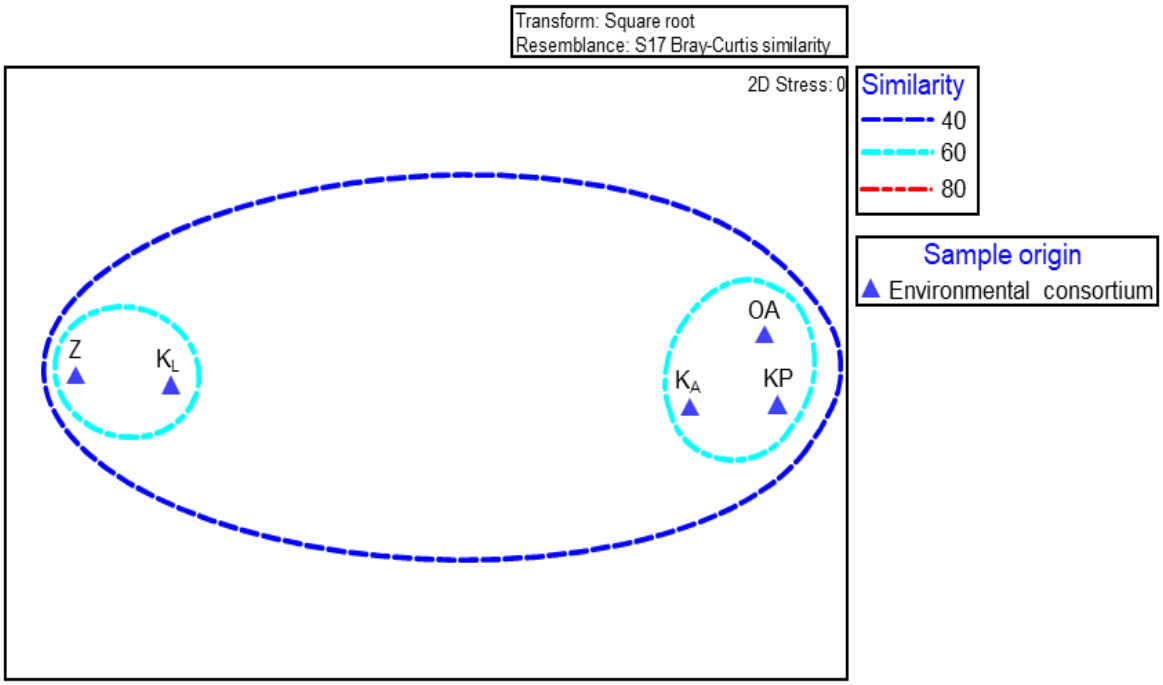

Figure 20: nMDS plot overlaid with cluster analysis of the Bray-Curtis similarity of relative abundance between bacterial species, based on dsr gene analysis, from enriched environmental

Consortia KP and OA show more than 60% similarity (Figure 20), which is not unexpected as the two samples were taken within 1.5 km of each other. Therefore, the environments are more likely to share similar biotic and abiotic characteristics. On the other hand, while originally from the same sample, enrichment consortia K_L and K_A are only within 40% similarity to one another, illustrating the effect substrate has on community development. The higher 60% similarity of Z to K_L and K_A to KP and OA is more surprising, considering their spatial separation (ca. 880 km and 400 km apart, respectively). However, despite the distance between samples, the sediment, plants, water characteristics, etc. may

be similar enough to have selected for similar communities. The in-depth characterisation of the sediments was beyond the scope of this study.

Figure 21A summarises relative abundance (RA) of bacterial species containing the *dsr*B gene for each of the enrichment consortia, with a RA cut-off of 4.2%. The data show that the 12 species that fit the criteria accounted for > 80% RA across all samples, with an average of 88% coverage. The unclassified *Desulfovibrio* sp. MCM B_508 was a dominant species across all consortia, with the greatest RA contribution in KP (56.9%) and OA (60.5%).

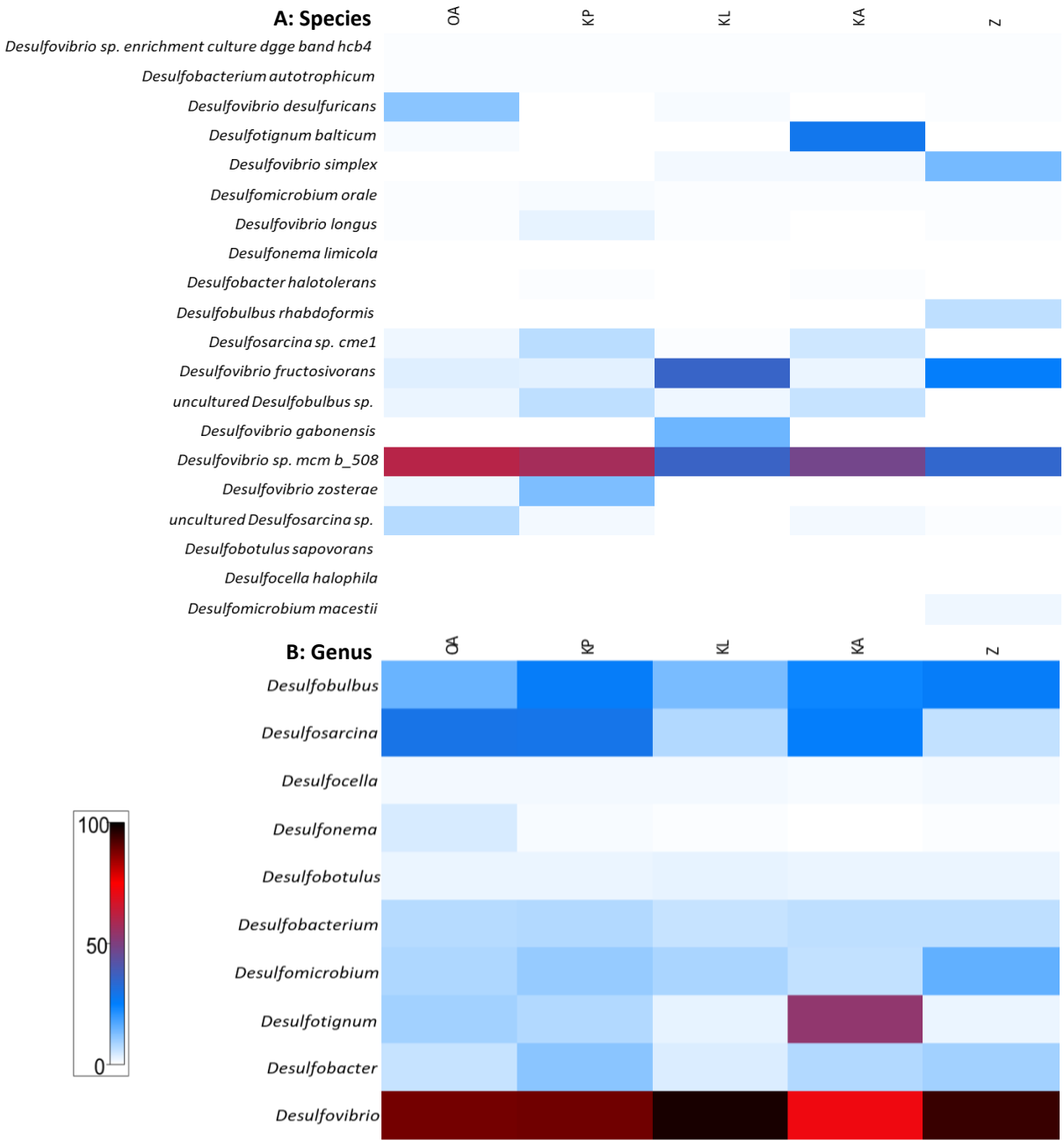

Figure 21: Amplicon sequencing results displayed as a shade plot of abundance of bacterial species containing dsr (A) and genus (B) in the enrichment consortia

Desulfovibrio sp. MCM B_508 was less prevalent in consortia K_L and Z (36.0% and 34.2%, respectively). For these consortia, *Desulfovibrio fructosivorans* made up a substantial fraction of the SRB (35.5% and 25.6%, respectively), while it was effectively insignificant (≤3% RA) in the others. These similarities

between consortia K_L and Z explain why they are classed as 60% similar by the cluster analysis in Figure 20. While known for its ability to utilise fructose, the presence of *D. fructosivorans* in consortia K_L and Z suggests its ability to utilise lactate as well. It is an incomplete oxidiser, leading to the production of acetate, which could be useful to other SRBs (Ollivier *et al*., 1988).

Desulfovibrio fructosivorans was absent from K_A, which instead had *Desulfotignum balticum* as the second most important species (28.3% RA). Consortium K_L also had *Desulfovibrio gabonensis* at 14.2% RA, while K_A had 0.1% RA. According to Kuever *et al*. (2001) and Tardy-Jacquenod *et al*. (1996), *D. balticum* and *D. gabonensis* are halotolerant, preferring 10-25 g NaCl/ℓ and tolerating up to 17% NaCl, respectively, which is consistent with the Kasouga estuary and artificial seawater (Table 14). Both have been shown to utilise a variety of simple organic compounds and fatty acids substrates, with Kuever *et al.* (2001) finding *D. balticum* to be a complete oxidiser, unless substrate concentrations were high. The results suggest that *D. balticum* preferred acetate over lactate. Tardy-Jacquenod *et al*. (1996) found that *D. gabonensis* could use SO_3^{2-}, $S_2O_3^{2-}$ and S^0 as electron acceptors in the presence of lactate.

Desulfovibrio simplex was present in the Z consortium sample at 13.4% RA. This species grows using a small variety of electron donors, including lactate, which is incompletely oxidised to acetate (Zellner *et al*., 1989). The original *D. simplex* strain XVI was isolated from the AD of sour whey and was found to prefer trace concentrations of NaCl (0-0.46 g/ℓ), with no growth observed for 18 g NaCl/ℓ, indicating that the strain found in consortium Z has adapted to tolerate higher NaCl concentrations.

The KP and OA consortia were dominated by the uncultured *Desulfovibrio* sp. MCM B_508, with the next most abundant SRB also being *Desulfovibrio* species. The KP consortium contained *Desulfovibrio zosterae* (12.6%), while *Desulfovibrio desulfuricans* (11.5%) was a significant component of the OA consortium. The species *D. zosterae* is found in marine environments and is capable of utilising lactate as well as various other organic acids as electron donors with SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ and S^0 as electron acceptors (Nielsen *et al*., 1999). The bacteria *D. desulfuricans* is well-researched in terms of its mercury methylation pathway and has maximum growth rates at NaCl concentrations of 170-342 mM, which are lower than the artificial seawater (409 mM NaCl). It can utilise lactate as an electron donor, along with other simple organics (Gilmour *et al*., 2011; Steger *et al*., 2002). The consortia contained smaller amounts (1.1-7.1% RA) of the complete acetate oxidiser *Desulfatiglans anilini* and uncultured *Desulfosarcina* sp. and *Desulfosarcina* sp. CME1*,* capable of lactate and acetate utilisation, as well as the incomplete lactate oxidiser, uncultured *Desulfobulbus* (Dworkin *et al*., 2006; Kuever *et al.*, 2015; Suzuki *et al.*, 2014).

Figure 21B shows that *Desulfovibrio* was the dominant genus in all cultures. In other studies describing enrichments of BSR using marine isolates, (e.g. Zouch *et al*., 2017), *Desulfovibrio* sp. also dominated, albeit with greater overall taxonomic diversity. This genus contains incomplete oxidisers with limited metabolic flexibility (Gilmour *et al*., 2011), with less substantial contributions by *Desulfobulbus*, *Desulfosarcina* and *Desulfomicrobium* across the consortia, while only consortium K_A contained a substantial amount of the genus *Desulfotignum* (28.3%). Therefore, in all consortia, the ratio of incomplete oxidisers to complete oxidisers was between 5.5-84.2, with the exception of consortium K_A, where they were only 1.6-times higher, due to the presence of acetate as an electron donor. The dominance of *Desulfovibrio* sp. MCM B_508 in the K_A consortium after over 200 days of enrichment on acetate alone indicates it must be able to grow on some product of acetate metabolism. The most likely explanation is that it can utilise hydrogen generated by other anaerobes (e.g. *Clostridium*

species) as the electron donor and CO_2 as the carbon source (Baffert *et al*., 2019; Benomar *et al*., 2015; Dworkin *et al*., 2006; Pankhania *et al*., 1986).

This means that there is likely to be an accumulation of acetate in the other four consortia reactors, unless there are un-sequenced community members that are able to fill this functional niche (Kuever *et al*., 2001). Fortunately, in application, accumulated acetate from the pre-treatment would be utilised by MA as it is the primary organic substrate in the production of methane (Bajpai, 2017; Deublein and Steinhauser, 2008).

5.3.3 *Semi-continuous artificial seawater hybrid linear flow channel reactors supplemented with lactate*

The batch tests showed the potential of the enrichment cultures to reduce SO_4^{2-} in artificial seawater, primarily with lactate as the carbon source (Section 5.3.1.2). The next step was a proof of concept evaluation using the HLFCR to test whether it could support simultaneous BSR and FSB formation in this pre-treatment system, using a mixed enrichment consortium (Figure 15: red border). The treatment of high salinity wastewater that is high in sulphur species has a broad range of possible applications.

5.3.3.1 Experimental run 1

The first experimental run of the seawater channel fed with lactate was inoculated with a mix of the Z : K_L: K_A : KP : OA consortia in the ratio of 1 : 1 : 0.8 : 0.2 : 0.2. The variation in HS^- and SO_4^{2-} concentration over time can be seen in Figure 22.

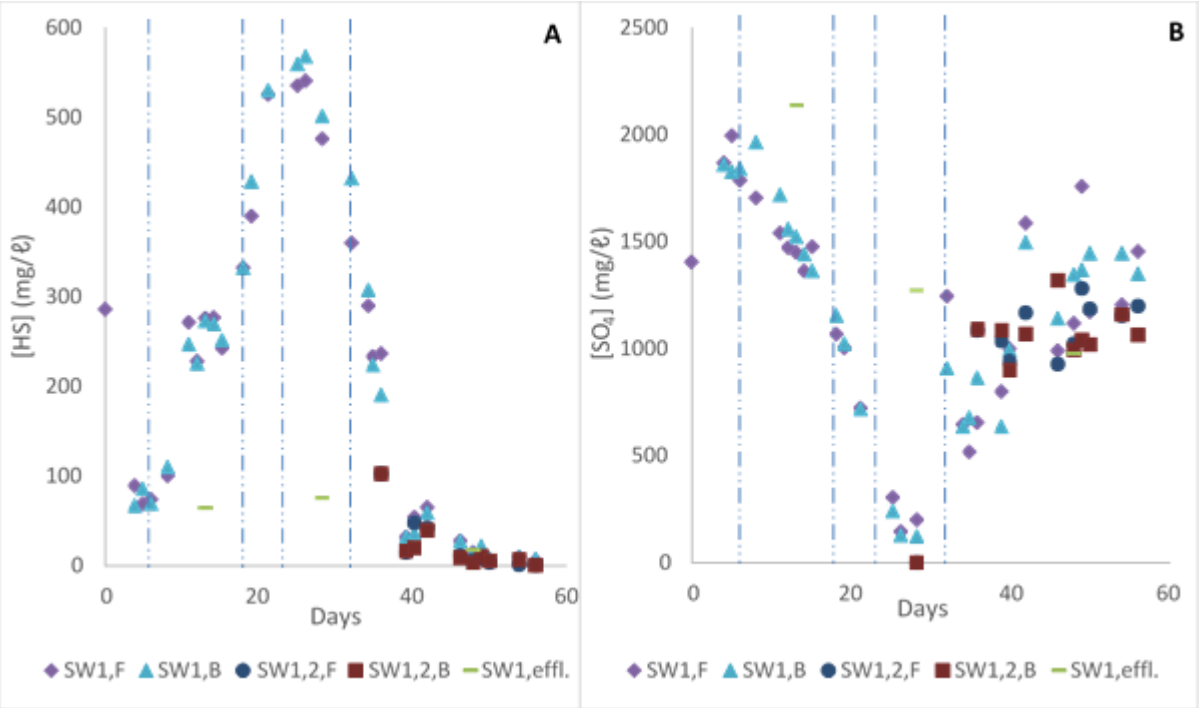

Figure 22: Change in HS^- (A) and SO_4^{2-} (B) concentrations over time for first seawater run HLFCR with lactate supplementation. $SW_{1,F}$ = 1st run of seawater channel, front sample port; $SW_{1,B}$ = 1st run seawater channel, back sample port; $SW_{1,2,F}$ = 1st run of seawater channel, 2nd channel in series, front; $SW_{1,effluent}$ = effluent of first run of seawater channels; dot-dash line indicates seeding with FSB.

Initially, the channel showed a decrease in the HS^- concentration. This was because the biofilm had not developed sufficiently to impede O_2 mass transfer from the reactor headspace into the bulk liquid, leading to rapid SO, both partial oxidation to colloidal S^0 and complete oxidation to SO_4^{2-}, hence the increase in SO_4^{2-} concentration (Marais *et al.*, 2020; Mooruth, 2013).

The enrichment cultures were maintained in closed bottles to maintain anaerobic conditions so are likely to have selected against sulphide oxidisers and aerobic heterotrophs, which are required for FSB development. After 6 days of operation, FSB from an artificial seawater culture was added to seed the channel. This was effective and elemental S^0 started to form around the channel edges. Biofilm formation allowed the HS^- concentration to increase enough to start continuous feeding (4-day HRT). The HS^- concentration in the effluent (64 mg/ℓ) was substantially lower than in the reactor, indicating successful SO at the surface. However, the SO_4^{2-} concentration in the effluent remained high indicating limited partial oxidation to elemental S^0, so more complete oxidation, resulting in a total SR of only 19%. A blockage in the effluent pipe led to an increase in reactor volume and then a rapid release of effluent, which caused a wash out of the FSB and resultant deceleration of HS^- production. However, the headspace was closed, so limited O_2 was available and BSR was not compromised.

On day 18, the channel was seeded with more exogenous FSB. However, still no FSB had formed by day 22, and the channel was re-seeded with fresh FSB from the channel effluent collection container. There was a period of instability, where the thin biofilm again washed out and the amount of HS^- remaining in the system decreased, but the fact that the reactor was sealed ensured high SRRs of 112-147 mg/ℓ.day were still maintained. Around 92% of the SO_4^{2-} present in the feed was reduced by day 28, a high removal efficiency showing high SRB activity.

On day 28, the HRT was reduced from 4 to 2 days. This resulted in a rapid increase in SO_4^{2-} and decrease in HS^-concentrations between days 28-32, as the culture appeared unable to adapt to the higher loading rate. It is possible that a significant portion of the planktonic SRB community was washed out during this time, as the HS^- concentration continued to drop and not exceed 65 mg/ℓ again, while the SO_4^{2-} concentration continued to increase.

The effluent from the channel was collected in an open 5 ℓ container that typically developed a thick biofilm over the surface. This was collected and used to re-seed the channel on day 32, this time with moderate success and a day later, there was a 50% complete biofilm. As the collected effluent appeared to contain active SOB, its contents were used to inoculate a second HLFCR in series. Three days later, both channels had complete thin biofilms, resulting in a slight increase in HS^- and an acceleration of BSR in the first channel (SRR of 14 mg/ℓ.day from day 36-39 and 270 mg/ℓ.day from day 39-40) and the second channel (SRR of 9 mg/ℓ.day from day 36-39 and then 285 mg/ℓ.day from day 39-40).

Unfortunately, on day 41 the effluent pipe from the first channel blocked again and when it was released the majority of biofilm from both channels was lost. The system did not recover from this and the experiment was ended two weeks later.

Throughout the run, the pH of the bulk liquid and effluent remained over 7.0, with an average of 7.95 for the first channel and 8.36 for the second channel. Similarly, the redox potential of both channels was maintained below -426 mV for the duration.

In summary, despite the challenges experienced the system did briefly show near complete SR at a 4-day HRT, achieving HS^- concentrations above 500 mg/ℓ. Biofilm formation was inconsistent, although selected seeding assisted with the formation of a FSB. The fact that the reactor was operated as a sealed unit with the aeration ports closed may have compromised biofilm formation.

5.3.3.2 Experimental run 2

This seawater HLFCR was inoculated with volumes of the Z : K_L: K_A : KP : OA consortia in the ratio of 0.3 : 0.7 : 0.5 : 1 : 1, to see whether a change in inoculum allowed improved BSR, SO and biofilm formation. As with the first run, there was an initial decrease in the HS^- concentration as the O_2 present in the reactor headspace caused oxidation due to the lack of biofilm (Figure 23). The bulk liquid developed a cloudy yellow appearance by day 7 indicating the presence of colloidal sulphur and some polysulphides, the latter formed by sulphur disproportionation as the pH became > 8.0 (Brüser *et al.*, 2000; Lenk, 2011; Mooruth, 2013).

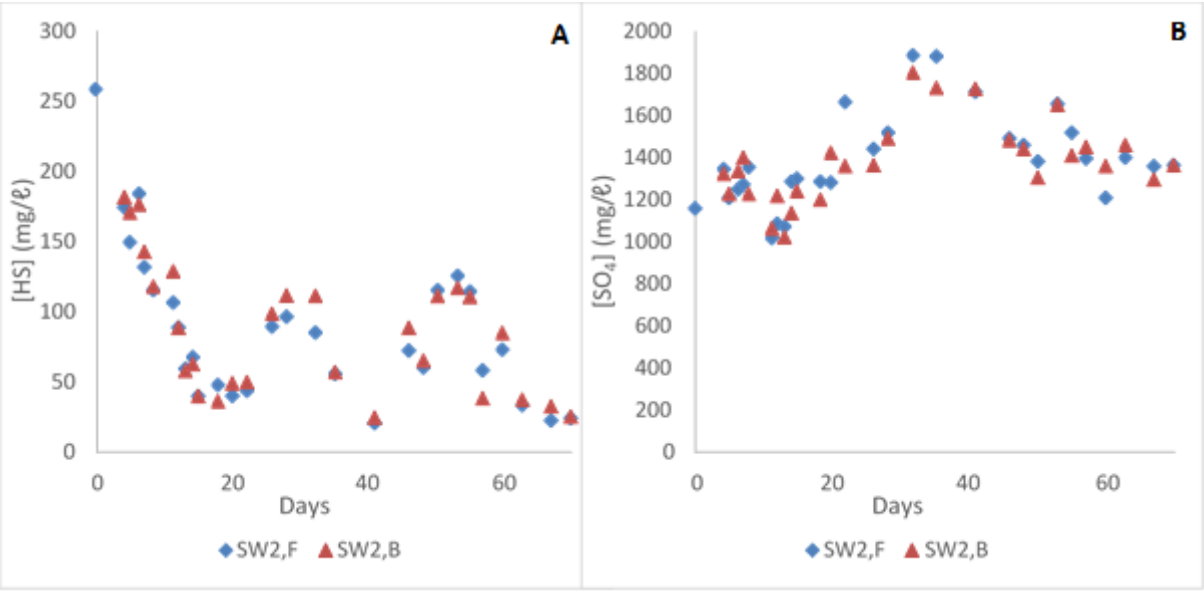

Figure 23: Change in HS^- (A) and SO_4^{2-} (B) concentrations over time for second seawater HLFCR with lactate supplementation. $SW_{2,F}$ = 2nd seawater channel, front; $SW_{2,B}$ = 2nd seawater channel, back

This led to a decrease in the HS^- concentration until day 11, a period almost twice as long as the first experimental run, by which time the bulk liquid was fully beige and cloudy and had a full thin FSB. As HS^- concentrations were decreasing, the channel was run in batch mode until they increased and a biofilm formed.

This slight browning of the bulk liquid could have been the first signs of the presence of green/brown sulphur bacteria (GSB), as on day 28 a significant opaque brown colouring of the bulk liquid had developed, corresponding with an increase in HS^- (up to 111 mg/ℓ), and four days later the channel bulk liquid was deep brown in colour. The brown-coloured GSB contain the isorenieratene pigment and along with other GSB species, are often found at greater depths than PSB with lower light intensities and possess chlorosomes, large antenna pigment structures that amplify light due to aggregated bcl *e* properties (Dworkin *et al.*, 2006; Frigaard and Bryant, 2004; Kimble-Long and Madigan, 2002; Madigan and Jung, 2008; van Gemerden, 1986). The first 32 days had an average SRR of 2 mg/ℓ.day and the maximum SR achieved was only 24% between days 8-11. On day 41, a pink-red colouring appeared, as can be seen in Figure 24, suggesting the presence of PSB (Section 5.3.1). On

day 46, a full thick biofilm had formed, aligning with a corresponding decrease in SO_4^{2-} and an increase in HS^- in the preceding days.

Figure 24: Purple-brownish colour of second seawater HLFCR on day 47, with an almost complete FSB

This finally allowed commencement of feeding on day 48 at a 4-day HRT. After an initial increase in HS^- (up to 117 mg/ℓ) and SO_4^{2-} (increase of about 350 mg/ℓ), both concentrations fell again and then the SO_4^{2-} did not reduce further and the HS^- concentration fell to < 25 mg/ℓ (Figure 23). From day 32-70, the channel was able to achieve an average SRR of 71 mg/ℓ.day, and the maximum SR of 27% between days 57-60.

5.3.3.3 Overall performance of hybrid linear flow channel reactors grown on lactate

The preceding sections show a successful proof of concept, illustrating that concurrent BSR and SO can be performed in the channel under saline conditions, using microbial consortia enriched from natural environments. Additionally, biofilm formation was demonstrated in this system, although a number of challenges were experienced that affected the rate of formation and stability of the biofilm. Biofilm formation is particularly sensitive to wash out and blockages and is therefore considered the part of the process that poses the greatest risk.

From a performance perspective, it was difficult to collect stable rate data as the reactors did not maintain steady state for long periods. The average SRRs for the experimental runs were 83 and 41 mg/ℓ.day respectively, although during times of stable performance (e.g. day 22-30 in run 1) the SRR increased to almost 150 mg/ℓ.day.

Sulphide oxidation was also effective, with effluent concentrations consistently below 70 mg HS^-/ℓ, while the concentration in the bulk liquid was above 400 mg/ℓ. Due to problems with biofilm formation, a lot of the sulphur formed as colloidal sulphur, rather than in the FSB, which was not ideal.

5.3.4 *Summary of findings*

Six consortia were isolated in a variety of coastal marine environments and enriched in batch reactors to evaluate their ability to perform SR. Batch enrichment consortia reactors with the highest SRR and HS^- production were with consortia from the Knysna Estuary, specifically, Old Airstrip and Kathy Park consortia. Some of their enrichment reactors reached SRRs of 550 mg/ℓ.day. These consortia all had conductivities ranging 40-45 mS/cm and saw a pH drop from 7.4 to around 6.5, dropping to below

neutral after 100 days. This pH drop coincided with a drop in activity for all consortia from average SRRs of 40-116 mg/ℓ.day to 5-28 mg/ℓ.day. The top performers were capable of complete SR from initial SO_4^{2-} concentrations of around 2100 mg/ℓ, reaching a maximum HS^- concentration of 664 mg/ℓ. The most active enrichment consortia at the time were chosen to inoculate continuous reactors.

While three seawater HLFCR runs were performed, the first run saw the highest maximum SRR (634 mg/ℓ.day) as well as attained the highest average SRR (83 mg/ℓ.day), the highest HS^- concentrations (568 mg/ℓ) and exhibited 92% SR. This indicates high SRB activity and therefore, capable consortia as an inoculum for BSR. The first segment of the reactor operation (until day 48) had much better performance than the latter (days 48-108), with an average SRR of 129 mg/ℓ.day in comparison to 11 mg/ℓ.day, respectively. This indicates a decreasing activity of the reactor with time. Furthermore, a correlation of SRR activity with the feeding cycles was found, indicating a near first order relationship of BSR to SO_4^{2-} concentration, due to the fresh supply of SO_4^{2-} in the feed. Lastly, biofilm formation was found to be the rate-limiting step, which limited the volume of feed supplied and the anaerobic environment of the reactor and therefore, the reduction of SO_4^{2-} and oxidation of HS^-. Issues aside, these seawater HLFCRs grown on lactate provide a basic proof of concept for the pre-treatment of sulphur species under saline conditions in the semi-passive HLFCR and potential suitability to treat tannery effluent.

6 Ostrich tannery effluent studies

6.1 Introduction

The previous chapter demonstrated that the environmental enrichments were able to reduce SO_4^{2-} and grow in a highly saline environment. However, the salinity of the OTE was far lower than that of the enrichment reactors and required acclimation. Similarly, a host of new compounds were found in the OTE, some in potentially inhibitive concentrations, while it was deficient in other key nutrients. Therefore, the next step was to see whether the enrichment cultures could be adapted to the OTE and whether treatment of the sulphur species in a HLFCR system could be successful with the adapted consortia or the microbial community endogenous to the OTE.

6.2 Research approach

With the enrichment consortia ready and the HLFCR concept proven using artificial seawater, the OTE was first characterised and then the evaluation of growth in the OTE in batch cultures could begin (Figure 25: red border) using the same labelling convention as before (Section 5.2). For example, Z_{OTE501} = Zandvlei acclimation reactor grown on OTE in second 500 mℓ reactor. Once BSR was achieved, a HLFCR was inoculated with the consortia and compared to a HLFCR that contained only the OTE and its existing endogenous community.

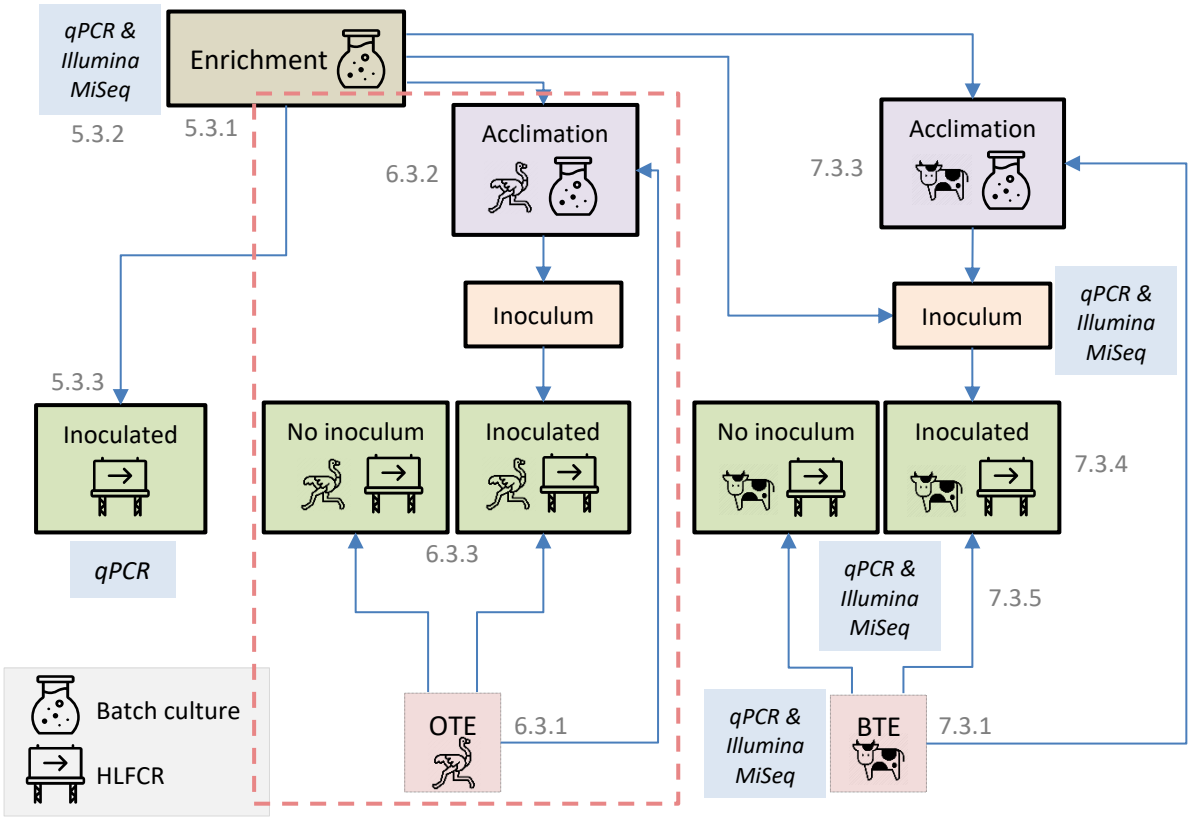

Figure 25: Flow diagram depicting the relationship of OTE studies to rest of the study (Step 2: Acclimation to OTE and treatment in HLFCR)

Concentrations of sulphur species were low in the OTE and required supplementation of Na_2SO_4 to ~2000 mg/ℓ in each reactor. These reactors were regularly monitored in terms of pH, redox potential, HS^- and SO_4^{2-}.

6.3 Results and discussion

6.3.1 *Ostrich tannery effluent characterisation and notable findings*

Effluent streams were obtained from an ostrich tannery for six weeks. These streams were characterised with specific attention to sulphur species and organic load. The full characterisation is shown in Table 23.

As anticipated, each batch of OTE had a significantly different composition. These fluctuations were assumed to be due to variations in process parameters, further influenced by environmental factors and microbial activity. For example, the batches had an average conductivity of 7.47 mS/cm with a standard deviation (SD) of 3.13 mS/cm, and an average COD of 8.55 g/ℓ with a SD of 3.77 g/ℓ. This shows that properties can vary by almost 50% from their average value.

Changes in feed composition can lead to unstable reactor performance (Buljan and Král, 2011). A resilient microbial consortium will support more stable performance in the face of variable feed (Mpofu *et al*., 2019a). The impact can be further mitigated through pulse-feeding and placing a balancing tank upstream of the reactor (Welz *et al*., 2018).

The pH of all batches was close to neutral (6.92) with a low SD (0.26). The COD:SO_4^{2-} ratio in batches 2-5 ranged from 13 to 37. However, the COD:SO_4^{2-} ratios of 7.1 and batch 6.1 were measured in batches 1 and 6, respectively. This was due to high SO_4^{2-} concentrations (1110 and 1190 mg/ℓ, respectively), not low COD concentrations (Table 23).

Active competition between SRBs and MAs has been reported in AD reactors at COD:SO_4^{2-} ratios of 1.7-2.7, with MA dominating at higher ratios (Burton *et al.*, 2009; Choi and Rim, 1991) (Section 2.4.3.5). However, this is not always the case. In an ASBR treating high SO_4^{2-} wastewater, Sarti and Zaiat (2011) found a 99% removal efficiency of SO_4^{2-} and 32% for COD removal at a COD:SO_4^{2-} ratio of 3.67, and Vossoughi *et al*. (2003) found a higher SO_4^{2-} removal efficiency (97%) than COD (>85%) at a ratio of 6 in an anaerobic baffled reactor treating synthetic wastewater. These efficiencies vary widely across literature and depend on a wide variety of parameters, including sulphate and COD concentration, carbon source, initial microbe populations, type of seed sludge and degree of acclimation (Chen *et al.*, 2008; Lens *et al.*, 1998a) (Section 2.5.2.4).

A common recommendation to guarantee the outcome of the competition is to operate the AD with wastewater containing a COD:SO_4^{2-} ratio exceeding 10, where the H_2S formed will never exceed the critical inhibiting value in the reactor, due to the stripping effect of the biogas produced (Rinzema and Lettinga, 1988; Sabumon, 2016). It is possible that pre-treatment to reduce the SO_4^{2-} of OTE with lower COD:SO_4^{2-} ratios, as in batches 1 and 6, could enhance the competitive advantage of MA over SRB, thereby promoting methanogenesis over sulphidogenesis during AD of OTE (Swartz *et al*., 2017). Unless a methanogenic community that is robust and adapted to lower COD:SO_4 ratios can be established, pre-treatment to reduce sulphate may be vital to ensure they are able to reliably out-compete SRBs.

Most metals were present in concentrations substantially below (by a factor of 10-10,000) the IC_{50} values for MA and acetogens (Abdel-Shafy and Mansour, 2014; Lin, 1992; Zayed and Winter, 2000). The concentrations of Fe, Zn, Cu, Cd, Ni, Pb and Al were generally higher in the first two batches than

the second four. Some metals, including Ca, Co, Cu, Ni and Zn, are necessary as metabolic co-factors and were found to be within or below the optimal range for AD (Thanh *et al.*, 2016).

Table 23: Characterisation of each batch of ostrich tannery effluent

Parameter	Unit	1	2	3	4	5	6	Mixed	Average	SD
pH		6.49	6.73	7.33	7.09	6.92	6.93	6.66	6.92	0.26
EC	mS/cm	8.22	8.27	8.81	11.87	4.04	3.61	3.50	7.47	3.13
TOC	mg/ℓ	2467	3380	4530	9080	485	820	ND	3460	3148
COD	mg/ℓ	7945	8143	7903	15690	4387	7235	2721	8551	3768
BOD	mg/ℓ	3532	1472	1542	1515	1531	1552	ND	1857	821
VOA_t	mg/ℓ AAE	3070	2800	2440	2480	2120	1800	ND	2452	456
TN	mg/ℓ	440	235	180	260	220	530	ND	311	140
TAN	mg/ℓ NH_3-N	18.8	13.5	16.2	41	13.2	9.6	ND	18.7	11.3
NO_3	mg/ℓ	143.8	39	28.6	18.7	11.5	54.7	ND	49.4	48.7
TP	mg/ℓ PO_4^{2-}-P	6.65	5.1	5.05	17.8	5	4.9	ND	7.41	5.11
SO_4	mg/ℓ	1114	626	352	424	173	1186	ND	646	417
HS^-	mg/ℓ	ND	2.38	5.7	2.2	0	0.12	2.61	2.08	2.31
Cl	mg/ℓ	2038	1547	1294	1022	911	2369	ND	1530	576
TS	g/ℓ	7.85	8.07	8.38	19.4	5.53	4.69	ND	8.98	5.3
TVS	g/ℓ	3.61	4.06	4.97	14.6	2.82	2.32	ND	5.4	4.61
K	mg/ℓ	11.7	19.9	13.2	12.3	10.7	6.6	ND	12.4	4.3
Na	mg/ℓ	1477	1315	1953	2789	964	754	ND	1542	740
Fe	µg/ℓ	3272	3081	606	497	193	282	ND	1322	1446
Ca	mg/ℓ	11.7	24	24.2	6.9	17.6	16.8	ND	16.9	6.8
Mg	mg/ℓ	19.4	15.5	39.5	55.3	14.9	13.2	ND	26.3	17.2
Zn	µg/ℓ	1568	674	439	401	229	198	ND	585	511
Cu	µg/ℓ	304	136	16.6	65.9	12.7	12.1	ND	91.2	115
Co	µg/ℓ	7.7	4.7	1.6	1.9	44.2	1.9	ND	10.3	16.8
Cd	µg/ℓ	2.27	1.08	0.18	0.27	0.18	0.18	ND	0.69	0.85
Ni	µg/ℓ	73.1	18.4	18.7	21.3	5.6	8.4	ND	24.2	24.7
Cr	µg/ℓ	766	57	1094	350	584	136	ND	498	395
Pb	µg/ℓ	8.4	2.3	4.8	6.8	5.2	4.1	ND	5.3	2.1
Al	µg/ℓ	1798	2366	583	624	85	101	ND	926	941
Alk	g/ℓ $CaCO_3$	245	236	330	264	297	308	ND	280	37
$COD:SO_4$		7.1	13	22.4	37	25.3	6.1	ND	18.5	10.9
TVS:TS		0.46	0.5	0.59	0.76	0.51	0.49	ND	0.55	0.10
BOD:COD		0.44	0.18	0.2	0.1	0.35	0.21	ND	0.25	0.11
C:N		5.61	14.4	25.2	34.9	2.20	1.55	ND	14.0	12.4
VFA:Alk		12.5	11.9	7.39	9.39	7.14	5.84	ND	9.03	2.48
COD:TVS		2.2	2	1.59	1.07	1.56	3.12	ND	1.92	0.64

AAE – ascorbic acid equivalents; EC – electrical conductivity; ND – no data; SD – standard deviation; TAN – total ammonia nitrogen; TN – total nitrogen; TOC – total organic carbon; TP – total phosphates; TS – total solids; TVS – total volatile solids, VOA – volatile organic acid.

The total nitrogen (TN) concentrations measured in this OTE were typically higher than those previously reported by Mannucci *et al*. (2014) (190 mg/ℓ) in their batch BSR studies.

Reactors fed with effluent that has lower than optimal C:N ratios (20-30) are likely to suffer NH_3 inhibition during AD (Sri Bala Kameswari *et al.*, 2014). In the OTE, most batches exhibited below optimal C:N ratios (1.6-14.4), with batch 4 marginally above optimal (34.9) and only batch 3 within the optimal range for AD processes (25.2).

Based on the analyses of the OTE it appears as though pre-treatment before AD may not be necessary, as the HS^- concentrations were below the published inhibitory ranges of 50-250 mg/ℓ (IC_{50}) for unacclimated MA (Burton *et al*., 2009; Chen *et al.*, 2008; McCartney and Oleszkiewicz, 1993). If all the SO_4^{2-} (646 mg/ℓ average, Table 6) was converted to H_2S, this would equate to approximately 220 mg/ℓ HS^-, which could be inhibitory. However, as the pH was near-neutral (pH 6.5-7.3, Table 23), the H_2S concentration would be < 110 mg/ℓ (Figure 6) and other less inhibitory sulphur species would also be present (Sabumon, 2016). Furthermore, microorganisms can adapt to H_2S (Section 2.4.2.5) and acetoclastic and hydrogenotrophic MA may only become significantly inhibited at concentrations greater than 1000 mg/ℓ free H_2S (Deublein and Steinhauser, 2008; Isa *et al.*, 1986a). It was therefore hypothesised that if an acclimated inoculum was used, there would be minimal chance of inhibition AD by HS^- for the OTE batches that were characterised.

Therefore, in this study the OTE was supplemented with SO_4^{2-} (Section 4.5.2.1) to assess whether the pre-treatment would be effective should SO_4^{2-} concentrations increase above the measured values (Table 23).

6.3.2 *Batch anaerobic studies with sulphate supplementation*

Each of the active consortia were first inoculated into 250 mℓ batch reactors and their sulphur species concentrations tested. The original 250 mℓ OTE reactor, inoculated with the enriched Zandvlei consortia (Z_{OTE}) had a SO_4^{2-} concentration of 341 mg/ℓ, which was adjusted to a concentration (1818 mg SO_4^{2-}/ℓ), more similar to artificial seawater, using $NaSO_4$ (Figure 26). The same supplementation was performed for all inoculated OTE batch reactors to achieve initial SO_4^{2-} concentrations close to 2000 mg/ℓ. The change in the HS^- and SO_4^{2-} concentrations for OTE batch reactors is shown in Figure 26. Only the results for Zandvlei consortia on OTE are presented and discussed, as reactors inoculated with the other enriched consortia displayed similar trends.

The batch reactors followed a familiar pattern, with the highest activity observed in the original 250 mℓ reactor, decreasing through consecutive sub-cultures and scale-ups. The maximum sulphate reduction rate (SRR) of 161 mg/ℓ.day was achieved by Z_{OTE}. Of the 500 mℓ reactors, $Z_{OTE,501}$ reached the highest SRR of 112 mg/ℓ.day after 31 days of operation (day 64). However, following this, the highest SRR it was able to reach was 13 mg/ℓ.day. Furthermore, the highest total SR reached was 65% by Z_{OTE} after 180 days, while other reactors were only able to reduce less than half of the SO_4^{2-} present. The sulphate reduction efficiency was substantially lower than for the enrichment cultures on lactate and poor in comparison to the batch reactors of Sabumon (2008) and Wang *et al*. (2008), who achieved >90% and >94.6% SR on sucrose and molasses, respectively, with initial SO_4^{2-} concentrations of 1900 and 742 mg/ℓ (Table 13).

This decrease in activity over time may be related to a reduction in pH. After 33 days, all reactors had a pH below 7.0, which had decreased to 6.5 and below after 75 days. This suggests that > 90% of sulphide in the reactors would have been present in the more toxic H_2S form (Figure 6), and could also be indicative of organic acid accumulation. While the residual COD in the reactors ranged from 3.3 to

4.1 g/ℓ, it is possible that the readily biodegradable organic fraction was depleted, causing the BSR to slow, as the SRBs were unable to metabolise the more recalcitrant fraction (Colleran *et al.*, 1995).

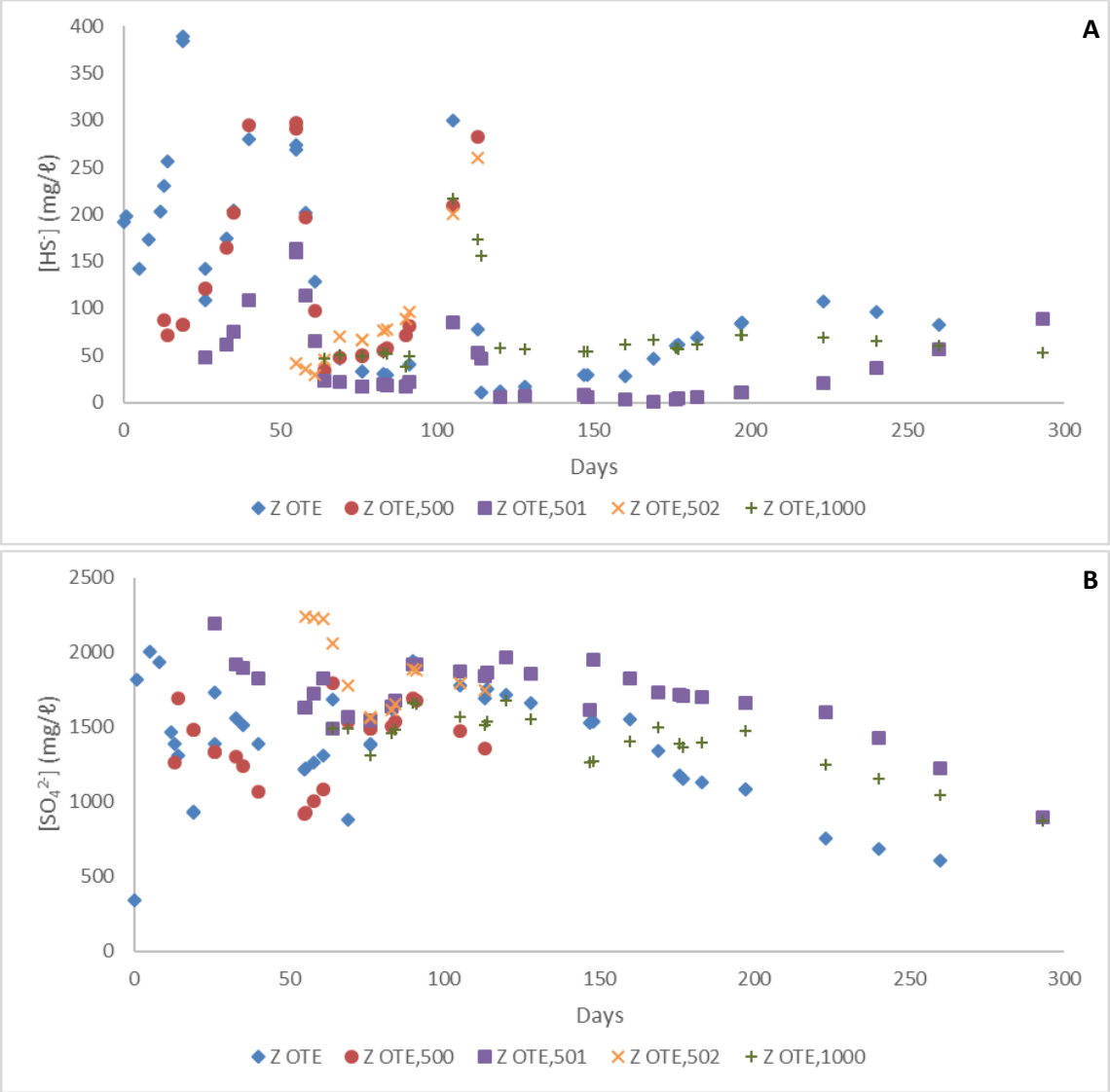

Figure 26: A) Change in HS^- concentration with time for Zandvlei consortia grown on OTE. B) Change in SO_4^{2-} concentration over time for Zandvlei consortia grown on OTE.

500, 501 and 502 refer to the 1st, 2nd and 3rd 500 mℓ subcultures.

6.3.3 *Semi-continuous hybrid linear flow channel reactor studies with sulphate supplementation*

The batch experiments showed that the enrichment cultures were able to grow on raw OTE, even though the long-term performance in batch reactors was not as good as when lactate was used as the carbon source. Nonetheless, the results suggested that the cultures should perform adequately in a continuous system, where conditions would be more similar to the first few days of batch operation. Two HLFCRs were set up; the first inoculated with the enrichment consortia grown on OTE, while the second was not inoculated, relying only on the microbial communities present in the OTE.

As seen in Table 23, the batches of OTE exhibited neither high HS^- nor very high SO_4^{2-} concentrations (646 mg SO_4^{2-}/ℓ, on average). Therefore, to simulate an effluent requiring pre-treatment, the initial

reactor volume and subsequent feed was supplemented with $NaSO_4$, to give them a similar SO_4^{2-} concentration to the artificial seawater. This provided the SRBs with sufficient SO_4^{2-} to produce enough HS^- so that a FSB might form.

6.3.3.1 Experimental run 1: with inoculum

The first experimental run of the channel operated using OTE was inoculated with volumes of the Z : K_L: K_A : KP : OA consortia grown on OTE in the ratio of 1 : 0.1 : 0.4 : 0.1 : 0.1. The HS^- and SO_4^{2-} concentrations over the duration of the experiment are shown in Figure 27. Initially, there was evidence of BSR, with relatively stable HS^- concentrations. A complete FSB was observed after six days and continuous feeding was started. However, by the following day most of the biofilm had washed out and the feed was stopped to allow it to reform. By day 12, the biofilm had reformed and continuous feeding was re-started. The effluent from this channel was sampled periodically and showed consistently low concentration of HS^- (Figure 27A). However, only the first effluent sample showed a SO_4^{2-} concentration lower than in the bulk liquid (Figure 27B), as there was a complete biofilm in place. Otherwise, the final effluent SO_4^{2-} concentrations were higher than in the bulk liquid, indicating limited BSR and that HS^- present in the bulk liquid was being fully oxidised. Further, it is possible that the OTE feed contained other reduced sulphur species (not measured) and these species were oxidised at the air-liquid interface, resulting in elevated effluent SO_4^{2-} concentrations.

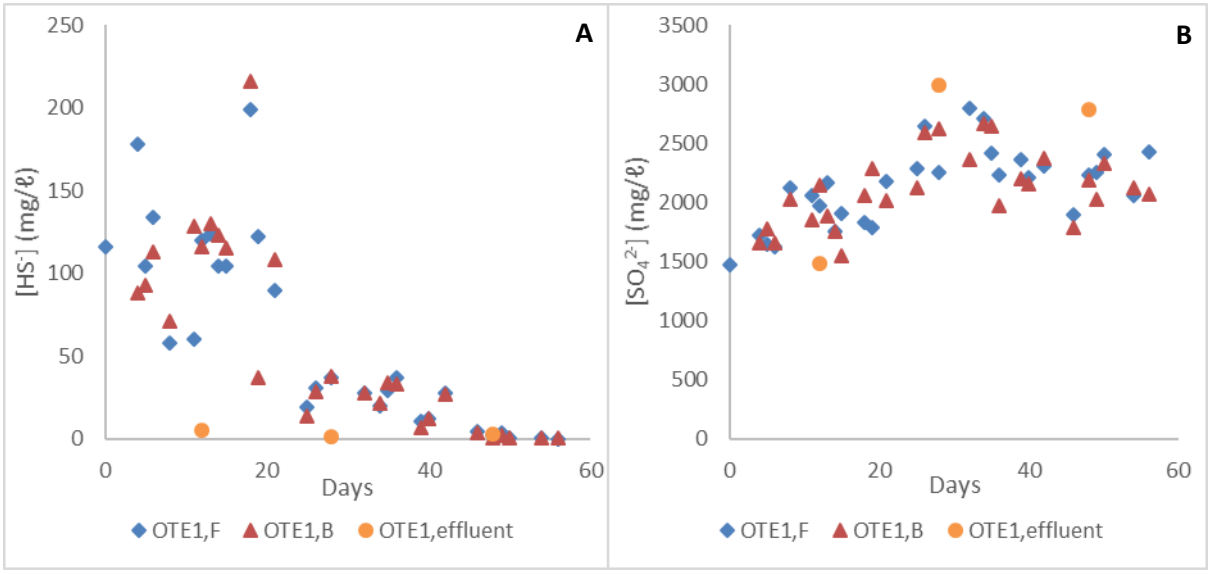

Figure 27: Change in HS^- (A) and SO_4^{2-} (B) concentration over time for first HLFCR operated on OTE and inoculated with enriched consortia.

$OTE_{1,F}$ = front sampling port of channel 1 operated using OTE; $OTE_{1,B}$ = back sampling port of OTE channel 1; $OTE_{1,effluent}$ = effluent sample of OTE channel 1.

The HLFCR is intended to be an open system, with the FSB acting as the barrier to oxygen penetration. However, to compensate for the slower FSB formation the reactor was operated with a lid to limit oxygen in the headspace. This presented another challenge as the experiment progressed. From day 18, there was disruption of the biofilm from condensation falling from the Perspex lid. These occurrences were followed by a sudden fall in HS^- concentration and an increase in SO_4^{2-} (Figure 27).

The trend of decreasing HS^- and increasing SO_4^{2-} concentrations persisted, even with a reduction in feed concentration from 2500 mg/ℓ to 1000 mg SO_4^{2-}/ℓ. During this period, the bulk liquid turned

purple in colour, indicating colonisation by purple sulphur bacteria. The PSB would have contributed to the complete oxidation of HS^- to SO_4^{2-} under conditions of abundant light and CO_2 (Larsen, 1952).

The initial soluble COD in the channel at the start of the experiment was 1663 mg/ℓ, while the feed had a concentration of 1875 mg/ℓ. The average COD concentration of the effluent was 1648 mg/ℓ, indicating low heterotrophic microbial activity, which would include SRB. The experiment was terminated on day 58 mainly due to the colonisation by the PSB.

6.3.3.2 Experimental run 2: endogenous microbial consortium

For the second experimental run, new batches of high and low HS^- OTE were blended in the feed to give higher concentrations of HS^- than the blend used for the first run. The important parameters for the new blend are provided in Table 24. Channel 2 was initially filled with only high HS^- OTE. The second run was used to determine whether the endogenous microbial community within the OTE was capable of performing BSR and SO when high HS^- and SO_4^- concentrations were present. Therefore, although the channel was seeded with powdered FSB at the start, to accelerate biofilm formation, no enriched exogenous consortia were added.

Table 24: Properties of the new blend of OTE

Parameter	Unit	High HS^-	Low HS^-
pH		9.06	4.99
EC	mS/cm	19.4	4.9
Redox	mV	-465	-42
NO_3	mg/ℓ	3.0	0
SO_4	mg/ℓ	1859	918
HS^-	mg/ℓ	30.2	1.1

The channel was initially operated in batch mode and a thin FSB was established by day 7. The FSB did not become thick and complete over the run period. Despite this, the HS^- concentration slowly increased to its maximum on day 41 (273 mg/ℓ) and SO_4^{2-} concentration decreased until day 88, when the maximum SR of 58% was achieved (Figure 28B).

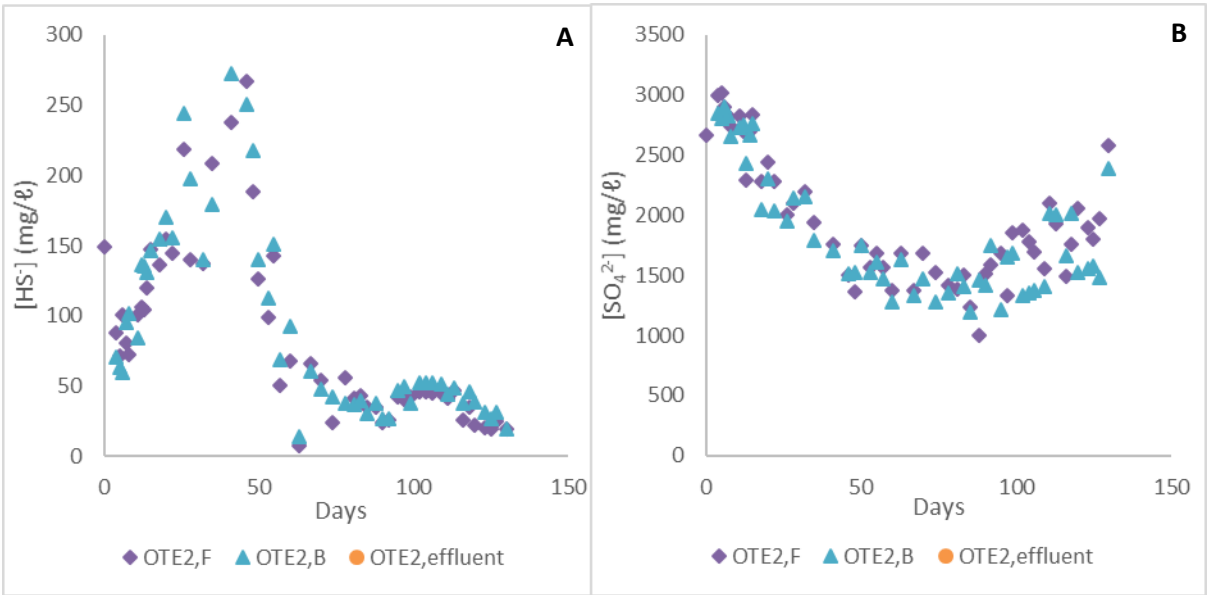

Figure 28: Change in HS^- (A) and SO_4^{2-} (B) concentration over time for second HLFCR operated on OTE with only endogenous consortia

After this, the SO_4^{2-} concentrations slowly increased and HS^- was depleted further. This suggested that suitable conditions to promote biofilm formation (Figure 7) could not be maintained and/or that the functional microbial community required was not present. The increase in SO_4^{2-} at the end of the run, while operating in batch mode, indicated complete sulphide oxidation.

6.3.3.3 Overall performance

Throughout both runs, the pH was maintained above a pH of 7.0, increasing to pH 8.0 after 6-7 weeks and as such, H_2S toxicity was limited (Figure 6). Conductivity started at ~20-22 mS/cm in each run and slowly decreased by 3-6 mS/cm as they were fed or refilled with OTE that had a lower conductivity than the original channel contents (Table 23). The redox potential of both runs was maintained below -350 mV and was favourable for BSR.

It was identified that initial HS^- concentrations of the OTE were too low (≤30 mg/ℓ) to support complete biofilm formation. The time required for BSR to result in enough HS^- generation to support SO may have been too long to allow the SO community to establish. This limiting HS^- concentration compromised biofilm formation, allowing too much oxygen ingress and resulting in a drop in overall BSR activity (Janssen *et al*., 1997, 1995).

Channel 1 was able to achieve a maximum SRR of 438 mg/ℓ.day and channel 2, 359 mg/ℓ.day during batch operation. However, these rates were not sustained and due to unstable performance, continuous operation was rarely employed, if it could be operated at all (run 2). Therefore, average SRRs were a fraction of these maximums, at 109 mg SO_4^{2-}/ℓ.day and 2 mg/ℓ.day, in channel 1 and 2, respectively. These SRRs were below those achieved by Boshoff *et al*. (2004) in tannery effluent (600 mg/ℓ.day) and Omil *et al*. (1996) using acetate, butyrate and propionate (805 mg/ℓ.day) with feed concentrations of 1800 and 1195 mg SO_4^{2-}/ℓ (Table 13).

It is possible that the slowing in activity observed with time in both channel runs was because there was not a sustained release of organic acids from degrading complex organic matter and the bioavailable substrate present was rapidly depleted (Mooruth, 2013). Soluble COD measurements were not performed during this study, so this could not be confirmed.

As part of a parallel Doctoral study, Mpofu *et al.* (2019) has demonstrated that this OTE does not require pre-treatment. He was able to achieve successful AD of this blended effluent using an acclimated sludge as an inoculum, reaching 176 mℓ CH_4/g volatile solids with a biogas composition of 58% CH_4 (Mpofu *et al.*, 2019). The OTE used in this study had a SO_4^{2-} concentration of 1202 mg/ℓ at the time of treatment, indicating that AD is successful for the OTE even at concentrations higher than the concentrations during this study.

6.3.4 *Summary of findings*

The characterisation of the OTE revealed that in most cases it would probably not require pre-treatment. The batch reactors needed to be supplemented with SO_4^{2-} to increase their initial concentration from 646 mg/ℓ to ~2000 mg/ℓ, while their initial HS^- concentration was ~6 mg/ℓ. This meant there was insufficient sulphide to form a biofilm in the channel reactors and performance was compromised.

Further, each of the reactors grown on OTE, both in batch and continuous mode, showed reduced activity within 24-48 days and then failed to produce HS^- and reduce SO_4^{2-}. The maximum SRR reached in the batch reactors was 161 mg/ℓ.day. The HLFCR inoculated with enriched consortia achieved a maximum SRR of 438 mg/ℓ.day, while, the channel grown solely on OTE, without enriched consortia, was unable to maintain a FSB and reached a maximum SR of only 58% and a maximum of SRR of 359 mg/ℓ.day. Therefore, the endogenous community within the OTE was not particularly active or robust.

The parallel Doctoral research by Mpofu, looking specifically at the AD of OTE has showed that biogas generation is possible on this OTE, without pre-treatment (Mpofu *et al.*, 2019a). Therefore, the focus of the research on pre-treatment will be on the bovine/ovine tannery effluent.

7 Bovine tannery effluent studies

7.1 Introduction

The pre-treatment of OTE was shown to be largely unsuccessful (Section 6.3.3). Subsequently, a parallel study concluded that AD of the untreated effluent was viable, as the sulphur species concentrations were low, making the pre-treatment of OTE unnecessary (Mpofu *et al.*, 2019a). However, the characteristics of the bovine/ovine tannery effluent (BTE) (Section 7.3.1), showed substantially higher concentrations of sulphide and sulphate than the OTE (average 699 vs. 2.61 mg HS^-/ℓ and 1951 vs. 646 mg SO_4^{2-}/ℓ, respectively). Therefore, the pre-treatment of the BTE prior to AD was considered crucial. This hypothesis was confirmed by preliminary AD studies of the raw and partially treated BTE (Section 7.3.2).

7.2 Experimental approach

Characterisation of the raw and partially treated BTE batches was performed. Batch AD tests were carried out on the raw and partially treated BTE to justify the need for pre-treatment to reduce sulphur species. Once the necessity of pre-treatment of the BTE was demonstrated, batch acclimation reactors, inoculated with enrichment consortia, were set up in order to determine whether the BTE streams contained substances inhibitory to BSR and assess the need for readily metabolisable substrate supplementation. This is illustrated in Figure 29.

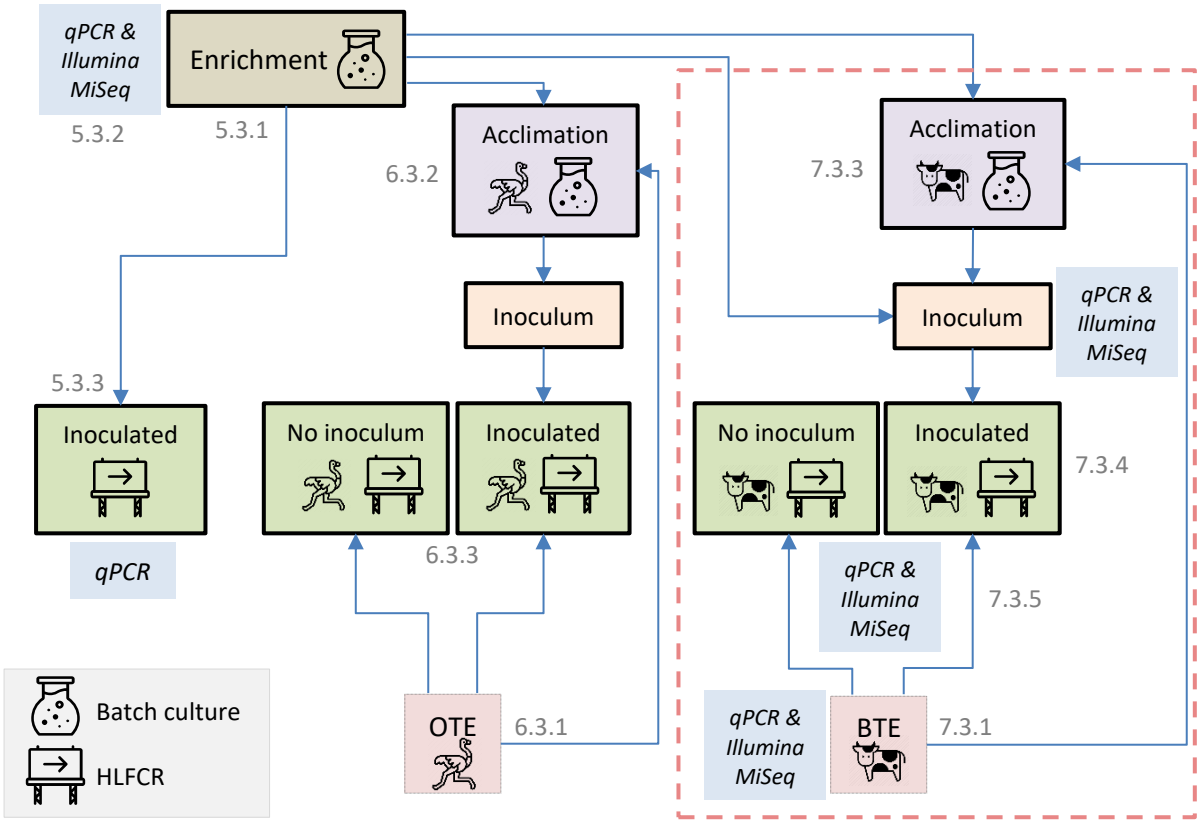

Figure 29: Flow diagram depicting the relationship of BTE studies performed to rest of the study (Step 3: BTE studies)

Following this, two HLFCRs, the first containing only populations present in the BTE (endogenous) and the second supplemented with enrichment consortia inoculum, were set up as a proof of concept and to evaluate whether sufficient sulphur species removal could be achieved to make AD of the pre-

treated effluent viable. Initially these HLFCRs were run in batch mode until the majority of SO_4^{2-} present had been reduced. The reactor containing the endogenous community (channel 1) was switched to continuous operation at a 4-day HRT after 33 days. Once the HLFCR supplemented with the enrichment cultures (reactor 2) reached the end of the batch phase (day 68) it was connected in series to reactor 1, receiving the effluent from reactor 1 as feed. The reactors were monitored for pH, redox potential, HS^- and SO_4^{2-} daily, and COD once per residence time. The biofilm was monitored visually. The first batch of raw BTE had an acceptable pH (8.5) and HS^- concentration (549 mg/ℓ), but these were too high (627-888 mg/ℓ) in subsequent batches. Therefore, after the first batch was depleted, subsequent batches were blended with partially treated BTE to maintain some consistency in feed composition.

The HLFCRs were sampled for DNA extraction after at least a week of continuous operation each time the feed was changed to a new batch of BTE and these samples analysed for their *dsr*B gene copy numbers and their community structure.

7.3 Results and discussion

7.3.1 *Bovine tannery effluent characterisation and notable findings*

Five batches of raw and partially treated BTE samples (25 L) were obtained from Cape Produce over the duration of the study. Upon arrival, the effluent was immediately tested to determine the sulphur species concentrations and the organic load. The results for the raw BTE are shown in Table 25 and the partially treated BTE in Table 26. Selected parameters are discussed in terms of their impact on the amenability of the BTE for pre-treatment, as well as for AD without pre-treatment (Sections 7.3.1.1 - 7.3.1.4).

The analyses showed large variations across each of the batches, similar to what was observed with the OTE. The scale of variability means that even if a balancing tank was installed before the reactor, the microbial consortia would still need to be resilient enough to withstand shock loads of certain components in the treatment of this effluent (Mpofu *et al.*, 2019a; Welz *et al.*, 2018).

7.3.1.1 pH, redox potential and salinity

The pH is an important parameter as it affects the degree of dissociation of molecules such as sulphide and ammonia. The pH of the raw BTE samples was consistently in the alkaline range, although there was considerable variation (pH 8.5-12.3). Therefore, the majority of the sulphide would have been present as HS^- ($pK_1 = 7$) in all batches. Literature values for the second dissociation constant (pK_2) range from 12 to 18, so the S^{2-} ion is unlikely to exist in significant amounts in the BTE (Migdisov *et al.*, 2002). Conversely, the pH of the partially treated BTE remained relatively constant across the batches at pH 7.81 $\pm$ 0.52, again with most of the sulphide present as HS^- (Table 26).

The dissociation constant of ammonia has been reported as 5.01×10^{-10} ($pK_a = 9.3$) for dilute aqueous solution, but can be significantly affected by solution composition. Liang *et al*. (2011) calculated that the dissociation constant in broiler litter slurry was only 20% of the value in aqueous solutions (1.02×10^{-10}). Based on the pH of the BTE streams, the ammonia speciation in the raw BTE was likely > 75% NH_4^+ in batches 1 and 3, with the remainder as NH_3, while NH_3 was likely dominant in batches 2,4 and 5 (Huang and Shang, 2007). The lower pH in the partially treated BTE meant the majority (>95%) was likely present as NH_4^+ (Huang and Shang, 2007). High NH_3 concentrations are problematic

for AD (Section 2.4.3.4) (Deublein and Steinhauser, 2008). The first batch of raw and partially treated BTE had the highest concentration of NH_3 (865 mg/ℓ and 760 mg/ℓ, respectively, Table 25 & 26) and were therefore, most likely to have inhibitory effects. However, all batches were below the AD inhibiting range, where a 50% reduction in CH_4 production occurs (1.7-14 g NH_3-N/ℓ) identified by Chen *et al*. (2008). This inhibition is dependent on substrates, inocula, pH, temperature and acclimation periods. The effect of ammonia on SRB is not well documented but is considered to be a key issue requiring investigation (Mannucci *et al*., 2014).

The redox potential of all batches was consistently negative (< -478 mV and < -397 mV in raw and partially treated BTE respectively). This redox potential would support BSR (Figure 7).

The salinity of both types of BTE (ca. 32 mS/cm, Table 25 & 26) was much closer to the salinity of the consortia enriched in artificial seawater (43.1 mS/cm), which makes the consortia better suited to the conditions of the BTE than they were to the salinity of the OTE (7.5 mS/cm, Table 23).

7.3.1.2 Organic fraction

There was considerable variability across all the organic parameters for both the raw and partially treated BTE, with batch 3 having the highest COD concentration for both streams (9.8 and 31.4 g/ℓ, respectively (Table 25 & 26). On average, the raw BTE stream had a total COD concentration almost 6 times higher than the partially treated BTE stream and the TOC concentration was almost 7 times the amount in the partially treated BTE (0.89 g/ℓ) in the raw BTE (6.1 g/ℓ). This disparity is most likely due to settling and the aerobic treatment that had occurred in the ETP up until this point. The majority of the proteins (89%), organic carbon (86%), TVS (85%), COD (83%), BOD (75%) and VOA_t (64%) were removed, as well as a significant portion of the TS (50%), TN (46%) and TAN (43%) (Table 25 & 26.).

In both BTE streams, the BOD concentration was far lower (BOD:COD = 0.23-0.37 on average) than the COD (Table 25 & 26). This indicates that the majority of the organic matter present is not readily metabolisable by the specific BOD consortium in 5 days. While the ratio increases in the partially treated BTE, there is still the risk of the SRB and SOB community requiring more easily metabolisable substrates (Figure 4). Once again, a functional microbial community that had adapted to these less than ideal properties would be more likely to thrive.

Reis *et al*. (1990) observed an IC_{50} for SRB at an undissociated acetic acid concentration of 54 mg/ℓ and for a pH range of 5.8-7.0. However, the pK_a of 4.76 at 37 ˚C has been reported for acetic acid. Therefore, neither BTE streams were likely to be inhibitive to pre-treatment as the pH of the effluent was consistently > 8.4 and > 7.2 in raw and partially treated BTE, respectively (Table 25 & 26).

7.3.1.3 Metal composition

The effluent was dominated by Na, Ca and Mg as these are present in the chemicals used in the tanning and treatment processes. The variation in concentrations were consistent with the measured EC (Table 25 & 26).

Inhibitory metal concentrations cited in literature vary greatly because of differences in SRB and AD microbial community structures, temperature, synergistic and antagonistic effects, and reactor configurations (Chen *et al*., 2008; Oleszkiewicz and Sharma, 1990). Many microbial communities have

the ability to adapt to modify trace metal availability at the biological interface and thereby change the rate-limiting flux to prevent trace metal deficiency and toxicity (Thanh *et al*., 2016).

Table 25: Characterisation of each raw batch of bovine tannery effluent

Parameter	Unit	Batch					Average	SD
		1	2	3	4	5		
pH		8.53	9.96	8.48	11.9	12.3	10.2	1.62
EC	mS/cm	29.8	29.2	33.2	33.1	34.8	32.01	2.16
Redox	mV	-478	-543	-480	-618	-615	-547	62
TOC	mg/ℓ	9250	7240	5160	4710	4220	6116	1875
COD	mg/ℓ	27468	30974	31364	29675	21364	28169	3665
BOD	mg/ℓ	7500	6500	5000	6000	6000	6200	812
VOA_t	mg/ℓ AAE	2637	3108.5	4172.5	2674	2007	2920	718
Proteins	mg/ℓ	1562	2610	2090	3931	4184	2875	1024
TN	mg/ℓ	1035	1320	1020	1315	1600	1258	215
TAN	mg/ℓ NH_3-N	865	96	135.5	230	176	301	286
NO_3	mg/ℓ	60.6	62.2	101.6	95.8	33.6	70.76	25.04
NO_2	mg/ℓ	0.3	12.2	10.6	1.1	1.3	5.10	5.18
PO_4	mg/ℓ	0.0	0.0	0.0	0.0	0.0	0.00	0.00
SO_4	mg/ℓ	2935	1964	2094	1459	1304	1951	574
HS^-	mg/ℓ	549	687	627	888	743	699	114
Cl	mg/ℓ	7290	7261	7836	8523	7808	7744	460
TS	g/ℓ	31.4	30.5	38.0	46.5	33.9	36.1	5.8
TVS	g/ℓ	11.6	9.8	14.4	18.9	10.9	13.1	3.3
K	mg/ℓ	45.6	78.5	99.6	129.0	126.0	95.7	31.1
Na	mg/ℓ	5552	6446	6058	6820	7184	6412	571
Fe	mg/ℓ	0.05	0.03	0.08	0.26	0.13	0.11	0.08
Ca	mg/ℓ	497.4	254.2	198.8	1091.2	1419.4	692.2	481.9
Mg	mg/ℓ	375.0	60.3	151.2	3.7	11.8	120.4	137.7
Mn	mg/ℓ	0.23	0.06	0.40	0.60	1.23	0.50	0.41
Zn	mg/ℓ	0.17	0.43	0.16	0.74	1.00	0.50	0.33
Cu	mg/ℓ	0.02	0.01	0.01	0.03	0.17	0.05	0.06
Co	mg/ℓ	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cd	mg/ℓ	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ni	mg/ℓ	0.03	0.03	0.03	0.02	0.03	0.03	0.00
Cr	mg/ℓ	0.14	0.06	0.16	0.07	0.04	0.09	0.05
Pb	mg/ℓ	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Al	mg/ℓ	0.04	0.01	0.01	0.05	0.06	0.03	0.02
Alkalinity	mg/ℓ $CaCO_3$	2425	4425	3030	4200	2200	3256	907
$COD:SO_4$		9.4	15.8	15.0	20.3	16.4	15.4	3.5
TVS:TS		0.37	0.32	0.38	0.41	0.32	0.36	0.03
BOD:COD		0.27	0.21	0.16	0.20	0.28	0.23	0.05
C:N		8.9	5.5	5.1	3.6	2.6	5.1	2.2
VFA:Alk		1.09	0.70	1.38	0.64	0.91	0.94	0.27
COD:TVS		2.4	3.2	2.2	1.6	2.0	2.2	0.52

Table 26: Characterisation of each partially treated batch of bovine tannery effluent

Parameter	Unit	Batch					Average	SD
		1	2	3	4	5		
pH		7.28	8.80	7.55	7.71	7.71	7.81	0.52
EC	mS/cm	31.4	ND	31.1	33.9	29.4	31.5	1.59
Redox	mV	-397	ND	-445	-443	-451	-434	22
TOC	mg/ℓ	930	594	1330	875	700	886	253
COD	mg/ℓ	3312	4625	9805	4675	2078	4899	2633
BOD	mg/ℓ	1500	1650	2443	900	1200	1539	520
VOA_t	mg/ℓ AAE	1458	531	1985	1062	167	1041	647
Proteins	mg/ℓ	255.7	281.5	203.6	377.4	434.6	310.6	83.9
TN	mg/ℓ	765	580	910	545	595	679	138
TAN	mg/ℓ NH_3-N	760	101.5	153	315	422	350	235
NO_3	mg/ℓ	53.4	0.6	58.2	63.4	26.8	40.5	23.6
NO_2	mg/ℓ	0.01	5.5	5.65	0.3	0.3	2.35	2.63
PO_4	mg/ℓ	0.0	5.05	0.0	0.0	1.0	1.21	1.96
SO_4	mg/ℓ	3945	4200	3581	3063	3645	3687	383
HS^-	mg/ℓ	184	5.7	62	160	2.1	83	76
Cl	mg/ℓ	8220	7500	7364	7514	7965	7713	325
TS	g/ℓ	20.06	16.89	17.52	22.46	12.85	17.96	3.23
TVS	g/ℓ	2.34	1.50	2.51	2.02	1.29	1.93	0.47
K	mg/ℓ	85.7	ND	80.3	105.2	130.0	100.3	19.5
Na	mg/ℓ	6235	ND	6072	6664	5928	6225	276
Fe	mg/ℓ	0.27	ND	0.04	0.34	0.11	0.19	0.12
Ca	mg/ℓ	198.4	ND	212.0	330.6	182.6	230.9	58.5
Mg	mg/ℓ	218.3	ND	215.2	263.2	186.0	220.7	27.6
Mn	mg/ℓ	11.26	ND	5.90	20.14	23.27	15.14	6.92
Zn	mg/ℓ	0.11	ND	0.08	0.19	0.38	0.19	0.12
Cu	mg/ℓ	0.02	ND	0.03	0.02	0.24	0.08	0.09
Co	mg/ℓ	0.00	ND	0.00	0.00	0.00	0.00	0.00
Cd	mg/ℓ	0.00	ND	0.00	0.00	0.00	0.00	0.00
Ni	mg/ℓ	0.02	ND	0.01	0.02	0.02	0.02	0.00
Cr	mg/ℓ	0.13	ND	0.12	0.28	0.27	0.20	0.08
Pb	mg/ℓ	0.00	ND	0.00	0.00	0.01	0.00	0.00
Al	mg/ℓ	0.03	ND	0.02	0.03	0.04	0.03	0.01
Alk	mg/ℓ $CaCO_3$	2010	1730	2705	1960	1590	1999	385
$COD:SO_4$		0.8	1.1	2.7	1.5	0.6	1.4	0.76
TVS:TS		0.12	0.09	0.14	0.09	0.10	0.11	0.02
BOD:COD		0.45	0.36	0.25	0.19	0.58	0.37	0.14
C:N		1.2	1.0	1.5	1.6	1.2	1.3	0.21
VFA:Alk		0.73	0.31	0.73	0.54	0.11	0.48	0.24
COD:TVS		1.4	3.1	3.9	2.3	1.6	2.5	0.93

AAE – acetic acid equivalents; EC – electrical conductivity; ND – no data; SD – standard deviation; TAN – total ammonia nitrogen; TN – total nitrogen; TOC – total organic carbon; TP – total phosphates; TS – total solids; TVS – total volatile solids, VOA – volatile organic acid.

It was postulated that microbial adaptation to metal deficiency may be very important due to the composition of the BTE used in this study. This is due to the interaction of metals with neutral solutions of a redox potential below -300 mV which may result in their precipitation as metal-sulphides or carbonates, making their mobilisation more difficult (Thanh *et al.*, 2016).

The low Cr concentration in the raw BTE (Table 25) was due to the samples being taken before the tanyard stream was mixed in, while in the partially treated BTE stream (Table 26) the low Cr demonstrated that the Cr removal process of the ETP was effective.

The high HS^- concentration of the raw BTE ensured that the concentration of transition metals in solution was very low, due to metal sulphide precipitation. Therefore, no metal concentrations were likely to be inhibitory (Abdel-Shafy and Mansour, 2014; Lin, 1992; Mekonnen *et al.*, 2017; Zayed and Winter, 2000). In both the raw and partially treated BTE streams, the concentrations of Fe, Co, Ni and Pb were lower than the trace nutrient requirements of most methanogens and, excluding Pb, these are considered the most essential trace nutrients for AD (Thanh *et al.*, 2016).

In both BTE streams, Zn, Cu, Pb, Fe, Cr, Ni, Cd and Mn metal concentrations were below the toxic concentrations cited in Brahmacharimayum *et al.* (2019) for specific and mixed culture SRBs. The exception was Mn in the partially treated BTE, which was > 10 mg/ℓ on average and may be inhibitory for *Desulfovibrio* sp. (Cabrera *et al.*, 2006). The precipitation of metal sulphides reduced trace nutrient concentrations, so there could have been a deficiency for SRB too (Loka Bharathi *et al.*, 1990). Therefore, to mitigate against the potential deficiency of essential trace nutrients it would be favourable to use well adapted endogenous consortia.

7.3.1.4 Sulphur species variation

There was a wide range of SO_4^{2-} and HS^- concentrations across each BTE stream, which could be attributed to variations in the tanning operations, rather than the performance of the ETP. Much of the HS^- was attributed to the addition of chemical Na_2S during the tanning process and not necessarily due to BSR. Batch 4 of raw BTE had the highest HS^- concentration (888 mg/ℓ) while batch 2 of the partially treated BTE had the highest SO_4^{2-} concentration (4200 mg/ℓ). The average HS^- (700 and 83 mg/ℓ for raw and partially treated BTE, respectively) and SO_4^{2-} (3700 and 1950 mg/ℓ for raw and partially treated BTE, respectively) concentrations of the BTE were notably higher than in the OTE, supporting the need to pre-treat the BTE to reduce sulphur species. Similarly, the presence of high concentrations of sulphur species also makes BTE a better test case for pre-treatment. A higher concentration of HS^- at the start of operation impacts the reaction kinetics positively, allowing the reactor to readily produce a FSB (Janssen *et al.*, 1997, 1995). The results obtained with artificial seawater, containing SO_4^{2-} alone, and OTE, with minimal initial HS^- (Table 23) (Sections 5.3.3 & 6.3.3, respectively) illustrated the difficulty in obtaining good FSB formation without sufficient HS^- in the feed.

The COD:SO_4 ratio of every batch of partially treated BTE (0.6-2.7) was within the range for dominance of sulfidogenesis over methanogenesis, due to the presence of high SO_4^{2-} levels (Table 26) (Burton *et al.*, 2009). On the other hand, the COD:SO_4 ratio of every batch of raw BTE (9.4-20.3) was above the cited ratio (>10) to favour methanogenesis (Table 25). This favourable ratio for methanogenesis was due to the very high COD in the effluent, although much of this may not have been bioavailable. While the COD:SO_4 ratio was suitable for AD, the high HS^- concentration (549-888 mg/ℓ; Table 25) could have

a severe inhibitory effect, especially for non-acclimated AD communities as a wide range of inhibitory concentrations (150 to 1100 mg/ℓ) have been reported (Omil et al., 1995; Sabumon, 2016).

A decrease in SRB activity has been found when H_2S levels are higher than 60-70 mg/ℓ (Hao *et al*., 2014). van Houten *et al*. (1997, 1994) reported that SRB growth becomes inhibited at 250 mg/ℓ H_2S, but that growth was possible up to 450 mg/ℓ and noted that H_2S inhibition was reversible upon its reduction in the reactor, which was confirmed by Okabe *et al*. (1995). Others have measured H_2S inhibition at 258-303 mg/ℓ (Brahmacharimayum *et al.*, 2019). Therefore, provided the pH remains above or close to neutral, there should be no problem with H_2S inhibition of SRBs. These inhibition levels vary based on consortium and reactor configuration. However, this variation is even more pronounced in dissolved sulphide (HS^-) inhibition. Reis *et al.* (1992) found a HS^- inhibition level of 547 mg/ℓ for SRBs growing on lactate and SO_4^{2-}, while in continuous reactors a wide inhibition range of 100-2737 mg HS^-/ℓ for mixed cultures has been reported (Brahmacharimayum *et al*., 2019).

7.3.2 *Anaerobic digestion of bovine/ovine tannery effluent before pre-treatment in batch reactors*

The evaluation of the HLFCR for the pre-treatment of tannery effluent was based on the hypothesis that raw and partially treated effluent would be unsuitable as a substrate for AD. To test this, a series of batch AD tests were conducted. Methane generation was used as the performance indicator and the overall specific CH_4 yields for the experimental reactors and positive controls are shown in Table 27. These tests indicated a complete lack of methanogenic activity on both the raw and partially treated BTE, even at a 50% dilution or with acetate supplementation. The controls showed high activity when the same AD sludge was used with acetate or microcrystalline cellulose (MCC) as the substrate (Figure 30).

Table 27: Overall specific methane generation of anaerobic digestion reactors on BTE before pre-treatment (units: mℓ/g $COD_{consumed}$)

Control Acetate$_1$	**Control Saline + Acetate**	**1 A**	**1 B**	**2 A**	**2 B**	**3 A**	**3 B**
381	208	0	0	0	0	0	0

1: undiluted BTE before pre-treatment; 2: undiluted BTE before pre-treatment with 3.8 g/ℓ acetate supplementation; 3: half diluted BTE before pre-treatment; A: raw BTE; B: partially treated BTE.

Detailed AD studies were out of the scope of this study. However, the next step would be a comprehensive test, using replicate reactors, where COD and volatile solids of the inoculum as well as the BTE are quantified and compared with a final measurement of each reactor. Negative controls, using BTE without an inoculum would confirm the absence of an endogenous community, while controls without BTE would allow quantification of biogas produced using residual volatile solids in the inoculum sludge.

Methane generation from acetate provides an indication of methanogenic activity only (Bajpai, 2017; Deublein and Steinhauser, 2008), while methane generation from MCC indicates that hydrolytic and acetogenic microbial communities are also active: in contrast to acetate that can be directly converted into methane, MCC first needs to be hydrolysed, and the hydrolysis products then need to be converted into methanogenic substrates (Section 2.4) (Ahring *et al.*, 2018; Ferrara *et al*., 1984). The

rate of CH_4 generation in the MCC control was, as expected, slower than in the acetate control, due to hydrolysis being the rate limiting step, with 137 mℓ methane being generated between days 3-4. These different rates are shown in Figure 30. The positive control grown on acetate showed the methanogenic community to be very active, producing a maximum of 294 mℓ of CH_4 in its first 24 hours. However, both these positive controls had a similar overall specific methane generation (372 mℓ/g COD for MCC compared to the first acetate control, 381 mℓ/g COD; Table 27), showing that MCC is just slower to get to a similar overall specific CH_4 generation.

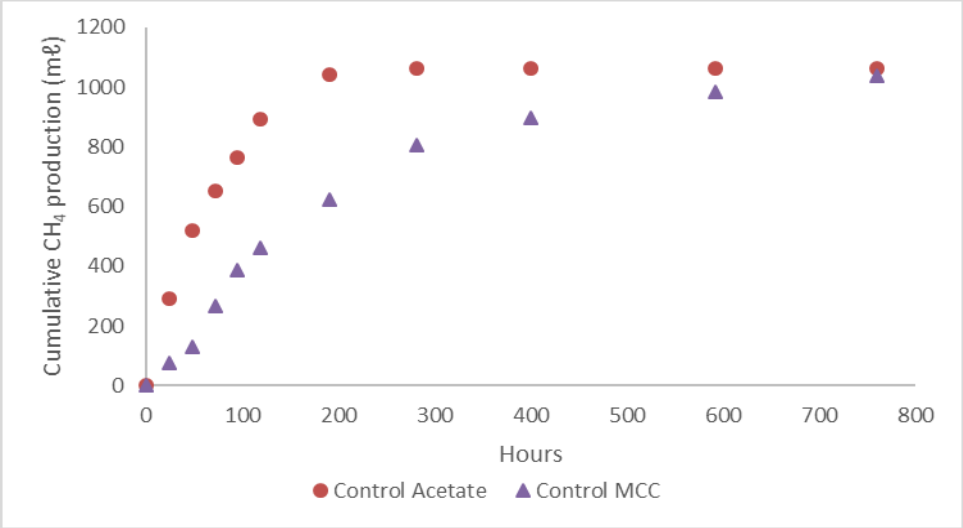

Figure 30: Cumulative methane production in preliminary anaerobic digestion studies for positive controls tested

The data suggest that there was no active endogenous methanogenic community in either the raw or partially treated BTE and that the effluent contained components, most likely sulphide, that completely inhibited an otherwise active inoculum (Table 27). This supported the hypothesis that the BTE requires pre-treatment to reduce the sulphur species concentration.

7.3.3 *Batch sulphate reduction studies with and without lactate supplementation*

The reactors used in the batch OTE acclimation studies tended to lose activity after a few weeks, possibly due to the hydrolysis of complex substrates becoming rate limiting. This is consistent with observations made by Mooruth (2013). The BTE reactors were tested both with and without supplementation with a readily available carbon source, using lactate as a model substrate. Both raw and partially treated BTE batches were assessed using two of the most active enrichment consortia available, Kathy Park (KP) and Old Airstrip (OA). One duplicate contained only the enrichment consortium and BTE, while the other was also supplemented with 2.55 g/ℓ lactate, as in the majority of previous batch reactor tests.

The results for the KP and OA consortia were similar and supplementation with lactate did not appear to significantly improve long-term performance (Figure 31A & B). The major difference in performance was between the raw and partially treated BTE, as expected, due to their differing sulphur species concentrations. During the first week, some reactors (OA_R, $OA_{PT, lactate}$, KP_{PT} and $KP_{R, lactate}$) reached their highest sulphate reduction rate (SRR), but exhibited low HS^- concentrations, suggesting some speciation of the BSR product. Thereafter, all reactors growing on partially treated BTE as well as reactor $KP_{R, lactate}$ had the thickest FSB seen on anaerobic batch reactors to date. This indicated the presence of an active SOB community in both BTEs, and that the HS^- present was being partially

oxidised to S^0, keeping HS^- concentrations lower (Figure 31A). After day 60, the reactors were occasionally topped up with BTE to maintain the adapted cultures.

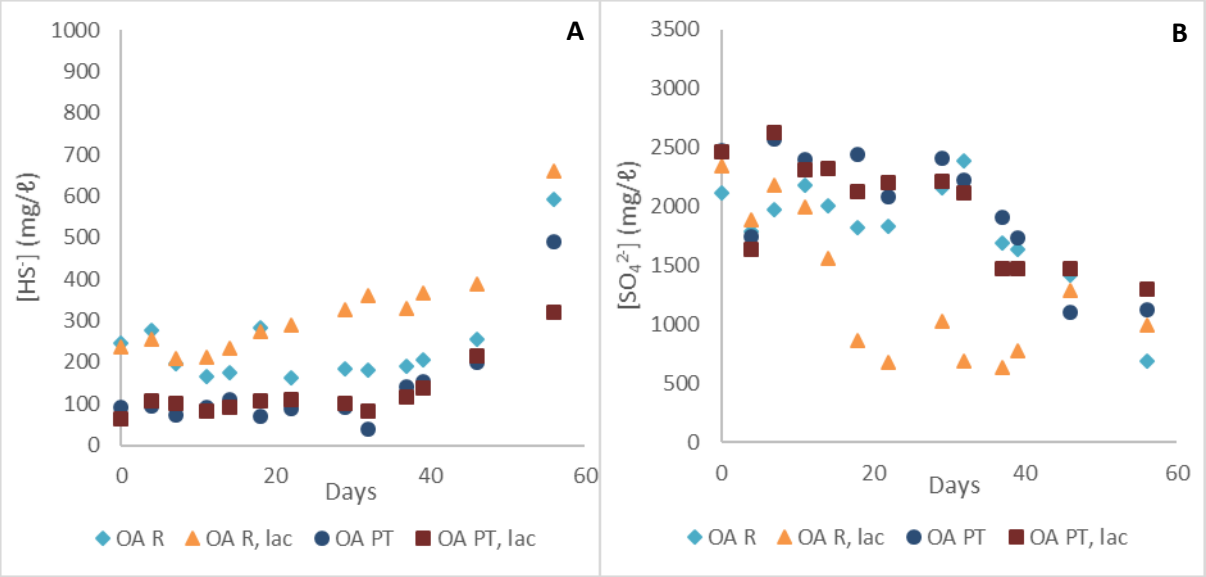

Figure 31: Change in HS^- (A) and SO_4^{2-} (B) concentration over time for the Old Airstrip (OA) enriched consortia batch reactors.

OA_R = OA consortium grown on raw BTE; $OA_{R, lac}$ = OA grown on raw BTE supplemented with lactate; OA_{PT} = OA grown on partially treated BTE; $OA_{PT, lac}$ = OA grown on partially treated BTE supplemented with lactate.

As shown in Table 28, when comparing the maximum SRRs of the consortia supplemented with lactate as opposed to those without, the OA consortia were able to reach higher maximum SRRs when supplemented with lactate (21% and 13% higher for raw and partially treated BTE, respectively, for 175 and 209 mg/ℓ.day). By comparison, the KP consortia were able to reach higher maximum SRRs without lactate supplementation (32% and 8% higher for raw and partially treated BTE, respectively, for 252 and 259 mg/ℓ.day). However, when looking at average SRR over the first 56 days of operation, the raw BTE showed better performance when supplemented with lactate (Table 28).

Table 28: Maximum and mean sulphate reduction rates of anaerobic BTE reactors with and without lactate supplementation and how much higher was the supplemented or unsupplemented reactor

Consortium	SRR (mg/ℓ.day)	% Greater*	SRR (mg/ℓ.day)	% Greater*
	Max	Max	Mean	Mean
KP_R	252	32%	6.3	83% lactate
$KP_{R, lactate}$	170		38	
KP_{PT}	259	8%	19	3%
$KP_{PT, lactate}$	238		19	
OA_R	139	21% lactate	26	49% lactate
$OA_{R, lactate}$	175		52	
OA_{PT}	182	13% lactate	23	29%
$OA_{PT, lactate}$	209		16	

Mean given for days 29-56, apart from raw BTE reactors supplemented with lactate calculated over days 0-32.
*what percentage greater the SRR of the lactate supplemented or unsupplemented counterpart was

Figure 31B shows limited BSR over the first 20-30 days for all reactors but $OA_{R,lactate}$. The mean SRR taken between days 0 and 32 for both reactors with raw BTE and lactate supplementation was about double the rate of other reactors taken between days 29 and 56 (Table 28). This demonstrates that the other reactors took about 30 days to acclimatise to the BTE. Therefore, in the absence of lactate, the raw BTE needed to undergo some conversion of organics to be readily used and was not substantially inhibitory. However, there was something in the partially treated BTE that made it more difficult to utilise lactate. It is possible that this could be an inhibitory substance, such as the Mn, which was > 10 mg/ℓ in all batches (Table 26), the limit found to inhibit *Desulfovibrio* sp. by Cabrera *et al.* (2006) and therefore, may have inhibited other members in the community until they adapted to it.

Generally, reactors containing partially treated BTE obtained higher SRRs than those containing raw BTE (Table 28). This is expected, due to higher initial SO_4^{2-} concentrations and lower potentially inhibitive HS^- concentrations.

In summary, these batch tests proved that BSR was possible within reactors containing either type of BTE. Lactate supplementation was found to be more beneficial to the raw BTE than to the partially treated BTE as only the raw BTE reactors with lactate supplementation did not have an extended lag period and had average SRRs more than double the other reactors' rates. The results suggest there was something within the partially treated BTE that was inhibitory and required acclimation. Nevertheless, after an initial acclimation period, it appeared that lactate supplementation was not necessary to achieve satisfactory BSR activity.

7.3.4 *Continuous hybrid linear flow channel reactor studies with bovine/ovine tannery effluent*

The preceding sections demonstrated that enrichment cultures from saline environments contained BSR and SO communities that could be adapted to grow on BTE. The final part of the research was to evaluate the performance of the HLFCR in batch and ultimately continuous mode. Initially, a single HLFCR was used to test the proof of concept. The results are presented in the section below. Partway through this study a second reactor was connected in series. Section 7.3.4.2 describes the data for the second channel.

7.3.4.1 Proof of concept: Treatment of bovine/ovine tannery effluent using the hybrid linear flow channel reactor

Based on the characterisation of the BTE and empirical observations there was a strong suspicion that the effluent contained endogenous SOB and SRB communities. To assess this, a HLFCR was loaded with raw BTE and not inoculated with the adapted enrichment cultures. The pH of batch 1 (pH 8.53) was within the acceptable range for SRB so no adjustment or blending of the effluent was required.

A thin biofilm formed on the surface of the reactor overnight and a week later a noticeable reduction in odour had occurred. At this point the biofilm was disrupted and the reactor switched to continuous mode with a 1-day HRT. This proved too ambitious and the high flowrate resulted in a drop in HS^- and increase in SO_4^{2-} concentrations in the bulk liquid. The reactor was switched back to batch mode until almost all the SO_4^{2-} had been reduced, after which continuous feeding was restarted, initially at a 2-day HRT, but ultimately a 4-day HRT, where the most stable performance was achieved. The performance data are discussed in more detail in the sections that follow.

7.3.4.1.1 pH and redox potential

The pH in the bulk liquid remained relatively stable above neutral for the duration of the experiment, due to the high alkalinity of the BTE and additional bicarbonate generated during BSR (Figure 32). The average pH was around pH 7.5, meaning that the majority (70%) of the sulphide in the system would present as HS^- rather than the more toxic H_2S. The pH of the channel effluent was consistently higher (0.6 pH units, Figure 32) than the bulk liquid due to SO chemistry, with partial oxidation releasing OH^- ions (Equation 7).

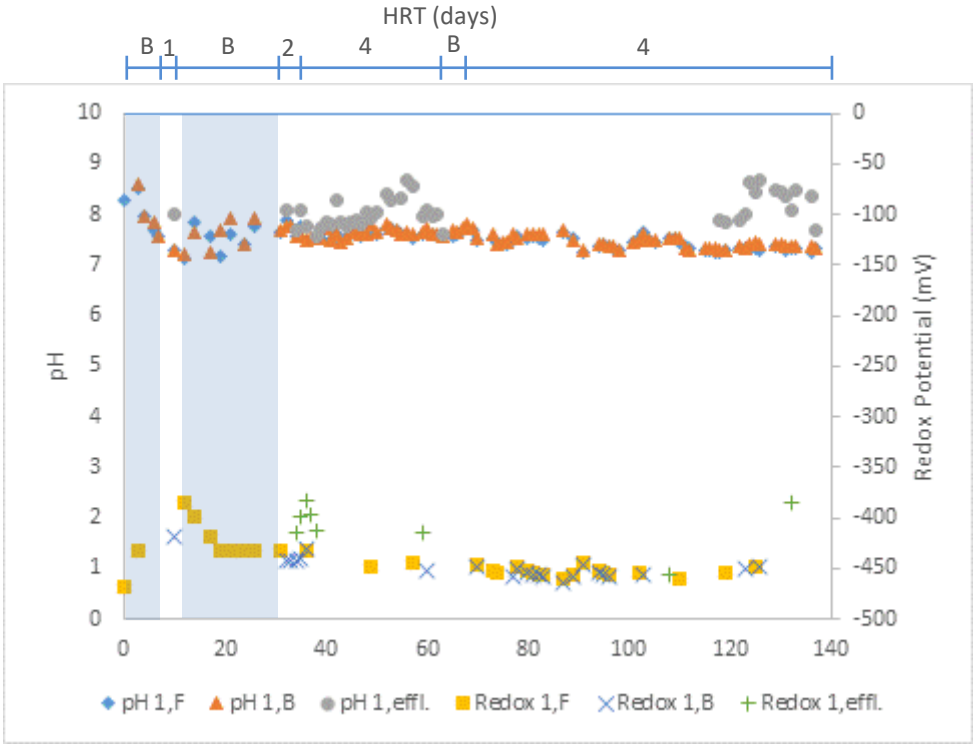

Figure 32: Change in pH and redox potential with time for first HLFCR grown on BTE.
Shaded = batch period (B).

Similarly, the redox potential in the bulk liquid remained below -380 mV for the duration of the run due to the presence of HS^-, indicating the bulk liquid was maintained completely anaerobic (Figure 32). The redox potential of the effluent was marginally higher on average, primarily due to the lower sulphide concentration (Figure 7).

7.3.4.1.2 Sulphur species removal

The HS^- and SO_4^{2-} concentrations measured immediately after the reactor was loaded with BTE were 187 mg/ℓ and 3200 mg/ℓ, respectively (Figure 33A & B). The HS^- concentration decreased rapidly over the first few days when the biofilm was absent or very thin.

Biofilm formation is crucial to the reactor's operation, such that O_2 mass flux to the bulk liquid is limited and anaerobic BSR can occur. For channel 1, the FSB initially formed overnight (Figure 34A). On day 7, the FSB was partially harvested and the rest was collapsed. Again, the FSB reformed overnight. This indicates that this first batch of raw BTE was rich in SOB.

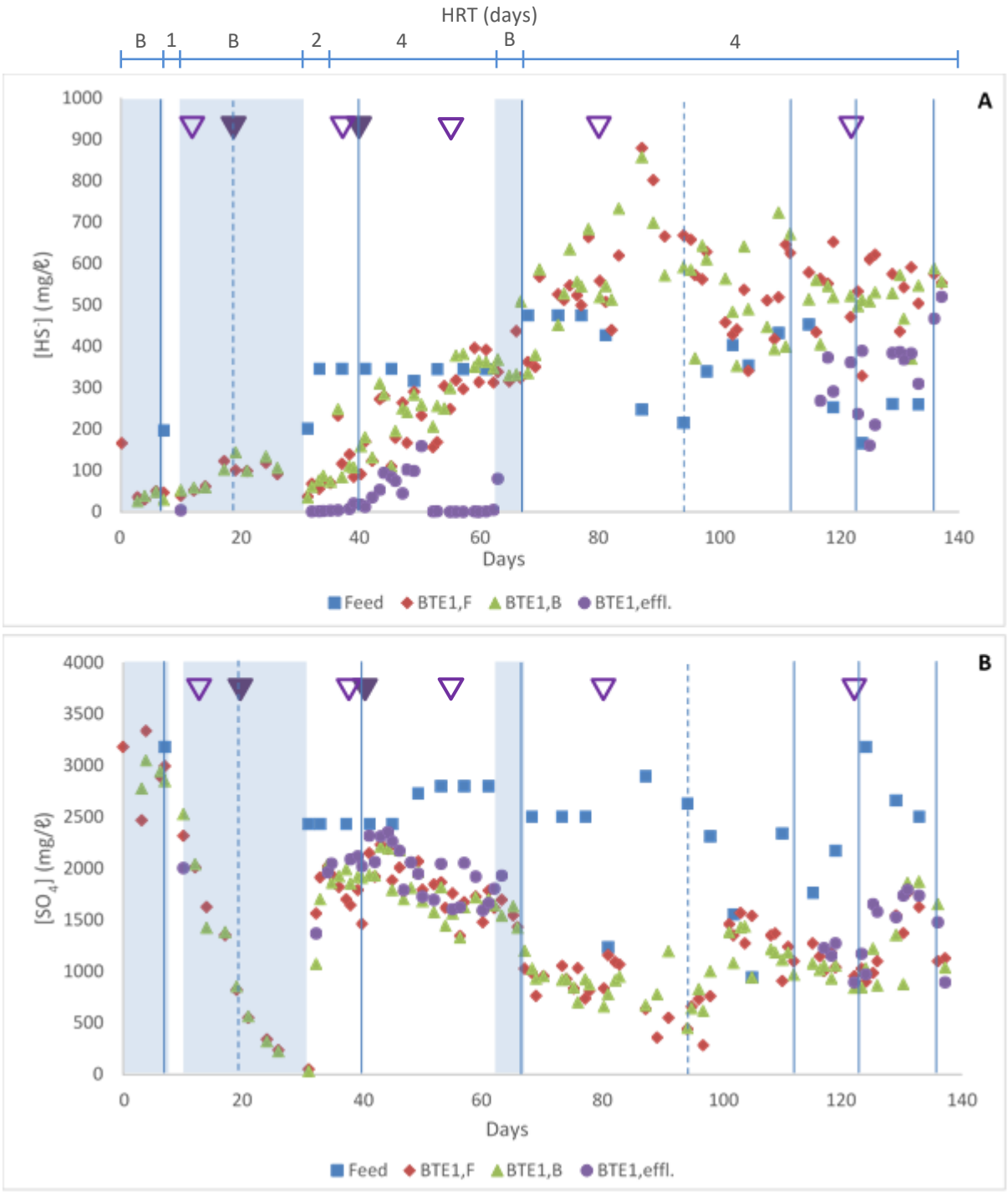

Figure 33: Change in HS^- (A) and SO_4^{2-} (B) concentrations with time for first HLFCR grown on BTE. *$BTE_{1,F}$ = first run of channel grown on BTE alone, bulk liquid, front sample port; $BTE_{1,B}$ = first run of channel grown on BTE, bulk liquid, back sample port; $BTE_{1,effl.}$ = first run of channel grown on BTE, effluent sample. Shaded = batch period (B); open triangle = bulk liquid DNA sample; closed triangle = FSB solids DNA sample; solid vertical line = FSB harvested; dashed vertical line = FSB disrupted.*

After the initial attempt to run continuously (day 7-9) the channel was run in batch mode for 21 days, until it had achieved near complete SR. The mean SO_4^{2-} concentration in the bulk liquid decreased from 2923 mg/ℓ to 41 mg/ℓ (Figure 33B). Sulphate reduction rates will be discussed in Section 7.3.4.1.3. Sulphate reduction began almost immediately after the reactor had been switched back to batch

mode, confirming that the raw BTE contained an active BSR community. There was no acclimation period as was seen when the enrichment cultures were switched onto BTE.

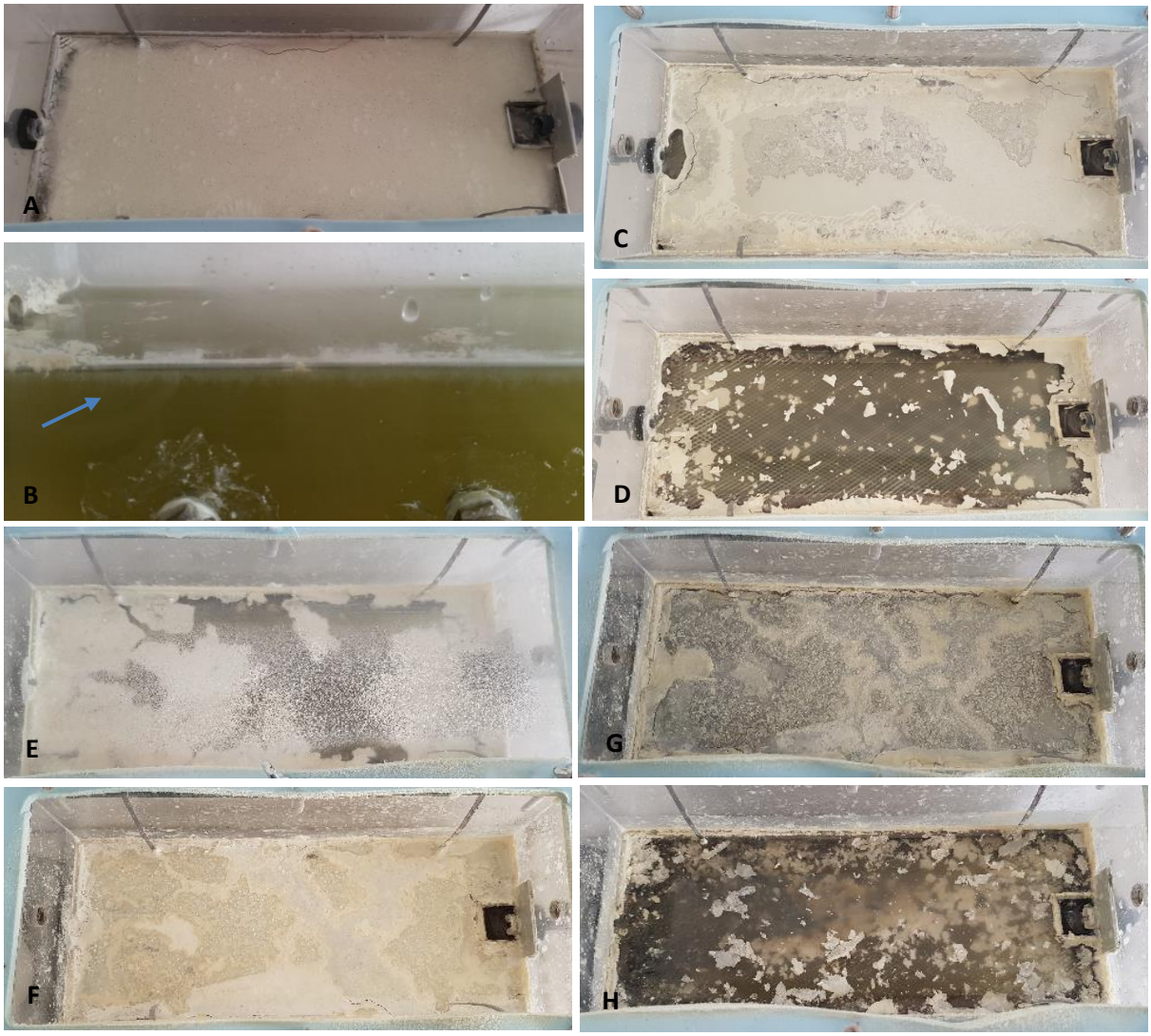

Figure 34: FSB formation on first BTE channel. A) First FSB self-formed. B) EPS strands forming beneath surface. C) Day 40 before harvest. D) Day 40 after harvest. E) 5 days after harvest of day 40. F) Day 52. G) Before collapse on day 66. H) After FSB was collapsed on day 66, note layer of FSB beneath surface.

During the batch phase, measured HS^- was no higher than 144 mg/ℓ, despite the near complete reduction of SO_4^{2-}. This suggests the majority of the HS^- generated was partially oxidised, either in the developing biofilm or to form colloidal S^0 in the bulk liquid.

By the time the channel had reached 99% SR, the biofilm was full and thick and the feed was started (2-day HRT instead of 1 day). The FSB remained full but thicker in the middle until most of it washed out when new feed was started on day 33 at a 4-day HRT. On day 40, the FSB was harvested and had not reformed a day later when a fresh feed was started (Figure 34C & D). Where the HLFCR has been used to assess the potential to treat mining-impacted water, a significant drop in HS^- concentration in the bulk liquid follows a biofilm disruption event, with the concentration increasing once the biofilm reforms (Marais *et al*., 2020). In this case, the high sulphide concentration in the feed mitigated against a more significant decrease in HS^- concentration within the reactor.

By day 45, the FSB was patchy, with thick biofilm covering 60-70% of the channel surface (Figure 34E), while the remainder was covered by a thin film. After an additional 24 hours thick biofilm covered 80% of the surface. An intact biofilm resulted in a steady decrease in SO_4^{2-} concentration and accompanying increase in HS^- in the bulk liquid.

Between day 52 and 66 (Figure 34F & G) an interesting phenomenon was observed. The biofilm began to lose its white colouring and appeared to be getting thinner, despite no apparent loss of performance. This was most likely due to the reaction of S^0 in the biofilm with HS^- in the bulk liquid, resulting in greenish coloured, soluble polysulphides (Kleinjan *et al.*, 2005). This suggests that too much time had elapsed between biofilm harvesting events and that an optimum frequency of harvesting needed to be determined. Mooruth (2013) showed that if the biofilm becomes too thick, oxygen ingress is reduced to the point where partial oxidation in the biofilm slows down. The reaction between aqueous HS^- and S^0 in the biofilm may become dominant under these circumstances.

On day 66, the biofilm was disrupted and allowed to reform (Figure 34H). The next day a very thin biofilm had reformed and by day 80, the FSB was again thick and covered the entire surface.

Biological SO_4^{2-} reduction performance continued to improve and the sulphide concentration reached a maximum of 879 mg/ℓ on day 87 (Figure 33A). At this time, the pH was 7.7, which equates to approximately 79% present as HS^- (Figure 6), resulting in a H_2S concentration of 186 mg/ℓ. This concentration is acceptable for an adapted consortia (Deublein and Steinhauser, 2008; Isa *et al.*, 1986a; Omil *et al.*, 1995). Further, concentrations of HS^- were similar in the batches, so the endogenous community is unlikely to be inhibited in the channel.

In general, high bulk liquid HS^- concentrations coincided with a minimum in the residual SO_4^{2-} concentration, such as on day 89 (359 mg SO_4^{2-}/ℓ) and the minimum for the continuous phase on day 97 of 282 mg/ℓ (89% SR) (Figure 33B).

The tannery effluent feed contained suspended solids and this, combined with some S^0 formation in the silicone tubing used to feed the reactor, occasionally led to the feed tube blocking. This occurred twice between days 87-97, resulting in two separate days without feed, so effectively batch operation. This could account for the particularly low SO_4^{2-} concentrations on days 89 and 97.

Following the biofilm disruption on day 97 there was a period of 3-4 days, while the biofilm reformed, where the residual SO_4^{2-} concentration increased and the HS^- decreased. Biofilm formation took longer in this system than was observed in the model system, with simple, defined growth media (Marais *et al*., 2020).

The HS^- concentration stabilised to around 520 mg/ℓ in the bulk liquid, with the SO_4^{2-} concentration settling at around 1000 mg/ℓ in the bulk liquid. This equated to a conversion of approximately 56% of the feed SO_4^{2-} between days 101-126. This is lower than the 60-80% SR efficiency reported by Boshoff *et al*. (2004) treating tannery effluent, although the feed concentration was higher in this case (Table 13). Comparing the actual SO_4^{2-} removal, Boshoff *et al*. (2004) were able to achieve a maximum between 1.08-1.44 g/ℓ, while channel 1 achieved an average of 1.23 g/ℓ, indicating similar performance. In addition, the majority of studies were not focussed on integrated SO_4^{2-} and HS^- removal, but solely on SR. Xu *et al*. (2012) did perform integrated sulphur species treatment and

achieved 81.5% SR (0.82 g/ℓ SR), but were using a readily available substrate (lactate) and were not performing treatment under saline conditions. Therefore, these results compare favourably.

Towards the end of the run, some challenges were experienced in terms of FSB formation. An accumulation of S^0 at the exit caused the outlet flow to be restricted, resulting in some accumulation of volume in the reactor. When the increased pressure forced the outlet to clear, the accumulated liquid flowed out rapidly, washing out most of the partially formed biofilm. As a consequence, there was an extended period without a complete FSB and the SO_4^{2-} concentration in the bulk liquid increased to above 1700 mg/ℓ (day 126 onwards). Without the FSB, excess O_2 entered the bulk liquid and caused the full oxidation of HS^- present to SO_4^{2-}, which coincided with a short drop in the HS^- concentration during this time and then stabilised to 550 mg HS^-/ℓ in the bulk liquid for the remainder of the run (Figure 33A).

Generally, biofilm formation was relatively rapid, with full FSB formation in 1-2 days. Following complete harvesting, the addition of saline-adapted dried FSB increased the speed at which the biofilm reformed. The biofilm was generally pale yellow and consistently brittle, which indicated a high portion of S^0 and relatively low organic carbon (Mooruth, 2013). Detailed biofilm composition will be discussed in Section 7.3.4.2.6.

While the effluent HS^- concentration from channel 1 was consistently < 5 mg/ℓ during stable operation, the SO_4^{2-} concentration was still in excess of 1500 g/ℓ. If all of this SO_4^{2-} was reduced to HS^- in an AD reactor, this would result in a HS^- concentration of over 500 mg/ℓ, which exceeds the threshold (150 mg HS^-/ℓ) previously found to inhibit non-acclimated methanogens (Omil *et al*., 1995). This served as motivation to include a second reactor in series, discussed in detail in Section 7.3.4.2.

7.3.4.1.3 Sulphate reduction rates

Channel 1, with the endogenous community alone, achieved an average SRR of 150 mg/ℓ.day during the batch phase (day 0-31). Following the day of continuous operation at a 1-day HRT, the SRR exceeded 200 mg/ℓ.day, until day 14, after which it decreased as the SO_4^{2-} concentration decreased, supporting the assumption that SRR approaches first order with respect to SO_4^{2-} concentration.

Continuous operation commenced on day 31, at a 2-day HRT, but was increased to a 4-day HRT on day 33, where it remained for the duration of the study. The reactor was operated for 22 HRTs at 4 days and data from this period was used to calculate the SRRs during continuous operation. These are presented in Figure 35. There was an increase in SRR once feeding commenced, after which the average SRR for each HRT was relatively consistent within the range of 450-700 mg/ℓ.day. The mean SRR was calculated as 550 ± 150 mg/ℓ.day.

Five of the 22 data points fell outside this range (3 lower and 2 higher, Figure 35). Excluding these points increases the mean SRR marginally, to 577 mg/ℓ.day, but significantly reduces the standard deviation from 150 to 87 mg/ℓ.day.

There are a number of possible explanations for the observed variation. The feed composition was changed several times during the experiment, which required blending of different batches of raw and partially treated BTE. The primary aim of the blending was to achieve a level of consistency in the feed in terms of pH, SO_4^{2-} and HS^- concentrations. Due to the variability in composition of the different

batches of BTE received the blended feed could vary significantly in terms of salinity, COD, light metal ions and nitrogen compounds.

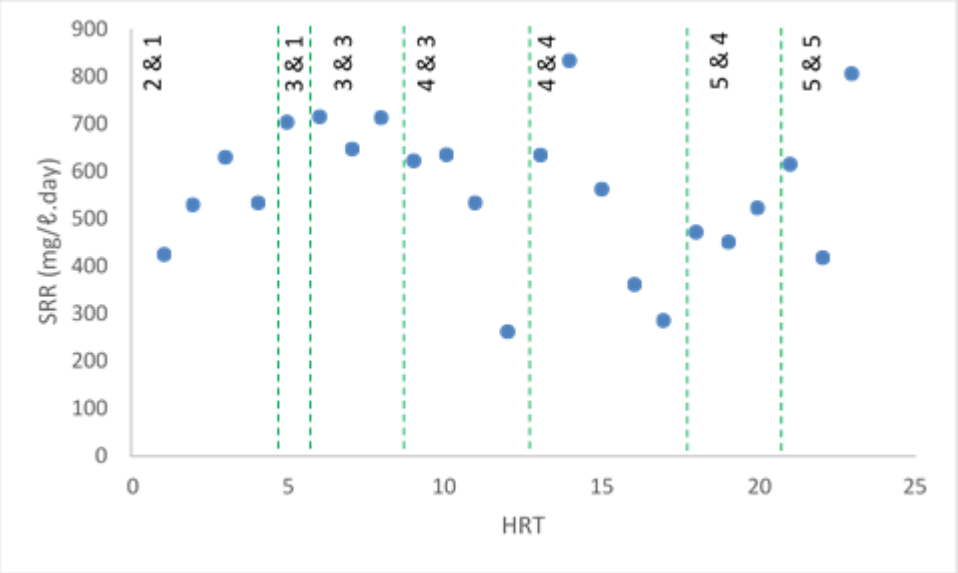

Figure 35: Average sulphate reduction rate for each HRT within the bulk liquid of the first BTE HLFCR. Dotted lines represent changes in feed, with the numbers indicating the batches of raw and partially treated BTE blended to prepare the feed, respectively.

As demonstrated, the BTE contained an endogenous BSR community. The BTE was not sterilised in any way, so some BSR did occur in the feed bottles. From day 75, a sample of feed was taken each residence time to determine the SO_4^{2-} concentration and the SRRs were adjusted to account for any decrease. This could explain the decrease in calculated SRR towards the end of a batch of feed.

Additional factors that could have affected the SRR were the nature of the biofilm, biofilm disruption and harvesting events and periodic blockages in the feed or effluent pipes. Beyond physicochemical characteristics and biofilm changes, it is possible that changes in community structure could have affected reactor performance. These community variations will be discussed in Section 7.3.5.2.1.

The measured SRRs achieved within the bulk liquid of channel 1 were encouraging, especially for passive treatment, when compared to those in Table 13. Waybrant *et al.* (2002) were able to achieve 120 mg SO_4^{2-}/ℓ.day using leaf compost and sawdust as substrates in a passive up-flow packed bed reactor (UF-PBR) (Table 13).

The primary aim of this study was to perform a proof of concept study. Subsequent research will focus on a more detailed determination of reaction kinetics. This study identified a number of challenges related to reactor setup and operation, as well as feed management, that would need to be addressed to allow collection of more accurate data.

7.3.4.1.4 Dissimilatory sulphite reductase gene abundance in the bulk liquid

The concentration of DNA extracted from a specific sample volume has been used as a proxy to semi-quantitatively compare microbial abundance where other methods of biomass quantification, such as direct counting or gravimetric analysis are not possible (Blagodatskaya *et al.*, 2003; Joergensen and Emmerling, 2006; Marstorp *et al.*, 2000; Muñoz *et al.*, 2017; Welz *et al.*, 2018). This approach could not be used in the current study as the majority of the DNA extracted from the BTE-fed reactor would

likely be eukaryotic. Bovine cells have far more genetic material, so it would require several hundred bacterial cells to get a comparable amount of DNA.

However, it is possible to get information on specific groups by targeting genes unique to that group, using specific primer sets for PCR. The bacterial community responsible for metabolising sulphur species can be probed by testing for the dissimilatory sulphite reductase (*dsr*) gene that codes for the enzyme involved in both BSR and SO, as well as other sulphur species removal.

Table 29 shows the concentration of DNA extracted from samples taken from channel 1 on specific days and the relative abundance of the *dsr*B gene, compared to the SRR measured at the time of sampling, while the specific *dsr*B gene copy number for all samples is shown in Figure 36.

Table 29: Rate of sulphate reduction of each sample extracted from the bulk liquid of channel 1 and corresponding dsrB abundance and DNA concentration

Run day	**DNA concentration**	**SRR**	***dsr*B gene abundance**
Days	ng/µℓ	mg/ℓ.day	Copies/ng
12	24.9	198	210,553
38	96.2	674	364,096
55	87	537	449,341
80	63.9	643	384,594
122	185.9	568	364,974

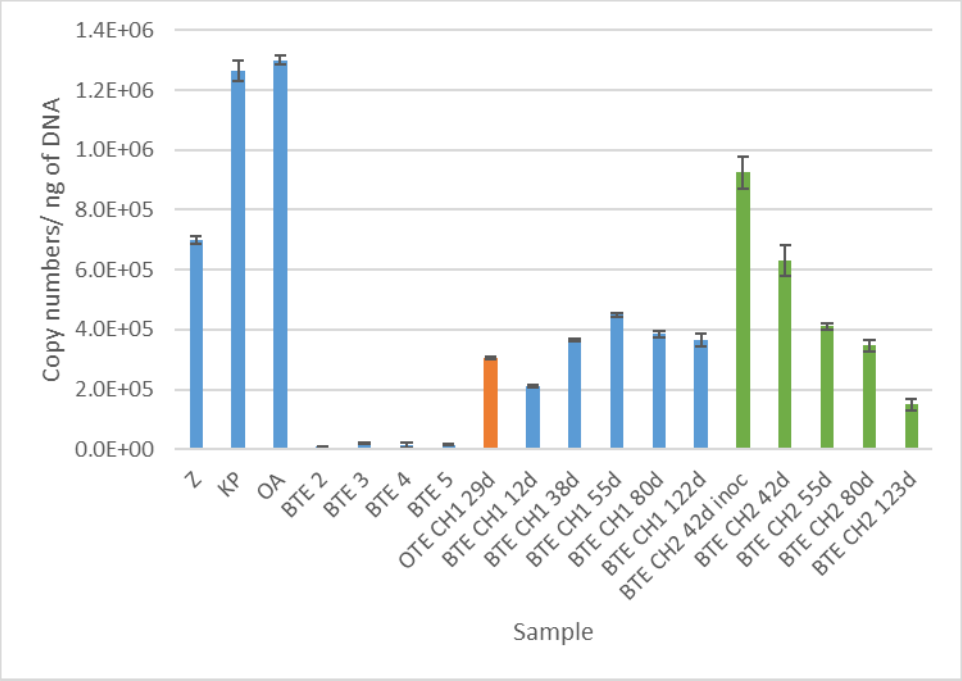

Figure 36: Number of copies of the dsrB gene per ng of DNA from samples taken of the bulk liquid of BTE HLFCRs

The enrichment cultures (Z, KP and OA; Figure 36) had been maintained on a simple organic carbon source (lactate) for many days under anaerobic conditions, so were dominated by SRBs, resulting in the highest *dsr*B gene concentrations of all bulk liquid samples. As anticipated, the raw BTE batches contained DNA from a more diverse range of species, most likely dominated by bovine DNA, so had a

far smaller proportion of the bacteria with the *dsr*B gene. This will be discussed further in Section 7.3.5.1.1. Therefore, in Figure 36, the abundance of *dsr*B gene copy numbers per ng DNA were much lower in the BTE batches than in the other samples.

The BTE used to feed the channels consisted mainly of the liquid fraction, with much of the solids having settled out. As a result, it was likely to contain less bovine tissue, so the relative proportion of SRB, based on relative *dsr*B gene copy number was higher. Initially, the was an increase in *dsr*B gene concentration in channel 1 as colonisation occurred. The extracted DNA concentration increased, as did the proportion of *dsr*B. From day 12 to 38, there was a 73% increase in the abundance of *dsr*B genes per ng of DNA, with almost 800 mg of SO_4^{2-} reduced and the concentration of HS^- almost tripling during this period. The SRB community continued to proliferate between days 38 to 55, seeing a 23% increase in *dsr*B gene copies per ng DNA.

The general trend for channel 2 was different. Half of the initial inoculum consisted of enrichment cultures dominated by SRB, hence the high *dsr*B concentration for the first sample (d42 inoc). The specific *dsr*B gene concentration decreased in subsequent samples as the SRB were progressively washed out or diluted with eukaryotic DNA of the tannery effluent in the feed (Figure 36).

There was then a minor 14% decline in the abundance of *dsr*B gene copies per ng DNA in channel 1 (Figure 36), between days 55 to 80, possibly relating to community stabilisation. As the SRR continued to increase there may have been selection for better adapted SRB species. It is important to note that *dsr*B gene concentration does not necessarily denote specific bacterial metabolic activity, as Marais *et al*. (2020) showed in their LCFR.

On day 122, the specific *dsr*B gene concentration had decreased by just another 5% since day 80, suggesting relative stability in terms of community function.

Overall, when comparing the SRRs at the time of the DNA extractions in channel 1 to the number of copies of the *dsr*B gene per ng of DNA (Table 29), the Pearson's correlation coefficient is as high as 80.2%. The strong correlation indicated that the number of copies of *dsr*B per ng DNA was an accuratemeasure of the channel's activity at the time, although the correlation between total DNA extracted and SRR was less consistent, particularly at the end of the experiment. This suggests that SRR is affected by more than just the number of SRB present.

7.3.4.1.5 Soluble COD utilisation

Minimising COD utilisation is in the best interest of this pre-treatment, as this will result in more organic matter available for AD and therefore, greater potential for CH_4 production.

On average, the feed contained 8.7 g/ℓ of soluble COD, while the channel contained 7.0 g/ℓ of soluble COD at the start of operation. This average COD:SO_4^{2-} ratio within the bulk liquid of the channel was 7.1, which decreased to 3.2 in the channel effluent. This was primarily as a result of some complete oxidation of HS^- to SO_4^{2-}, rather than a substantial decrease in COD. The COD concentration decreased by 1.5 g/ℓ, on average. At this ratio, there is still a good chance of methanogenic archaea (MA) outcompeting SRBs for available organic carbon in a downstream AD reactor (Burton *et al*., 2009).

On average, this channel used 15.7% of the soluble COD that was in the feed within the bulk liquid and a further 13.5% was utilised between the bulk liquid and the effluent, with the majority utilised

by heterotrophs for forming EPS in the biofilm. This left 70.8% of the soluble COD initially present in the feed for downstream applications.

Further, the change in soluble COD was not proportional to SO_4^{2-} reduced. Some COD was consumed for BSR, but could also be created as more complex COD particles are hydrolysed by other members of the community. To quantify how much COD was used during BSR and whether the amount of soluble COD being formed by the community was significant, Equation 14 was used (Lu *et al*., 2012):

$$127.8\ g\ COD + 192\ g\ SO_4^{2-} + 55.8\ g\ H_2O \rightarrow 68\ g\ H_2S + 2.4\ g\ sludge + 244\ g\ HCO_3^- \quad (14)$$

The soluble COD was measured once per HRT for channel 1. Equation 14 was applied to assess the COD material balance, per HRT, while the performance was relatively stable. Concentration data were multiplied by reactor volume to determine actual mass of soluble COD (Table 30). On average, 4.36 g more COD was consumed than would be required for BSR, according to Lu *et al*. (2012). Therefore, while complex organic matter may have been hydrolysed into soluble COD, this was far outweighed by the COD utilised by SRB, heterotrophs and other organisms. Utilisation of COD by this channel and the hybrid BTE channel in series will be discussed further and compared in Section 7.3.4.2.6.

Table 30: Measured soluble COD values and theoretical utilisation by SRB per HRT. Average values during stable performance

Property	Channel 1	SD
COD_{feed} (g)	17.40	2.02
COD_{bulk} (g)	14.74	2.15
$COD_{effluent}$ (g)	12.34	1.30
$COD_{consumed,\ theoretical}$ (g)	2.93	1.35
$COD_{consumed,\ actual}$ (g)	6.59	1.74
COD_{excess} (g)	4.36	0.87
Actual:theoretical	3.18	0.91
Excess*:theoretical	2.18	

*excess = actual - theoretical

7.3.4.2 Two channels treating bovine/ovine tannery effluent in series: HLFCR with endogenous community followed by HLFCR with hybrid community

The single channel, containing only the endogenous BTE community, was not able to consistently achieve a SR efficiency in excess of 50%. It was postulated that a second channel, inoculated with a mixed consortium containing the endogenous community and enrichment consortia, connected in series with the first channel, would allow more effective BSR across the system and offer protection against shock loads and biofilm harvesting.

The second channel was partially filled (1082 mℓ) with the liquid fraction (after solids settling) of the second batch of BTE. The remainder was the inoculum made up with the most active of the enrichment consortia (Zandvlei (Z), Kathy Park (KP) and Old Airstrip (OA)), using a ratio of 0.56 : 1 : 1. The KP and OA consortia in the inoculum had previously been grown on the first batch of raw BTE (the

same batch the first BTE channel was started with) for 39 days and supplemented with lactate. Therefore, at the start, HLFCR 2 contained endogenous populations from the first two batches of BTE, along with the three enrichment consortia.

The channel was initially operated in batch mode (days 42-68) until the majority of the SO_4^{2-} present at the start had been reduced and the HS^- concentration consistently increased, confirming SRB activity (Figure 38B). Settled sludge from the first batch of BTE was injected into the reactor on day 47 (20 mℓ) and day 49 (100 mℓ) to give the channel a similar solids composition to the first channel.

This set up did not allow a direct comparison between the endogenous community and the hybrid community, as the second reactor received the overflow from the first, but could provide some insights into the relative performance of the enrichment culture. In addition, the molecular analysis could provide information on the persistence, or elimination, of the species from the enrichment cultures in the second HLFCR.

7.3.4.2.1 pH and redox potential of second hybrid linear flow channel reactor treating bovine/ovine tannery effluent

The pH measured in the second channel mirrored that of the first, remaining relatively consistent above pH 7.2 (Figure 37). Generally, the pH in the second channel was 0.2 pH units higher than the first, most likely due to hydroxide ions released during partial SO. A similar trend was observed in pH between the bulk volume in HLFCR 2 and the effluent. The stable, slightly alkaline pH mitigated against H_2S toxicity, as the majority of the sulphide remained as HS^- (Figure 6).

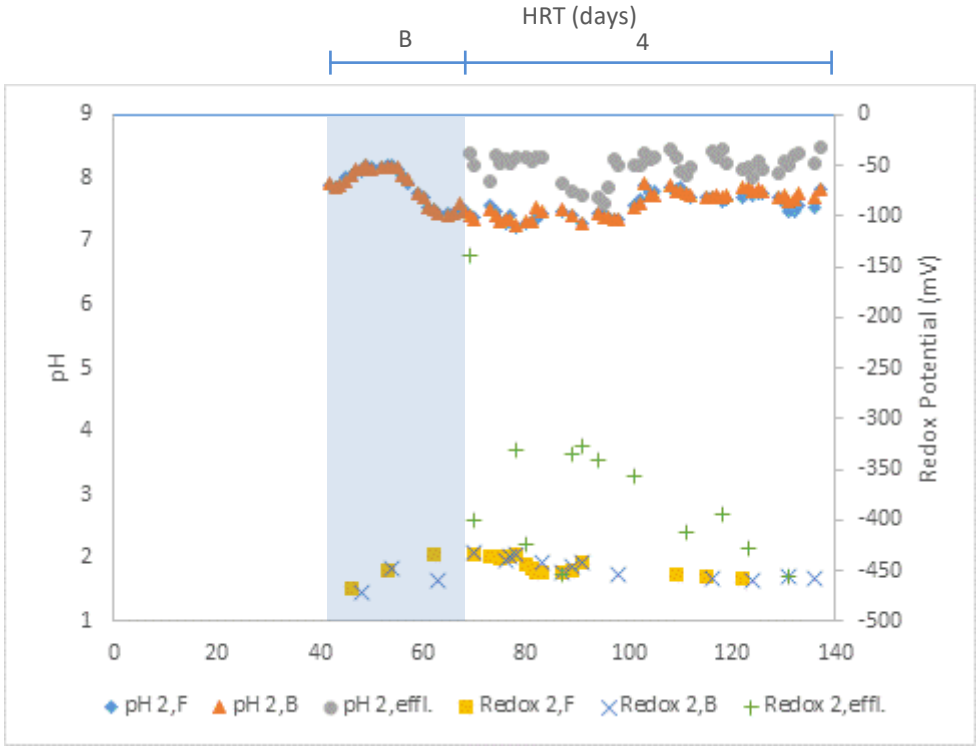

Figure 37: Change in pH and redox potential with time for second HLFCR grown on BTE. Shaded = batch period (B).

The mean redox potential in the bulk liquid was -447 mV (Figure 37), indicative of high levels of HS^- and an anaerobic environment. The first effluent sample collected from the second channel, once continuous operation commenced, had a redox potential of -139 mV, most likely due to very low HS^-

concentration of 0.6 mg/ℓ. The effluent redox potential then decreased to -400 mV the next day and ranged between -320 mV and -420 mV for the remainder of the study, as the HS^- concentration fluctuated.

7.3.4.2.2 Sulphur species removal for reactors in series

The data show a period of adaptation during the start of the batch phase, where very little sulphate reduction occurred. During this time the HS^- concentrations decreased from 534 mg/ℓ on day 43 to a minimum of 84 mg/ℓ on day 56, after which it began to rise once again (Figure 38A).

The increase in HS^- concentration in the bulk liquid accelerated after the reactor was switched to continuous operation, in part due to an increase in the HS^- concentration of the feed, reaching a maximum on day 87 (658 mg/ℓ; Figure 38A).

The minimum bulk liquid SO_4^{2-} concentration (109 mg/ℓ) was measured on day 76 and represented a sulphate reduction efficiency across the two channels of 96.6%. The biofilm was then disrupted, which was followed by a period of increase in SO_4^{2-}, until day 104, after which it remained relatively stable at < 1100 mg/ℓ. At this point the system had reached a degree of stability and more frequent biofilm harvesting was started.

Connecting the two reactors in series meant it was no longer possible to collect effluent from channel 1 directly from the effluent pipe as this was fixed onto the feed port of channel 2. Instead a sample was carefully drawn from within the weir around the effluent port using a syringe and hypodermic needle. This introduced some inaccuracy, particularly for HS^-, as it was impossible not to draw up some liquid that was more characteristic of the bulk volume, which had a high HS^- concentration. As a result, the HS^- in the "effluent" sample was typically around 35% lower than in the bulk liquid, which was much higher than measured from the effluent pipe when channel 1 was operated by itself (Figure 33).

Similarly, the SO_4^{2-} measured in the "effluent" was typically lower (as low as 895 mg/ℓ) than previously measured, so was likely an underestimation. As a result, the second channel appears to get a feed of lower SO_4^{2-} than it actually receives. This results in a higher calculated SR in channel 1 than actually occurs and a lower calculated SR in channel 2.

When looking at the combined performance of the channels, the HS^- concentration in the bulk liquid was generally higher in channel 1, an average of 551 mg/ℓ from day 74-137, compared to channel 2, 420 mg/ℓ from day 80-137. This was likely due to the higher SRRs in the first channel, possibly influenced by SO_4^{2-} concentration and a higher concentration of preferred organic substrate.

The HLFCRs grown on BTE showed that both the endogenous and hybrid community are capable of BSR and SO. Similarly, both communities are capable of complete removal of HS^- from the effluent, provided blockages do not cause sudden expulsion of large amounts of effluent and that FSB regeneration is fast enough.

On average, for the duration of the experimental run, the channels in series were able to reduce 42% of SO_4^{2-} in the feed. Disrupting or harvesting the FSB reduced performance until the biofilm reformed. Considering only the data when the system was operating at steady-state the SR efficiency increased to 66%.

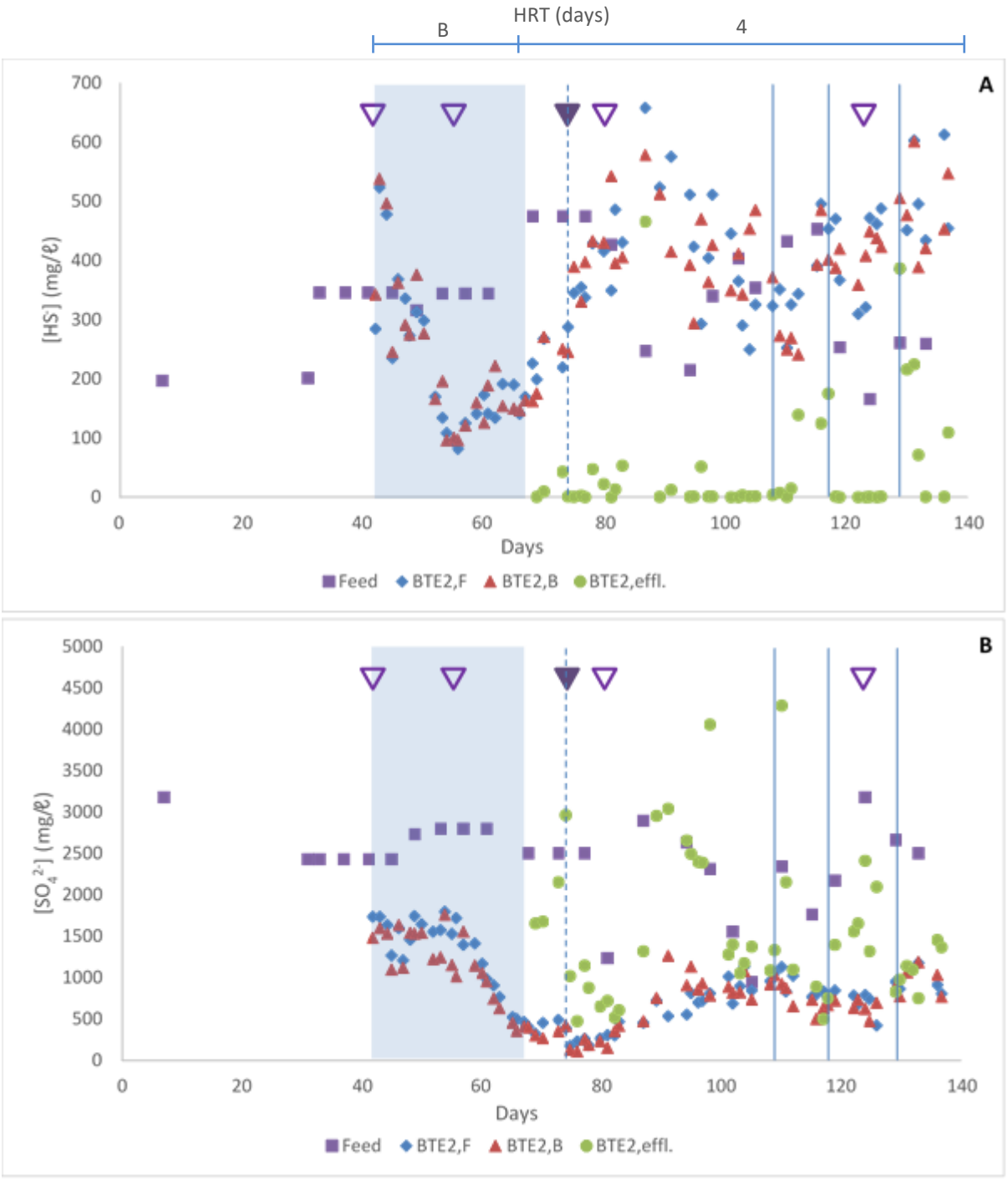

Figure 38: Change in HS^- (A) and SO_4^{2-} (B) concentration with time for second HLFCR grown on BTE with hybrid community.

$BTE_{2,F}$ = second run of channel grown on BTE, bulk liquid, front sample port; $BTE_{2,B}$ = second BTE channel, bulk liquid, back sample port; $BTE_{2,effl.}$ = second BTE channel, effluent sample.
Shaded = batch period (B); open triangle = bulk liquid DNA sample; closed triangle = FSB solids DNA sample; solid vertical line = FSB harvested; dashed vertical line = FSB disrupted.

The overall performance of the channels in series was similar to Boshoff *et al*. (2004), who were able to reach an average SR of 60-80% when treating tannery effluent with a CSTR and 80% with a UASB. A direct comparison is difficult due to the differences in composition of the tannery effluent used, the reactor configurations and operating conditions. The feed SO_4^{2-} concentration in their study went up

to 1800 mg/ℓ, while the BTE treated in this study averaged 2500 mg/ℓ. When looking at the actual reduction in concentration, Boshoff *et al*. (2004) were able to remove between 1080 and 1440 mg/ℓ, while the channels in this study achieved an average BSR of 1202 mg/ℓ SR.

Much of the published work on tannery effluent treatment, such as Boshoff *et al*. (2004), used active reactor systems like the UASB or CSTR, while the HLFCR used in this study is essentially a passive reactor. Passive BSR systems have mainly been used to treat mine water, so direct comparison is again difficult. Molwantwa *et al*. (2010) and Waybrant *et al.* (2002) were able to achieve 1.8% and 31.6% SR in down- and up-flow packed bed reactors (DF-PBR and UF-PBR), respectively, for feed SO_4^{2-} concentrations of 1090 and 243 mg/ℓ (Table 13). These values are considerably lower than in the current study. However, the mine water studies were not performed with tannery effluent and their relatively poor performance was most likely influenced by the substrates used: wood chips, manure and primary sewage sludge, and leaf compost and sawdust, respectively. Both mixes were primarily composed of complex organic matter, for which hydrolysis has been shown to be the rate limiting step (Mooruth, 2013), while the BTE used in this study was still rich in soluble COD (Table 25 & 26).

It was difficult to accurately measure the contribution of each channel to the overall performance or assess whether the endogenous community or enrichment community performed better. To overcome this, a better way of sampling the first channel's effluent needs to be developed or both channels should be started at the exact same time, with the same BTE batch and each fed separately with a feed of the exact same composition, as far as possible.

7.3.4.2.3 Sulphate reduction rate for reactors in series

The previous section highlighted the challenge of getting consistently accurate measurements of the effluent from channel 1 when the reactors were operated in series. This affected the determination of the SRRs. Despite this, it is clear that the SRRs for both channels were relatively high for passive treatment, relative to other studies (Table 13).

The maximum SRR the first channel achieved in the bulk liquid (1049 mg/ℓ.day) was twice as high as the second channel (513 mg/ℓ.day). Comparing the calculated SRR over each HRT, as shown in Figure 39, it is clear that the average SRR is significantly higher in channel 1 than channel 2 for most of the study. Previous work using CSTRs with increasing feed SO_4^{2-} concentrations showed that the relationship between feed concentration and SRR was close to first order until substrate inhibition occurred (Moosa *et al.*, 2002). Therefore, the disparity in SRR performance was likely influenced by the higher concentration of SO_4^{2-} received by channel 1 (2400 mg/ℓ, on average), while channel 2 received the partially treated BTE from channel 1, with a substantially lower sulphate concentration (<1700 mg/ℓ, on average), as well as the possibility that some of the most readily available carbon source was depleted in channel 1.

Figure 39 also shows when the reactor feed was changed and which batches of raw and partially treated BTE were blended to make up the feed. While the change in feed does not appear to have a consistent impact on the SRRs it does appear as though the performance decreases once the batch of feed gets older. This is particularly apparent for the two blends using batch 4 of the raw BTE. This was consistent across channel 1 and channel 2.

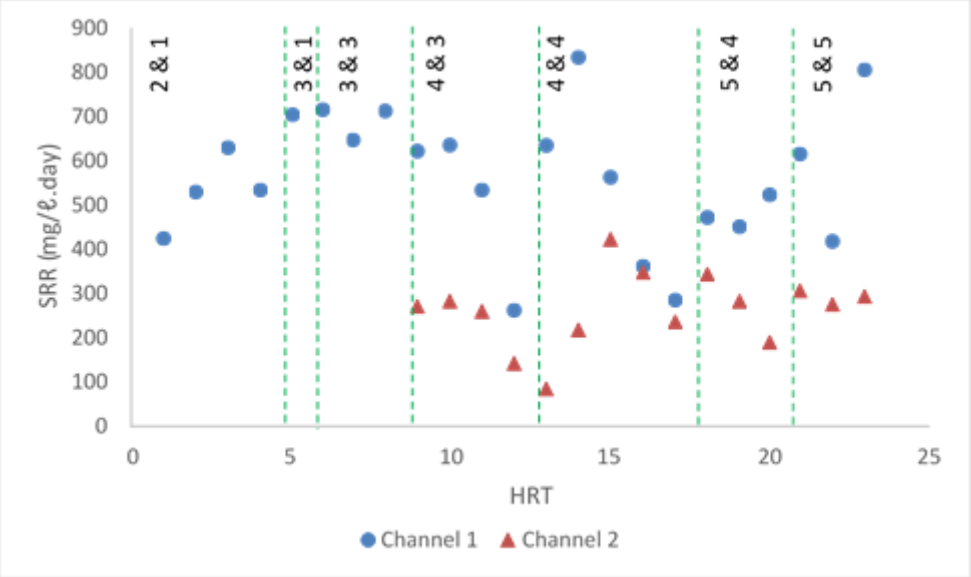

Figure 39: Average SRR within the bulk liquid across each HRT for each channel.
Dotted lines divide each HRT for raw & partially treated BTE batches, respectively.

Batch 4 of raw BTE was the first batch to have a pH as high as pH 11.94 and had the highest HS^- concentration seen by the channels (888 mg/ℓ) and one of the lower SO_4^{2-} concentrations (1459 mg/ℓ). The raw BTE was blended with partially treated BTE to try and achieve a consistent feed composition, but the high pH and HS^- may have affected the endogenous community, which could have impacted the performance within the HLFCR.

The measured SRRs were significantly higher than those for the passive DF-PBR and UR-PBR of Molwantwa *et al.* (2010) and Waybrant *et al*. (2002), who were able to achieve SRRs of 13 and 120 mg/ℓ.day, respectively. The SRRs reached in this study were more similar to those of Boshoff *et al*. (2004), who were able to achieve rates of 600 mg/ℓ.day treating tannery effluent in active reactor systems. This illustrates the potential of the HLFCR as a system for SO_4^{2-} reduction in BTE, when inoculated with a suitably adapted SRB consortium, such as the endogenous community present in the BTE samples received.

Therefore, the performance of each channel is highly dependent on feed composition and while performance was relatively consistent with each batch cycle, each component's effect on reactor performance should be evaluated. Reactor feed and postulated effects would need to be monitored in a real-world tannery.

Both channels grown on BTE performed better than the channels operated using ASW with lactate and with OTE, in terms of their SRRs. The BTE-fed channels reached higher maximum and average SRRs than both OTE channels. This demonstrates the suitability of BTE to this pre-treatment technique.

7.3.4.2.4 Changes in DNA concentration and dissimilatory sulphite reductase gene prevalence in channel reactors

The *dsr*B gene copy numbers were previously presented in Figure 36 in Section 7.3.4.1.4, while the extracted DNA concentrations and SRR at the time of sampling are presented in Table 31. The starting inoculum for channel 2 had a *dsr*B gene copy number over 30% higher than the first sample from the

channel. The majority (78%) of the channel 2 inoculum was taken from raw BTE batch reactors (Section 7.3.3), which initially contained 40% (vol./vol.) of their respective enrichment consortia reactor (lactate grown) contents (Section 5.3.1.2.3). The other 22% (vol./vol.) of the inoculum consisted of enrichment consortia Z reactor contents (Section 5.3.1.2.1). Therefore, a significant proportion of the inoculum came from enrichment reactors operated specifically to select for SRB community members, rather than raw BTE, which had a far more diverse community.

The *dsr*B gene copy number per ng DNA continued to decrease in the samples from channel 2 as the experiment progressed, despite an increase in the extracted DNA concentration between day 42 and day 80, suggesting a relative decrease in the proportion of SRB making up the community. The *dsr*B gene copy number in the day 80 sample from channel 1 was similar, so it is unlikely that the decrease was due to the carryover of bovine cells from channel 1. A more likely explanation is either the washout of slow growing SRB species or the increased attachment of SRB to the carbon fibres, which would mean they were not detected in the planktonic sample.

The DNA concentration of the final DNA sample from both channels was more than double the previous sample, taken on day 80 (Table 31). Despite this, the *dsr*B gene copy number per ng DNA was lower, particularly in channel 2. This indicates that the increase in DNA extracted was most likely not due to a substantial increase in SRB, but rather non-sulphate reducing species.

Table 31: DNA concentrations of each sample extracted from the bulk liquid of channel 1 and 2 and corresponding SRRs

Channel	**Run day**	**DNA concentration**	**SRR**
	Days	ng/µℓ	mg/ℓ.day
1	12	25	198
	38	96	674
	55	87	537
	80	64	643
	122	186	568
2	42 (inoculum)	237	-
	42	137	-
	55	184	439
	80	113	182
	123	230	281

Further evidence for this can be seen when looking at the Pearson's correlation coefficient for the SRR and *dsr*B gene copies per ng of DNA, which was only 36.0% for channel 2, compared to 62.7% across the two reactors in series. This indicates that the day 123 data had a large effect on the relationship of SRR to the *dsr*B concentration.

7.3.4.2.5 Sulphide oxidation rate for reactors in series

The average SOR, measured for each residence time, is plotted in Figure 40. The SOR was calculated from the difference between the bulk liquid concentration and that in the collected effluent, initially for channel 1 alone (HRT 1-8), then from the effluent from the second channel in series (HRT 9-18).

From HRT 19 to the end of the experiment an "effluent" sample was extracted from the weir in channel 1.

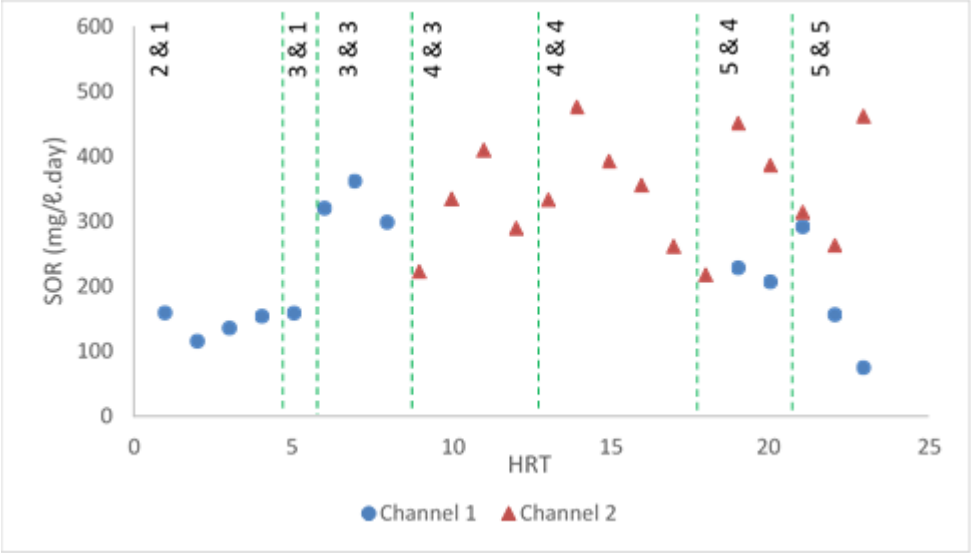

Figure 40: Average SOR calculated from the difference between bulk liquid and effluent concentrations for each channel. Dotted lines indicate changes in feed composition.

At the start of the experiment, the HS^- concentration in the bulk volume of channel 1 was relatively low (Figure 33), so despite all the sulphide being oxidised the SOR averaged around only 130 mg/ℓ.day. This increased to 320 mg/ℓ.day as the rate of BSR in the channel accelerated and there was more sulphide available for oxidation. The SOR remained relatively stable for the next two HRTs.

Aside from the periods following a disruption in stable operation, usually caused by a blockage in the effluent pipe that led to volume accumulation in the channel, followed by the outflow of a portion of the bulk volume, the HS^- concentration in the effluent was insignificant. This showed that there was a well-established SOB community and the actual SOR was determined by the amount of sulphide available largely as a result of SRB activity, rather than some limiting factor.

Overall, the system showed an average SOR of 305 mg/ℓ.day, which was higher than that shown by Mooruth (2013), who managed a maximum of 184 mg/ℓ.day in a LFCR. Similarly, Xu *et al*. (2012) were able to achieve an SOR of 183 mg/ℓ.day in a micro-aerophilic expanded granular sludge bed (EGSB) reactor with lactate as a carbon source when at a dissolved oxygen (DO) level of 0.8-1.0 mg/ℓ, optimised for S^0 recovery. The fact that the HLFCR system exceeded those values confirmed the potential of the HLFCR for treating BTE.

7.3.4.2.6 COD utilisation across the reactors in series

For the system to be effective for the pre-treatment of BTE as a substrate for AD there needs to be enough soluble COD in the effluent to make AD viable. The first BTE channel started off with a total COD concentration of 27 g/ℓ, while the second channel started at 17 g/ℓ. The soluble fractions were 7.0 g/ℓ in channel 1 and 8.3 g/ℓ in channel 2. Soluble COD could be consumed by the SRB during sulphate reduction as well as by non-SRB in the bulk volume and aerobic and micro-aerobic autotrophs within the FSB. At the same time, hydrolysis and acidogenesis reactions could generate more soluble COD from the partial digestion of organic solids (sludge). Therefore, a definitive relationship between SO_4^{2-} reduced and soluble COD consumed was not possible.

Figure 41 shows the soluble COD concentration of the feed ranged between 7-10 g/ℓ, while the effluent from channel 2 was significantly lower.

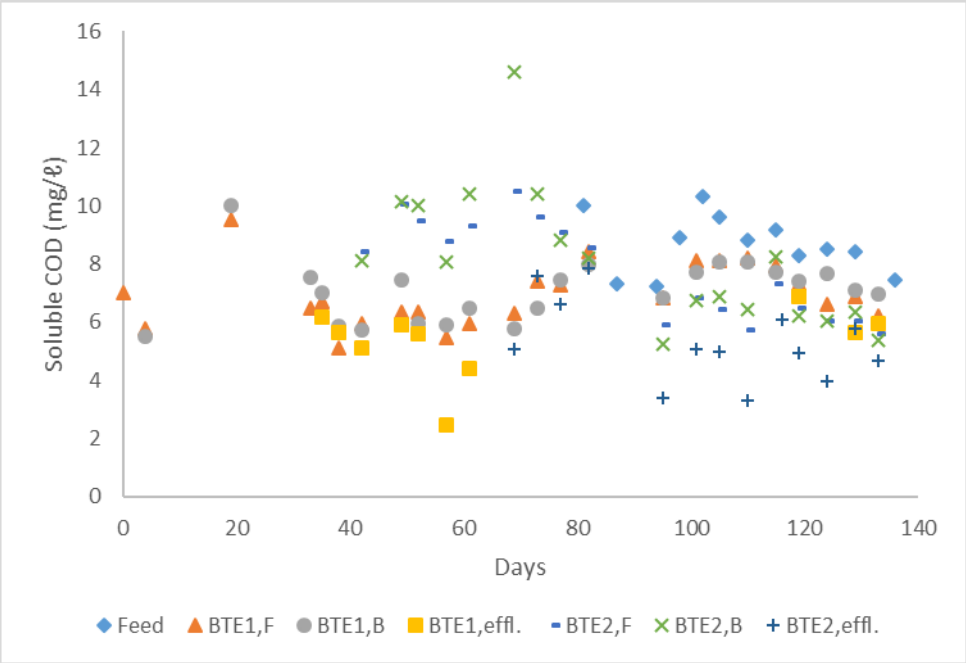

Figure 41: Change in concentration of soluble COD with time for both HLFCRs grown on BTE.

$BTE_{1,F}$ = first channel grown on BTE, bulk liquid sample, front sample port; $BTE_{1,B}$ = first channel, back port; $BTE_{1,effl.}$ = first channel, effluent sample; $BTE_{2,F}$ = second channel, bulk liquid sample, front, $BTE_{2,F}$ = second channel, back; $BTE_{2,effl.}$ = second channel, effluent sample.

Once the channels were connected in series, the majority of the COD consumption took place in the first channel (29% reduction), with COD consumption in channel 2 approximately half that of channel 1. This correlates with the majority of the BSR taking place in the first channel, as discussed in Section 7.3.4.1.3. The effluent had an average soluble COD concentration of 5.3 g/ℓ. This means that, on average, around 44% of the soluble COD in the feed was used across the two channels, assuming limited hydrolysis of organic solids. More than half of the soluble COD remained available for downstream AD. Despite the reduction in soluble COD during the pretreatment, the residual soluble COD in the effluent is still sufficiently high to make AD viable and worthwhile.

The COD mass balance across the two channels was estimated, based on the amount of COD required for the measured sulphate reduction. This showed that, over each HRT, around double the amount of soluble COD was consumed, compared to what was theoretically expected for channel 1 (Table 32), based on Equation 14. Across the reactors in series, an excess of 1.19 g of COD $\pm$ 5 g was utilised, which equates to a 38% excess. However, the large SD indicates how much variation there was. What was clear was that the second channel was using far less COD than the first channel, suggesting there may have been more hydrolysis of solid COD in channel 2.

This greater demand for substrate in the first channel could be attributed to its higher proportion of BSR (Section 7.3.4.2.2) or more diverse microbial community, with non-SRB heterotrophs responsible for some of the COD reduction.

Table 32: Soluble COD measurements and theoretical consumption values for channel 1 and overall system. COD values represent total mass per HRT

Property	Channel 1	SD	Series	SD
COD_{feed} (g)	17.35	2.02	17.35	2.02
COD_{bulk} (g)	14.74	2.15	29.49	2.97
$COD_{effluent}$ (g)	12.34	1.30	10.01	2.72
$COD_{consumed,\ theoretical}$ (g)	2.93	1.35	4.58	1.30
$COD_{consumed,\ actual}$ (g)	6.59	1.74	8.70	4.85
COD_{excess}(g)	4.36	0.87	1.19	5.00
Actual:theoretical	3.18	0.91	1.99	1.22
Excess*:theoretical	2.18	-	0.38	-

*excess = actual - theoretical

Further, it was found that the COD in the effluent was significantly lower, by on average 24% and 33% in the first and second channel, than in the bulk volume (Figure 41). This was due to consumption by the aerobic heterotrophs at the air liquid interface, with some of the consumed substrate metabolised into the EPS that helped form the floating biofilm.

7.3.4.2.7 Biofilm formation and regeneration

Biofilm formation in the second channel did not occur as rapidly as in the first channel and seeding with powdered biofilm was required to speed up the process. This suggests that the microbial consortium used to inoculate the channel may not have been as rich in species needed to promote biofilm formation. The environmental sample enrichments were performed in sealed reactors so would have selected against aerobic or microaerobic species.

Initial seeding with dried FSB obtained from reactors treating less saline AMD had little impact, while seeding with dried saline-adapted FSB from channel 1 (Figure 42B) speeded up the process, with an EPS network visible after two days and a full, thin FSB covering the surface after another day (Figure 34B).

On day 62, both channels had complete biofilms and there was a distinct lack of odour from the reactors, indicating that when running smoothly, this treatment method can help control odour problems associated with HS^- treatment.

The use of silicon tubing, which is oxygen permeable, for inlet and outlet pipes created an environment where partial oxidation of any residual sulphide could occur. As a consequence, the effluent pipes were periodically blocked by the accumulation of sulphur, which caused disruptions in performance of the system. Typically, there was an accumulation of bulk volume and when released the loss of some, or occasionally all, of the FSB. This also accounted for the spikes in effluent HS^- concentration, as observed on day 96 (Figure 38A).

Both biofilms were full and thick on day 101 (Figure 42C). The biofilms did not become as thick, over time, as had been seen in previous studies (Mooruth, 2013), so did not severely limit O_2 mass transfer and inhibit SO in the lower part of the biofilm. If this occurred, a progressive increase in HS^- in the effluent would be seen.

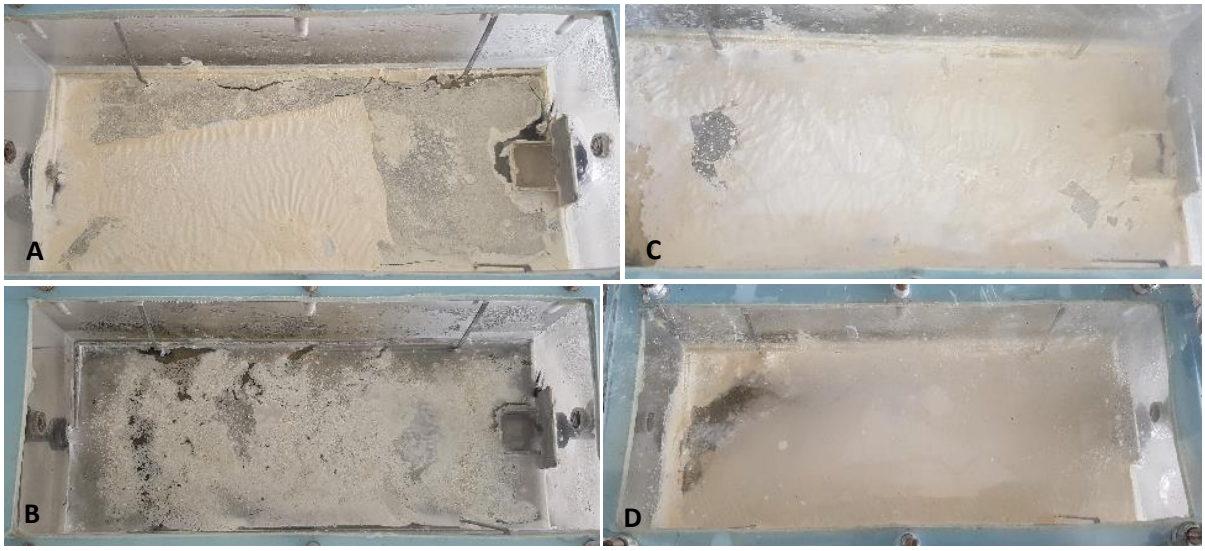

Figure 42: FSB formation of second BTE channel. A) Example of biofilm recovery after partial washout. B) Day 53, four days after initial seeding with saline-adapted FSB. C) One week before harvest on day 108. D) 95% complete biofilm two days after harvesting on day 108.

7.3.4.2.8 Biofilm characterisation and implications for system performance

Once the channels were running smoothly, the biofilms were harvested alternately (from day 108). If the channel was seeded with saline-adapted FSB right after harvesting, the FSB would reform in 2-3 days. This rate was longer if a blockage in the pipe led to washout of FSB or if very little FSB was left in the channel after harvesting. Harvested and dried biofilm was flaky and had a characteristic yellow-cream colour (Figure 43), suggesting a high S^0 content.

Figure 43: Harvested and dried FSB

The harvested biofilm composition was analysed in terms of the elements listed in Table 33. The proportion of sulphur was higher in the second channel's FSB while the amount of organic matter (hydrocarbons: C and H) was higher in channel 1. This may have been due to the first channel receiving the raw effluent, which contained some suspended sludge material. A small portion of the sludge was caught on top of the screen and was unavoidably collected when harvesting the biofilm.

Table 33: Amount of FSB harvested and its composition

Channel	Date	Run day	Harvested (g)	Seeded (g)	Mass analysed (mg)	QC Analysed (%)			
						N	C	H	S
2	12-Aug	108	1.79	0.509	9.17	1.1	6.1	0.6	62.2
1	16-Aug	112	2.05	0.200	6.84	1.0	7.8	0.5	57.7
2	21-Aug	117	1.06	0.148	9.12	1.0	6.2	0.6	76.5
1	27-Aug	123	0.44	0.254	16.27	1.7	11.0	1.1	37.9

QC – quality control

Typically, the sulphur content of the biofilm was in the region of 60%, reaching almost 80% in some of the samples from channel 2. While this is lower than the average sulphur content obtained by Mooruth (2013), when the LFCR was operated exclusively as a SO unit, it was higher than sulphur content reported by Marais *et al.* (2020), who operated a HLFCR on modified Postgate media.

The biofilm composition data were used to try and close a sulphur species balance across the system. It was found that for channel 1, around 42% of sulphur species could not be accounted for between the biofilm and effluent over a harvest cycle. For channel 2, 39% of the sulphur species were missing from the effluent (Table 34). Taking into account what portion of the biofilm was S^0, as given by Table 33, S^0 recovery by each channel and the share of the missing sulphur species was found. On average, for channel 1, it was found that 18.9 mmoles of sulphur species were missing from the effluent per day and of these, the amount S^0 in the biofilm harvested was 21.0 mmoles. However, this biofilm was formed and finally harvested over a period of 11-13 days. By comparison, channel 2 was missing 14.5 mmoles of sulphur species per day and the average amount of S^0 within biofilm harvested was 25.7 mmoles over 9-12 days. Therefore, 6.9% and 10.2% recovery of sulphur species as S^0 occurred on average for channel 1 and 2, respectively. Portions of missing sulphur species, which includes the S^0 in the FSB, is shown for each channel and each sampling in Table 34 as well as their S^0 recovery.

Table 34: Sulphur recovery and portion of mmoles of sulphur species missing between the bulk liquid and effluent of each channel

Sampling	Channel sulphur species missing in effluent (%)		S^0 recovery (%)	
	CH1	CH2	CH1	CH2
1	46*	31	11.6	6.6
2	37	45	2.3	15.1
3		42		8.9
Mean	41.9	39.2	6.9	10.2

*no effluent data at this time: mmoles of HS^- in effluent was taken as an average of later effluent data

There are four possible explanations for the unaccounted sulphur species. The majority is most likely in the form of colloidal S^0 in suspension in the effluent (Figure 44). The HS^- data confirm that almost all the sulphide present in the bulk liquid has been removed in the effluent, suggesting SO is efficient. Ideally, the majority of the elemental sulphur formed would be deposited in the FSB, but due to some of the challenges with FSB formation this is unlikely and there was probably a lot of partial oxidation occurring in the liquid just below the interface.

Secondly, effluent samples often contained small pieces of FSB that the weir was unable to retain and these were washed out of the channel and settled to the bottom of the sample or effluent collection container. This was partly due to unstable nature of the FSB at times.

Thirdly, the effluent sample was occasionally more yellow in appearance than in Figure 44, such as on days 112-118, which indicates the presence of polysulphides (S_n^{2-}). Generally, when the pH is higher there will be more polysulphides present. Polysulphides are green-yellowish to orange coloured aqueous solutions and their presence has been found to accelerate the dissolution of S^0 (Kleinjan *et al*., 2005). Additionally, this S^0 could be colloidal S^0 or S^0 that has been deposited on the surface of microorganisms' cells.

Figure 44: Image of an effluent sample containing colloidal sulphur

Lastly, there is also the possibility of some speciation to SO_3^{2-}, $S_2O_3^{2-}$ and $S_4O_6^{2-}$. The IC tests performed would have picked up any $S_2O_3^{2-}$ and the other species are quantifiable by HPLC, but these substances are less stable intermediates and would oxidise biotically or abiotically into SO_4^{2-} relatively quickly (Mooruth, 2013), so these are unlikely to have been a significant contribution.

Understanding which of these species were most abundant would help with the development of downstream applications for its recovery. The S^0 fragments can be filtered out of the effluent, while pH adjustment can cause colloidal sulphur to aggregate and settle out. Fortunately, both colloidal S^0 and polysulphides should not inhibit downstream AD, as S^0 is the most stable form of sulphur and polysulphides are unlikely to penetrate microbial cells (Mooruth, 2013).

Therefore, these experiments have shown that the channels inoculated with an endogenous BTE community and a hybrid community were both able to maintain a FSB, although seeding with saline-acclimated FSB was required to accelerate regeneration following the harvest of the FSB.

As consistent BSR and SO are highly dependent on the presence of the FSB, the FSB formation rate has been identified as the performance limiting aspect of the process. The SO component was also identified by Xu *et al*. (2012) to be the rate limiting step in their micro-aerophilic integrated BSR and SO reactor.

Mooruth (2013) used an LFCR as a dedicated SO reactor to remove HS^- from the effluent of a packed bed BSR reactor treating simulated AMD. He reported that an SOR of 5.6 mmol/ℓ.day could be achieved at a 1 day HRT, with an 82.2% HS^- conversion and 92% of the converted HS^- reporting as S^0 to the biofilm. In this study, channel 2 reached a maximum SOR of 14.4 mmol/ℓ.day. This shows a high SO efficiency, but close to half was oxidised to SO_4^{2-} and a significant portion of the other half became

colloidal S^0, primarily due to slow and occasionally incomplete FSB formation. Therefore, it is hypothesised that the heterotrophic organisms responsible for making the organic fraction of the biofilm that gives it its structural integrity are not present in sufficient amounts or are inhibited, possibly by the high salinity, resulting in slow biofilm scaffold formation and low S^0 deposition in the biofilm (Mooruth, 2013). Alternatively, it is possible that these microorganisms depend on others to break down complex carbon sources and this is happening too slowly for them to get the substrate required to make the scaffolding fast enough. Mooruth showed that acetate supplementation was required to ensure good biofilm formation in his LFCRs. Detailed characterisation of the soluble organics was not performed in this study.

The species required for biofilm formation present in this study may be slower growing than lower salinity counterparts. If this pre-treatment were investigated further, it would be beneficial to look at the SOB community and whether adaptation may benefit them. While some aspects of the SOB community performance were favourable, the true measure of HLFCR performance is the conversion of HS^- to S^0 in the biofilm, as it is more beneficial to be able to harvest the S^0 than for it to remain in the effluent as colloidal S^0 (Mooruth, 2013).

The quantification of polysulphides and other short-lived intermediates is particularly challenging, but more accurate quantification of the colloidal sulphur fraction, not accounted for in this study, would help to close the sulphur balance. This can be achieved by precipitating colloidal sulphur from the collected effluent and quantifying gravimetrically.

7.3.5 *Microbial community structure characterisation*

7.3.5.1 Endogenous bovine tannery effluent communities

7.3.5.1.1 Copy numbers of *dsr*B genes in batches of bovine/ovine tannery effluent

It was postulated that due to the presence of high concentrations of sulphur species in the BTE, a well-adapted bacterial community, capable of sulphur species metabolism, may already be present in the BTE. Light microscopy confirmed the presence of large numbers of bacterial cells. To identify the presence of SRB, DNA extractions were performed and the DNA was used as template for qPCR using primers specific for the *dsr*B gene, which codes for an enzyme that plays a key role in BSR. The results for copy numbers of the *dsr*B gene are presented in Figure 45.

The raw BTE would have contained residual animal tissue, so a significant portion of the extracted DNA was likely to have been eukaryotic, which explains the relatively low *dsr*B gene copy number, as a function of template DNA, compared to the similar analysis on the enrichment cultures.

The exception was the sample for BTE batch 1, which was collected after the effluent had been loaded into channel 1 and allowed to settle for 12 days. No inoculum was added to this channel. During this time, the community present in the raw BTE had a chance to proliferate under controlled, anaerobic conditions, while some of the heavier animal tissue would have settled out.

The qPCR analysis confirmed the presence of SRB within each sample of BTE received from the tannery. The efficiency of qPCR for *dsr*B genes is commonly 93-107% (Bae *et al.*, 2014; Jochum *et al*., 2017), however some studies have reported near 90% efficiency (Faulwetter *et al.*, 2013). In this study, the efficiency of the assay was 73.1%. The lower efficiency was primarily due to the low concentration

of DNA in BTE batches 2, 3, 4 and 5, and that a significant portion of this was likely eukaryotic DNA, leaving SRB in very low concentration, causing them to be out of range for the standard.

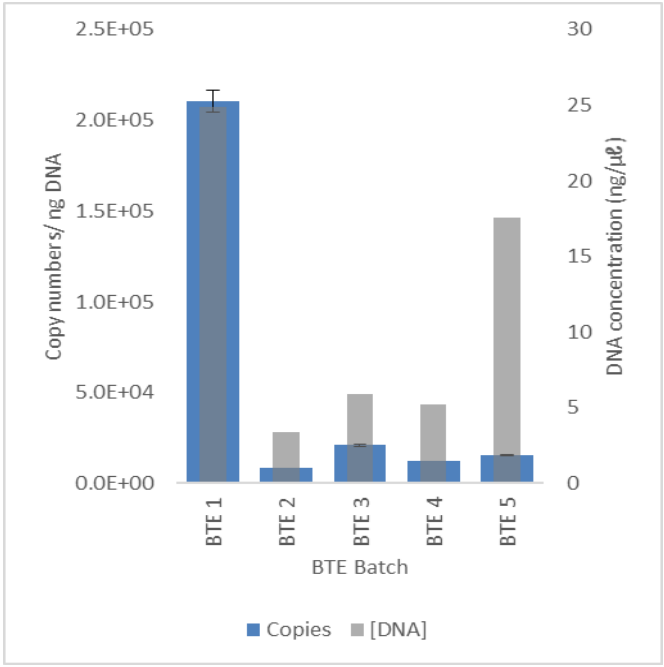

Figure 45: Number copies of dsrB gene per nanogram of DNA as well as concentration of DNA for each batch of BTE

7.3.5.1.2 Characterisation of endogenous SRB community based on *dsr*B gene sequencing

The microbial community containing the *dsr*B gene was characterised based on sequence analysis. The *dsr*B gene codes for an enzyme capable of catalysing both the forward and reverse reaction, so it was hoped that sequencing would identify species involved in sulphide oxidation, but this did not appear to be the case.

The community structure across the batches was unexpectedly dissimilar, having an average similarity of 52.5% across all batches using the SIMPER analysis, considering that the samples were taken at the same effluent outlet over at intervals of between 18 and 35 days (Table 36). This suggested that the changing composition of the effluent had an effect on the bacterial community capable of sulphur species removal.

The spatial distribution of points representing the BTE batches in the non-metric multidimensional scaling (nMDS) plot overlaid with cluster analyses in Figure 46 shows that most batches' potential sulphur species removal communities were within 40% similarity to one another.

Batches 3 and 4 were considered to be 60% similar, which may be due to them having the shortest time between sampling (18 days). Again, the 2D stress on this plot indicates that this is an excellent representation of the BTE batch similarities.

From Figure 47, it can be seen that the 20 most dominant species, selected of the 158 identified bacterial species containing the *dsr*B gene, account for 92.3-99.7% relative abundance in each BTE batch. Further, Figure 47 shows that an unnamed member of the genus *Desulfovibrio* (*Desulfovibrio* sp. enrichment culture DGGE band HCB4) was present in significant numbers across all BTE batches

with 18.1-59.3% relative abundance (RA), dominating three of the five samples. Batch 2 and 3 had different dominant species.

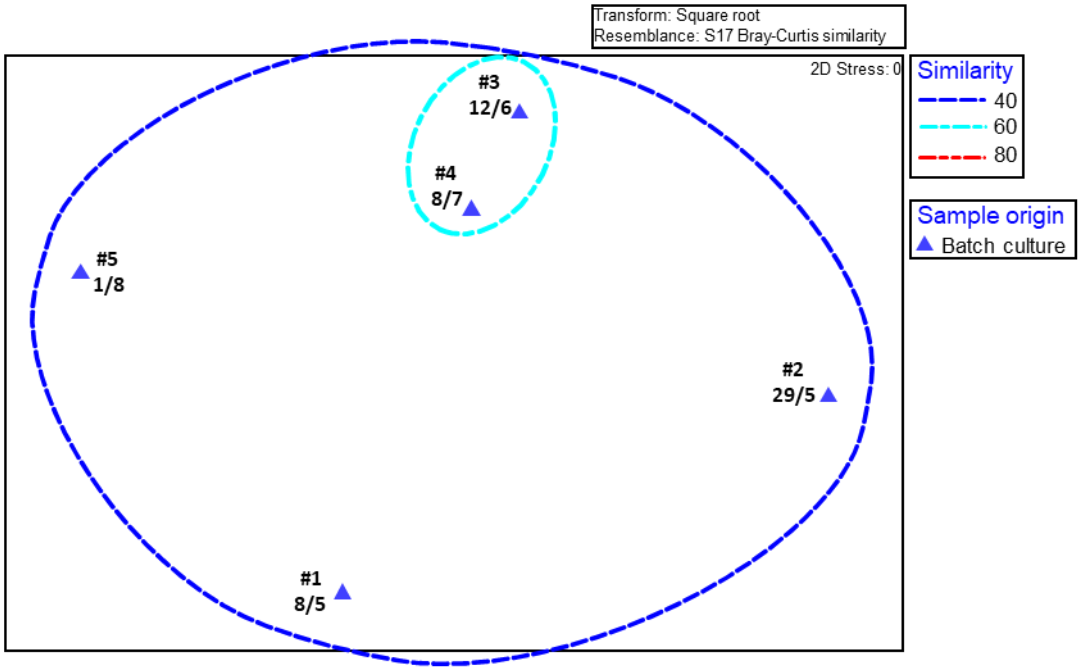

Figure 46: nMDS plot overlaid with cluster analysis of the Bray-Curtis similarity of the relative abundance between bacterial species containing dsr from raw BTE batches

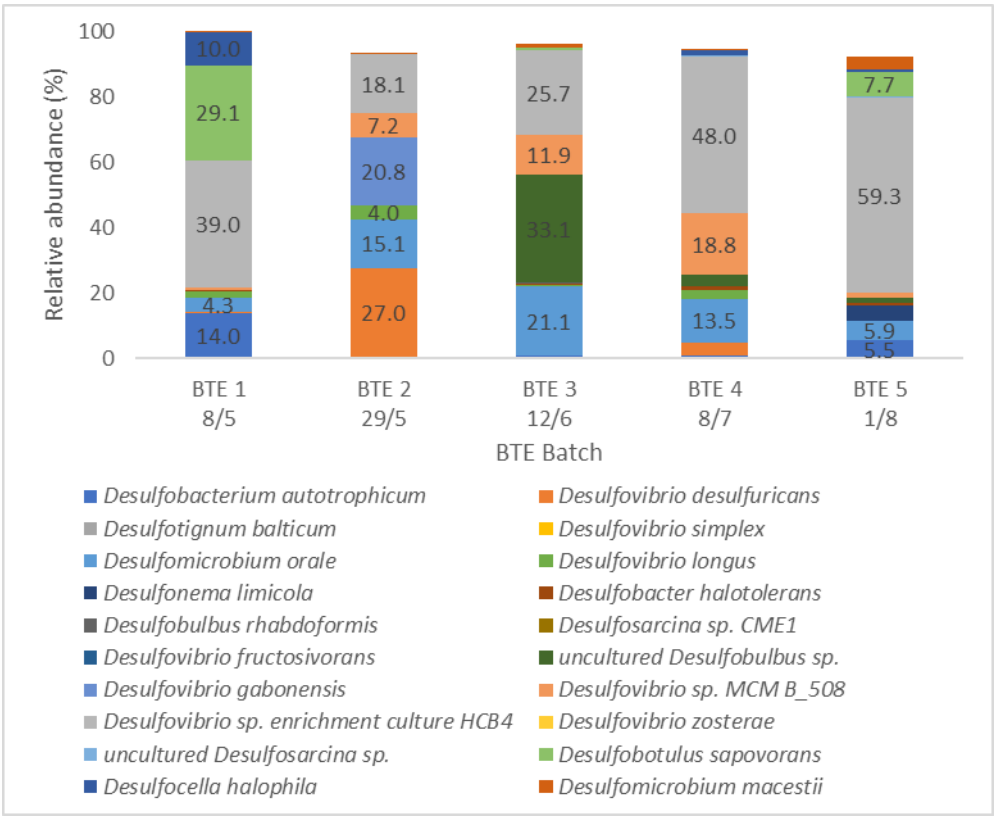

Figure 47: Amplicon sequencing results of the relative abundance of 20 highest bacterial species contributors containing dsr in the raw bovine tannery effluent batches

Batch 1 of raw BTE was dominated by *Desulfovibrio.* sp. HCB4 (39.0% RA) and *Desulfobotulus sapovorans* (29.1% RA), with smaller amounts of *Desulfobacterium autotrophicum* (14.0% RA) and

Desulfocella halophila (10.0%). Batch 2 had a wide variety of species in similar proportions, with *Desulfovibrio desulfuricans* (27.0% RA) and *Desulfovibrio gabonensis* (20.8% RA) with the highest abundance and *Desulfovibrio* sp. HCB4 (18.1% RA) and *Desulfomicrobium orale* (15.1% RA) in significant proportions. Uncultured *Desulfobulbus* sp. (33.1% RA), *Desulfovibrio* sp. HCB4 (25.7% RA) and *D. orale* (21.1% RA) dominated batch 3, with a small proportion of *Desulfovibrio* sp. MCM B_508 (11.9% RA) present. Batch 4 was similar to batch 3 but excluded the uncultured *Desulfobulbus* sp. and contained a larger proportion of *Desulfovibrio* sp. HCB4 (48.0% RA) with *Desulfovibrio* sp. MCM B_508 (18.8% RA) and *D. orale* (13.5% RA) in the background. Finally, the majority of the community containing the dsr enzyme of batch 5 consisted of *Desulfovibrio* sp. HCB4 (59.3% RA).

7.3.5.1.3 Characteristics of microbial species identified

Desulfovibrio sp. HCB4 is an uncultured *Desulfovibrio* species, as is *Desulfovibrio* sp. MCM B_508 (maximum of 59.3% and 18.8% RA, respectively, Figure 47), so only genus level attributes are discussed. As discussed in Section 5.3.2.2, the *Desulfovibrio* genus are incomplete organics oxidisers with limited metabolic flexibility (Gilmour *et al*., 2011). *Desulfovibrio* spp. are commonly found in a variety of marine, estuarine and freshwater environments, confirming the tolerance of these species to saline environments (Widdel and Pfennig, 1984).

Other *Desulfovibrio* species found in the batches were *D. gabonensis* (20.8% RA in batch 2) and *D. sulfuricans* (27.0% RA in batch 2), which were discussed briefly in Section 5.3.2.2 (Figure 47). It is advantageous for the pre-treatment system that both have been found to utilise a variety of carbon sources, as the BTE contains organic matter in various stages of break down (Gilmour *et al*., 2011; Tardy-Jacquenod *et al.*, 1996).

There is extensive literature on *D. desulfuricans*, including its use in the treatment of ARD and in its control in wastewater treatment (Arslan *et al.*, 2004; Liao *et a*l., 2011; Liu *et al*., 2015; Sani *et al.*, 2001). Okabe *et al.* (1995) found that cell yield reduced by half at 250 mg HS^-/ℓ at pH 7.0, as well as seeing a reduced maximum specific growth rate, whereas specific lactate utilisation rate increased with elevated HS^- concentrations. This shows that there was an increase in the relative energy required for maintenance in order to overcome HS^- inhibition and that growth has to be uncoupled from energy production at high HS^- concentrations (Okabe *et al.*, 1995). Seeing that HS^- concentrations within the raw batches were 700 mg/ℓ on average, the SRB community may utilise more COD than is theoretically required for BSR as was found in Section 7.3.5.1 (Lu *et al*., 2012).

In terms of species within the *Desulfobacterales* order, *Desulfomicrobium orale* appeared appreciably in all batches, with an overall contribution of 14.9% of the total group (Appendix D: SIMPER analysis), with its largest population in batch 3 (21% RA, Figure 47). Other *Desulfomicrobium* species found in harbour water and estuarine areas, are obligate anaerobes and incomplete lactate oxidisers (Langendijk *et al*., 2001). *Desulfobacterium autotrophicum* is a complete oxidiser and is capable of growing chemolithoautotrophically with H_2, CO_2 and SO_4^{2-}, hence its name (Strittmatter *et al*., 2009). *D. autotrophicum* was most dominant in BTE batch 1 (14.0% RA). The presence of over 250 proteins from the sensory/regulatory protein families theoretically enables *D. autotrophicum* to efficiently adapt to changing environmental conditions, making this species a good candidate for selection within the channels (Strittmatter *et al*., 2009). Also part of the metabolically versatile *Desulfobacteraceae* family, *Desulfobotulus sapovorans* (29.1% RA in batch 1) is abundant in marine sediments and predisposed to highly saline environments (Strittmatter *et al.*, 2009).

Desulfobotulus sapovorans is an incomplete organics oxidiser, using SO_4^{2-} and SO_3^{2-} as electron acceptors and is unable to use $S_2O_3^{2-}$, S^0, fumarate and NO_3^{2-} (Widdel, 1988).

Of the *Desulfobulbaceae* family, uncultured *Desulfobulbus* sp. was found in batch 3 in 33.1% RA. All species are incomplete organics oxidisers, although H_2 can be utilised as an electron donor in the presence of acetate (chemolithoheterotrophic growth) (Kuever *et al*., 2015). Most commonly, SO_4^{2-} is used as an electron acceptor and often SO_3^{2-} and $S_2O_3^{2-}$ but S^0 is never reduced by *Desulfobulbus* sp. (Kuever *et al*., 2015).

Therefore, most BTE batches were significantly populated with the genus *Desulfovibrio* (38.7-79.1% RA) with *Desulfomicrobium* having the next highest contribution overall (4.4-23.1% RA) (Table 35). Both of these genera are incomplete oxidisers, utilising lactate as an electron donor and breaking it down into acetate (Gilmour *et al.*, 2011). From Table 35, these incomplete oxidisers by far outweigh the complete oxidisers that can oxidise acetate or lactate and acetate into CO_2 (3.2-96.2 times), except for batch 1 where the complete oxidisers dominated (0.8 times) (Dworkin *et al.*, 2006).

Table 35: Relative abundances of complete and incomplete oxidising genera of the BTE batches

Genus	BTE 1 8/5	BTE 2 29/5	BTE 3 12/6	BTE 4 8/7	BTE 5 1/8
Complete oxidisers					
Desulfosarcina	0.03	0.02	0.1	0.1	0.5
Desulfobacterium	14.0	0.5	0.8	1.1	5.5
Desulfotignum	0.1	0.03	0.05	0.04	0.3
Desulfobacter	0.2	0.2	0.6	1.1	0.9
Desulfocella	10.0	0.02	0.1	1.8	0.7
Desulfonema	0.01	0.001	0.01	0.01	4.5
Desulfobotulus	29.1	0.1	0.9	0.1	7.7
Total	53.3	0.8	2.6	4.2	20.0
Incomplete oxidisers					
Desulfobulbus	0.1	0.1	33.1	3.4	1.5
Desulfomicrobium	4.4	15.1	23.1	15.5	12.6
Desulfovibrio	42.0	79.1	38.7	74.9	63.4
Total	46.5	94.2	94.9	93.8	77.5
Incomplete:Complete	0.8	96.2	15.1	17.9	3.2

Once again, as with the environmental enrichment consortia (Section 5.3.2.2), this could lead to an accumulation of acetate in the system when batches 2-5 are fed to the channels (Kuever *et al.,* 2001).

7.3.5.2 Microbial community characterisation in samples from the hybrid linear flow channel reactor

7.3.5.2.1 Relationship between microbial community structure in the feed and channels

As the BTE HLFCRs attained consistent BSR and SO performance and were able to maintain biofims, these channels were periodically sampled and the metagenomic DNA sent for NGS sequencing. Samples were taken from both the bulk liquid and FSB, with the latter to try and characterise the SO community. However, the results did not identify putative sulphide oxidisers, so the FSB data are reported, but not discussed in detail.

The 20 most abundant bacterial species were selected from the 158 identified species across all samples. Average similarity percentages (SIMPER) are given in Table 36 for each of the sample groups according to their Bray-Curtis similarity resemblance using one-way analysis and a 70% cut off for low contributions. Average dissimilarity percentages between the various sample groups are given in Table 37, based on their Bray-Curtis similarity resemblance. These similarity relationships are displayed visually in the spatial distribution of points representing the BTE batches and channel samples in the nMDS plot overlaid with cluster analyses in Figure 48.

Table 36: Average similarity percentages within entire sample groups

Group	Average similarity (%)
Environmental consortium	56.2
Batch culture	52.5
Channel 1	79.7
Channel 2	61.3
Channel 1 FSB	76.4

Table 37: Average dissimilarity percentages between different sample groups

Groups	Average dissimilarity (%)	Groups	Average dissimilarity (%)
Channel 1		Channel 2	
BTE & CH1	50.4	BTE & CH2	47.9
		BTE & CH2 inoc	39.4
		CH2 inoc & CH2	33.5
CH1 & CH2	32.9		
Channel 1 FSB		Channel 2 FSB	
BTE & CH1 FSB	47.2	BTE & CH2 FSB	53.2
CH1 & CH 1 FSB	27.4	CH2 & CH2 FSB	29.8
		CH1 & CH2 FSB	21.3
CH1 FSB & CH2 FSB	25.3		

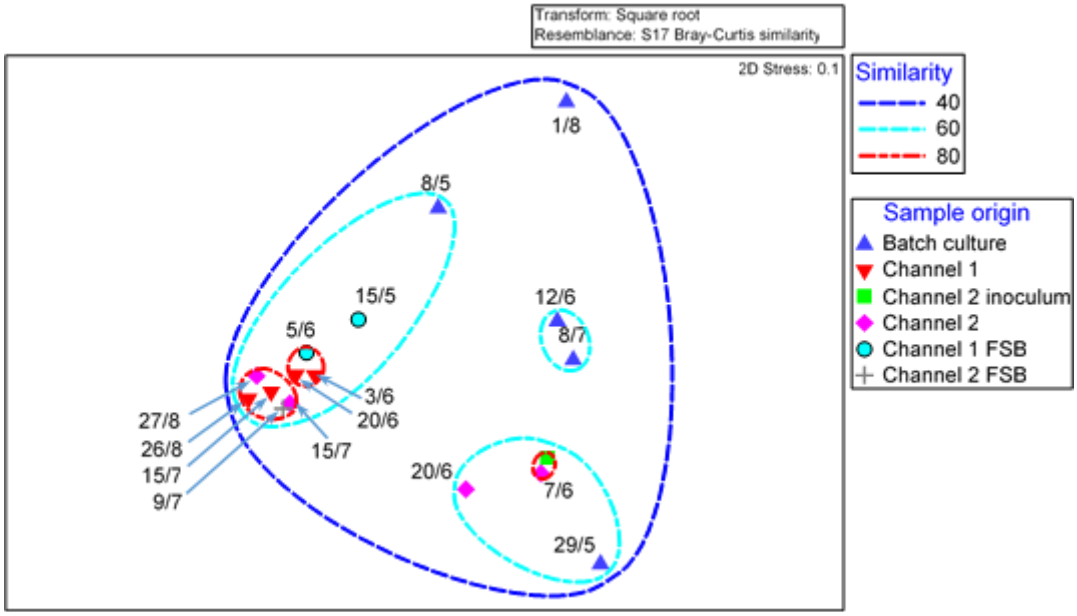

Figure 48: nMDS plot overlaid with cluster analysis of the Bray-Curtis similarity of the relative abundance between bacterial species containing dsr from BTE batch culture and both channels

Over the duration of the experiment different batches of BTE were used to make up the feed. The endogenous community within the feed had an impact, at least initially, on the microbial community structure within the channel. The was typically a 60% similarity between the feed batch community and the channel sample taken after a week or more of receiving that feed (Figure 48). This becomes less apparent in the final two samples from each channel.

The community within channel 2 displays a greater diversity and more development with time than channel 1, as channel 1 samples show near 80% similarity to one another while samples from channel 2 are only 61% similar to one another (Table 36).

Each of the channel samples taken on the same day are commonly 60-80% similar to one another (Figure 48), showing that the community of channel 2 is strongly linked to channel 1. This is not unexpected as channel 2 receives effluent from channel 1. This is emphasised by the low dissimilarity (33%) of the communities of channel 1 and channel 2 (Table 37).

Channel 1 and channel 2 are each initially in their own corner in the nMDS plot of Figure 48 and have a similarity of only 40%, considering batch 1 of 8/5 as the first channel 1 sample. The similarity between samples quickly converges into a third corner, opposite to the BTE batches, as time passes until the last two samples from each channel are within 80% similarity. This indicates that community members emerge that are best suited to the conditions within the channel and flow from channel 1 to channel 2, regardless of the enriched consortia inoculum given to channel 2.

The BTE batches are similarly dissimilar to the channel 1 group (50%) as to the channel 2 group (48%), demonstrating this convergence of the channel samples away from the BTE batches with time. A channel community emerged and remained almost 80% similar to itself after 55 days in channel 1 and 38 days in channel 2. It is likely that the stability of the community within channel 1 caused the stability of the community of channel 2.

7.3.5.2.2 Changes in community structure with time in the channel reactors

As expected, there was large diversity between BTE batches which translates through each of the channel samples. Bacterial species were selected with a relative abundance (RA) contribution of > 4.0% across all metagenomic DNA samples sequenced. The programme, PRIMER 7, was used when analysing the abundance of the species present in the selected metagenomic DNA samples. The changes in community composition, with the emergence of dominant species and the disappearance of others, within the HLFCRs is demonstrated in the shade plot (Figure 49A). The plot includes the results for the BTE batches and enrichment culture samples.

The major contributions of these species in each sample can be seen in Appendix E: Microbial community analysis graphs.

As discussed in Section 7.3.5.1.2, the BTE batches were primarily made up from contributions of four species: *Desulfovibrio* sp. enrichment culture DGGE band HCB4 (15.0, i.e. 28.6% of total), *Desulfomicrobium orale* (7.8, i.e. 14.9%), *Desulfovibrio* sp. MCM B_508 (4.5, i.e. 8.61%) and *Desulfobacterium autotrophicum* (2.9, i.e. 5.5%) (Appendix D: SIMPER analysis). However, *Desulfovibrio* sp. HCB4 appears to be selected against in the channels (decreasing significantly in approx. 2 months for channel 1 and 1 month in channel 2), with an overall contribution of 6.7% and 9.6% in channel 1 and 2, respectively.

On the other hand, *Desulfovibrio* sp. MCM B_508 was dominant in the enrichment consortia (34.2-60.5% RA; Section 5.3.2.2) and appeared in increasing RA in the BTE batches (0.5-18.8% RA) until batch 5 (1.6% RA). Both channels mirrored the RA variance of the influent, with channel 1 presenting a progressive increase (0.5-12.2% RA) until the last batch (3.7% RA) (Figure 49A).

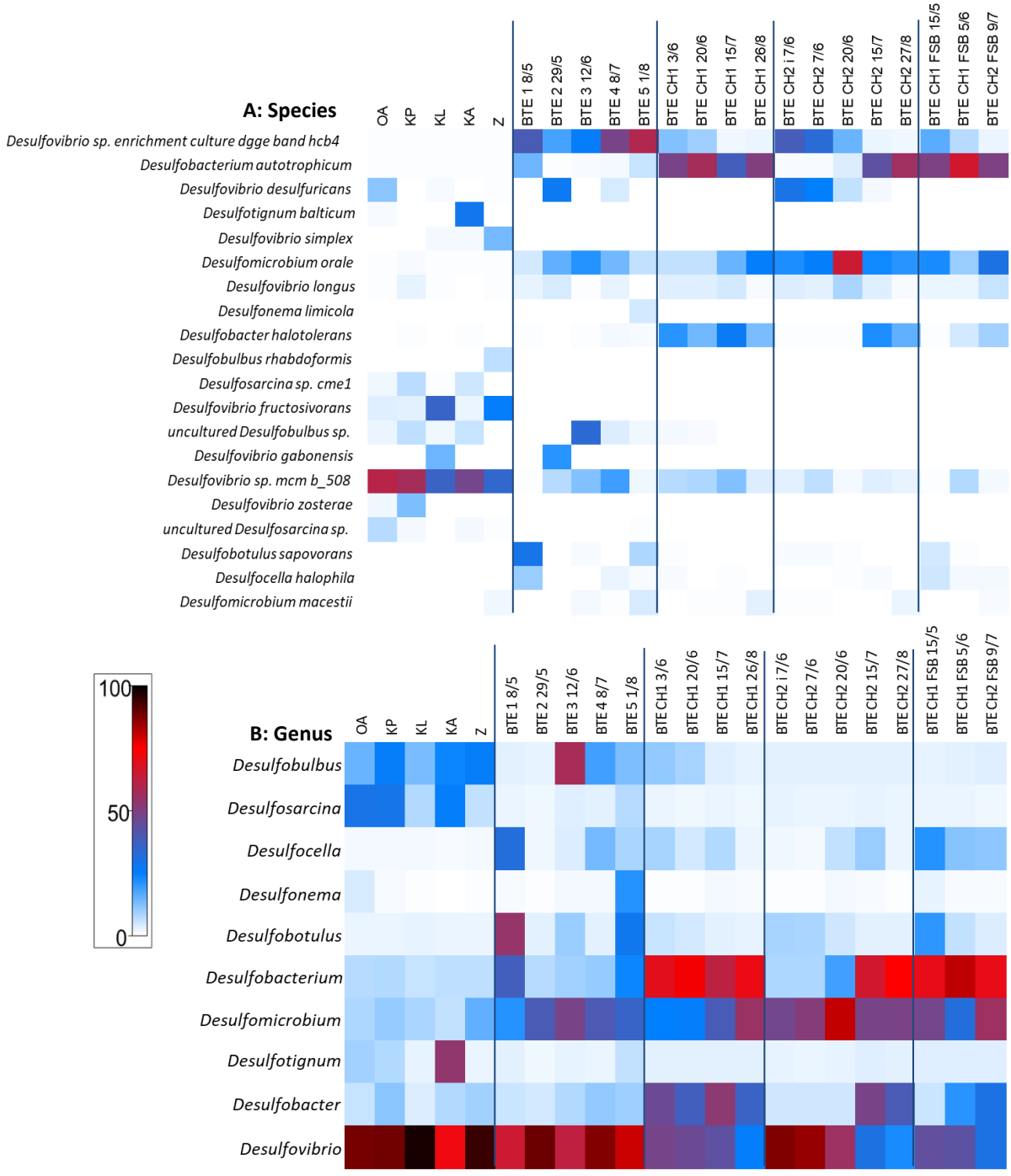

Figure 49: Amplicon sequencing results displayed as a shade plot of abundance of bacterial species containing dsrB (A) and genera (B) in the samples

In contrast, the conditions within the channel appeared to favour *Desulfomicrobium orale*. This species becomes more important with each successive sample of channel 1 (4.3-25.3% RA). This was similar for channel 2, where *D. orale* reaches a maximum in the second sample (64.6% RA), but then decreased to 20.5-23.0% RA as *Desulfobacterium autotrophicum* proliferated to in excess of 40% RA

(Figure 49A). The first channel 1 sample had *D. autotrophicum* present in 14.0% RA and a sharp rise was seen to sample 2 (47.9% RA), reaching a maximum (57.2% RA) after being fed batch 3 of the raw BTE. As no other batches had > 5.5% RA, this large increase may have been influenced by the microbial community within the partially treated BTE that was blended with the raw BTE to make up the feed, which was not characterised.

From Figure 49A, it can be seen that this species took longer to dominate in channel 2. Throughout operation, but most so towards the end, the conditions within both channels' FSB appeared to select for to *D. autotrophicum* (48.8-65.6% RA). Channel 1 reached its second highest average SRR (806 mg/ℓ.day) during HRT 23 (Figure 35) and it is postulated that the larger relative population of *D. autotrophicum* in batch 5, compared to previous batches, as well as in the channel itself contributed to this positive effect.

Therefore, two of the four dominant species of the BTE are selected out within the channels, while in the final sample of each channel *Desulfovibrio* sp. HCB4 and *Desulfovibrio* sp. MCM B_508 are both below 3.7% RA. The selection of *Desulfobacterium autotrophicum* is likely influenced by its metabolic versatility, enabling it to efficiently adapt to changing influent conditions (Strittmatter *et al*., 2009).

Otherwise, there was a relatively strong presence of *Desulfobacter halotolerans* in all channel 1 samples (12.8-26.7% RA) except the first (0.2% RA). When contrasted to the low RA of this species in the BTE batches (0.2-1.1% RA), this indicates that the partially treated BTE, whose community was not characterised, may have had a higher portion of *D. halotolerans* in each batch.

Channel 1 had a contribution of 15.8% of the total group, the second highest contribution, while channel 2 had only a 5.8% contribution of the total (Appendix D: SIMPER analysis), again seeing a delay in its proliferation with a maximum of 22.0% RA in the sample following the feeding of batch 4 (Figure 49A).

Desulfovibrio longus, while in relatively small proportions, saw increasing RA in channel 1 (2.2-4.2%) until the last sample and in channel 2 (3.5-8.2%), falling off from the third sample. *Desulfovibrio desulfuricans* was present in batch 2 (27.0% RA) and therefore, was present in the inoculum (29.3% RA) and initial sample of channel 2 (24.9% RA). However, populations of *D. desulfuricans* had fallen to only 1% RA by the second last sample.

7.3.5.2.3 Characteristics of dominant species within the channel reactors

Channel 1 was initially rich in *Desulfovibrio* sp. HCB4 and *Desulfobotulus sapovorans.* While, the inoculum of channel 2 and its first sample had *Desulfovibrio* sp. HCB4 and *Desulfovibrio desulfuricans* in large proportions. All these species were selected against as the experiment progressed and both channels selected for *Desulfobacterium autotrophicum*, *Desulfomicrobium orale* and *Desulfobacter halotolerans* (Figure 49A).

Desulfovibrio longus was also present, albeit in smaller amounts. It appeared in increasing abundance in each channel and FSB (2.2-8.2% RA), while the highest abundance in the influent was 4.0% RA in batch 2. After the fourth sample, abundances within the channel began to reflect that of the influent. *D. longus* is an incomplete oxidiser, utilising $S_2O_3^{2-}$, SO_3^{2-}, S^0 and fumarate as well as SO_4^{2-} in the presence of lactate (Magot *et al*., 1992). Growth was recorded between NaCl concentrations of 0-8%, with optimum conditions of 1-2% NaCl and pH of 7.4 (Magot *et al*., 1992). Therefore, if given the

chance, it is possible *D. longus* could have proliferated, had the other dominant species not been present.

Desulfomicrobium orale has been described in literature as an oral SRB involved in human peridontal disease and therefore, is usually found in human hosts and not in wastewater endogenous microbial communities (Langendijk *et al*., 2001). It has been found to oxidise lactate and pyruvate incompletely to acetate, with pyruvate being poorly fermented (Langendijk *et al*., 2001). *D. orale* has been found to exhibit slow growth rates which have been postulated to be due to direct inhibition by HS^-, trace metal deficiency due to precipitation by HS^- and/or syntrophic growth with other bacteria (Langendijk *et al*., 2001). This could explain its slow emergence within the channels, which both commonly saw HS^- concentrations of 400-500 mg/ℓ (Figure 33A & Figure ***38***A), as its population acclimated and/or enjoyed syntrophic growth with other community members.

As discussed briefly in Section 7.3.5.1.3, *Desulfobacterium autotrophicum* is a complete organic substrate oxidiser, capable of degrading a variety of carbon sources, including long chain fatty acids, and completely oxidise acetyl-CoA, used for energy production, to CO_2 (Strittmatter *et al*., 2009). It is also able to grow chemolithoautotrophically, with H_2, CO_2 and SO_4^{2-}. This metabolic versatility, combined with its abundance of sensory/regulatory protein families, enables *D. autotrophicum* to be well-suited to adapting to its environment (Strittmatter *et al*., 2009). Therefore, its selection within the channels and FSB, where conditions are continuously changing with each batch of BTE was highly probable. Additionally, *D. autotrophicum* can utilise only $S_2O_3^{2-}$ as well as SO_4^{2-} and therefore, was not responsible for poor biofilm formation through the use of S^0 as an electron acceptor (Dworkin *et al.*, 2006).

D. halotolerans, like *D. autotrophicum*, is also of the *Desulfobacteraceae* family and is also a complete acetate oxidiser, further, it is able to utilise ethanol and pyruvate as electron donors (Brandt and Ingvorsen, 1997; Dworkin *et al*., 2006). Originally, *D. halotolerans* was enriched from hypersaline environments, able to tolerate high concentrations of salt (< 13% NaCl and < 4.5% $MgCl_2 \cdot 6H_2O$) (Brandt and Ingvorsen, 1997). This species exhibited the highest NaCl tolerance reported for members of the *Desulfobacter* genus, however, saw maximum growth in medium with 1-2% NaCl (Brandt and Ingvorsen, 1997). Hence, its discovery in tannery effluent with < 2% NaCl.

Overall, unlike previous characterisations performed (Sections 5.3.2.2 & 7.3.5.1), there was a significant dominance of complete oxidisers over incomplete oxidisers in both channels, in about twice the abundance, after operating for at least over two weeks (Table 38).

In terms of community structure at a family level, initially channel 1, as with the BTE batches, had large proportions of *Desulfovibrionaceae* (39-83% RA; Figure 50). However, sample 1 of channel 1 had the second lowest abundance (42% RA) and a slightly larger proportion of *Desulfobacteraceae* (53% RA). Even in the beginning, the *Desulfobacteraceae* family was emerging in channel 1.

The development of the SRB community within channel 2 demonstrates this competition between the *Desulfovibrionaceae* and the *Desulfobacteraceae* families further. The inoculum of channel 2 was heavily dominated by *Desulfovibrionaceae* (76% RA) as a result of their dominance of the enrichment consortia (51-94% RA, average 78% RA) (Figure 50). This dominance dropped only slightly for the initial channel 2 sample (72% RA), due to the high abundance in batch 2 of raw BTE (83% RA. The second channel 2 sample saw the emergence of *Desulfomicrobium* (65% RA) and the decline of

Desulfovibrionaceae. By the third sample, *Desulfomicrobium* too had declined (23% RA) and the *Desulfobacteraceae* family had been selected for (67% RA).

Table 38: Relative abundances (%) of complete and incomplete oxidising genera of SRB in the BTE-fed HLFCRs

Genus	BTE CH1 8/5	BTE CH1 3/6	BTE CH1 20/6	BTE CH1 15/7	BTE CH1 26/8	BTE CH2 i 7/6	BTE CH2 7/6	BTE CH2 20/6	BTE CH2 15/7	BTE CH2 27/8
Complete oxidisers										
Desulfosarcina	0.03	0.03	0.03	0.04	0.03	0.05	0.04	0.04	0.05	0.04
Desulfobacterium	14.0	47.9	57.2	38.0	49.9	0.60	0.62	3.35	42.9	55.8
Desulfotignum	0.1	0.08	0.07	0.08	0.08	0.06	0.04	0.05	0.09	0.08
Desulfobacter	0.2	20.8	14.0	27.4	13.0	0.21	0.24	0.22	23.2	15.4
Desulfocella	10.0	0.74	0.19	0.57	0.04	0.02	0.01	0.33	0.94	0.04
Desulfonema	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00
Desulfobotulus	29.1	0.30	0.17	0.09	0.06	0.74	0.67	0.23	0.07	0.09
Total	53.3	69.9	71.7	66.2	63.1	1.69	1.62	4.23	67.3	71.4
Incomplete oxidisers										
Desulfobulbus	0.1	1.1	0.8	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Desulfomicrobium	4.4	6.0	6.5	15.0	30.3	22.1	25.8	64.8	23.2	23.3
Desulfovibrio	42.0	22.8	20.9	18.4	6.4	75.9	72.2	30.7	9.3	5.0
Total	0.9	30.0	28.1	33.4	36.8	98.1	98.1	95.6	32.6	28.4
Incomplete:Complete	0.8	0.43	0.39	0.50	0.58	58.2	60.4	22.6	0.48	0.40

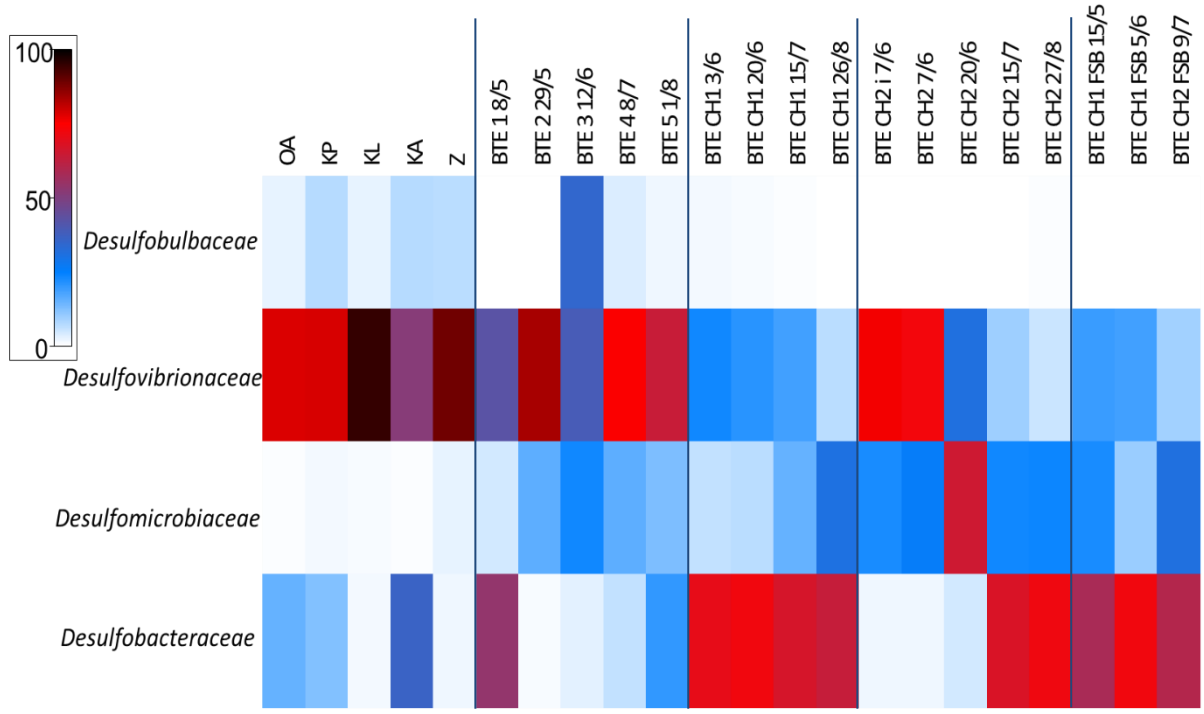

Figure 50: Taxonomic family classification of the community containing dsrB present in all samples

In summary, species *Desulfobacterium autotrophicum* and *Desulfomicrobium orale* emerged as best suited to the conditions within the HLFCRs treating BTE and are worthy of further investigation.

7.3.6 *Anaerobic digestion tests on bovine/ovine tannery effluent pre-treated in hybrid linear flow channel reactors*

Anaerobic digestion (AD) tests were once again carried out in 1 ℓ reactors, this time using BTE that had passed through the channels and had therefore, undergone pre-treatment. Three experimental reactors, using undiluted pre-treated BTE, pre-treated BTE diluted 1:1 with tap water and diluted pre-treated BTE, supplemented with acetate, were set up and compared to two positive controls, one containing water and acetate and the second using acetate-supplemented artificial seawater. The setup and monitoring were similar to the preliminary tests (Section 7.3.2) with CH_4 generation as the performance indicator.

In Figure 51B, the positive acetate control under saline conditions (average 26.3 mS/cm) demonstrated the inhibition of salinity on methanogenic activity, indicating that the AD sludge community requires acclimation to higher salinities as well as to HS^-. The preliminary results on undiluted BTE that was treated in the HLFCR system ("treated BTE" in Figure 51) (Section 7.3.4.2) exhibited the lowest overall specific CH_4 generation (38.4 mℓ/g COD; Table 39) with an average EC of 28.1 mS/cm, as expected for the treatment of effluent made inhibitory by its salinity as well as other inhibitory compounds beyond just HS^-.

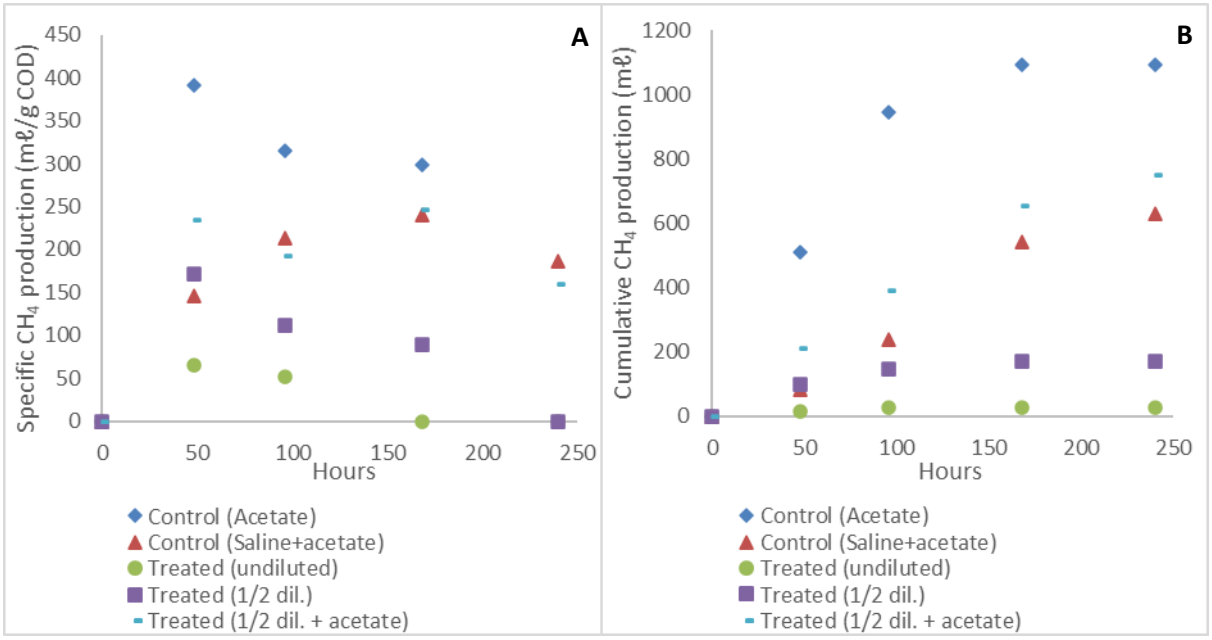

Figure 51: Specific (A) and cumulative (B) methane production in preliminary anaerobic digestion studies with BTE treated in HLFCR system

Only when the treated BTE was diluted by half and supplemented with acetate was the AD reactor able to achieve a specific CH_4 generation of 214 mℓ/g COD (Table 39) at an average EC of 18.1 mS/cm, essentially equal to the generation the control grown on acetate under saline conditions (EC 26.3 mS/cm) was able to achieve. When compared to the results obtained with raw and partially treated BTE (Table 27), this suggests that the pre-treatment shows definite potential, especially if the AD culture can be slowly acclimatised.

Table 39: Overall specific methane generation and portion of biogas present as methane of anaerobic digestion reactors on BTE after pre-treatment

	Control Acetate$_2$	**Control Saline + Acetate**	**Treated BTE** (undiluted)	**Treated BTE** (½ dilution)	**Treated BTE** (½ dilution + acetate)
Specific CH_4 gen. (mℓ/g $COD_{consumed}$)	342	208	38.4	130	214
Biogas % CH_4 (%)	69.7	42.45	13.7	32.5	46.8

The portion of the biogas that was present as CH_4 (Table 39) was similarly low (14% CH_4) in the undiluted pre-treated BTE AD reactor and finally exceeded the positive saline control (42%) when the pre-treated BTE was diluted and supplemented with acetate (47%). The excess was likely due to the lower salinity of the diluted pre-treated BTE than the positive saline control.

While the true measure of a HLFCR is the recovery of S^0 to the biofilm, in this study, increasing the amenability of the tannery effluent to AD was the aim of the research. These preliminary results indicate that the BTE was made more amenable to AD through pre-treatment in HLFCRs. With acclimation of the AD sludge to salinity and trace nutrient deficiencies, and optimisation of the pre-treatment, this pre-treatment could perform even better.

7.3.7 *Summary of findings*

Five batches of raw and partially treated BTE were obtained from an active tanning operation in South Africa. On average, each of the effluent types were found to contain 28.2 g/ℓ and 4.9 g/ℓ of COD and had a conductivity of 32.0 mS/cm and 31.5 mS/cm, respectively. Concentrations of HS^- were, on average, 699 mg/ℓ and 83 mg/ℓ, with corresponding SO_4^{2-} concentrations of 1951 mg/ℓ and 3687 mg/ℓ, respectively.

Batch AD tests, using an inoculum that was not adapted to saline or high sulphide conditions, showed that the effluent was not a suitable substrate, with no gas production recorded.

A qPCR analysis using primers specific for the *dsr*B gene showed that the effluent contained microbes that possessed the gene, which were most likely SRB. This was confirmed by subsequent sequence analysis. In contrast to the HLFCRs operated using OTE as a substrate, the channels grown on BTE showed that both the endogenous community (channel 1) and the hybrid community, with endogenous and enriched consortia (channel 2), were capable of BSR and SO and supported the formation and maintenance of a FSB.

Channel 1 was able to reach 99% SO_4^{2-} reduction during 21 days of batch operation and channel 2 attained 80% SR during its batch period and the reactors in series, a maximum SR of 96.6% in the bulk liquid during continuous operation at a 4-day HRT. The two reactors in series reached a maximum reduction of influent SO_4^{2-} of 81%, with an average SR of 47% SR. The average feed SO_4^{2-} concentration was 2408 mg/ℓ.

Both channels were consistently capable of complete HS^- removal, with negligible concentrations measured in the effluent under normal operating conditions. However, the reactor design and setup require optimisation to prevent blockages and biofilm wash out events, which caused a build-up of

volume in the reactor and the sudden expulsions of large amounts of bulk liquid, resulting in elevated HS^- concentrations in the effluent.

The HLFCR feed composition varied with each batch of BTE treated. The fact that successful BSR and SO were maintained, demonstrated that the microbial communities were able to withstand rapid changes in feed composition. This would be the requirement for treating real-world tannery effluent.

The presence of a complete FSB resulted in substantial odour reduction and the channels could be operated, uncovered, on the laboratory bench. Furthermore, small breaks in the FSB did not have adverse effects on BSR performance. The harvested biofilm samples had a high sulphur content, with average S^0 and C values for channel 1 of 47.8% and 9.4%, respectively, and 69.3% S^0 and 6% C for channel 2. Once the FSB was harvested, it did not reform rapidly under normal conditions. Seeding the surface with saline-acclimated biofilm improved the rate and completeness of FSB formation. This indicates that background SOB concentration is not high enough after a full harvest.

A sulphur mass balance across the system showed that of the HS^- and SO_4^{2-} measured in the bulk liquid, a substantial portion (31-46%) of the sulphur species in the effluent could not be accounted for and was most likely present as washed out FSB (identified in most effluent samples), colloidal S^0 (identified in Figure 44), and small amounts of polysulphides and other soluble intermediates. Finally, from the analysis of the biofilm samples, it was found that 6.9% and 10.2% of sulphur species were recovered as S^0 in the biofilm for channel 1 and 2, respectively.

The majority of the soluble COD reduction took place in the first channel (29%) and almost half the amount in the second channel (15%). This correlates with the majority of the BSR taking place in the first reactor. The effluent had an average soluble COD concentration of 5.3 g/ℓ, so more than half of the soluble COD remained available for downstream AD. Channel 1 utilised three times more COD than theoretically required for BSR, while overall the reactors in series utilised 2.3 times more COD than the theoretical. Some COD would have been utilised by the heterotrophs involved in FSB formation, but it is likely that the endogenous BTE community contains other, non-SRB, anaerobic organisms that were not as prevalent in the enrichment cultures.

The batches of BTE contained a large and diverse endogenous microbial community. In terms of SRB, the community structure differed between batched. However, the greatest contribution to the group came from *Desulfovibrio* sp. HCB4 (18-59% RA, 28.6% of total). Batch 2 and 3 also had large populations of *Desulfovibrio desulfuricans* (27% RA) and uncultured *Desulfobulbus* sp. (33% RA). The second greatest contributor across all batches was *Desulfomicrobium orale* (4.3-21% RA, 14.9% of total). Only batch 1 had a slightly higher proportion of complete oxidisers (53% RA), while the other batches were significantly dominated by incomplete oxidisers (78-95% RA).

During continuous operation, there was a clear evolution in terms of SRB community structure in the two channels, although it was influenced by the inoculum. Channel 1 saw fairly rapid selection of *Desulfobacterium autotrophicum* as a dominant SRB, rising from 14% RA to 57% RA within 6 weeks. *Desulfomicrobium orale* was also selected for over time, with 14% RA after 8 weeks, increasing to 25% RA after 15 weeks. Channel 2 saw populations of *Desulfobacterium autotrophicum* proliferate after 5 weeks (43% RA), despite an RA of only 0.6% in the inoculum. This demonstrated the carry-over of the community from channel 1 to channel 2. *Desulfovibrio desulfuricans* initially abundant (26% RA), but was selected out in under 2 weeks, as was *Desulfovibrio* sp. HCB4. Most members of the

enrichment consortia, inoculated into channel 2, were quickly selected against, as were the most prominent members of the BTE community, with species initially presented in smaller numbers selected for, due to their suitability to the conditions. Both channels were dominated by the *Desulfobacteraceae* family at the end of the run, with smaller amounts of the *Desulfomicrobiaceae* family. Both channels started off with appreciable amounts of the *Desulfovibrionaceae* family, which were largely eliminated after 5-8 weeks.

In summary, the reactor system was run continuously for over 100 days and achieved relatively consistent BSR and effective removal of practically all HS^-, except at times where technical challenges affected stable operation. Therefore, this research demonstrated the technical proof of concept for the process, using the endogenous and enriched SRB/SOB community to reduce SO_4^{2-} under saline conditions.

Biofilm formation was the performance-limiting step and could be due to poor biofilm scaffold formation, also limiting S^0 recovery. The removal of SO_4^{2-} was incomplete and could be further optimised in future investigations, where BSR and SO kinetics are tested under stable operating conditions.

The residual SO_4^{2-} could negatively impact the potential of the pre-treated BTE as a substrate for AD, although preliminary AD studies did show that the pre-treated BTE was made more amenable to AD. More research, both optimising the HLFCR system and developing a saline-tolerant AD community, is required to improve this further.

8 Conclusions and Recommendations

8.1 Conclusions

Based on the data presented in the preceding chapters, a number of conclusions can be drawn from this research.

Six consortia capable of biological sulphate reduction (BSR) were isolated from natural marine environments and enriched in batch anaerobic reactors using artificial seawater as the base medium. Lactate proved to be the most effective carbon source. Activity levels varied, with some enrichment cultures achieving sulphate reduction rates (SRRs) of 550 mg/ℓ.day in batch reactors and near complete SO_4^{2-} reduction, from initial SO_4^{2-} concentrations of around 2100 mg/ℓ. Sulphide concentrations of up to 664 mg/ℓ were achieved.

The SRB fraction of the microbial communities were characterised using molecular biology techniques, using the dissimilatory sulphite reductase (*dsr*B) gene. The enrichment consortia were dominated by an uncultured *Desulfovibrio* species (MCM B_508), at a 34.2-60.5% relative abundance (RA) across the different cultures and *Desulfovibrio fructosivorans* (1.8-35.5% RA).

The most active enrichment cultures were used to inoculate hybrid linear flow channel reactors (HLFCRs) fed with SO_4^{2-} enriched artificial seawater and lactate as the carbon source. A maximum SRR of 634 mg/ℓ.day was achieved, with average SRRs of 83 mg/ℓ.day. Sulphide concentrations of up to 568 mg/ℓ were recorded, with a 92% sulphate reduction efficiency when operated under stable conditions. Floating sulphur biofilm (FSB) formation was found to be the limiting step, and incomplete biofilm formation reduced overall performance. However, when operating continuously, effluent HS^- concentrations were below 76 mg/ℓ. Therefore, robust consortia, capable of BSR and sulphide oxidation (SO) under saline conditions in the HLFCR were successfully developed.

Seven batches of OTE and five batches of raw and partially treated bovine/ovine tannery effluent (BTE) were obtained from active tanning operations in South Africa and characterised. On average, the OTE had an electrical conductivity of 7.5 mS/cm and a total COD of 8.6 g/ℓ COD. The sulphate and sulphide concentrations were relatively low, with mean values of 646 and 2.1 mg/ℓ respectively.

The characterisation of the OTE indicated that it would not require pre-treatment, based on the low SO_4^{2-} and HS^- concentrations. The average COD:SO_4^{2-} ratio was 18.5, making it more suitable for AD. Limited BSR took place in the hybrid LFCR and biofilm formation was largely unsuccessful due to the low HS^- concentrations. A parallel study showed that untreated OTE was suitable as a substrate for AD, using a suitably adapted culture.

Several batches of raw and partially treated BTE were received and characterised. The BTE was significantly more saline than the OTE, with mean EC values of 32.0 mS/cm and 31.5 mS/cm for the raw and partially treated effluents, respectively. Average COD values were 28.2 g/ℓ and 4.9 g/ℓ of COD. The raw effluent batches had particularly high HS^- concentrations (average of 699 mg/ℓ) and relatively high SO_4^{2-} concentrations (1951 mg/ℓ), while the partially treated BTE had lower HS^- (83 mg/ℓ), but higher SO_4^{2-} concentrations (3687 mg/ℓ).

Batch anaerobic digestion (AD) tests, using a non-adapted inoculum, on the raw and partially treated BTE showed no biogas production, confirming the need for pre-treatment.

The BTE was shown to contain an active sulphate reducing community, with all batches tested dominated by an uncultured *Desulfovibrio* sp. (enrichment culture DGGE band HCB4) at 18.1-59.3% RA and *Desulfomicrobium orale* (4.3-21.1% RA). There was substantial diversity among other minor contributors between batches.

The HLFCRs treating BTE demonstrated that both the endogenous community (channel 1) and the hybrid community, with endogenous and enriched consortia (channel 2), were capable of BSR and SO and were able to maintain a FSB. Channel 1 was able to reach 99% reduction of SO_4^{2-} over 21 days of batch operation and channel 2 attained 80% SR during its batch period. Together, when operating continuously in series at a 4-day HRT, the reactors achieved a maximum SR of 96.6% in the bulk liquid and reached a maximum of 81% reduction of influent SO_4^{2-}.

The reactors in series achieved an average SRR of 170 mg/ℓ.day and a maximum of 444 mg/ℓ.day. The SRR improved during continuous operation due to the near first order relationship of BSR to SO_4^{2-} concentration.

Both channels were consistently capable of near-complete HS^- removal during stable operation. The effluent from channel 1 had an average HS^- concentration of 8.4 mg/ℓ when operated alone, while the average HS^- concentration in the effluent from channel 2 was 9.7 mg/ℓ when the reactors were operated in series.

The composition of the reactor feed affected performance. There was no effective way of preserving feed integrity over time and there was generally decreasing performance trend in terms of both SRRs and SORs towards the end of each BTE batch used in the feed.

The rate of biofilm formation and subsequent integrity had a major impact on system performance. The rate of formation was slower than has been reported on defined growth media and once the FSB was harvested, it was necessary to seed the reactor with saline-acclimated FSB to improve FSB formation rate. Therefore, it was found that FSB formation was the performance-limiting process.

Analysis of the harvested FSB showed that the biofilm from channel 1 consisted of an average of 47.8% S^0 and 9.4% C and the biofilm of channel 2 of 69.3% S^0 and 6% C. A recovery of 6.9% and 10.2% of sulphur species as S^0 to the biofilm occurred on average for channel 1 and 2, respectively. It was found that blockages and biofilm harvesting had a large effect on the performance of the reactors.

The majority of the COD reduction (29%) took place in the first channel and almost half the amount (15%) in the second channel. Therefore, the majority of COD present in the feed would still be available for downstream AD.

Samples taken from the channels as the experiment progressed showed changes in the composition of the SRB community over time. Channel 1 was initially dominated by the uncultured *Desulfovibrio.* sp. HCB4 and *Desulfobotulus sapovorans* (39.0% and 29.1% RA, respectively), while in channel 2, the inoculum was dominated by *Desulfovibrio* sp. HCB4 and *Desulfovibrio desulfuricans* (38.2% and 29.3% RA, respectively). The subsequent sample from channel 2 still saw *Desulfovibrio* sp. HCB4 dominating (32.9% RA), but the emergence of *Desulfomicrobium orale* in almost equal abundance to *D. desulfuricans* (25.5% and 24.9% RA, respectively). However, by the end of the operational period, all of these species, except *D. orale*, had decreased to less than 2% RA. Instead, *Desulfobacterium autotrophicum* and *D. orale* were selected for both channels (49.9% and 25.3% RA for channel 1, and

55.8% and 20.5% RA in channel 2, respectively). The community displayed stabilisation with time and the results indicate that the unique conditions within the HLFCR treating BTE favoured *D. autotrophicum*.

Preliminary batch studies on the AD of the pre-treated BTE showed it to be more amenable to digestion than the raw effluent, with some biogas and methane formation observed. The AD culture used in the tests was not adapted to high salinity, so the diluted pre-treated BTE performed better.

Therefore, this pre-treatment system showed potential for tannery effluent treatment, provided that sulphur species concentrations are high enough to warrant it and that the composition of the tannery effluent used has similar properties to the bovine/ovine tannery effluent used in this study. The generation of biogas using treated BTE supports the hypothesis that pre-treatment to reduce sulphur species can make the effluent more amenable to downstream AD.

8.2 Recommendations

As a result of this research, several recommendations can be made for future studies to build on the foundation of this work.

8.2.1 *Design and operation of the hybrid linear flow channel reactor*

- Quantification of polysulphides and colloidal S^0 is recommended for future studies to get a clear picture of speciation happening within the system such that AD amenability can be better guaranteed and S^0 deposition to the biofilm optimised.
- Investigation into the prevention of blockages of the effluent port should be undertaken, for example, by using a larger weir pipe adapter.
- Precipitation of essential metal ions by HS^- can lead to a decrease in metabolic activity. Therefore, the effect of the addition of trace elements on activity should be investigated.
- Reactor activity may have been inhibited by by-products of substrate breakdown, so a more detailed quantification of the organic fraction is suggested during experimental runs.

8.2.2 *Treatment of sulphur species under saline conditions in hybrid linear flow channel reactors grown on lactate*

- The feed should contain HS^- to improve reaction kinetics to most effectively test the efficacy of BSR and SO. Thereby, biofilm formation will not be dependent on BSR activity.
- For further exploration of saline treatment environments, tap water should be used throughout when making up artificial seawater, for nutrient augmentation.

8.2.3 *Optimisation of the pre-treatment of tannery effluent with hybrid linear flow channel reactors*

Optimisation of the pre-treatment system is the best next step for sulphur species treatment of tannery effluent with this reactor.

- A detailed kinetic study should be performed to try and optimise performance of the reactor.
- Raw BTE had initially high HS^- concentrations which were advantageous for the pre-treatment system, however, this BTE was generally high in sludge. Investigation into the supplementation of hydrolysing and fermentative bacteria to the consortia to break down

complex long chain organics to produce more soluble COD is suggested. This would help with blockages and avoiding organic matter waste.

- The performance of each reactor was dependent on feed composition and so, each component's effect should be investigated and quantified. This could be due to how active the community in the batch was which led to feed composition changes and often resulted in decreasing feed quality. Reactor feed and postulated effects would need to be monitored in a real tannery. For example, assess whether refrigeration of several HRTs worth of blended effluent allows for a consistent feed composition and whether the endogenous microbial community is able to recover.
- Ongoing adaptation of enrichment consortia to the tannery effluent for more efficient BSR should be evaluated.
- An extension of the molecular work to focus on the 16S is suggested, rather than *dsr*B gene specifically to get a sense of the non-SRB portion of the community and its possible major functions.

Floating sulphur biofilm formation was found to be the rate-limiting process, therefore, it is suggested that a number of areas can be focused on in future studies:

- Investigation into sourcing a more halotolerant SO community or assessing whether the existing SOB culture can be systematically acclimated to make it more effective.
- The optimisation of these SOB species could also be evaluated in an aerated reactor, not as a biofilm but as a planktonic community, to see how quickly they can perform SO relative to low salinity communities.
- Specific studies on biofilm structural integrity which could be performed, including:
    - As efficient SO was seen, it is likely the heterotrophic organisms responsible for making the organic fraction of the biofilm that gives it its structural integrity were slow. Therefore, the configuration should be tested with acetate supplementation to assess whether stimulated scaffold formation increases biofilm formation rate and S^0 recovery, as Mooruth (2013) found.
    - It is suggested that a more detailed characterisation of the soluble organic fraction within the reactors be performed, this time with an emphasis on ascertaining what proportion of it are VFAs, which are used to make the scaffold, versus the longer chain fatty acids or proteins, etc.

9 References

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10 Appendices

10.1 Appendix A: Analytical methods

Sulphide assay: N,N-dimethyl-p-phenylenediamine (DMPD) hydrochloride solution method (APHA, 2012)

200 µℓ 10 g/ℓ Zinc acetate
20 µℓ sample
4780 µℓ water
500 µℓ DMPD reagent
500 µℓ $FeCl_3$ reagent

500 mℓ DMPD reagent prepared with:
2 g DMPD
287.5 mℓ 32% HCl
212.5 mℓ H_2O

500 mℓ $FeCl_3$ reagent prepared with:
8 g $FeCl_3$
287.5 mℓ 32% HCl
212.5 mℓ H_2O

Sulphate assay: Barium chloride technique (APHA, 2012)

4900 µℓ water
100 µℓ sample
500 µℓ conditioning reagent
10 µℓ saturated $BaCl_2$ solution (0.3 g $BaCl_2$ in 1 mℓ water, vortexed, prepared fresh every time)

500 mℓ conditioning reagent prepared with:
75 g NaCl
30 mℓ 32% HCl
50 mℓ glycerol
100 mℓ 70% ethanol
300 mℓ H_2O
The sulphate assay is more of an indication of the sulphate concentration as opposed to an exact measurement, as it measures the absorbance of the amount of white precipitate at a wavelength of 420 nm. The solution may not be well mixed at the point of measurement, even with vortexing immediately before reading, as settling begins immediately. However, it is assumed that the concentration calculated from this assay is accurate for calculation purposes.

Ammonia assay: Merck reagent Spectroquant® test kit, low and high concentration ranges (Table 40)

Low concentration range:
5 mℓ sample
400 µℓ reagent NH_4-1
1 blue microspoon (given) reagent NH_4-2
4 drops NH_4-3

High concentration range:
5 mℓ reagent NH_4-1

100 µℓ sample (or 200 µL for 2-75 mg/ℓ NH_4-N)
1 blue microspoon (given) reagent NH_4-2

Chemical oxygen demand: Merck reagent test technique, low and high concentration ranges (Table 40)

Low concentration range:
150 µℓ low concentration COD reagent A
1.15 mℓ low concentration COD reagent B
1.5 mℓ sample

High concentration range:
2.2 mℓ COD reagent A
1.8 mℓ COD reagent B
1 mℓ sample

Table 40: Details for assays performed for ammonia and COD

Parameter	Concentration range	Reagent	Catalogue number	Wavelength
Ammonia	0.010-3.00 mg/ℓ NH_4-N	Kit	1.14752.0001	690
	5-150 mg/ℓ NH_4-N	Kit	1.00683.0001	690
COD	100-1500 mg/ℓ COD	A	1.14538.0065	610
		B	1.14680.0495	
	500-10000 mg/ℓ COD	A	1.14679.0495	610
		B	1.14680.0495	

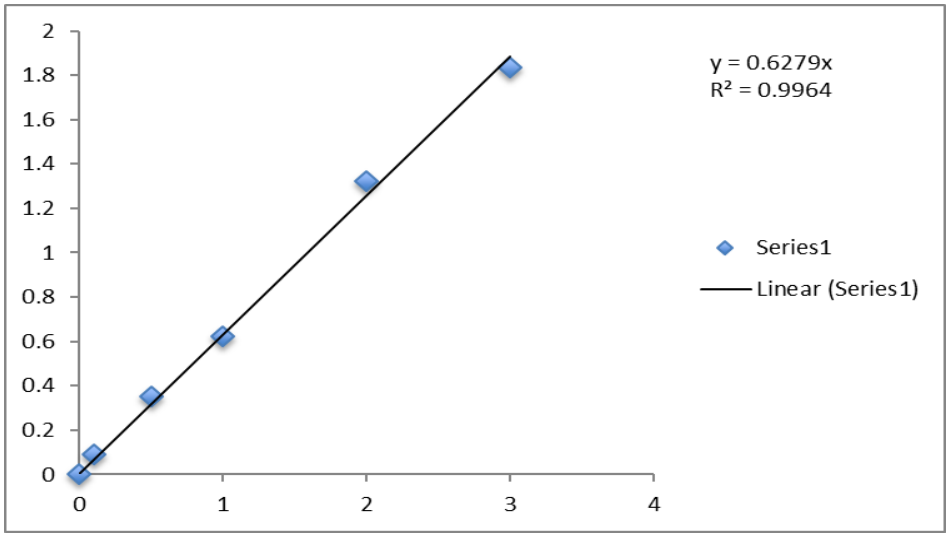
DNA Extraction: Qiagen PowerLyzer Ultraclean Microbial DNA isolation kit (Cat No. 12224-50) and Qiagen PowerLyzer PowerSoil DNA isolation kit (Cat No. 12855-50)

10.2 Appendix B: Standard curves

Ammonium standard curve: Low concentration

- Prepare 1 g/ℓ NH_4 solution by dissolving 1.485 g NH_4Cl in 500 mℓ deionised water
- Dilute to 50 mg/ℓ by adding 100 µℓ stock solution to 1900 µℓ deionised water
- Prepare standard curve from 0.1-3 mg/ℓ NH_4

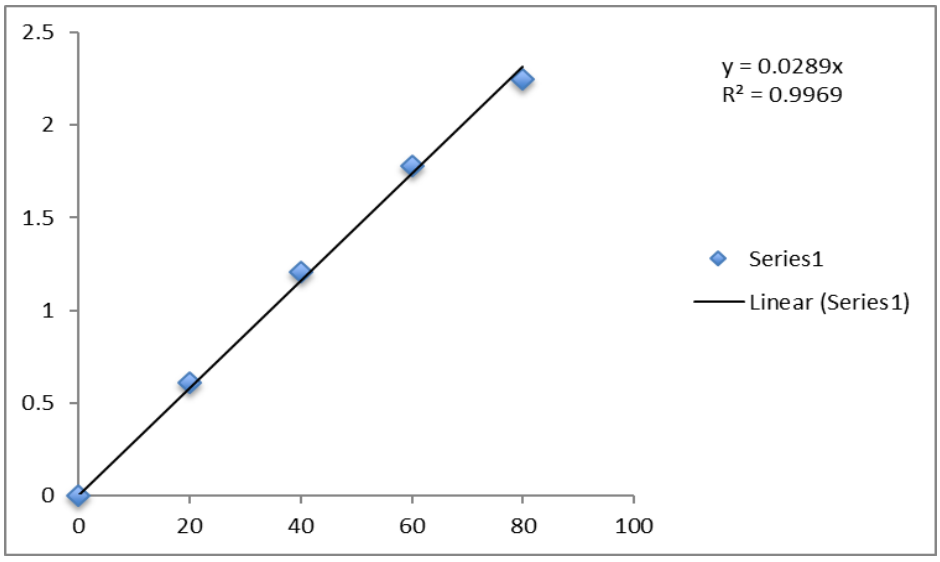
Concentration (mg/ℓ)	$V_{50\ ppm}$	V_{ddH2O}	A_{690}
0	0	5000	0
0.1	10	4990	0.093
0.5	50	4950	0.352
1	100	4900	0.624
2	200	4800	1.323
3	300	4700	1.833



Ammonium standard curve: High concentration

- Prepare standard curve from 0-80 mg/ℓ NH_4

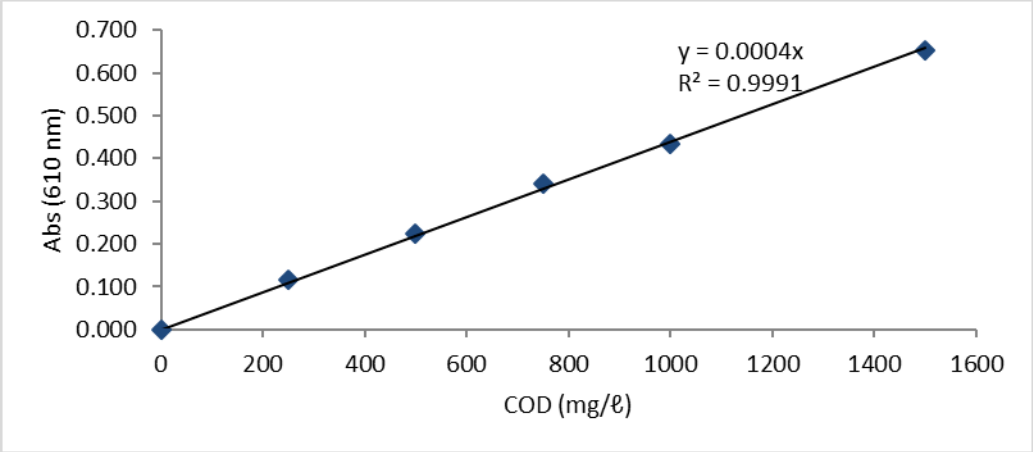
Concentration (mg/ℓ)	$V_{1\ g/l}$	V_{ddH2O}	A_{690}
0	0	2000	0
20	40	1960	0.61
40	80	1920	1.208
60	120	1880	1.78
80	160	1840	2.248



Chemical oxygen demand standard curve: Low concentration

- Autozero on blank (0 mg/ℓ COD)
- Prepare a standard curve from 0-1500 mg/ℓ COD

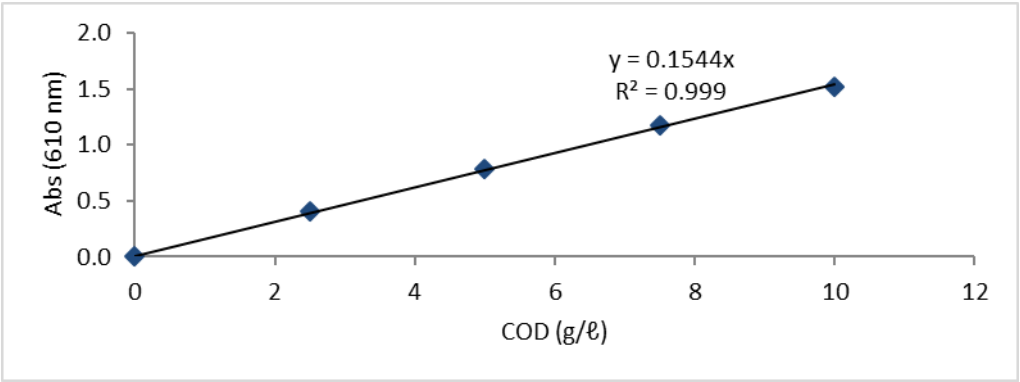
Concentration (mg/ℓ)	A_{610}	A_{610}	Mean
0	0	0	0.000
250	0.117	0.117	0.117
500	0.228	0.220	0.224
750	0.337	0.342	0.340
1000	0.437	0.428	0.433
1500	0.637	0.668	0.653



Chemical oxygen demand standard curve: High concentration

- Prepare a standard curve from 0-10 g/ℓ COD

Concentration (mg/ℓ)	A_{610}	A_{610}	Mean
0	0	0	0.000
2.5	0.400	0.420	0.410
5	0.778	0.793	0.786
7.5	1.171	1.170	1.171
10	1.521	1.522	1.522



Sulphate standard curve:

- Make up a 10 g/ℓ SO_4^{2-} stock solution

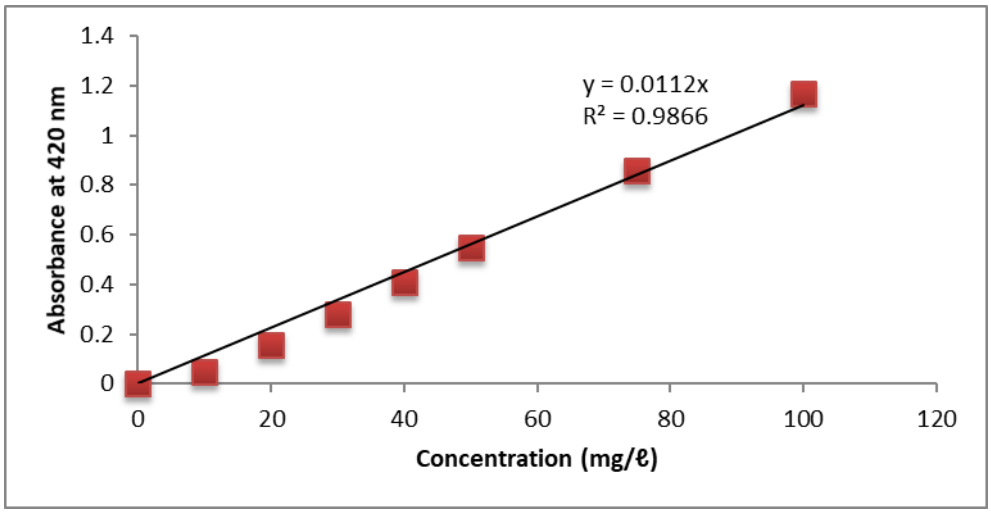
$$\frac{10\ g/l}{96.06\ g/mol} = 0.104\ M$$

- To make up 500 mℓ of 0.104 M anhydrous sodium sulphate requires

$$0.104\ mol/l \times 142.02 g/mol \div 2 = 7.39\ g\ salt$$

- Prepare a standard curve for 0-100 mg/ℓ SO_4^{2-}

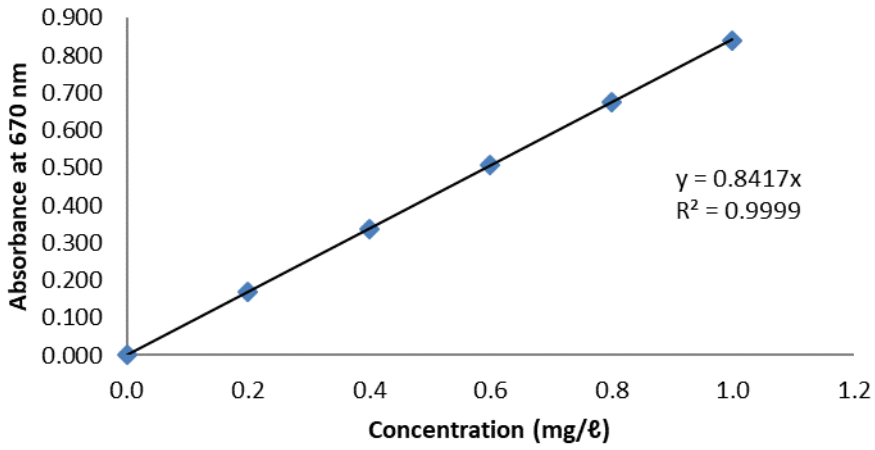
Concentration (mg/ℓ)	$V_{stock\ soln}$	V_{ddH2O}	A_{420}
0	0	5000	0.000
10	50	4990	0.050
20	100	4980	0.156
30	150	4850	0.279
40	200	4800	0.411
50	250	4750	0.547
75	375	4625	0.857
100	500	4500	1.166



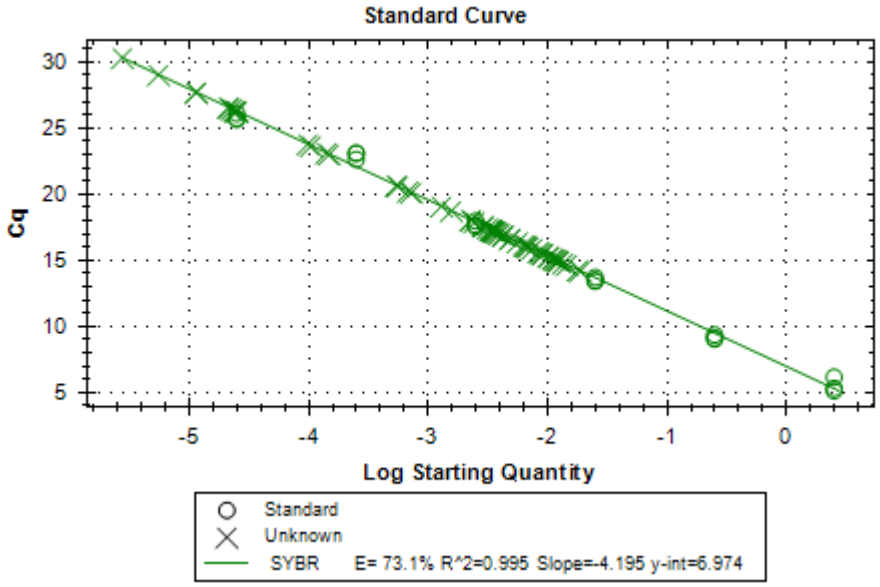
Sulphide standard curve:

- Prepare a 0.1 g/ℓ HS^- stock solution
- Prepare standard curve for 0-1 mg/ℓ HS^-

Concentration (mg/ℓ)	$V_{stock\ soln}$	V_{ddH2O}	A_{670}
0	0	5000	0.000
0.2	10	4990	0.169
0.4	20	4980	0.338
0.6	30	4970	0.506
0.8	40	4960	0.676
1.0	50	4950	0.838



qPCR standard curve:



10.3 Appendix C: Sample calculations

$Carbon\ source\ solution$: $0.02\ l\ water, 2.43\ g\ Na\ lactate\ 60\%$

$$M_{Na\ lactate,\ \ carbon\ source\ soln} = 2.43\ g \times 60\% = 1.46\ g$$

$$M_{lactate,\ \ carbon\ source\ soln} = 1.46\ g \times \frac{112.06 - 22.99\ g/mol}{112.06\ g/mol} = 1.16\ g$$

$$V_{final,\ \ carbon\ source\ soln} = 0.02\ l + \frac{2.43\ g}{1.31\ g/ml} \times \frac{1\ l}{1000\ ml} = 0.0219\ l$$

$$M_{lactate,\ \ 0.26\ l\ reactor} = 1.16\ g\ \times \frac{0.0125\ l}{0.0219\ l} = 0.66\ g$$

$$C_{lactate,\ \ 0.26\ l\ reactor} = \frac{0.66\ g}{0.26\ l} = 2.55\ g/l$$

Mass of S^0 in biofilm formed during batch phase in BTE channel 1

$$\Delta C_{HS} = \frac{C_{HS,2} - C_{HS,1}}{number\ of\ days} = \frac{(39-47)\ mg/l}{3\ days} = -2.5\ mg/l.day$$

$$\Delta n_{HS} = \frac{\Delta C_{HS}}{M_{HS}} = \frac{-2.5\ mg/l.day}{33.1\ mg/mmol} = -0.07\ mmol/l.day$$

Similarly for Δn_{SO_4} for a molar mass of 96.06 g/mol $= 2.35\ mmol/l.day$

$$\Delta n_{SO_4} = \Delta n_{HS,\ theoretical} = 2.35\ mmol/l.day$$

Assuming:

$$\Delta n_{polysulphides} = 0$$

Then:

$$\Delta n_S = \Delta n_{HS,\ theoretical} - \Delta n_{HS,\ actual} = 2.35 - (-0.07) = 2.42\ mmol/l.day$$

$$\therefore for\ V_{reactor} = 2.2\ l:$$

$$\Delta m_S = \Delta n_S \times \Delta M_S \times V_{reactor} = 2.42\ mmol/l.day \times 32.07\ mg/mmol \times 2.2\ l = 171\ mg/day$$

$$\therefore \Delta m_{S,\ batch\ phase\ CH1} = \sum_{n=10}^{31} \Delta m_{S,n} = 902\ mg$$

To find the number of copies (or molecules) of the *dsrB* gene from qPCR data:

$$number\ of\ copies_{KP} = \frac{Starting\ quantity\ \times Avagadro's\ number}{length\ \times M_{dsDNA}}$$

$$= \frac{0.01236\ ng\ template\ \times 6.022 \times 10^{23}\ \frac{molecules}{mol}}{350\ base\ pairs\ of\ template \times 660\ \frac{g}{mol\ base\ pair} \times 10^9\ \frac{ng}{g}}$$

$$= 3.22\ \times 10^7\ copies$$

$$\frac{3.22\ \times 10^7\ copies}{5\ \frac{ng}{\mu l} \times 5 \mu l} = 1.29\ \times 10^6\ copies/ng\ DNA$$

Calculating SRR:

Batch reactors

$$SRR_{batch} = \frac{SO_{4,i} - SO_{4,0}}{day_0 - day_i} = \frac{1591\ \frac{mg}{l} - 1400\ \frac{mg}{l}}{3\ days}$$

Continuous reactors

Assume:

- Same amount fed each day unless specifically stated (If 4 day HRT, then 500 ml fed each day) (such as due to load shedding)

- Effluent sample is representative of that whole day (500 ml for 4 day HRT)
- When channel 1 effluent was not sampled, assume SO_2^{2-} concentration of channel 1 effluent is 150 mg/l higher than the average of the bulk liquid when fed to channel 2

$$SRR_{Channel\ 1} = \frac{V_{feed,o-i} \times [SO_{4,feed}] + V_{reactor} \times \left(\frac{[SO_{4,bulk,F,i}] + [SO_{4,bulk,B,i}]}{2} - \frac{[SO_{4,bulk,F,o}] + [SO_{4,bulk,B,o}]}{2}\right)}{day_o - day_i}$$

Calculating the SOR:

$$SOR_{Channel\ 2} = \frac{2 \times V_{effluent,daily} \left(\frac{[HS_{bulk,CH2,F}] + [HS_{bulk,CH2,B}]}{2} - [HS_{effluent}]\right) mg/l}{days}$$

10.4 Appendix D: SIMPER analysis

Similarity Percentages - species contributions

One-Way Analysis
Data worksheet
Data type: Abundance

Parameters
Resemblance: S17 Bray-Curtis similarity
Cut off for low contributions: 70.00%

Group Environmental consortium
Average similarity: 56.23

Species	Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfovibrio sp. mcm b_508	6.81	16.86	13.58	29.98	29.98
desulfovibrio fructosivorans	3.15	5.12	1.57	9.11	39.09
uncultured desulfobulbus sp.	1.6	2.86	1.73	5.09	44.19
desulfosarcina sp. cme1	1.34	1.87	1.11	3.32	47.5
uncultured desulfosarcina sp.	1.14	1.7	2.25	3.02	50.52
desulfarculus baarsii	1.04	1.68	1.32	2.99	53.51
desulfobacterium autotrophicum	0.65	1.61	16.14	2.86	56.37
desulfovibrio sp. enrichment culture dgge band hcb4	0.58	1.48	13.52	2.64	59.01
desulfomicrobium orale	0.62	1.39	11.27	2.48	61.49
desulfovibrio simplex	1.26	1.28	0.96	2.28	63.77
desulfovibrio longus	0.67	1.21	9.05	2.15	65.92
desulfovibrio desulfuricans	1.08	1.1	2.27	1.96	67.88
desulfotalea arctica	0.56	1.09	2.42	1.93	69.81
desulfobacter halotolerans	0.47	0.98	4.44	1.75	71.56

Group Batch culture
Average similarity: 52.46

Species	Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfovibrio sp. enrichment culture dgge band hcb4	6.04	14.98	5.74	28.55	28.55
desulfomicrobium orale	3.33	7.81	3.33	14.89	43.45
desulfovibrio sp. mcm b_508	2.5	4.52	1.51	8.61	52.06
desulfobacterium autotrophicum	1.74	2.87	2.15	5.46	57.52
desulfovibrio longus	1.2	2.36	1.41	4.5	62.02
desulfobotulus sapovorans	1.93	1.9	0.86	3.63	65.65
uncultured desulfobulbus sp.	1.85	1.61	0.95	3.07	68.72
desulfobacter halotolerans	0.72	1.55	3.45	2.96	71.68

Group Channel 1

Average similarity: 79.73

Species	Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	6.93	21.86	12.8	27.41	27.41
desulfobacter halotolerans	4.25	12.56	10.84	15.75	43.16
desulfomicrobium orale	3.42	8.85	4.82	11.1	54.27
desulfovibrio sp. mcm b_508	2.72	7.69	5.64	9.64	63.91
desulfovibrio sp. enrichment culture dgge band hcb4	2.29	5.31	2.26	6.66	70.57

Group Channel 2

Average similarity: 61.29

Species	Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfomicrobium orale	5.6	16.13	23.53	26.32	26.32
desulfobacterium autotrophicum	4.16	7.23	0.94	11.8	38.13
desulfovibrio sp. enrichment culture dgge band hcb4	3.05	5.87	1.67	9.57	47.7
desulfovibrio sp. mcm b_508	1.74	4.71	5.49	7.68	55.38
desulfovibrio longus	1.77	4.28	2.89	6.98	62.36
desulfobacter halotolerans	2.38	3.56	0.74	5.81	68.17
desulfovibrio desulfuricans	2.2	3.11	1.12	5.08	73.25

Group Channel 1 FSB

Average similarity: 76.35

Species	Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	7.54	24.57	SD=0!	32.18	32.18
desulfomicrobium orale	3.93	11.05	SD=0!	14.47	46.65
desulfovibrio sp. enrichment culture dgge band hcb4	3.33	9.38	SD=0!	12.28	58.93
desulfovibrio longus	1.41	4.94	SD=0!	6.46	65.39

desulfocella halophila	1.65	4.11	SD=0!	5.38	70.78

Groups Batch culture & Channel 1
Average dissimilarity = 50.42

Species	BTE Av.Abund (%)	CH1 Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	1.74	6.93	8.05	3.89	15.97	15.97
desulfovibrio sp. enrichment culture dgge band hcb4	6.04	2.29	5.75	2.49	11.41	27.38
desulfobacter halotolerans	0.72	4.25	5.5	4.42	10.91	38.29
desulfobotulus sapovorans	1.93	0.38	2.62	0.8	5.2	43.49
uncultured desulfobulbus sp.	1.85	0.6	2.45	0.8	4.85	48.34
desulfovibrio desulfuricans	1.61	0.28	2.11	0.68	4.18	52.52
desulfovibrio sp. mcm b_508	2.5	2.72	1.94	1.53	3.86	56.38
desulfomicrobium orale	3.33	3.42	1.81	1.29	3.59	59.96
desulfovibrio gabonensis	1.07	0.15	1.48	0.52	2.93	62.9
desulfocella halophila	1.16	0.56	1.43	0.88	2.83	65.73
desulfovibrio longus	1.2	1.6	1.07	1.4	2.12	67.84
desulfomicrobium macestii	0.8	0.79	1.05	1.26	2.09	69.93
desulfomicrobium baculatum	0.62	0.41	0.81	1.25	1.61	71.55

Groups Environmental consortium & Channel 2 inoculum
Average **dissimilarity** = 66.84

Species	Cons Av.Abund (%)	CH2 inoc Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfovibrio sp. enrichment culture dgge band hcb4	0.58	6.18	8.43	27.49	12.61	12.61
desulfovibrio sp. mcm b_508	6.81	1.66	7.73	6.9	11.56	24.17
desulfovibrio desulfuricans	1.08	5.41	6.53	3.27	9.76	33.93
desulfomicrobium orale	0.62	4.67	6.09	12.69	9.11	43.05
desulfovibrio fructosivorans	3.15	0.32	4.31	1.27	6.45	49.5
uncultured desulfobulbus sp.	1.6	0.25	2	1.61	2.99	52.49
desulfovibrio longus	0.67	1.87	1.83	2.58	2.73	55.23
desulfotignum balticum	1.39	0.24	1.79	0.54	2.68	57.91
desulfovibrio simplex	1.26	0.13	1.74	0.78	2.6	60.51
desulfosarcina sp. cme1	1.34	0.17	1.74	1.18	2.6	63.11
uncultured desulfosarcina sp.	1.14	0.14	1.49	1.12	2.24	65.34
desulfovibrio zosterae	1.03	0.15	1.31	0.63	1.96	67.3
desulfovibrio sp. enrichment culture dgge band ycb1	1.04	0.53	1.21	0.58	1.81	69.11
desulfovibrio gabonensis	1.01	0.25	1.17	0.49	1.75	70.86

Groups Batch culture & Channel 2 inoculum
Average **dissimilarity** = 39.40

Species	BTE Av.Abund (%)	CH2 inoc Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfovibrio desulfuricans	1.61	5.41	5.96	1.75	15.12	15.12
uncultured desulfobulbus sp.	1.85	0.25	2.56	0.7	6.49	21.61
desulfobotulus sapovorans	1.93	0.86	2.49	0.8	6.32	27.93
desulfovibrio sp. mcm b_508	2.5	1.66	2.16	1.56	5.48	33.41
desulfomicrobium orale	3.33	4.67	2.1	1.23	5.32	38.73
desulfovibrio sp. enrichment culture dgge band hcb4	6.04	6.18	1.65	1.48	4.19	42.92
desulfocella halophila	1.16	0.15	1.65	0.79	4.18	47.1
desulfobacterium autotrophicum	1.74	0.77	1.59	0.74	4.05	51.15
desulfovibrio gabonensis	1.07	0.25	1.46	0.47	3.7	54.84
desulfovibrio oxyclinae	0.23	1.09	1.34	4.53	3.41	58.25
desulfovibrio longus	1.2	1.87	1.09	1.23	2.76	61.01
desulfomicrobium macestii	0.8	0.52	0.82	1.1	2.08	63.09
desulfomicrobium baculatum	0.62	0.12	0.79	1.07	2	65.09
desulfobaculum xiamenense	0.54	0.13	0.75	0.56	1.89	66.98
desulfovibrio sp. dmss_1	0.17	0.58	0.65	2.06	1.66	68.64
desulfonema limicola	0.48	0.04	0.62	0.5	1.57	70.2

Groups Channel 2 inoculum & Channel 2
Average **dissimilarity** = 33.54

Species	CH2 inoc Av.Abund (%)	CH2 Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	0.77	4.16	5.82	1.01	17.36	17.36
desulfovibrio desulfuricans	5.41	2.2	5.53	1.56	16.5	33.85
desulfovibrio sp. enrichment culture dgge band hcb4	6.18	3.05	5.38	1.46	16.04	49.89
desulfobacter halotolerans	0.45	2.38	3.3	0.87	9.84	59.73
desulfomicrobium orale	4.67	5.6	1.75	0.63	5.21	64.94
desulfovibrio oxyclinae	1.09	0.63	1.53	11.69	4.56	69.5
desulfovibrio longus	1.87	1.77	1.05	1.25	3.12	72.62

Groups Environmental consortium & Channel 2
Average dissimilarity = 68.54

Species	Cons Av.Abund (%)	CH2 Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfovibrio sp. mcm b_508	6.81	1.74	7.63	5.53	11.14	11.14
desulfomicrobium orale	0.62	5.6	7.54	3.2	11.01	22.14

desulfobacterium autotrophicum	0.65	4.16	5.33	1.18	7.78	29.92
desulfovibrio fructosivorans	3.15	0.28	4.39	1.4	6.41	36.33
desulfovibrio sp. enrichment culture dgge band hcb4	0.58	3.05	3.7	1.31	5.4	41.73
desulfobacter halotolerans	0.47	2.38	2.99	1.03	4.36	46.09
desulfovibrio desulfuricans	1.08	2.2	2.73	1.14	3.99	50.08
uncultured desulfobulbus sp.	1.6	0.24	2.02	1.77	2.95	53.02
desulfotignum balticum	1.39	0.22	1.81	0.59	2.64	55.66
desulfovibrio longus	0.67	1.77	1.77	1.5	2.58	58.24
desulfosarcina sp. cme1	1.34	0.15	1.76	1.3	2.56	60.81
desulfovibrio simplex	1.26	0.16	1.71	0.84	2.5	63.3
uncultured desulfosarcina sp.	1.14	0.13	1.52	1.24	2.22	65.53
desulfovibrio zosterae	1.03	0.14	1.32	0.68	1.93	67.45
desulfarculus baarsii	1.04	0.23	1.29	1.41	1.88	69.33
desulfovibrio sp. enrichment culture dgge band ycb1	1.04	0.37	1.28	0.63	1.87	71.2

Groups Batch culture & Channel 2

Average dissimilarity = 47.94

Species	BTE Av.Abund (%)	CH2 Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfovibrio sp. enrichment culture dgge band hcb4	6.04	3.05	5	1.68	10.43	10.43
desulfobacterium autotrophicum	1.74	4.16	4.98	1.28	10.4	20.82
desulfomicrobium orale	3.33	5.6	3.61	1.27	7.54	28.36
desulfovibrio desulfuricans	1.61	2.2	3.22	1.14	6.71	35.07
desulfobacter halotolerans	0.72	2.38	3.04	1.09	6.35	41.42
desulfobotulus sapovorans	1.93	0.46	2.63	0.81	5.48	46.91
uncultured desulfobulbus sp.	1.85	0.24	2.57	0.76	5.36	52.26
desulfovibrio sp. mcm b_508	2.5	1.74	2.16	1.52	4.51	56.77
desulfocella halophila	1.16	0.46	1.53	0.9	3.19	59.96
desulfovibrio gabonensis	1.07	0.2	1.46	0.51	3.05	63.01
desulfovibrio longus	1.2	1.77	1.36	1.3	2.84	65.85
desulfomicrobium macestii	0.8	0.66	0.97	1.26	2.02	67.88
desulfovibrio oxyclinae	0.23	0.63	0.82	0.74	1.71	69.59
desulfobaculum xiamenense	0.54	0.07	0.78	0.62	1.62	71.21

Groups Channel 1 & Channel 2

Average dissimilarity = 32.94

Species	CH1 Av.Abund (%)	CH2 Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	6.93	4.16	5.21	1.17	15.83	15.83
desulfomicrobium orale	3.42	5.6	3.86	1.3	11.72	27.54

desulfobacter halotolerans	4.25	2.38	3.72	1.3	11.31	38.85
desulfovibrio desulfuricans	0.28	2.2	3.19	1.05	9.7	48.55
desulfovibrio sp. enrichment culture dgge band hcb4	2.29	3.05	2.95	1.25	8.94	57.49
desulfovibrio sp. mcm b_508	2.72	1.74	1.79	1.58	5.44	62.92
desulfovibrio longus	1.6	1.77	1.19	1.22	3.61	66.53
desulfomicrobium macestii	0.79	0.66	0.93	1.13	2.81	69.35
desulfovibrio oxyclinae	0.14	0.63	0.83	0.65	2.52	71.87

Groups Batch culture & Channel 1 FSB
Average dissimilarity = 47.20

Species	BTE Av.Abund (%)	CH1 FSB Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	1.74	7.54	9.24	4.13	19.58	19.58
desulfovibrio sp. enrichment culture dgge band hcb4	6.04	3.33	4.25	1.96	9	28.58
desulfobotulus sapovorans	1.93	1.31	2.74	1.05	5.8	34.38
uncultured desulfobulbus sp.	1.85	0.24	2.6	0.74	5.51	39.89
desulfovibrio sp. mcm b_508	2.5	1.72	2.35	1.25	4.98	44.88
desulfovibrio desulfuricans	1.61	0.25	2.2	0.67	4.65	49.53
desulfocella halophila	1.16	1.65	1.83	1.67	3.88	53.41
desulfomicrobium orale	3.33	3.93	1.83	1.43	3.87	57.28
desulfovibrio gabonensis	1.07	0.13	1.54	0.51	3.26	60.54
desulfobacter halotolerans	0.72	1.27	1.3	1.25	2.76	63.3
desulfovibrio vulgaris	0.16	0.91	1.19	5.5	2.52	65.82
desulfomicrobium macestii	0.8	0.21	0.94	0.97	1.99	67.81
desulfomicrobium baculatum	0.62	0.1	0.81	1.14	1.72	69.53
desulfovibrio longus	1.2	1.41	0.8	1.39	1.7	71.24

Groups Channel 1 & Channel 1 FSB
Average **dissimilarity** = 27.37

Species	CH1 Av.Abund (%)	CH1 FSB Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacter halotolerans	4.25	1.27	5.06	2.8	18.5	18.5
desulfovibrio sp. enrichment culture dgge band hcb4	2.29	3.33	2.27	1.5	8.31	26.81
desulfovibrio sp. mcm b_508	2.72	1.72	2.09	1.27	7.63	34.44
desulfomicrobium orale	3.42	3.93	2.08	1.53	7.59	42.03
desulfocella halophila	0.56	1.65	1.85	1.86	6.77	48.8
desulfobotulus sapovorans	0.38	1.31	1.58	1.19	5.79	54.59
desulfobacterium autotrophicum	6.93	7.54	1.33	1.26	4.85	59.44
desulfovibrio vulgaris	0.3	0.91	1.04	2.87	3.78	63.22
desulfomicrobium macestii	0.79	0.21	1.03	1	3.75	66.97

desulfovibrio longus	1.6	1.41	0.86	3.65	3.13	70.11

Groups Batch culture & Channel 2 FSB
Average dissimilarity = 53.18

Species	BTE Av.Abund (%)	CH2 FSB Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	1.74	7.02	8.39	3.97	15.78	15.78
desulfovibrio sp. enrichment culture dgge band hcb4	6.04	1.38	7.34	3.98	13.8	29.58
desulfobacter halotolerans	0.72	2.98	3.64	5.2	6.84	36.42
desulfomicrobium orale	3.33	5.5	3.45	1.98	6.48	42.9
desulfobotulus sapovorans	1.93	0.35	2.69	0.73	5.06	47.96
uncultured desulfobulbus sp.	1.85	0.32	2.58	0.71	4.85	52.8
desulfovibrio sp. mcm b_508	2.5	1.04	2.53	1.21	4.75	57.55
desulfovibrio desulfuricans	1.61	0.4	2.14	0.64	4.03	61.58
desulfovibrio longus	1.2	2.37	1.82	1.86	3.42	65
desulfovibrio gabonensis	1.07	0.17	1.49	0.47	2.8	67.8
desulfocella halophila	1.16	1.08	1.43	1.08	2.7	70.5

Groups Channel 2 & Channel 2 FSB
Average dissimilarity = 29.81

Species	CH2 Av.Abund (%)	CH2 FSB Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfobacterium autotrophicum	4.16	7.02	5.33	1.01	17.87	17.87
desulfobacter halotolerans	2.38	2.98	3.31	2.5	11.1	28.97
desulfovibrio desulfuricans	2.2	0.4	3.13	0.92	10.51	39.48
desulfovibrio sp. enrichment culture dgge band hcb4	3.05	1.38	3.01	0.87	10.11	49.59
desulfomicrobium orale	5.6	5.5	2.04	1.2	6.84	56.43
desulfovibrio longus	1.77	2.37	1.48	1.8	4.98	61.4
desulfovibrio sp. mcm b_508	1.74	1.04	1.2	1.21	4.01	65.41
desulfocella halophila	0.46	1.08	1.07	1.59	3.59	69.01
desulfovibrio oxyclinae	0.63	0.13	0.85	0.57	2.84	71.85

Groups Channel 1 & Channel 2 FSB
Average dissimilarity = 21.32

Species	CH1 Av.Abund (%)	CH2 FSB Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfomicrobium orale	3.42	5.5	3.53	1.67	16.57	16.57
desulfovibrio sp. mcm b_508	2.72	1.04	2.86	2.67	13.41	29.98
desulfobacter halotolerans	4.25	2.98	2.15	1.71	10.06	40.04

desulfovibrio sp. enrichment culture dgge band hcb4	2.29	1.38	1.65	0.91	7.74	47.78
desulfovibrio longus	1.6	2.37	1.32	1.36	6.2	53.99
desulfocella halophila	0.56	1.08	0.89	1.67	4.17	58.16
desulfomicrobium macestii	0.79	0.83	0.82	1.4	3.83	61.98
desulfobacterium autotrophicum	6.93	7.02	0.65	1.01	3.06	65.04
uncultured desulfobulbus sp.	0.6	0.32	0.59	1.01	2.76	67.8
desulfomicrobium baculatum	0.41	0.13	0.53	0.62	2.48	70.28

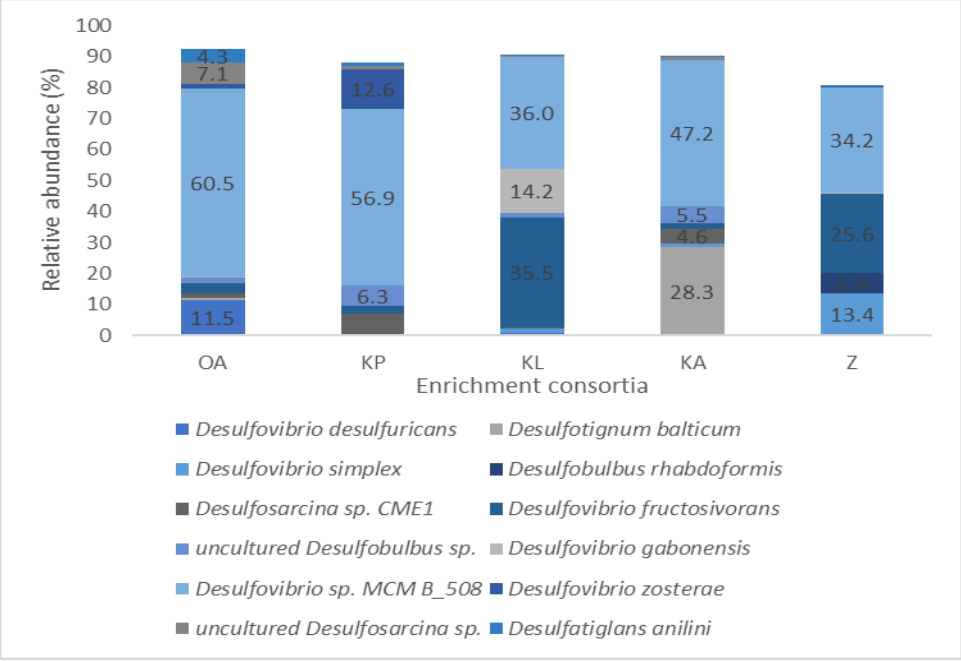
Groups Channel 1 FSB & Channel 2 FSB
Average dissimilarity = 25.30

Species	CH1 FSB Av.Abund (%)	CH2 FSB Av.Abund (%)	Av.Sim	Sim/SD	Contrib (%)	Cum.(%)
desulfovibrio sp. enrichment culture dgge band hcb4	3.33	1.38	3.4	2.13	13.46	13.46
desulfobacter halotolerans	1.27	2.98	3	1.55	11.84	25.3
desulfomicrobium orale	3.93	5.5	2.77	1.4	10.94	36.24
desulfovibrio sp. mcm b_508	1.72	1.04	1.79	1.06	7.06	43.3
desulfovibrio longus	1.41	2.37	1.69	233.14	6.69	49.98
desulfobotulus sapovorans	1.31	0.35	1.68	0.94	6.63	56.62
desulfovibrio vulgaris	0.91	0.11	1.41	5.57	5.57	62.18
desulfomicrobium macestii	0.21	0.83	1.1	7.72	4.36	66.54
desulfocella halophila	1.65	1.08	0.99	0.84	3.91	70.45

10.5 Appendix E: Microbial community analysis graphs

Amplicon sequencing results of RA of highest contributing bacterial species containing *dsrB*.

Enrichment consortia



BTE batches and HLFCRs