The Microbial Devulcanisation of Waste Ground Tyre Rubber Using Acidophilic Microorganisms

by

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Abstract

The structural stability provided by vulcanisation presents a major hurdle in the reclamation of tyre rubber. Devulcanisation processes aim to break the sulphur crosslinks in the tyre matrix, while preserving the rubber properties of the polymer. However, most mechanical, thermal and chemical devulcanisation techniques result in unselective breakage of both the sulphur crosslinks and the carbon chains in the rubber polymer. Disrupting the carbon chains causes deterioration of the mechanical properties of the rubber, resulting in a less valuable product. Conversely, microbial devulcanisation using sulphur oxidising acidophilic microorganisms has been reported to selectively break the sulphur crosslinks in tyre rubber without causing polymer degradation. Although promising, microbial devulcanisation is not yet industrially viable and the performance needs to be improved through increased toxin resistance and sulphur oxidation activity of the microbes.

Acidithiobacillus ferrooxidans DSMZ 14882 and an acidophilic biomining consortium have been identified as promising cultures suitable for ground tyre rubber (GTR) devulcanisation. The sulphur oxidising, autotrophic acidophile, *At. ferrooxidans* has been investigated extensively in devulcanisation literature and displays good devulcanisation performance and toxin resistance. Furthermore, co-culturing with another sulphur-oxidising autotroph, *At. thiooxidans,* has been shown to further improve devulcanisation performance. Similarly, mixed cultures of acidophiles are known to improve sulphur oxidation activity of the autotrophs in biomining, particularly through symbiotic relationships with organic toxin consuming heterotrophic cells. However, the devulcanisation performance of complex acidophilic consortia containing heterotrophs has not yet been reported in literature.

The devulcanisation and cell growth performance of active cultures of *At. ferrooxidans* DSMZ 14882 and a biomining consortium on GTR manufactured from waste tyre rubber is investigated. The sulphur oxidation activity of the devulcanisation cultures was maximised by developing culture preparation methods that ensured adequate biomass concentration and sulphur oxidising activity. Active cultures of *At. ferrooxidans* DSMZ 14882 and the biomining consortium are demonstrated to change the properties of industrial GTR within 15 to 30 days of incubation. *At. ferrooxidans* DSMZ 14882 is conclusively shown to achieve devulcanisation in the absence of polymer degradation, leading to a 1.09 ± 0.02 % GTR sol fraction increase. The biomining consortium devulcanisation performance is less conclusive, as microbial treatment only increased the GTR sol fraction by 0.56 ± 0.01 %. Additionally, polymer degradation and increased chemical additive extraction from the GTR is observed in biomining consortium GTR, and is attributed to the activity of heterotrophic microorganisms. Cell lysis is observed in devulcanisation cultures of both *At. ferrooxidans* DSMZ 14882 and the biomining consortium, and was attributed to acetone-extractable toxins in the GTR. Extensive cell death was caused by non-acetone-extractable toxins, and these are identified as the greatest

challenge to improved growth performance. Attached cells in the non-sulphur-oxidising component of the biomining consortium demonstrated the greatest toxin resistance.

At. ferrooxidans DSMZ 14882 was conclusively shown capable of devulcanisation of the industrial GTR used in this study. Despite the poorer devulcanisation performance of the biomining consortium, the increased toxin resistance compared to *At. ferrooxidans* DSMZ 14882 makes it a promising culture for further research. Additional investigation is required to improve understanding of the effect of the biomining consortium on GTR properties and the effect of GTR on the culture ecology. The toxicity of non-acetone-extractable-compounds suggests that further work should be conducted to increase toxin resistance of the microbes used in devulcanisation. Alternatively, detoxification techniques targeting the non-solvent-extractable-component of the toxins should be investigated to supplement solvent extraction methods usually used in microbial devulcanisation.

Uittreksel

Die strukturele stabiliteit wat deur vulkanisering aan rubber verleen word, bied ’n groot struikelblok tydens voertuigbuitebande herwinning. Devulkaniseringsprosesse het die breking van swaelkruisbindings in die rubbermatriks ten doel, terwyl die eienskappe van die rubberpolimeer bewaar moet word. Meeste meganiese, termiese en chemiese devulkaniseringstegnieke lei egter tot nie-selektiewe afbraak van die swaelkruisbindings asook die koolstofkettings in die rubber in die voertuigbuiteband. Ontwrigting van die koolstofkettings veroorsaak agteruitgang van die rubberpolimeer wat tot ’n laer kwaliteit produk kan lei. Daarenteen is gevind dat wanneer devulkanisering deur middel van swael-oksiderende, asidofiliese mikroörganismes uitgevoer word, die swaelkruisbindings in buitebandrubber selektief gebreek kan word om sodoende rubberpolimeerafbraak te bekamp. Alhoewel belowend, is mikrobiese devulkanisering nog nie ’n industrieel werkbare tegnologie nie, hoofsaaklik weens onvoldoende toksienweerstand asook lae swael-oksidasie aktiwiteit van mikroorganismes wat tot dusver bestudeer is.

Acidithiobacillus ferrooxidans DSMZ 14882 en ‘n asidofiliese biomyn konsortium is as belowende kulture geïdentifiseer om devulkanisering op gemaalde buitebandrubber (‘ground tyre rubber’, GTR) uit te voer. Die swael-oksiderende outotrofiese asidofiel, *At. ferrooxidans,* is ekstensief in devulkaniseringsliteratuur ondersoek en daar is bevind dat dié organisme uitstekende devulkaniseringsvermoëns en toksienweerstand vertoon het (5-9). Verder is daar bewys dat gesamentlike kweking met ’n ander swael-oksiderende outotroof, genaamd *At. thiooxidans*, die devulkaniseringsvermoë van *At. ferrooxidans* kon verbeter. Dit is ook bekend dat gemengde kulture van asidofiliese organismes die doeltreffendheid van swael-oksidasie deur outotrofiese organismes tydens biologiese verwerking van mynmateriaal verbeter, hoofsaaklik omdat daar ’n simbiotiese verwantskap met organiese toksien-verterende heterotrofiese organismes bestaan. Die devulkaniseringsvermoë van komplekse asidofiliese konsortiums wat heterotrofiese organismes bevat is egter nog nie in die literatuur beskryf nie.

Die devulkanisering- en selgroeivermoë van aktiewe kulture van *At. ferrooxidans* DSMZ 14882 en ’n biomynkonsortium op GTR wat van afval buitebandrubber vervaardig is, is in hierdie studie ondersoek. Die swael-oksiderende aktiwiteit van die devulkaniseringskulture is geoptimeer deur kultuurbereidingsmetodes te ontwikkel ten einde voldoende biomassa-konsentrasie en swael-oksiderende aktiwiteit te bewerkstellig. Daar is getoon dat aktiewe kulture van *At. ferrooxidans* DSMZ 14882 en ‘n biomynkonsortium die eienskappe van industriële GTR binne 15 tot 30 dae van inkubasie kon verander. Daar word ook bewys dat *At. ferrooxidans* DSMZ 14882 devulkanisering in die afwesigheid van polimeerdegradasie kon bewerkstellig, wat in ’n 1.09% toename in die GTR oplosbare fraksie waarneembaar was . Daarteenoor het die biomynkonsortium die GTR oplosbare fraksie met slegs 0.56% verhoog, maar het tot polimeerdegradasie gelei weens die heterotrofiese mikrobes se aktiwiteit. Sel lise is in devulkaniseringskulture van beide *At.*

ferrooxidans DSMZ 14882 en die biomynkonsortium waargeneem en is aan asetoon-ekstraheerbare toksiene in die GTR toegeskryf. Ekstensiewe seldood is deur toksiese komponente, wat nie tydens asetoon-ekstraksie verwyder kon word nie, veroorsaak. Hierdie toksiene is gevolglik as die grootste uitdaging ten opsigte van verbeterde groeivermoë geïdentifiseer. Selle in die nie-swael-oksiderende komponent van die biomynkonsortium en wat aan die substraat aangeheg was, het die beste toksienweerstand getoon.

Daar is bewys dat *At. ferrooxidans* DSMZ 14882, die tipestam van *At. ferrooxidans,* in staat was om die industriële GTR wat in hierdie studie gebruik is, te devulkaniseer. Ten spyte van die swakker devulkaniseringsvermoë van die biomynkonsortium vergeleke met *At. ferrooxidans* DSMZ 14882, is eersgenoemde ’n belowende kandidaat vir toekomstige navorsing weens die hoer toksienweerstand wat die kultuur vertoon het. Verdere ondersoek is nodig om die effek van die biomynkonsortium op GTR eienskappe en die effek van GTR op die ekologie van die kultuur beter te verstaan. Die toksiese effek van die toksiene wat nie asetoon-ekstraheerbaar is nie, impliseer dat verdere werk gedoen ook moet word om toksienweerstand van die mikrobes wat in devulkanisering gebruik word, te verhoog. As alternatief behoort detoksifiseringstegnieke wat op die nie-oplosmiddel-ekstraheerbare komponent van die toksiene gemik is ondersoek te word ten einde oplosmiddel-ekstraksiemetodes wat gewoonlik in mikrobiese devulkanisering gebruik word, aan te vul.

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Definitions, Nomenclature and Abbreviations

Table i: Definitions

Term	Definition
Acidophile	Microbe with growth optimum below pH 3
Desulphurisation	Removal of sulphur from ground tyre rubber (GTR) resulting in devulcanisation
Devulcanisation	Breakage of sulphur bonds in vulcanised polymer component of GTR
Tyre Rubber	Refers to the portion of the rubbery portion of the tyre once it has been stripped from supporting textile and steel frame
Ground Tyre Rubber (GTR)	Tyre rubber which has been ground, either at ambient temperatures or under cryogenic conditions to different crumb sizes
Rubber	The pure polymer portion of the tyre rubber, *i.e.* poly-isoprene or styrene butadiene rubber
Sol Fraction	The portion of the tyre rubber which is removed during solvent extraction, expressed either as a mass fraction or a mass percentage. For the purpose of this study, the sol fraction includes both the portion of the rubber polymer which can be removed and the other extractable compounds in the rubber
Extractables	Chemical components which can be removed by solvent extraction but are not rubber polymer. These may include a variety of complex organic chemicals, oils and waxes
Autotroph	A microbe which utilises carbon dioxide as carbon source for growth
Chemotroph	A microbe which uses inorganic molecules as a source of energy
Heterotroph	A microbe which uses organic carbon as carbon source for growth and energy
Mixotroph	A microbe which can use both an autotrophic and heterotrophic carbon metabolism

Table ii: Nomenclature

Symbol	Definition
X	Cell concentration
T	Time
M	Mass
M	Molar (mole/litre)
ρ	Density
μ	Growth rate = ln(X)/dt
V	Volume
X	Mass fraction
C	Concentration
E_0	Standard electrode potential
G^0	Standard Gibbs free energy
R^2	Correlation coefficient
SE	Standard error
RSD	Relative standard deviation = $\sigma/\bar{x}$
$\bar{x}$	Mean
σ	Standard deviation
σ^2	Variance
n	Sample size
phr	Parts per hundred rubber

Table iii: Abbreviations

Abbreviation	Longhand
ASTM	American Standard Testing Methods
GTR	Ground tyre rubber
cGTR	Control ground tyre rubber
dGTR	Devulcanised ground tyre rubber
aGTR	Acetone leached ground tyre rubber
DSMZ	*Deutsche Sammlung von Mikroorganismen und Zellkulturen*
EtOH	Ethanol
RISC	Reduced inorganic sulphur compounds
RSC	Reduced sulphur compounds
NR	Natural rubber (polyisoprene)
SBR	Styrene butadiene rubber
BR	Butadiene rubber
S-S, S-C	Single bonds between two sulphur atoms and a sulphur atom and a carbon atom, respectively
C-C, C=C	Carbon-carbon single double bonds, respectively
FTIR-ATR	Fourier transform infrared spectroscopy attenuated total reflection
SEM	Scanning electron microscope or microscopy
SEM-EDS	Scanning electron microscopy energy dispersive X-ray spectroscopy
P.	*Picrophilus*
T.	*Thermoplasma*
At.	*Acidithiobacillus*
A.	*Acidiphilium*
Ac.	*Acidomicrobium*
Ad.	*Acidocella*
Ab.	*Acidobacterium*
Al.	*Alicyclobacillus*
Am.	*Acidomonas*
Af.	*Acidoferrobacter*
F.	*Ferromicrobium*
L.	*Leptospirillum*
S.	*Sulfolobus*
Th.	*Thiomonas*

1 Introduction

1.1 Background and Motivation

Tyres play an integral role in the transportation of goods and people worldwide. Over one hundred years of careful formulation has produced a material that is strong, flexible, and above all durable. The durability of tyre rubber makes both its disposal and reclamation very challenging. It is estimated that 1.4 billion tyres were produced worldwide in 2010 (1), and that the demand will increase to 2.5 billion in 2022 (2). Increased production results in increased demand for raw materials, and increases the numbers of tyres that have reached the end of their lifespan. This puts pressure on the supply of raw materials as well as waste management systems.

In South Africa alone, 11 million waste tyres are produced every year (3). Currently, waste tyres enter one of four waste streams: recycling, energy recovery, pyrolysis or landfills (4). Of these, only recycling has the potential to produce a fraction of rubber polymer which can be reused in tyres, while the remaining routes are destructive and unable to decrease demand for raw materials (Figure 1-1). Due to technological and economic constraints, the recycling stream of the rubber polymer that can be used in the production of new tyres is very small. Only 1% to 5% of reclaimed polymer can currently be used in the production of new tyres without impairing the quality of the tyres (5,6). This means that the tyre manufacturing industry operates in a linear manner: 95% to 99% of the raw polymer materials consumed are used only once before effectively becoming waste. This is a highly unsustainable practice, since 60% of the rubber used in tyre manufacturing is produced using non-renewable fossil fuels, and moreover, production of natural rubber cannot be expanded without drastic environmental impact (7,8). It is, therefore, critical that processing methods are developed which can produce a high quality reclamation product in a relatively inexpensive manner, thereby increasing the polymer reclaim stream.

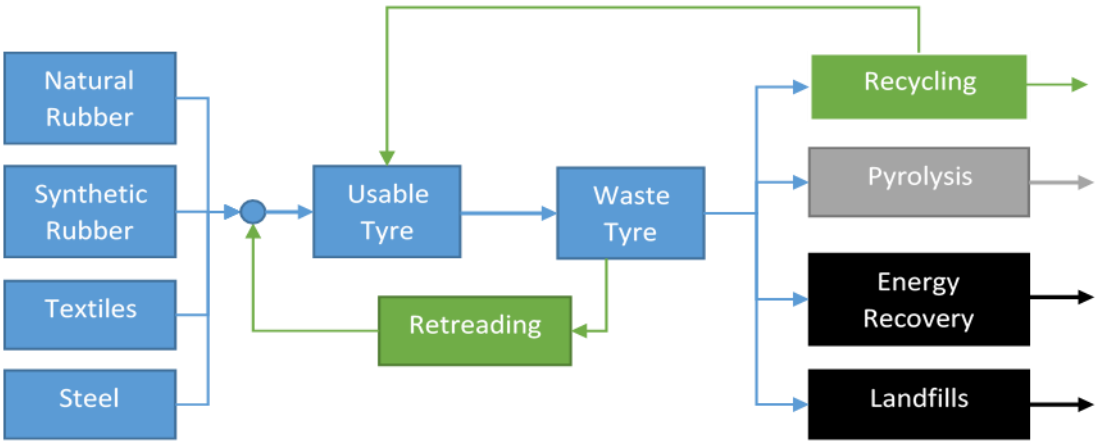

Figure 1-1: Basic overview of the current lifecycle of a tyre adapted from literature (4)

A major hurdle in the reclamation of tyre rubber is the structural stability brought about by vulcanisation with sulphur (6,9). During the vulcanisation process, the tyre rubber compound is heated and sulphur crosslinks form a three dimensional network with the rubber polymer chains. These sulphur crosslinks are very stable and require a large amount of energy to break. As a result, most established devulcanisation techniques are harsh and result in unselective breakage of the carbon chains of the rubber polymer as well as the sulphur crosslinks, resulting in deterioration of the rubber properties (4,6,9).

Several microorganisms have been shown capable of breaking the sulphur crosslinks in rubber while leaving the carbon chains intact (10–12). Crosslink disruption can be achieved through either sulphur oxidation (10,11) or reduction (13), which result in the breakage of sulphur crosslinks at the surface of the rubber without causing deterioration of its physical properties. The removal of sulphur and, in the case of sulphur oxidising microbes, oxidation of sulphur at the particle surface, creates active points where a new polymer matrix can chemically bond to the particle, improving the strength of the compound material (11,14). In addition to the potential to produce higher quality rubber reclaim than established devulcanisation techniques, this treatment occurs under fairly mild physiological conditions, and has the potential to be less costly (11,15,16).

1.2 Problem Statement

Microbial devulcanisation has been proposed as a potential method to valorise waste tyre rubber, since metabolic processes can specifically target the vulcanising sulphur bonds of the rubber (10,17). Although acidophilic autotrophs capable of devulcanisation have been identified in literature (11,18–21), the devulcanisation process is still poorly understood and is not extensive enough to produce an industrially viable product. Additionally, some components in the tyre rubber are toxic to the cells, further inhibiting performance, and usually need to be removed from the tyre rubber using a solvent extraction step prior to treatment (22,23). To increase the extent of devulcanisation and reduce process costs, the toxin resistance and performance of the cultures used should be improved and, where possible, the detoxification step should be eliminated.

At. ferrooxidans has shown good devulcanisation performance and toxin resistance across a number of strains (11,18–21,24). Furthermore, it is the most extensively characterised microbe and is one of few microbes demonstrated to devulcanise ground tyre rubber (GTR) that has not been detoxified (11,18,21). The extensive characterisation, good performance and toxin resistance of *At. ferrooxidans* makes it a promising microorganism for the devulcanisation of GTR which has not been detoxified. However, because previous strains characterised were non-type strains, some are no longer available. As such, it would be beneficial to characterise the devulcanisation performance of the type strain, *At. ferrooxidans,* DSMZ 14882*,* which is widely available.

Furthermore, literature reports improved devulcanisation performance of mixed cultures of acidophiles compared to pure cultures. However, to the author's knowledge, only simple mixed cultures containing the autotrophs *At. ferrooxidans* and *At. thiooxidans* have been investigated (11,20,21). The use of more complex acidophilic consortia containing a variety of autotrophic and heterotrophic species has demonstrated improved performance compared to pure cultures in biomining applications (25). The improved performance is due, in part, to heterotrophic organisms metabolising organic molecules that are toxic to the sulphur oxidising autotrophs. Since a number of the toxins present in tyre rubber are organic chemicals (22,23), the inclusion of heterotrophic acidophiles may also reduce the concentration of organic toxins leaching from the GTR. As such, there is scope for investigation into the devulcanisation performance of a biomining consortium.

Due to the hydrophobic nature of GTR, microbial devulcanisation only occurs in the outer 4 to 6 µm of the particle surface. Because the particle sizes of GTR used in microbial devulcanisation studies range from 80 to 400 µm, only a very small fraction of the total particle volume is affected. The resulting overall change in properties is small and can be challenging to quantify accurately. Consequently, the devulcanisation effect of the microbes also needs to be maximised to improve the quality of the results. Past studies have achieved this by including a preliminary batch growth phase (24,26,27), which ensures high cell concentration and limits any nutrients which could be used preferentially to the sulphur in the GTR. Although a preliminary growth stage has been included in the devulcanisation study of one strain of *At. ferrooxidans*,(24) the method has not been described for *At. ferrooxidans* DSMZ 14882 or any biomining consortium, and would need to be developed and tested prior to devulcanisation.

1.3 Aim

The purpose of this study is to compare the devulcanisation performance of a mesophilic biomining consortium of acidophiles to that of a pure culture of *At. ferrooxidans* DSMZ 14882 for untreated, industrially supplied ground tyre rubber (GTR).

1.4 Significance

Microbial devulcanisation is a promising method to produce reclaimed tyre rubber, but it requires significant additional research. This project contributes to the knowledge base by developing and assessing the performance of devulcanisation processes for *At. ferrooxidans* DSMZ 14882 and a biomining consortium, neither of which have been reported in literature.

1.5 Thesis Overview

The literature review, Chapter 2, provides an overview of devulcanisation, ground tyre rubber (GTR) material properties and devulcanisation characterisation techniques; followed by an investigation into reported microbial devulcanisation processes and the resultant growth and devulcanisation performance; and lastly,

an assessment of the potential microbial population of a biomining consortium and its expected interactions with GTR. Chapter 3 provides the research methodology, and as such, defines the research questions, objectives, scope for novelty and project limitations. Chapter 4 develops the culture preparation steps for *At. ferrooxidans* and the biomining consortium required to improve devulcanisation performance. Chapter 5 presents and compares the devulcanisation performance of *At. ferrooxidans* DSMZ 14882 and the biomining consortium. Lastly, Chapter 6 provides the concluding remarks and recommendations for the complete study. Appendix A, B and C provide supplementary information for Chapter 2, 4 and 5 respectively.

2 LITERATURE REVIEW

2.1 ABSTRACT

Industrially supplied waste ground tyre rubber (GTR) presents several challenges in terms of material properties, feed variation and microbial toxicity. An industrial scale microbial devulcanisation process would therefore require a culture which is both toxin resistant and robust to feed variation. Furthermore, the low industrial value of reclaimed GTR (4,6) would require a process in which both capital expenditure and process costs are limited. Although a full economic analysis is outside the scope of this project the literature review, GTR crumb and microbial selection have been conducted with process cost effectiveness in mind.

The first section of this chapter provides the required background information on GTR as a material along with the basic principles of devulcanisation, desulphurisation and analytical techniques used to quantify these parameters. The second section provides a detailed overview of microbial devulcanisation studies completed to date, with an aim to highlighting the impact of GTR on microbial growth performance and the most promising microbial devulcanisation performance. The last section assesses the potential of a mesophilic biomining consortium to improve devulcanisation performance by considering the ecology and metabolism of the acidophilic microorganisms known to occur under the specified conditions.

2.2 DEVULCANISATION: MATERIALS, MECHANISMS AND CHARACTERISATION

Devulcanisation is usually carried out on GTR, which is made from the rubbery portion of the tyre and has been stripped away from the metal and fabric frame. It may include material from the tread or the side walls of the tyre, but does not include any fibres or metal pieces. This section provides an overview ground tyre rubber composition, the mechanisms of devulcanisation, and the methods used to characterise the effects of microbial treatment on GTR.

2.2.1 GROUND TYRE RUBBER COMPOSITION

Ground tyre rubber is a composite material consisting of rubber polymers compounded with a filler material and chemical additives (Figure 2-1). Rubber polymers account for approximately 60-64% of the total mass of the tyre rubber, while fillers account for about 30% and chemical additives can account for around 6-10%. Tyre rubber can be ground either under ambient conditions, resulting in rough particles, or cryogenically, resulting in straight fractures. As a result, ambient ground rubber has a higher surface to volume ratio than cryogenically ground rubber. The cost of grinding increases drastically with decreasing particle size (6) and GTR ranging from 10 to 200 mesh (2 mm to 75 µm sieve size) can be used as a filler in tyre manufacturing (5).

The rubber polymer composition varies depending on the application of the tyre, with poly-isoprene (natural rubber or NR), styrene butadiene rubber (SBR), and butadiene rubber (BR) the most predominantly used

compounds (Figure 2-2). A relatively high proportion of natural rubber is typically used in truck- and off-road tyre treads, while car tyres tend to contain more SBR (28).

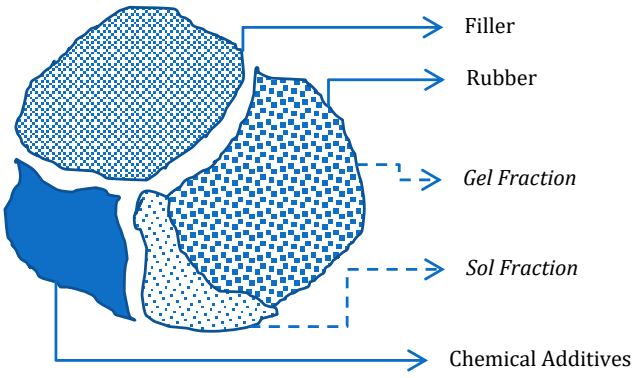

Figure 2-1: Graphical representation of the composition of ground tyre rubber, adapted from literature (29)

Sulphur vulcanisation is necessary in tyre applications as it increases the tensile strength and durability of the rubber and reduces its permanent elastic deformation. During vulcanisation the sulphur bonds with the carbon backbones of the polymers, to form three dimensional crosslinks between polymer macromolecules (Figure 2-3). Crosslinks can be monosulphidic or polysulphidic and in some cases may include sulphur pendant groups or cyclic formations. The presence of crosslinks increases the rigidity of the system, reducing permanent elastic deformation and increasing strength (30). Sulphur-vulcanised rubber is chemically very stable, which is desired for tyres-application, but presents challenges to recycling processes (31).

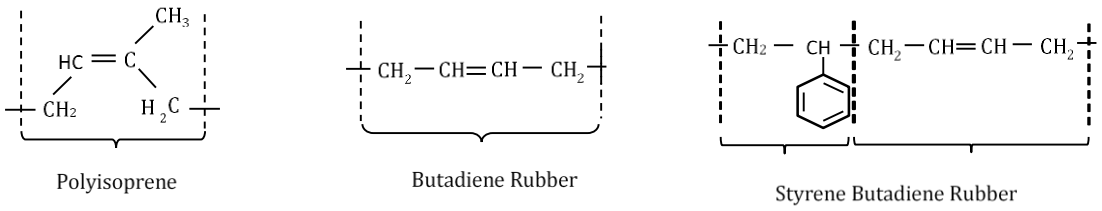

Figure 2-2: Common rubber polymers polyisoprene (NR), butadiene rubber (BR), and styrene butadiene rubber (SBR) (9)

The polymer component is often defined in terms of the soluble (sol) fraction and gel fraction. The gel fraction consists of rubber which is cross-linked by sulphur bonds (vulcanised) and cannot be removed by solvent extraction. The theoretical sol fraction consists of any remaining free polymers which can be removed from the matrix by organic solvents such as acetone, toluene and chloroform (32,33). Due to the presence of other solvent soluble chemicals in the GTR, the measured sol fraction is often inflated (29). Since the sol fraction includes free rubber polymers, an increase in sol fraction is often used as a metric to measure the extent of devulcanisation in microbial devulcanisation processes (12,26,34). It should be noted, however, that an

increase in free rubber polymers can be achieved by both carbon bond breakage and sulphur bond breakage. Hence, an increase in sol can also indicate carbon degradation.

Particulate fillers strengthen rubber compounds for use in tyres, increasing tear and abrasion resistance, and tensile strength. Fillers usually consist of a mixture of carbon black and silica, but can also include clays and limited amounts of fine GTR (35,36). Although chemical bonding is not necessary for reinforcement to occur, the complex surface interactions of carbon black and silicas with the surrounding rubber, lead to these two compounds displaying the best overall reinforcing capacity (35). Bonding between the rubber matrix and GTR filler can be improved by producing chemically active sites at the surface of GTR, and has the potential to increase the maximum allowable amount of reclaimed material in new tyres (11).

Chemical additives are not usually chemically bound to the rubber matrix and can leach out of the rubber over time, or be extracted using a solvent (26,32,37,38). The leaching of toxic additives presents a challenge to microbial devulcanisation, as they can inhibit cell growth and devulcanisation performance (13,22,24,37). Chemical additives are grouped in three classes: processing aids, curing agents, and stabilisation agents (30,36). Processing aids, such as oils and waxes or low quality rubber reclaim, are added to the virgin rubber to improve handling of the rubber mixture (36). The curing system is used to control the sulphur vulcanisation process and improve the properties of the final product. Curing systems typically include accelerators, retarders and zinc oxide, which control the rate and extent of crosslink formation in the rubber (30,36). Chemical additives in the stabilisation system assist in maintaining tyre mechanical properties throughout the lifecycle. These additives often include, but are not limited to, anti-oxidants, anti-ozonation agents and sun-checking agents, which aid in protection against UV radiation (36).There is a large amount of variation in the chemical additives in GTR, as they differ between tyre manufacturers and tyre rubber applications (9).

2.2.2 Devulcanisation Mechanism

Tyre rubber devulcanisation processes aim to produce a reclaimed product by reversing sulphur vulcanisation, the process in which sulphur chains chemically link with the polymer chains of the rubber to form a stable three dimensional network (Figure 2-3). The three dimensional network contributes to the material hardness, resistance to permanent elastic deformation and tensile strength. Therefore, high levels of devulcanisation should theoretically lead to GTR approaching the properties of virgin rubber-filler compounds prior to vulcanisation. Devulcanisation can occur with or without the removal of sulphur, specifically referred to as desulphurisation (39). For the purpose of this study, the term ‘desulphurisation’ is used only when sulphur removal is directly measured, while ‘devulcanisation’ refers to any measure of sulphur crosslink breakage.

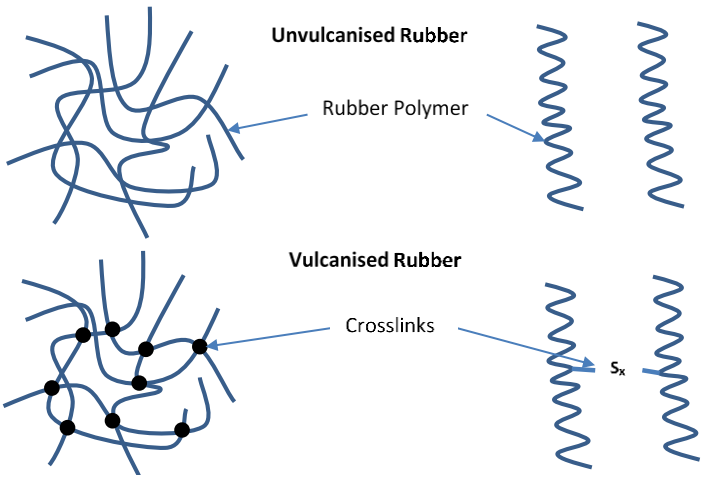

Figure 2-3: Sulphur vulcanisation of rubber polymers, adapted from literature (9)

Most microbial devulcanisation processes investigated in literature occur through sulphur oxidation, which has been proposed to proceed according to the 4S sulphur oxidative pathway (Figure 2-4). According to the mechanism, the attached sulphur is oxidised first to sulfoxide, then sulfone, and finally sulphate, at which point the sulphur-carbon bonds are broken and devulcanisation occurs. The mechanism was developed using the model compound dibenzothiophene, which contains a single monosulphidic crosslink (11,40). However, the occurrence of sulphoxide and sulphone intermediaries at the surface of microbially devulcanised GTR suggests that the 4S pathway can be applied to the breakage of the polysulphidic crosslinks found in vulcanised rubber (11,24). The simultaneous removal of sulphur and the breakage of sulphur-carbon bonds further means that desulphurisation can be used as an indirect measure of devulcanisation in sulphur oxidative microbial devulcanisation processes, as has been done in past studies (11,18,20,21,24). Interestingly, although the formation of sulphoxide or sulphone does not result in sulphur bond breakage (devulcanisation), it has been suggested that the increased reactivity of the oxidised sulphur can lead to improved bonding between the treated GTR and a new tyre rubber matrix (11).

The high specificity of the microbial metabolic process leads to improved selectivity of sulphur crosslink breakage compared to traditional mechanical, thermal and chemical methods, which tend to result in a high proportion of carbon bond breakage in the rubber polymer (6,9,11,18). Carbon bond breakage, or carbon chain degradation, leads to deterioration of the rubber properties and a decrease in the value of the GTR reclaim produced. As a result, most mechanically, thermally or chemically produced devulcanised GTR is used as a processing oil (6,9). By conserving the rubber properties of the GTR, it has been proposed that microbially devulcanised GTR could be used as a high quality filler, in doing so, reducing the portion of virgin rubber compound required in new tyres (10,11). Sulphur oxidising obligate autotrophs (carbon fixing organisms) are of particular interest, since they are unable to metabolise organic carbon, eliminating the potential for carbon chain degradation (16).

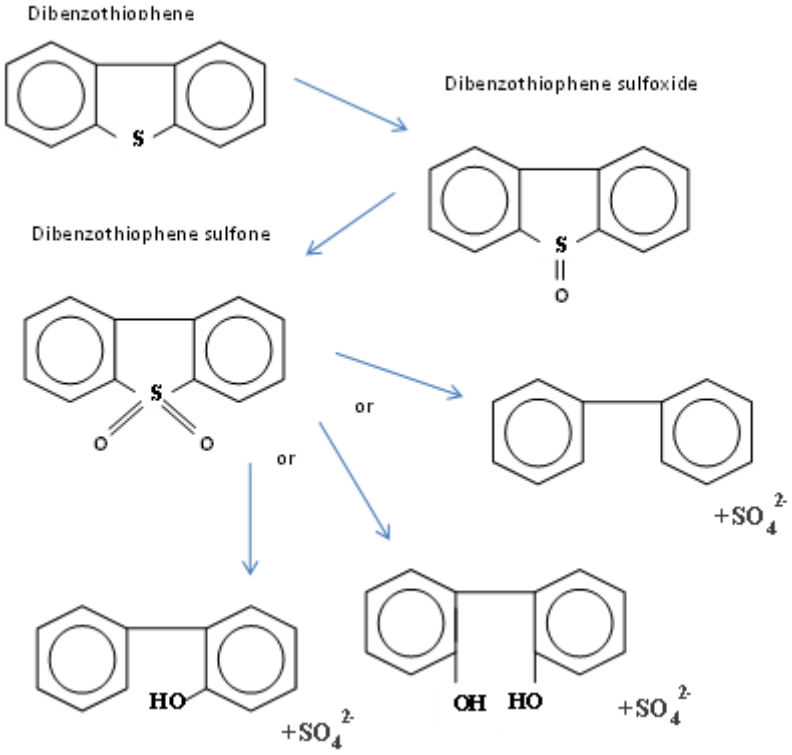

Figure 2-4: The 4S sulphur oxidative devulcanisation pathway illustrated for the model compound benzothiophene, adapted from literature (11,40)

2.2.3 Characterisation Methods for Microbial Devulcanisation

The characterisation of microbial devulcanisation processes is uniquely challenging because of the limited accessibility of the hydrophobic GTR particle. Due to the high cost of grinding, most microbial devulcanisation studies are conducted on GTR with particle sizes ranging from 80 to 200 µm (11,18,20,21,24,26,27,34,41,42), which are within the range used and produced in industry (5). Since the microbes are only able to penetrate the outermost 2 to 6 µm of the GTR particle (11,18,34), only a fraction of each particle is altered, resulting in small changes of the overall properties of the GTR particle and/or growth medium which are challenging to quantify accurately. The next three sub-sections discuss the advantages and challenges of the commonly used measures of microbial devulcanisation quantification and characterisation. The characterisation methods have been grouped as industrial measures of devulcanisation; measures of desulphurisation; surface property characterisation and compounding tests.

2.2.3.1 Industrially Recognised Devulcanisation Measures

American Standard Testing Method (ASTM) D6814 provides a standard method to determine the extent of devulcanisation of a sample of tyre rubber, using sol fraction and crosslink density measurements (33). The sol fraction is determined using solvent extraction and indicates the weight fraction of unbound polymer molecules in the rubber matrix, while the crosslink density is determined with toluene swelling tests.

Sol fraction extraction also extracts a portion of the added chemicals and processing aids, and as a result is usually measured as a change in sol fraction (Δsol). An increase in sol fraction is often used as a measure of devulcanisation in microbial devulcanisation studies (12,26,41,43). However, an increase in sol fraction may be the result of sulphur crosslink breakage (devulcanisation) or carbon bond breakage (carbon degradation). Consequently, sol fraction testing should be supplemented with crosslink density determination or surface characterisation when testing heterotrophic (organic carbon metabolising) microbes to determine whether carbon degradation has occurred. Additionally, the methods used to characterise the soluble fraction of GTR in microbial devulcanisation studies vary between literature (12,26,27,34,41,42) and standard testing methods (32,33) making direct comparisons between studies problematic. Although relatively rudimentary, sol fraction tests have the advantage of requiring very little specialised equipment. Furthermore, relatively small quantities of treated rubber can be used to determine these properties, making them convenient for characterisation during small scale laboratory testing.

The crosslink density is an indication of the number of sulphur crosslinks in the tyre rubber sample. This measure can be related to the mass of a rubber sample swollen in toluene using the Flory-Rehner equation. The standard testing methods used are designed for large pieces of rubber, which can be measured individually (33). Accurate measurement of the swelling of the fine GTR crumb used in microbial devulcanisation studies is challenging due to high levels of liquid entrainment (44). Only one study on microbial devulcanisation has reported change in crosslink density of the treated GTR without prior hot pressing or compounding of the GTR into new tyre rubber (27).

The sol fraction and the crosslink density can be related using a Horikx plot (Figure 2-5) to determine whether the devulcanisation process is more carbon-carbon bond or sulphur bond specific (45). A large increase in sol fraction and small decrease in crosslink density correspond to high carbon-carbon specificity (degradation). Conversely, a small increase in sol fraction and a large decrease in crosslink density indicate a high specificity to the sulphur crosslinks (devulcanisation). A number of studies considered sol fraction increase and crosslink density independently (12,26,32–34,41,43), while only one relevant study considered Horikx plot interactions (27).

The performance of microbial devulcanisation is not easily compared to traditional methods of devulcanisation because of the vast difference in the treatment aims and the properties of the final product. Microbial devulcanisation is a surface treatment which only affects the outer 2 to 6 µm of the GTR particle, resulting in a small overall change in the properties of the GTR (Figure 2-5). As such, microbially treated GTR has the potential to retain the rubber properties of the particle interior while allowing for improved bonding with a new rubber matrix at the surface of the particle. Traditional devulcanisation methods (mechanical, chemical, thermal or a combination), can cause bond breakages throughout the particle, resulting in much larger overall changes in sol fraction and crosslink density. Despite the apparent improved extent of

devulcanisation, traditional methods tend to cause excessive carbon bond degradation (Figure 2-5) resulting in a loss of property benefits associated with the polymeric structures of the rubber. As such, the final product of traditional devulcanisation can approach the properties of oil and cannot be used as a substitute for rubber in new tyres.

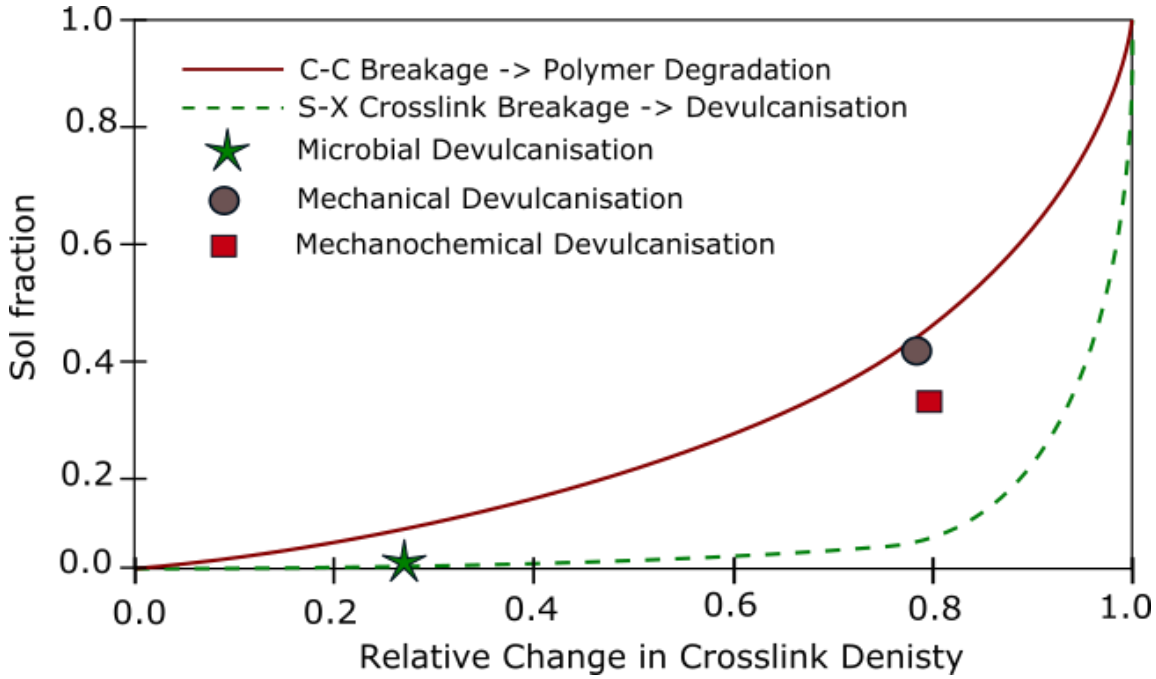

Figure 2-5: Horikx plot illustrating trends for carbon bond breakage and sulphur crosslink breakage. Sample points from literature included to illustrate the properties of mechanical (46)*, mechanochemical* (46) *and microbial devulcanisation (27).*

2.2.3.2 Desulphurisation

Desulphurisation can be used as a measure of devulcanisation by assuming that the removal of sulphur equates to the breakage of sulphur bonds, as suggested by the proposed sulphur oxidative mechanism (Figure 2-4). Desulphurisation is detected either indirectly or directly. Desulphurisation is detected indirectly by the emission of sulphate ions in oxidative processes (11,18,24). Direct detection of desulphurisation has been achieved by high precision elemental sulphur analysis, Scanning Electron Microscopy Electron Dispersive X-ray Spectroscopy (SEM-EDS) or X-ray photoelectron spectroscopy (XPS or ESCA) (11,12,18,34,41). Unfortunately, elemental analysis cannot be used in most cases of microbial devulcanisation since most GTR samples contain less than 2% sulphur (9). The expected change in sulphur content is therefore below the threshold where this method can be applied to accurately (18). SEM-EDS has been shown to be most successful when analysing flat flakes of homogenous rubber and can analyse at a depth of 1 to 2 µm (18,27,34,42). XPS can be used to determine elemental composition near the surface of irregular particle surfaces (47,48). SEM-EDS is cheaper, and requires less sample preparation compared to XPS (48,49).

2.2.3.3 Surface Properties

Chemical bonds at the surface of rubber are of particular interest as they can indicate carbon bond breakage, devulcanisation and the formation of oxidised active sites (11,12,18). Surface bonds have been characterised in literature using attenuated total reflection Fourier transform infrared spectroscopy (FTIR-ATR), XPS and x-ray absorption near edge spectra (XANES). FTIR-ATR can be used to detect the presence, increase and decrease in C=C, C-C and C-H bonds in the first few micrometres at the particle surface (47,50). A relative decrease in C=C bonds indicates the breakage of C=C bonds, and therefore polymer degradation (12,24,26,27,41). The bond state of carbon, sulphur and oxygen, as well as the relative elemental concentrations on the outer 2 to 10 nm of the GTR, can be determined using XPS (47). XPS can therefore be used as an additional confirmation of carbon bond degradation in conjunction with FTIR-ATR. Additionally, XPS can be used to determine the bond state of sulphur at the surface of the particle providing insight into the chemical intermediates formed during the process under consideration (12,18,24,26,34,41,43). XANES is less widely used, but has also been employed to determine the bond state of sulphur at the surface of rubber particles (11). Surface morphology of rubber flakes and microbial attachment during various stages of devulcanisation, has been characterised using SEM imaging (27,34).

Both FTIR-ATR and SEM analysis were selected because they can be conducted with relative ease on very small samples, with limited sample preparation and cost. Although XPS and XANES provide the most insight into surface bond properties, both these analysis techniques are costly and require the removal of all oils from the samples, which could affect the surface properties of the sample (48).

2.2.3.4 Compounded Rubber Properties

Compounding the treated GTR with new tyre rubber represents the final test to determine the value of the reclaimed rubber and the impact of the devulcanisation treatment on the GTR. Properties of the resulting tyre rubber can then be compared to tyre rubber without GTR filler or to tyre rubber containing the same concentration of untreated GTR filler. GTR is usually incorporated into new tyre rubber at concentrations of 5 to 40 per hundred rubber (phr) and the mechanical properties most often tested in microbial devulcanisation studies are: tensile strength, elongation at break, tear strength, shore A hardness, and crosslink density (11,12,23,24,26,34,41,43). Tensile strength is indicative of the maximum tensile stress the rubber compound can endure at the point just before failure, while elongation at break is the amount the rubber compound elongates at the point of breakage in the tensile strength test. Elongation at break is expressed as a percentage of the original length. Tear strength indicates resistance to tearing and Shore A hardness is a measure of hardness. The crosslink density of the final product is measured in the same manner as described in Section 2.2.3.1. SEM imaging is also used to compare fracture properties of rubber containing untreated and treated GTR. These tests require relatively large quantities of treated GTR in addition to extensive rubber compounding and testing facilities and, as such, fall beyond the scope of the current study.

2.3 Microbial Devulcanisation: Processes and Performance

Microbial devulcanisation is a relatively new technique, and as a result, the existing research is limited. This section provides an overview of the microbial devulcanisation processes investigated to date, followed by the effect of GTR and GTR toxicity on growth. Finally, the impact of microbial devulcanisation on GTR properties reported in literature is summarised.

2.3.1 Process Overview

The microbial devulcanisation processes reported in literature consist of up to three preparation steps for the GTR feedstock, followed by the microbial devulcanisation stage (Figure 2-6). The devulcanised GTR (dGTR) is then characterised, and in some instances compounding is conducted to investigate the effect of the inclusion of dGTR on fresh tyre rubber properties. Some studies also include an initial microbial culturing step to improve devulcanisation performance, or may be followed by analysis of sulphur compounds in the medium.

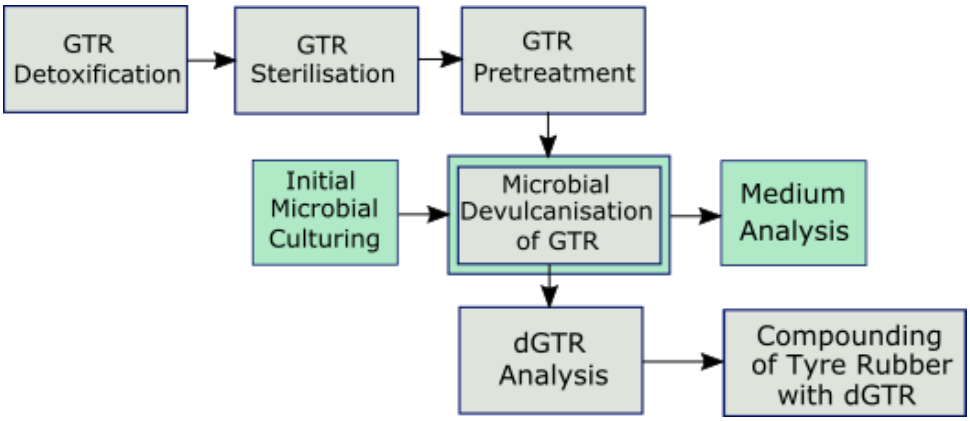

Figure 2-6: Overview of microbial devulcanisation processes used in literature

2.3.1.1 Preparation of GTR for Microbial Devulcanisation

Three stages of GTR feedstock preparation have been reported in literature: detoxification, sterilisation, and pretreatment. Detoxification has been shown to reduce cell death in cultures incubated in the presence of freshly manufactured GTR, and is usually achieved by removing a portion of the extractable organic chemicals in the GTR with solvent extraction. Sterilisation is conducted to ensure that there is no interference from contaminant cultures, and has been conducted using autoclaving at 121°C (11,18) or ethanol leaching (12,13,24,26,27,34,41,42). However, both methods cause fundamental changes to the properties of the GTR. Temperatures in excess of 80°C have the potential to effect the crosslink density and the integrity of the rubber polymers in GTR (32,33,44), while ethanol leaching removes a portion of the organic chemicals, resulting in concurrent detoxification. The pre-soaking of GTR in Tween surfactant to reduce hydrophobicity at the surface is the only GTR pretreatment that has been reported in literature to date (34).

2.3.1.2 Preparation of Microbial Culture for Devulcanisation

Microbial culturing prior to devulcanisation is conducted in literature using two methods. In the first, an inoculum is cultured and used to inoculate medium containing GTR (11,18). In the second, the culture is initially grown in batch without GTR and the GTR is added after a sufficient concentration of biomass has been achieved (24,26). Both methods are designed to limit the concentration of energy sources which could be used in preference to the sulphur in GTR, although the second method also includes cell counts and aims to maximise the number of viable cells, hence maximising the devulcanisation of the GTR.

2.3.1.3 Microbial Devulcanisation Process Variations

Previous microbial devulcanisation experiments have been conducted in batch experiments and usually range from seven (11) to thirty days (12). Experiments quantifying desulphurisation from the production of sulphate tend to last seven to twenty one days (11,18), while experiments quantifying devulcanisation in terms of GTR properties are sometimes extended to 30 days to maximise the effect of the microbes (12). GTR solids loading ranges from 20 to 50 g/l. One shortcoming of some early studies is that the effect of the sterile medium on the GTR was often not quantified, and any changes in properties were compared to the starting material (12,24,26,41). As such, the property changes attributed to microbial treatment in early studies may be overestimated because they are a combination of the medium effects and the microbial effects on the GTR. Later studies rectified this by including GTR treated with sterile medium (henceforth referred to as control GTR) to determine the influence of the medium on GTR properties, hence allowing for the quantification of the microbial effects alone (18,27,34,42).

2.3.2 Effect of GTR on Microbial growth

Ground tyre rubber contains a number of chemical constituents that can leach into microbial medium over time, some of which are known to be toxic to microorganisms. Only *At. ferrooxidans*, *At. thiooxidans*, *S. acidocaldarius* and *T. perometabollis* have been shown to devulcanise industrially-sourced waste tyre GTR that has not been detoxified, hereafter referred to as untreated GTR. No microbes have been shown to grow in the presence of GTR made from freshly manufactured tyre rubber (22).

Microbial devulcanisation GTR toxicity studies to date have tested the toxicity of a few compounds commonly used in tyre rubber manufacturing, but have not investigated the extent of leaching of these compounds from GTR under devulcanisation conditions. GTR component toxicity tests have been conducted on six microbes in literature and are summarised in Table 2-1. Aromatic organic chemicals and metal salts were identified as the most challenging toxins, since they caused the most widespread cell death (13,22,23). Overall, *At. ferrooxidans* was found to be the most toxin-resistant microbe due to its outstanding resistance to zinc compounds, coupled with good organic chemical tolerance (22,23). *At. ferrooxidans* DSMZ 583 was the only microbe shown capable of growth in the presence of elevated concentrations of both zinc oxide and zinc salts (22,23), and was the second most resistant to organic toxins. The most toxic organic molecules were

tetramethyl-thiuram-monosulfide (TMTM), dimethyl-phenyl-*p*-phenylenediamine (6PPD), cyclohexyl thiophthalimide (CTP) and stearic acid, only three of which affected *At. ferrooxidans* DSMZ 583. Although *Rhodococcus rhodochrous* DSMZ 43241 was inhibited by less organic carbon components than *At. ferrooxidans*, because of its sensitivity to zinc oxide it could not grow in the presence of GTR, even after leaching treatment with acetone (22). Consequently, *R. rhodochrous* is unsuitable for GTR devulcanisation. Natural rubber and butadiene rubber did not prevent growth in any of the microbes. However, only *R. rhodochrous* (22) and *At. ferrooxidans* YT-1 (23) were able to grow in the presence of styrene butadiene rubber. The improved resilience of the *At. ferrooxidans* YT-1 strain compared to DSMZ 583 may be due to variation in the toxin resistance of the microbial strain, or the type of SBR used.

Although the extent of leaching of GTR toxins under microbial devulcanisation conditions has not been investigated in literature, environmental studies on the leaching of GTR in building materials confirm that both inorganic and organic compounds are extracted under neutral to mildly acidic leaching conditions (38,51). Furthermore, the extent of leaching of the metals zinc, iron, aluminium and calcium was shown to increase with a decrease in pH, with calcium and zinc contributing the highest concentration to the liquid phase (38). The leaching of toxic heavy metals is problematic to microbial growth, and confirms the importance of resistance to zinc oxides and zinc salts, which can constitute up to 2% of tyre rubber (9). Although zinc solubility increases with pH, the superior zinc resistance of *At. ferrooxidans* (Table 2-1), makes it the most suitable devulcanising microbe tested to date. The organic components detected in environmental tests of GTR leachate included benzothiazole (38,51), quinoline (51) and benzothiazolone (51), suggesting that these compounds are also likely to be present during microbial devulcanisation. However, no information regarding the toxicity of these compounds is available in microbial devulcanisation literature.

Potential detoxification treatments were investigated when it was ascertained that freshly compounded tyre rubber inhibited growth in *At. ferrooxidans DSMZ 583, R. rhodochrous, As. brierleyi* and TH2 Lund (22). Solvent leaching pretreatment was met with a fair degree of success to mitigate the toxic effects of GTR constituents. Acetone leaching was most successful, and enabled the growth of *At. ferrooxidans, As. brierleyi* and TH2 Lund. Leaching with 96% ethanol was also successful and enabled the growth of *Pyrococcus furiosus, As. brierleyi* and *At ferrooxidans* (22). Ethyl acetate, acetone-water mixtures and boiling water were met with varying degrees of success (22). Ethanol leaching before microbial treatment has been the most widely adopted process, used in all microbial devulcanisation and desulphurisation studies since 2010. In one instance, pretreatment using the *Resinicium bicolor* instead of chemical agents also appeared promising (37).

Table 2-1: Studies on the toxicity of common tyre rubber constituents to microbes (G=grew, N= did not grow)

Type	Component	*Loading*[+] *(g/l)* (23)	*Loading* (g/l)* (13,22)	*As. brierleyi DSM 1651** (22)	*TH2 Lund* (22)	*R.rhodochrous DSMZ 43241**	*At. ferrooxidans DSMZ 583 ** (22)	*At. ferrooxidans* YT-1 [+] (23)	*Pc. furiosus* DSM 3538 * (13)
Elastomer	Natural Rubber	50	50	G	G	G	G	G	G
	Butadiene Rubber	50	50	G	G	G	G	G	G
	Styrene-butadiene rubber (unspecified)	50	-	-	-	-	-	G	-
	Styrene-butadiene rubber 1500	-	50	N	N	G	N	-	N
	Styrene-butadiene rubber 1712	-	50	N	N	G	N	-	N
Filler	Carbon black N330	15		-	-	-	-	G	-
	Carbon black N375	-	35	N	G	G	G	-	G
	Carbon black N660	-	35	G	G	G	G	-	G
Processing Aids	Paraffin	-	0.5	G	G	G	G	-	G
	Mineral Oil	-	0.4	N	N	G	G	-	G
Curing Agents	Stearic Acid	1	1	N	N	G	G	G	N
	Zinc oxide	2	2.5	N	G	N	G	G	N
	Zinc-salt	-	1.6	N	N	G	G	-	N
	Sulphur	1	2	G	G	G	G	G	G
	Tetramethyl-thiuram-monosulphide (TMTM)	-	0.5	N	N	N	N	-	G
	Tetramethyl-thiuram-disulphide (TMTD)	-	0.5	N	N	G	N	-	G
	Dibenzothiazyl-disulphide(MBTS)	0.6	0.5	G	G	G	G	G	G
	Cyclohexyl benzo-thiazole-sulfenamide (CBS)	-	0.5	N	G	G	G	-	G
	N-Oxydiethene-2-benzo thiazyl-sulfenamide (MBS)	-	0.5	N	N	G	G	-	N
	Diphenylguanidine	0.3	-	-	-	-	-	G	-
Protective chemicals and retarders	Dimethyl-phenyl-*p*-phenylenediamine (6PPD)	-	1	N	N	G	N	-	G
	Cyclohexyl thiophthalimide (CTP)	-	0.5	N	N	N	G	-	G
	Trimethyl-dihydroquinoline (TMQ)	-	1	G	G	G	G	-	G
	Anti-sunchecking agent	-	1	G	G	G	G	-	G

Relatively few studies have been conducted which characterise the impact of GTR on cell growth and growth conditions, and no studies have compared the cell growth performance on treated and untreated GTR. Only

two studies have determined the concentration of GTR that inhibits cell growth (20,41). A further four studies have monitored cell growth response to a set concentration of ethanol leached GTR (12,26,41,43). The GTR concentration had a marked effect on microbial growth, but also depended on the nature of the rubber and microbe tested. In an early study, solids loading of industrially sourced SBR-GTR in excess of 80 g/l inhibited growth of *At. ferrooxidans* and *At. thiooxidans* (20). A much lower toxicity threshold, 30 g/l, was reported for *Sphingomonas sp.* cultured with ethanol leached laboratory manufactured SBR-GTR (41). Variations in the type of SBR used or the resilience of the microbial strain may have contributed to the better performance of *At. ferrooxidans* and *At. thiooxidans. At. ferrooxidans* also outperformed *Sphingomonas* sp. in terms of cell growth in the presence of ethanol leached GTR (12,41). Although both cultures showed cell death after the addition of GTR, the cell concentration of *At. ferrooxidans* recovered after five days of incubation (12), while that of *Sphingomonas* sp. continued to decline (41). Later studies reported increased cell growth in heterotrophs when co-cultured on glucose (26,43). All experiments which characterised cell growth were conducted with an initial batch phase of growth in the absence of GTR, followed by the addition of ethanol leached GTR (12,26,41,43).

The overall hardiness, zinc salt and oxide resistance of *At. ferrooxidans* make it the most promising candidate for devulcanisation studies aiming to limit the requirement for pretreatment. Despite the susceptibility of some strains of *At. ferrooxidans* to styrene butadiene rubber and some organic constituents, it has been shown to survive exceptionally high concentrations of GTR in solution and has been demonstrated to devulcanise untreated GTR.

2.3.3 Impact of Microbial Devulcanisation on GTR properties

The change in GTR properties with microbial treatment has been characterised for eight pure microbial species and two mixed cultures (Table 2-2 and Table 2-3). Both autotrophic and heterotrophic species have been investigated. The devulcanisation performance of most autotrophic species is characterised using only desulphurisation measures, while the performance of heterotrophic species is characterised using industrial measures of devulcanisation, including sol fraction and crosslink density. The most extensively investigated species is the autotrophic acidophile *At. ferrooxidans*, with five strains investigated in various devulcanisation studies (11,18,19,21,24). *At. ferrooxidans* is also the only microbe which has had its performance characterised in terms of both desulphurisation and sol fraction measures. An overview of the microbial devulcanisation performance reported in literature to date is presented according to desulphurisation measures, industrial devulcanisation measures, Horikx plot characterisation, and surface characterisation. Lastly, a summary of the difference in the mechanical properties of tyre rubber containing treated GTR filler compared to untreated GTR filler is provided.

GTR desulphurisation caused by sulphur oxidising microbes is usually measured by the associated increase in sulphate ions in the medium solution, and has been used almost exclusively for the devulcanisation

performance characterisation of autotrophic microbes (Table 2-2). Reported sulphur removal ranged between 6% and 10%, except in the case of *At. thiooxidans* ATCC 15494 (18) where only 3% sulphur was removed and *Thiobacillus perometabollis,* which was reported to remove in excess of 30% sulphur (52). However, the experimental method used to determine sulphur content in the study on *Thiobacillus perometabollis* is not sufficiently detailed to assess the reliability of the result. *At. ferrooxidans* is the next best performing acidophilic autotroph, and the most extensively tested, consistently showing the ability to achieve desulphurisation across the strains investigated. *At. ferrooxidans* sp. was the best performing strain and achieved a 16% decrease in sulphur (24). However, since it was the only *At. ferrooxidans* study to use ethanol leaching as a detoxification and sterilisation step, the improved performance may indicate the beneficial effects of removing toxins from the GTR prior to microbial exposure, rather than improved strain performance alone. Two studies have been conducted on mixed cultures of *At. ferrooxidans* and *At. thiooxidans* (11,20,21), and found that the mixed cultures were more metabolically active (20,21) and removed a greater amount of sulphur (11), compared to the pure cultures. The improved performance of the mixed cultures corresponds to observations in biomining, where complex consortia of acidophiles used in industrial processes are reported to perform better than pure cultures (53). Improved performance is attributed to the symbiosis between autotrophs, as well as between heterotrophs and autotrophs (25). However, no devulcanisation studies on acidophilic consortia containing heterotrophs have been reported in literature. Consequently, research into the devulcanisation application of complex biomining consortia containing both heterotrophs and autotrophs may lead to further performance improvements.

The industrial devulcanisation measures, sol fraction and crosslink density, have only been determined for microbially treated GTR which was ethanol leached prior to treatment. Most studies used toluene Soxhlet extraction to determine the sol fraction of the GTR, without quantifying the GTR crosslink density. *At. ferrooxidans* sp. was found to increase the sol fraction of the treated GTR by 58% (12), making it both the only autotroph for which devulcanisation was characterised in terms of sol fraction, and the only microorganism for which devulcanisation was characterised using industrial and desulphurisation performance measures (24). The increase in sol fraction reported for heterotrophic microbes was larger than for *At. ferrooxidans* and ranged from 63% to 115% (Table 2-2). However, in the case of heterotrophs it should be noted that carbon bond degradation of the rubber polymer may contribute to the increase in sol fraction (Section 2.1.3.1). The largest increase in sol fraction, ~115%, was achieved by *Sphingomonas* sp. on GTR pretreated using Tween 20 surfactant (34). The increased change in sol fraction achieved by *Sphingomonas* sp. on Tween treated GTR compared to stock GTR (Table 2-2) suggests that pretreatment improved microbial attachment to the hydrophobic surface of the GTR while limiting the toxicity of the surfactant (34). As with desulphurisation studies, a lack of medium treated control GTR caused an inflation of the reported change in sol fraction attributed to microbial activity in some studies. The inflation of sol fraction due to the omission

of a control is illustrated by the difference between the change in sol fraction achieved by *Sphingomonas* sp. without a medium treated GTR control, 85% (26), and with a medium treated GTR control,~74% (34).

Only one study to date, on *Gordonia amicalisa,* has used Horikx plots (Section 2.1.3.1) to determine the sulphur-bond selectivity of a microbial process (27). Sol fraction and crosslink density were determined according to ASTM 6814, with acetone Soxhlet extraction followed by toluene swelling (27,33). According to the Horikx plot, a large change in sol fraction with a small decrease in crosslink density indicates polymer degradation, while a small change in sol fraction and a large decrease in crosslink density indicates selective sulphur bond breakage (devulcanisation). With a 100% increase in sol fraction and a 13% decrease in crosslink density, *G. amicalisa* was found to cause excessive polymer degradation to GTR made from natural rubber. Conversely, the same microbe caused highly selective devulcanisation in styrene butadiene derived GTR, causing a 22% decrease in crosslink density with no observable change in sol fraction (27). This further confirms that care should be taken when using sol fraction as a means of measuring devulcanisation performance of heterotrophic organisms, as it may indicate degradation of rubber polymers in GTR rather than devulcanisation. The devulcanisation performance of a mixed culture of *G. amicalisa* and *Sphingomonas* sp. was characterised using crosslink density (42). As was the case for the *Acidithiobacillus* mixed culture, the *G. amicalisa* and *Sphingomonas* sp. mixed culture also out-performed their pure cultures, achieving a 10% crosslink density increase on industrial GTR, compared to a 7% increase on the same GTR using the pure cultures (42).

Polymer degradation at the surface of the GTR using FTIR-ATR has only been investigated for three cultures, one autotroph, *At. ferrooxidans*, and two heterotrophs, *Sphingomonas* sp. and *Gordonia amicalisa* (Table 2-3). Degradation was detected in GTR treated by both *Sphingomonas* sp. and *G. amicalisa* (26,27,41). As is to be expected, carbon degradation was not detected in GTR treated by pure cultures of the autotroph *At. ferrooxidans* as it cannot metabolise organic carbon (12,24). XANES and XPS analysis at the surface of treated GTR particles (Table 2-3) shows sulphur oxidation, sulphur bond breakage and sulphur removal in GTR samples treated by *At. ferrooxidans* and *Sphingomonas* sp. (12,24,26,34,41). Interestingly, natural rubber GTR treated by *G. amicalisa,* which had been shown to be degraded rather than devulcanised*,* did not show any decrease in sulphur content, only an increase in oxygen at the surface. Conversely, the devulcanised SBR GTR showed a decrease in sulphur concentration (27). Oxidation of sulphur species at the surface of the GTR may indicate the formation of intermediate species in the devulcanisation pathway. It should be noted, however, that an overall increase in oxygen at the surface may also indicate oxidation of other species, including carbon, and as such, may indicate polymer degradation.

Table 2-2: Summary of devulcanisation performance of previously tested microbes grouped by metabolism and reported in terms of desulph urisation and industrial devulcanisation parameters

Reference	Microbe	Time (days)	Type of GTR	Ethanol Leached	GTR Loading (g/l)	Control GTR	(a) Desulphurisation		(b) Effect of microbe on industrial parameters	
							Medium Control	Microbe	Sol Fraction	Crosslink Density
Autotrophs										
Romine & Romine (11)	*At. ferrooxidans* ATCC 13661	7	Factory – 1.8% sulphur	No	NS	Yes	*NS*	6%		
Christiansson et al (18)	*At. ferrooxidans* DSM 583	20	Spent Tyre Rubber – 1.6% sulphur	No	50	Yes	3%	8%		
Jiang et al (24)	*At. ferrooxidans*	20	Lab Made– 1.8% sulphur	Yes	20	*NS*	*NT*	16%		
Li et al (12)	*At. ferrooxidans*	30	Factory sourced	Yes	50	*NS*			4.69 to 7.43% (58%)	Decrease
Romine & Romine (11)	*At. thiooxidans* ATCC 15494	7	Factory– 1.8% sulphur	No	*NS*	*NS*	*NS*	3%		
Li et al (43)	*Thiobacillus* sp.	20	Factory sourced	Yes	*NS*	*NS*			4.69 to 7.63% (63%)	*NT*
Romine & Romine (11)	*Sulfolobus acidocaldarius* ATCC 33909	7	Factory – 1.8% sulphur	No	*NS*	*NS*	*NS*	13%		
Romine & Romine (11)	*At. ferrooxidans* ATCC 13661 and *At. thiooxidans* ATCC 15494	7	Factory – 1.8% sulphur	No	*NS*	*NS*	*NS*	10%		
Raghavan & Torma (20,21)	*At. ferrooxidans* and *At. thiooxidans*	6	Factory - 15% sulphur	No	43	Yes	33 ppm	350 ppm		

* NT: not tested, NS: not specified, ~: approximation from data presented

Table 2.1 continued

Reference	Microbe	Time (days)	Type of GTR	Ethanol Leached	GTR Loading (g/l)	Control GTR	Media Control	Microbe	Sol Fraction	Crosslink Density
Heterotrophs										
Christiansson et al (18)	TH2 Lund Isolate	20	Spent Tyre Rubber – 1.6% sulphur	No	50	Yes	0.6%	1.6%		
Kim and Park (52)	*Thiobacillus perometabollis*	30	Spent Tyre Rubber – 1.33% sulphur	No	20	*NS*	8.3%	34%		
Jiang et al (41)	*Sphingomonas* sp.	10	Lab Made: SBR	Yes	25	*NS*			4.3% to 7.3% (70%)	*NT*
Li et al (26)	*Sphingomonas* sp.	20	Factory sourced	Yes	25	*NS*			4.69 to 8.68% (85%)	Decrease
Hu et al (34)	*Sphingomonas* sp.	20	Factory sourced	Yes	25	Yes			3.48% increase (~74%)	~4% decrease
Hu et al (34)	*Sphingomonas* sp.	20	Factory sourced, pre-soaked in Tween 20	Yes	25	Yes			5.44 sol fraction increase (~115%)	~8% decrease
Cui et al (42)	*Sphingomonas* sp.	10	Factory sourced	Yes	NS	Yes			*NT*	~7% decrease
Hu et al (27)	*G. amicalisa*	20	Lab Made: NR	Yes	50	Yes			~1.5 to 3% increase (100%)	~13% decrease
Hu et al (27)	*G. amicalisa*	20	Lab Made: SBR	Yes	50	Yes			~4.75% no change vis	~22% decrease
Cui et al (42)	*G. amicalisa*	10	Factory sourced	Yes	NS	Yes			*NT*	~7% decrease
Cui et al (42)	*Sphingomonas* sp. and *G. amicalisa*	10	Factory sourced	Yes	NS	Yes			*NT*	~10% decrease

* NT: not tested, NS: not specified, ~: approximation from data presented

Table 2-3: Summary of the effect of microbes on (a) GTR surface properties and (b) the effect of including microbially treated GTR on the mechanical properties of newly compounded tyre rubber compared to that of untreated GTR

Paper	Microbe	Time (days)	GTR Type	Ethanol Leached	GTR (g/l)	Control GTR	(a) Surface Properties		(b) Properties of compounded tyre rubber		
							C-Bond Breakage	Bonds at Surface	Compounding Rubber	Mechanical Properties Up	Mechanical Properties Down
Autotrophs											
Romine and Romine (11)	*Sulfolobus acidocaldarius* ATCC 33909	3	Factory sourced	No	*NS*	Untreated	*NT*	Sulphur oxidised	SBR	Elastic modulus (15%)	
Romine and Romine (11)	*At. ferrooxidans* ATCC 13661 and *At. thiooxidans* ATCC 15494	3	Factory sourced	No	*NS*	Untreated	*NT*	*NT*	SBR		Elastic modulus
Li et al (43)	*Thiobacillus* sp.	20	Factory sourced	Yes	*NS*	Untreated	*NT*	S-C, S-S bond breakage, sulphur oxidised	NR	Tensile strength, Tear strength, Elongation at break	Crosslink density
Jiang et al (24)	*Acidithiobacillus ferrooxidans*	20	Lab Made: NR	Yes	20	Untreated	No	S-C, S-S bond breakage, sulphur oxidised	SBR 1502	Tensile strength, tear strength, elongation at break	Shore A hardness , crosslink density
Li et al (12)	*Acidithiobacillus ferrooxidans*	30	Factory sourced	Yes	50	Untreated	No	O increase, S decrease	NR	Elongation at break, tensile strength	Crosslink density, Shore A hardness
Li et al (23)	*Acidithiobacillus ferrooxidans* YT-1	20	Factory sourced	Yes	50	Untreated	*NT*	*NT*	SBR	Elongation at break, tensile strength	

* NT: not tested, S: sulphur, C: Carbon, O: oxygen

Table 2.3 continued

Paper	Microbe	Time (days)	GTR Type	Ethanol Leached	GTR (g/l)	Control GTR	C-Bond Breakage	Bonds at Surface	Compound-ing Rubber	Mechanical Properties Up	Mechanical Properties Down
Heterotrophs											
Kim and Park (52)	*Thiobacillus perometabollis*	30	Spent Tyre Rubber	No	20	Untreated	*NT*	*NT*	NR	Elongation at break, tensile strength	Crosslink density
Bredberg et al (13)	*Pyrococcus furiosus* DSM 3538	10	Spent GTR	Yes	50	Untreated	*NT*	*NT*	NR		Crosslink density
Jiang et al (41)	*Sphingomonas* sp.	10	Lab Made: SBR	Yes	25	Untreated	Yes	S-C, S-S bonds broken	NR	Elongation at break, tensile strength	Crosslink density, Shore A hardness, Tear strength
Li et al (26)	*Sphingomonas* sp.	20	Factory sourced	Yes	25	Untreated	Yes	S decrease, O increase	SBR	Elongation at break, tensile strength	Crosslink density
Hu et al (34)	*Sphingomonas* sp.	20	Factory sourced	Yes	25	Media Leached	*NT*	O Increase, S decrease. S-S, S-C bonds broken, S-O formed.	SBR	Elongation at break, tensile strength	
Hu et al (27)	*Gordonia amicalisa*	20	Lab Made: NR	Yes	50	Media Leached	Yes	O increase, S unchanged	*NT*	*NT*	*NT*
Hu et al (27)	*Gordonia amicalisa*	20	Lab Made: SBR	Yes	50	Media Leached	No	S decrease, C increase	*NT*	*NT*	*NT*
Cui et al (42)	*Sphingomonas* sp. & *Gordonia amicalisa*	10	Factory sourced	Yes	NS	Media Leached	*NT*	S decrease, O increase	SBR	Elongation at break, tensile strength	Shore A hardness

* NT: not tested, S: sulphur, C: Carbon, O: oxygen

In the final test to determine effectiveness of devulcanisation treatment, the mechanical properties of tyre rubber compounded using a portion of microbially treated GTR are compared to tyre rubber compound containing an equal quantity of untreated GTR. Compared to tyre rubber compounded with untreated GTR, compounds containing microbially treated GTR commonly exhibit an increase in tensile strength, elongation at break, a decrease in Shore A hardness and crosslink density (Table 2-3). Improved bonding between the treated GTR particles and rubber compound has also been observed using SEM imaging (12,24,26,41,43). Tear strength was improved for GTR treated with the autotroph *Thiobacillus* sp. (43), but was decreased for *Sphingomonas* sp. treated GTR (41), indicating that the *Thiobacillus* sp. produced higher quality treated GTR. Mechanical properties are the most important performance parameters to measure the quality of the GTR, and literature indicates rubber compounds containing treated GTR outperform compounds containing untreated GTR. As a result, microbial devulcanisation has the potential to increase the maximum allowable inclusion of reclaimed GTR.

The extensive testing and devulcanisation characterisation of the microbe *At. ferrooxidans* makes it a suitable microbe on which to base a new devulcanisation study. Although all the strains of *At. ferrooxidans* investigated showed good devulcanisation performance and toxin resistance, the type strain, DSMZ 14882, is not among those tested to date. Consequently not all of the strains are prioritised for storage in culture banks, and some of the previously tested strains are no longer available.

2.4 Acidophiles: Investigation of the potential of a mesophilic biomining consortium to achieve devulcanisation

Acidithiobacillus ferrooxidans has been identified as an unusually toxin-resistant, mesophilic devulcaniser capable of devulcanising untreated GTR. Furthermore, the extent of devulcanisation can be improved by co-culturing *At. ferrooxidans* with *At. thiooxidans*, suggesting that further investigation into mixed cultures of acidophiles should be performed. However, no studies have been conducted on the devulcanisation performance of complex biomining consortia, which are already used in industrial acidophilic processes. This section investigates whether a complex biomining consortium cultured at 30°C on 0K elemental sulphur medium could further improve devulcanisation performance. The ecology sub-section investigates the type of organisms likely to occur in a sulphur maintained consortium and how they interact with one another to improve growth performance. The metabolism sub-section gives an overview of the most relevant sulphur, iron and carbon metabolisms of acidophiles to assess their possible effects on GTR. Lastly, the toxin resistance of acidophiles is investigated, providing insight into the possible effect of toxins present in the GTR on acidophilic consortia.

2.4.1 Ecology

Acidophilic ecosystems are shaped by two major factors: the interaction of members of the microbial population with the environment, and the interaction of the microbes with one another. In natural environments, autotrophic acidophiles aid dissolution of sulphide rock and maintain the acidic environment by producing sulphuric acid (54,55). The heterotrophic microorganisms scavenge the organic carbon molecules produced by the autotrophs (53). Since organic molecules are toxic to the autotrophs in high concentrations, the heterotrophic organisms are able to improve growth performance of the autotrophs by ensuring the concentration of organic by-products remains low (56). Because the mesophilic biomining consortium used was maintained on elemental sulphur at 30°C with pH 1 to pH 1.3, only microorganisms capable of growth under these conditions are considered. Mesophilic biomining cultures are typically dominated by microbes with temperature optima in the range of 20 to 40°C, although they may contain some moderate thermophiles growing under sub-optimum conditions. For this reason, both mesophiles and moderate thermophiles are discussed. A summary of the mesophilic and moderately thermophilic organisms and their growth conditions can be seen in Table A-1 and Table A-2, Appendix A.

The autotrophic component of the biomining culture is likely to be dominated by the sulphur oxidising prokaryotes *At. ferrooxidans, At. thiooxidans* and *At. caldus,* two of which have already been proven capable of microbial devulcanisation (11,12,18,23,24). *At. ferrooxidans, At. thiooxidans* and *At. caldus* are the only sulphur oxidising autotrophs reported to be capable of growth below pH 1.3 in the required temperature range. *At. ferrooxidans* and *At. thiooxidans* are most likely to dominate growth at 30 °C, since their optimums lie between 25 and 30 °C and 28 and 30 °C, respectively (Table A-1). At 45°C the optimum growth temperature of *At. caldus* is much higher, however it is also known to occur in significant concentrations in mesophilic cultures (53). Although a number of obligate ferrous iron oxidising microbes can occur under the stipulated pH and temperature conditions, the absence of ferrous iron in the medium would limit their occurrence.

The prokaryotic eubacteria *Acidiphilium cryptum* and *Acidiphilium symbioticum* are likely to dominate the heterotrophic component of the biomining consortium at the specified temperature and pH (Table A-2). However, the remaining members of *Acidiphilium* sp., *Acidocella* sp. and *Alicyclobacillus* sp. are unlikely to occur at such low pH (Table A-2). Although mesophilic consortia are usually dominated by rod-shaped gram negative eubacteria (56), they may include some moderately thermophilic heterotrophic archaea, such as *Picrophilus* sp. and *Thermoplasma* sp. (Table A-2). Because *Picrophilus* sp. and *Thermoplasma* sp. are particularly tolerant to extreme acidity (57), they may be more dominant in mixed cultures with a low pH, despite growth temperatures being well below their optimum. The interaction between acidophilic heterotrophs and the organic carbon in GTR has not been reported to date.

Acidophilic eukaryotic organisms have not been as extensively characterised as prokaryotic organisms, and most studies have researched the ecology of acid-mine drainage and naturally occurring acidic rivers (56,58–

60). Although eukaryotic acidophiles can be present in biomining consortium, very little information is available on the typical eukaryotic organisms present, and no information specific to GTR devulcanisation is available. Fungi, yeast, and protozoa have all been found capable of growth below pH 3 (58). Yeast found in acid mine drainage include *Rhodotorula* spp. and *Candida cryptococcus* (56). Some of the most acid-tolerant organisms reported to date are the obligately acidophilic filamentous fungi *Acontium cylatium, Trichosporon cerebriae* and *Cephalosprium* sp., which were found capable of growing near a pH of 0 (56,61). Protozoa, including *Eutropia* spp., *Cinetochilium* sp. and *Vahlkampfia* sp., have also been observed in acidophilic ecosystem and are predators of acidophilic bacteria (56).

2.4.2 Metabolism

The metabolism of the microbes most likely to occur in a biomining consortium is investigated to assess their possible effect on GTR properties. The sulphur oxidation mechanism of *At. ferrooxidans*, *At. caldus* and *At. thiooxidans* is investigated to improve understanding of the metabolic mechanisms used to oxidise the sulphur species, such as those present in GTR. Since iron can leach from GTR (38), and *At. ferrooxidans* oxidises ferrous iron preferentially to sulphur (62), the oxidative iron metabolism of *At. ferrooxidans* is also included. A brief overview of the carbon metabolism of heterotrophic acidophiles, and the known forms of organic molecules metabolised is included to determine their possible effect on the GTR polymer properties.

2.4.2.1 Oxidative Sulphur Metabolism

The oxidative sulphur metabolism of acidophilic autotrophs is still relatively poorly understood, although inorganic sulphur compounds are thought to be fully oxidised according to the mechanism presented in Figure 2-7 (53). Neither the sulphur oxidising (sox) pathway nor the sulphur oxygenase reductase (sor) pathway, used by bacterial and archaeal neutrophiles (63,64), can be used to fully describe the sulphur oxidative metabolism of acidophilic bacteria. Genetic studies suggest that *At. ferrooxidans* does not contain any of the sox or sor genes, while *At. caldus* and *At. thiooxidans* contain parts of both (53,64). The sulphur oxidative metabolism of *At. ferrooxidans* has been the most extensively characterised, followed by *At. caldus.* Relatively little information on the sulphur metabolism of *At. thiooxidans* is available. Although the sulphur oxidative metabolic pathway varies between acidophilic sulphur oxidisers, elements of the pathway appear to be common across the microbes under consideration.

A number of similarities across the oxidative metabolism of the acidophiles *At. ferrooxidans, At. caldus* and *At. thiooxidans* can be observed. The most complete models of oxidative sulphur metabolism, based on genetic information, are presented for *At. ferrooxidans* (Figure 2-8) and *At. caldus* (Figure 2-9). Genetic evidence suggests that both *At. ferrooxidans* and *At. caldus* use the enzymes tetrathionate hydrolase (tetH), sulphur quinone reductase (SQR) and heterodisulphide reductase (HDR) to metabolise tetrathionate, hydrogen sulphide, and elemental sulphur, respectively (63–65). Although the sulphur oxidative metabolism of *At. thiooxidans* has not yet been fully characterised, genetic sequencing of three different strains (66–68)

has found evidence of genes capable of coding SQR, tetH, and HDR, the same enzymes common to *At. ferrooxidans* and *At. caldus.* Furthermore, the glutathione transport mechanism (Figure 2-10) which assists elemental sulphur oxidation has been found to be common across all three microbes (64,65).

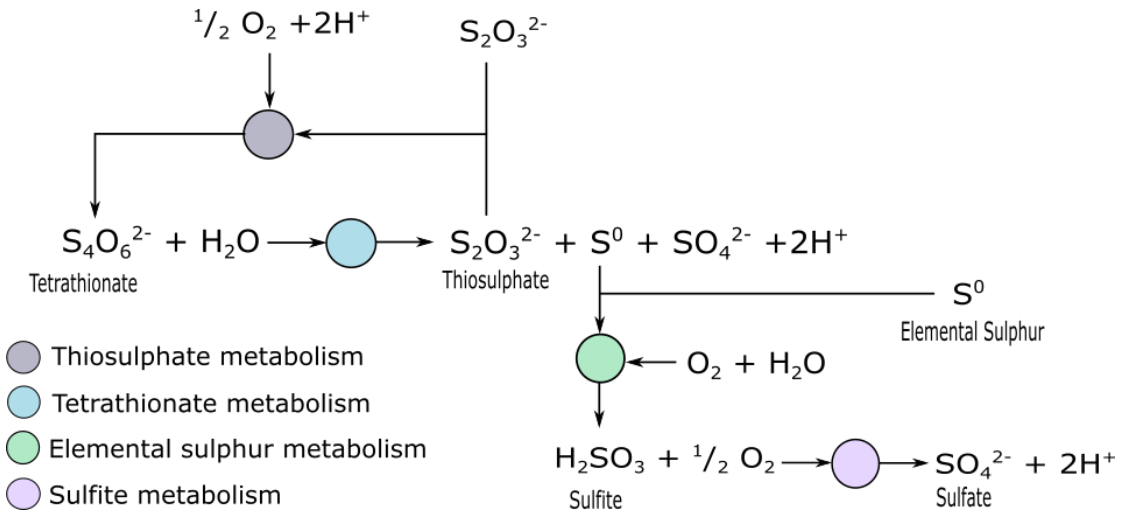

Figure 2-7: Overview of mechanisms of sulphur oxidation, adapted from literature (53)

However, differences in the remaining elements of the sulphur metabolisms suggest that *At. thiooxidans* is more similar to *At. caldus* than to *At. ferrooxidans.* Thiosulphate oxidation in *At. ferrooxidans* is catalysed by the enzyme thiosulphate quinone reductase (TQR). Conversely, evidence of sox gene clusters has been observed in both *At. caldus* (64) and *At. thioxidans* (66–68), suggesting the use of a sox complex to oxidise thiosulphate in these microbes. Furthermore, studies of *At. caldus* (64) and two strains of *At. thiooxidans* (66,68) found evidence of sor cluster genes, which would be capable of oxidising periplasmic elemental sulphur produced by the sox oxidative mechanism (Figure 2-9). Since both *At. ferrooxidans* and *At. thiooxidans* have been shown capable of ground tyre rubber (GTR) devulcanisation, and the sulphur oxidising metabolisms of *At. thiooxidans, At. ferroxidans*, and *At. caldus* are very similar, *At. caldus* would be a promising devulcanisation candidate to investigate. As such, the *At. caldus* present in a biomining consortium is likely to contribute to the devulcanisation of GTR.

Limited information regarding the specific metabolic pathway used by acidophiles to oxidise the sulphide bonds found in vulcanised rubber is available in literature. Most studies consider compounds found during mineral dissolution and do not consider organic molecules containing polysulphidic bonds, as found in GTR. However, based on the proposed mechanism for *At. ferrooxidans* (Figure 2-8), it could be tentatively suggested glutathione assisted HDR, or a similar enzyme, may be involved in the breakage of disulphide bonds at the GTR surface. Furthermore, no studies have been compared the devulcanisation performance of actively growing cells to resting cells. Most devulcanisation studies to date maximise the cell concentration

of an exponentially growing culture under the assumption that it will maximise the sulphur oxidation at the GTR surface, and therefore the devulcanisation performance (12,24,26,34,41).

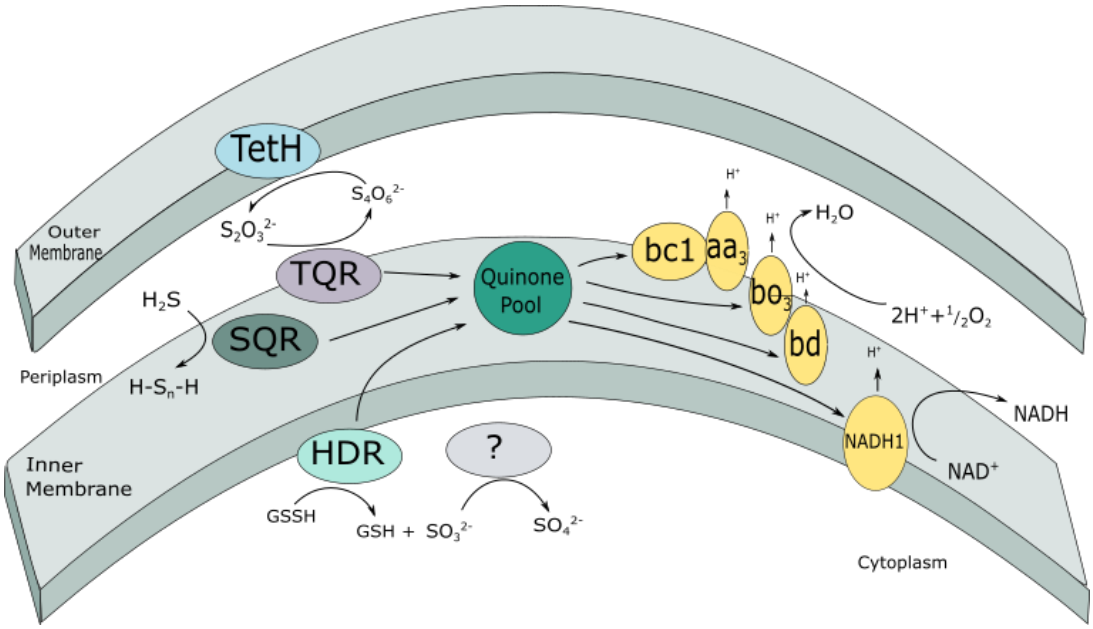

Figure 2-8: Proposed Acidithiobacillus ferrooxidans sulphur oxidative metabolic pathway. Abbreviations are as follows: TetH- Tetrathionate hydrolase, TQR- thiosulphate quinone reductase, SQR-sulphur quinone reductase, HDR – heterodisulphide reductase (sulphane assisted), NADH1 – NADH 1 complex. The pathway for metabolism of sulphite has not yet been fully characterised. Adapted from literature (63,69).

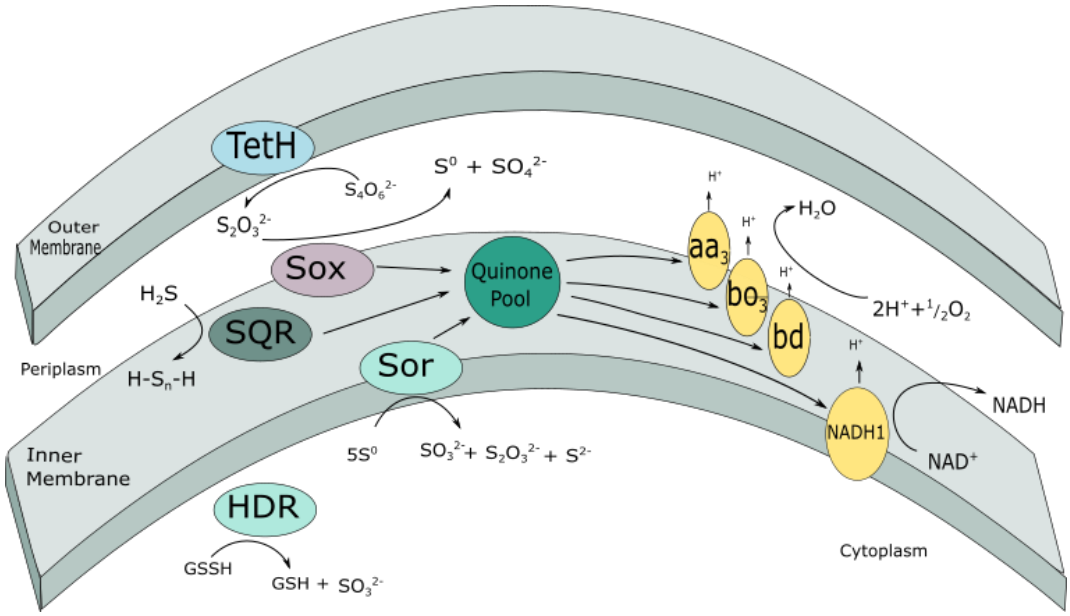

Figure 2-9: Proposed At. caldus sulphur oxidation metabolic pathway. Abbreviations are as follows: TetH – Tetrathionate hydrolase, Sox – sox sulphur oxidising complex, SQR – sulphur quinone reductase, Sor – sulphur oxidising reductase complex, HDR – heterodisulphide reductase (sulphane assisted), NADH1 – NADH 1 complex. Adapted from literature (64)

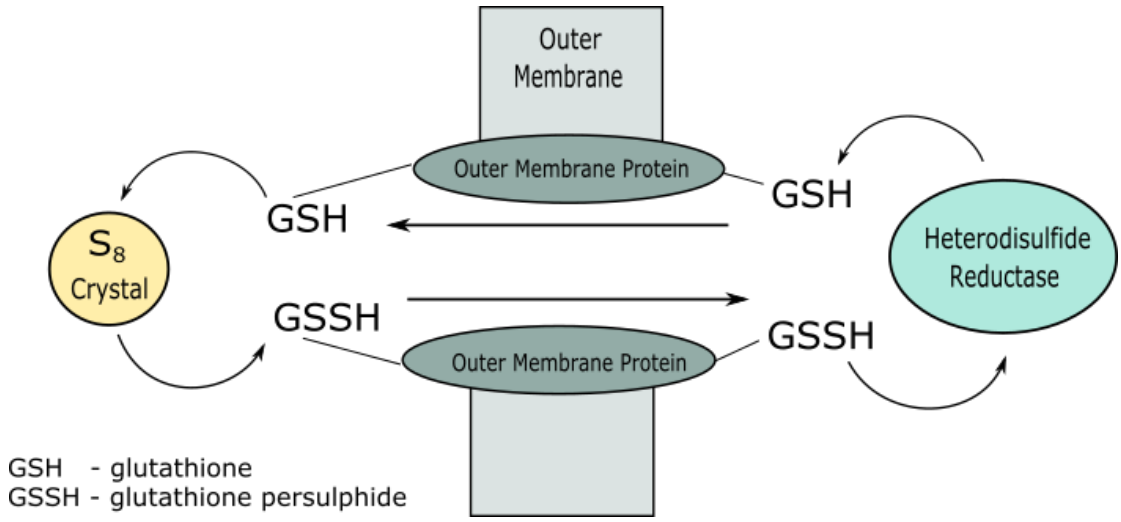

Figure 2-10: Role of membrane attached sulphane in sulphur oxidation metabolism, demonstrated using glutathione, however other sulphanes may be involved. Adapted from literature (63,65)

2.4.2.2 Ferrous Iron

At. ferrooxidans is able to use both ferrous iron and reduced sulphur as a source of energy. Iron has been noted to leach out of GTR (38) and, as such, may provide an alternative energy source to the sulphur during devulcanisation experiments. Since *At. ferrooxidans* is known to oxidise iron preferentially to sulphur (62), high levels of iron during devulcanisation experiments would be undesirable.

Iron oxidation occurs according to Equation 2-1 in which the energy source ferrous iron, Fe^{2+}, is oxidised to ferric iron, Fe^{3+} (55). The consumption of a proton and oxygen, to produce water results in a net decrease in the pH of the solution.

$$Fe^{2+} + H^{+} + 0.25O_2 \xrightarrow{Bacteria} Fe^{3+} + 0.5H_2O$$

Equation 2-1

The ferrous iron oxidative metabolism is considered to be less energetically favourable than the reduced sulphur oxidative metabolism because of the small Gibbs free energy (G') released per mole of iron oxidised and the low reducing potential of the electron released (62) compared to reduced sulphur. The low energy potential of the electron further means that ferrous iron oxidation cannot spontaneously reduce NAD^+ to NAD(P)H, which is required for various metabolic processes (Figure 2-11). Instead, an additional "uphill" electron transport chain (ETC) is required to increase the potential of the electron and facilitate synthesis of NADH in obligately autotrophic acidophiles (Figure 2-11 and Figure 2-12), such as *At. ferrooxidans* (63). Despite the energetic and thermodynamic challenges of ferrous iron oxidation, *At. ferrooxidans* has consistently been observed to consume ferrous iron in preference to reduced inorganic sulphur compounds, or RISCs (62). Literature found that this was most likely due to the rate at which *At. ferrooxidans* is able to metabolise the two different energy sources (62). Although RISCs yield more energetic electrons and a higher Gibbs energy, the metabolic process required is more complex. As a result, the rate at which energy is

produced from RISCs is roughly equivalent to that of ferrous iron (62). The complexity of the sulphur metabolism compared to the iron metabolism is illustrated by comparing Figure 2-8 and Figure 2-12.

Previous devulcanisation studies have not considered the impact of the iron present in the GTR on cell growth. However, some previous devulcanisation studies have used ferrous iron during the initial batch growth phase prior to the addition of GTR (12,23,24). Ferrous iron was found to provide good growth and, because it is soluble, the concentrations can be more easily controlled and monitored than insoluble elemental sulphur. Furthermore, by characterising the growth and ferrous iron concentration, the GTR could be added after the ferrous iron had been depleted, ensuring that it was not a competing energy source during devulcanisation.

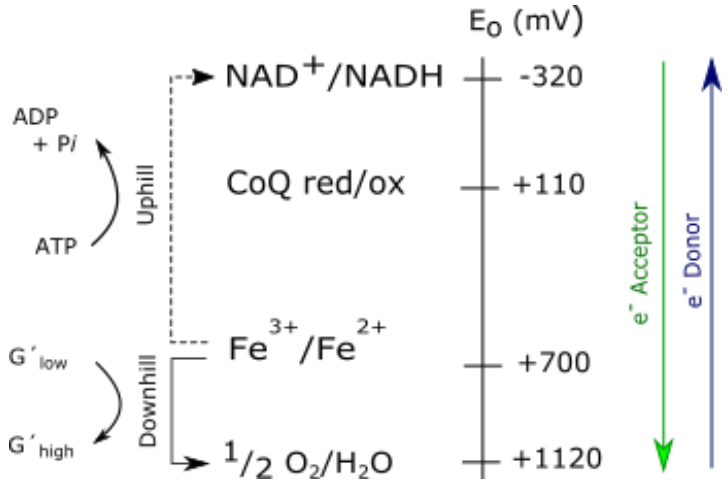

Figure 2-11: Redox reactions involved in iron oxidation electron transport chain of At. ferrooxidans. Adapted from literature (53,62,108–110)

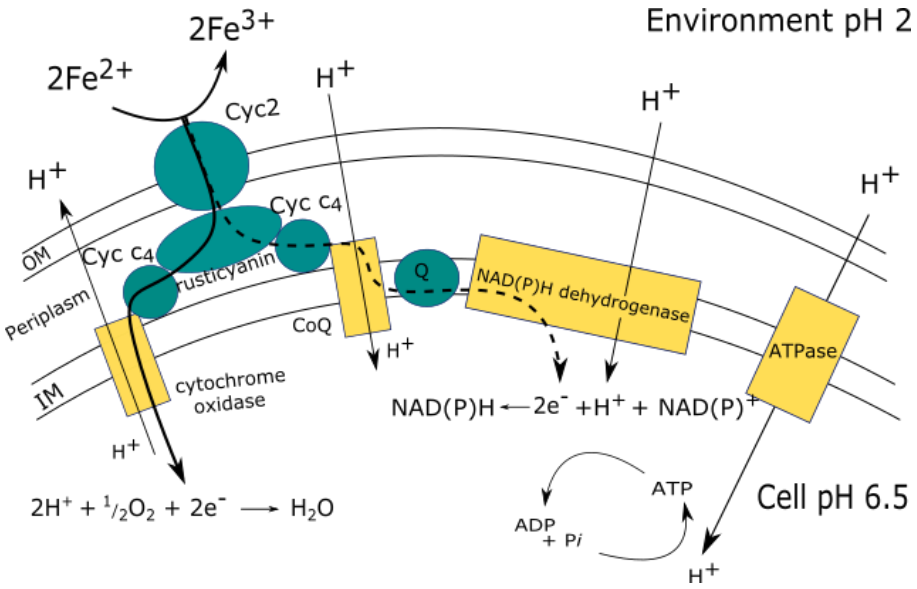

Figure 2-12: Model of ferrous iron oxidation in At. ferrooxidans including the downhill electron transport chain (ETC) which provides energy by reducing oxygen, and the uphill ETC which is used to synthesise NAD(P)H. The downhill ETC is indicated by a solid arrow, while the uphill ETC is indicated by dashed arrow. Adapted from literature (53,62,108,109).

2.4.2.3 Carbon

Autotrophic prokaryotes use carbon dioxide as their sole source of carbon, and act as the primary producers of the organic carbons utilised by heterotrophs and facultative heterotrophs in many acidic environments (53,70). It has been proposed that most autotrophic acidophiles use the Calvin Benson Bassham (CBB) cycle (53). As a result they are unable to metabolise organic forms of carbon during aerobic growth, and will not cause polymer degradation to the GTR during devulcanisation experiments.

Limited research has been conducted on the organic carbon metabolism of acidophiles, however evidence of the pentose phosphate and Entner-Doudroff pathways has been found in the gram positive eubacteria *Acidiphilium cryptum* (53). Heterotrophic acidophiles are known to metabolise a wide variety of simple organic carbons and, in some cases, more complex aromatic compounds. As such, it is possible that some acidophilic heterotrophs would be capable of metabolising some of the organic chemicals which leach out of GTR. Organic compounds commonly metabolised by acidophilic heterotrophs include monosaccharides, dicarboxylic acid, tricarboxylic acid, amino acids, fructose, glycerol, simple alcohols, organic acids and hexadecanoic acids. Additionally, *Acidocella aromatic* has been found to be capable of metabolising the aromatic compounds phenol, benzoic acid, and naphthalene and some gram positive acidophiles have been observed to hydrolyse polysaccharides (53). Although heterotrophic acidophiles may be able to metabolise carbon in the rubber polymer chains, the simpler autotrophic cell by-products or shorter chain chemical additives are likely to be used preferentially to the complex carbon chains in the rubber polymer.

2.4.3 Toxin Resistance in Consortia

The toxins that are most likely to occur in GTR devulcanisation experiments with biomining consortia include simple organic molecules formed by the autotrophs (25), the organic chemical additives present in the GTR, and zinc compounds in the GTR (12,13,22). Heterotrophic acidophiles consume the organic by-products of autotrophs and are known to metabolise some aromatic organic compounds (25,56), making their inclusion a promising method to reduce the concentration of organic toxins. Acidophiles are also often unusually heavy metal resistant, since their natural environments often contain high concentrations of dissolved metals (71).

The improved sulphide dissolution observed in biomining processes using consortia, compared to pure cultures, has been attributed to the presence of heterotrophic organisms (54). It has been suggested that the heterotrophic organisms consume organic acids produced by the autotrophic acidophiles, which are toxic to the autotrophs and can cause growth inhibition in high concentrations (53). By feeding on the organic acids the heterotrophs ensure that the concentration of organic toxins remains low and, therefore, does not inhibit the growth of the autotrophs (56). Furthermore, some heterotrophic acidophiles have been found to metabolise more short-chain organic compounds, including aromatic carbon compounds (53). Since the toxic chemical additives known to leach from GTR include similar organic compounds, heterotrophs present in

biomining consortium may be capable of metabolising some of the chemical additives, reducing their concentration.

Resistance to heavy metals, and especially, Zn^{2+} is important in the context of tyre devulcanisation, as GTR can contain up to 2 weight% zinc oxide (9), which is known to leach out during incubation (13,22,38). Although Zn^{2+} cannot undergo redox reactions under metabolic conditions, and as a result does not interfere with the transport chain, it can complex with components in the cell and has been observed to competitively inhibit enzymes (71). Due to the high concentration of heavy metals that occur in the natural environment of acidophiles, they are unusually heavy metal resistant (71). However, the extent of toxin resistance can also depend on the microbial strain, the metabolism being used by the microbe, and the type of growth. *At. ferrooxidans* is known to be particularly Zn^{2+} tolerant, with some strains capable of surviving concentrations of 1071 mM (71). Interestingly, *At. ferrooxidans* has been shown to be more tolerant of high concentrations of Zn^{2+} when using an iron metabolism than a RISC metabolism, suggesting that Zn^{2+} may have a greater effect on pathways in the RISC oxidising metabolism (57,72). Good resistance to Zn^{2+} has also been demonstrated for *At. ferrooxidans* strains tested in GTR devulcanisation studies (12,22). The Zn^{2+} resistance of *Acidiphilium cryptum* and *Acidiphilium symbioticum* was reasonable, with maximum tolerances of 125 mM and 150 mM, respectively (71). *At. caldus* has been reported to have a maximum tolerance of 200 mM Zn^{2+} (73), while some strains of *At. thiooxidans* have been reported to grow uninhibited with concentrations up to 250 mM of Zn^{2+} (74). Additionally, cells growing in attached biofilms have been observed to exhibit better toxin resistance than planktonic cells (71), but most devulcanisation studies to date have investigated the effects of planktonic cultures.

2.5 Conclusions

Microbial devulcanisation has been identified as a promising method to achieve selective breakage of vulcanising sulphur bonds at the surface of ground tyre rubber (GTR). The sulphur oxidative mechanism proposed in literature suggests that sulphur oxidative devulcanisation leads to the removal of sulphur (11,14). As such, microbial devulcanisation can be detected using both desulphurisation measures and industrial measures, such as change in sol fraction and crosslink density. However, care should be taken when using change in sol fraction as the exclusive measure for heterotrophic organisms, since it may also indicate polymer degradation (27,32,33).

Quantification and characterisation of microbial devulcanisation was found to be challenging because devulcanisation only occurs in the outermost 2 to 6 µm of the GTR particle. As such, the total change in GTR properties can be very small, and is difficult to measure accurately. Previous studies aimed to maximise the total change in GTR properties by maximising the concentration of newly cultured cells in contact with the GTR during devulcanisation. The most recent studies achieved this by including a preliminary batch culturing

step prior to the addition of GTR, while ensuring that no energy sources were present which could compete with the sulphur in the GTR.

At. ferrooxidans and *At. thiooxidans* have been shown to be unusually resistant to the toxins in GTR, both having been shown to devulcanise untreated, industrial GTR made from waste tyres. However, the cell growth performance of only one acidophile, *At. ferrooxidans,* has been characterised during devulcanisation, and only in the presence of devulcanised GTR. Toxins present in GTR that have been demonstrated to negatively impact microbes in past studies included zinc salts, zinc oxides, organic chemical additives and SBR. Some strains of *At. ferrooxidans* have demonstrated unusually high zinc resistance, both in biomining and devulcanisation studies (22,23,71). Other acidophiles identified as likely to occur in a mesophilic biomining consortium *At. thiooxidans, At. caldus*, *Acidiphilium cryptum,* and *Acidiphilium symbioticum*, also exhibit some zinc resistance. Additionally, the presence of heterotrophs will ensure that the concentration of organic growth by-products remains low, preventing growth inhibition of the sulphur oxidising autotrophs responsible for devulcanisation (53,69). These heterotrophic organisms may be capable of metabolising some more complex organic compounds (53), including some of the organic chemical additives present in GTR, reducing their toxicity to the consortium. Since SBR cannot be removed from the GTR and is toxic to some microbes tested (22), GTR containing limited amounts of SBR should be selected.

The type culture of *At. ferrooxidans,* DSMZ 14882, has not been tested for its devulcanisation performance. However, previous devulcanisation studies have shown good devulcanisation performance across a number of strains of *At. ferrooxidans*. Devulcanisation performance has been measured in terms of both desulphurisation, of which it achieved the highest reliable percentage removal of sulphur (24), and change in sol fraction. Although the change in sol fraction was lower than the other organisms characterised in this way, it was the only obligate autotroph, and therefore the only microbe to which a change in sol could be attributed purely to devulcanisation. The heterotrophic microorganisms tested were shown to cause polymer degradation at the surface, which would have increased the change in sol fraction. Although the devulcanisation performance of biomining consortia has not been reported in literature, findings suggest that the complex ecosystem of autotrophic sulphur oxidisers and heterotrophs may improve devulcanise performance. Mixed cultures of the autotrophs *At. ferrooxidans* and *At. thiooxidans*, both expected to occur in a mesophilic biomining consortium, have been found to improve devulcanisation performance compared to pure cultures. The heterotrophs likely to be present, *A. cryptum* and *A. symbioticum,* usually metabolise the simple organic compounds produced by autotrophic microbes, and as such are unlikely to cause carbon degradation of the complex rubber polymer in the GTR. Furthermore, it is hypothesised that the inclusion of heterotrophs could further improve devulcanisation performance by limiting toxic growth products, and potentially metabolising organic compounds which leach from the GTR.

3 Research Methodology

3.1 Research Approach

At. ferrooxidans was identified as an acidophilic microbe with proven devulcanisation capabilities and toxin resistance across a wide range of strains, which has been extensively characterised in literature. Because the strains tested to date are non-type cultures and are not always easily available, the type culture *At. ferrooxidans* DSMZ 14882 was selected for this study, and obtained from the *Leibniz-Institut DSMZ-Deutsch Sammlung von Mikroorganismen und Zellkulturen GmbH* (DSMZ). Sulphur oxidising, mesophilic biomining consortia were identified as promising cultures which have not yet been tested for devulcanisation performance. A mesophilic biomining consortium maintained at 30 °C in 0K elemental sulphur medium was obtained from the Centre for Bioprocess Engineering Research (CeBER) at the University of Cape Town (UCT) for testing.

Industrial ground tyre rubber (GTR) manufactured from waste truck tyres was selected for the devulcanisation testing due to the low content of styrene butadiene rubber, SBR, (9,75) and the reduced toxicity of industrial GTR reported in literature (22). The smallest available particle fraction, 40 mesh, was selected and the GTR was not detoxified (referred to as untreated GTR) to minimise material property changes and in order to determine the performance of the microbes on the raw material. A GTR concentration of 50 g/l was selected from literature for devulcanisation experiments (Table 2-2).

Preliminary culture preparation steps were developed for both *At. ferrooxidans* DSMZ 14882 and the biomining consortium, which aimed to ensure sufficient biomass concentration of sulphur oxidising organisms as well as conditions conducive to cell growth at the point of the addition of GTR (Chapter 4). Once achieved, the devulcanisation and growth performance of both *At. ferrooxidans* DSMZ 14882 and the biomining consortium was characterised (Chapter 5).

3.2 Scope for Novelty

Three potential areas of novelty were identified in the literature review. The first is the development of active, sulphur oxidising cultures of *At. ferrooxidans* DSMZ 14882 and the biomining consortium for the purpose of devulcanisation. The second is the testing of the *At. ferrooxidans* type culture, DSMZ 14882, devulcanisation performance. Despite numerous studies on different strains of *At. ferrooxidans*, it has not yet been proven that the ability to devulcanise GTR extends to the type species. The third potential area of novelty is the testing of a complex mesophilic consortium of acidophiles sourced from biomining application, as to the author's knowledge no consortium of similar complexity has been reported on in literature.

3.3 Research Questions

1. How can the culture conditions of *At. ferrooxidans* DSMZ 14882 and a mesophilic biomining consortium be altered to maximise devulcanisation of untreated GTR?
2. How does untreated, industrially sourced GTR affect the growth performance of *At. ferrooxidans* DSMZ 14882 and a mesophilic biomining consortium during devulcanisation experiments?
3. How does microbial devulcanisation using *At. ferrooxidans* DSMZ 14882 and the mesophilic biomining consortium affect the properties of industrially sourced, untreated GTR?

3.4 Objectives

The research objectives were divided into culture preparation and devulcanisation performance. The preliminary objectives were investigated in Chapter 4, while the main objectives are presented in Chapter 5.

(1) Culture Preparation
   a. Develop a culture preparation step which ensures sufficient biomass concentration and growth conditions conducive to growth on the sulphur in GTR for *At. ferrooxidans* DSMZ 14882 and a 30 °C acidophilic consortium.
   b. Confirm the viability of the cultures produced by the culture preparation step by characterising *At. ferrooxidans* growth on ferrous iron, elemental sulphur and tetrathionate and consortium growth on elemental sulphur and tetrathionate.

(2) Devulcanisation Performance
   a. Develop analytical techniques required for devulcanisation performance characterisation.
   b. Perform microbial devulcanisation experiments with *At. ferrooxidans* and the consortium using untreated industrial GTR.
   c. Investigate the impact of acetone leaching of the GTR on growth performance of *At. ferrooxidans.*
   d. Assess performance of both cultures in terms of microbial growth and devulcanisation performance.

3.5 Project Scope and Limitations

Research was limited to laboratory scale, batch microbial devulcanisation experiments using industrially manufactured GTR. The small quantities of GTR treated did not allow for a study on the effect of microbial devulcanisation on the physical properties of compounded tyre rubber containing treated and untreated GTR. Consortium studies were limited to a 30 °C elemental sulphur maintained acidophilic biomining consortium of unknown microbial population, which had not been selected to improve toxin resistance. Microbial population studies and genetic identification methods were not pursued as they required in-house method development and were not within the scope of the project.

4 Development of a Culture Preparation Step to Facilitate Devulcanisation of Untreated Ground Tyre Rubber using *At. ferrooxidans* DSMZ 14882 and a Mesophilic Biomining Consortium

4.1 Context

The culture preparation step is developed to ensure that there is sufficient biomass at the point of addition of untreated ground tyre rubber (GTR) to the culture, and that the conditions are conducive to utilisation of the sulphur in GTR. In doing so, this section answers the first research question, "How can the process conditions used for *At. ferrooxidans* DSMZ 14882 and a mesophilic biomining consortium be altered to maximise devulcanisation of untreated GTR?", and fulfils the first group of project objectives, the devulcanisation process development.

4.2 Abstract

Microbial devulcanisation studies of ground tyre rubber (GTR) in literature have used a preliminary culturing step to increase the extent of devulcanisation achieved by *At. ferrooxidans* (12,24). Maximising the extent of devulcanisation is not only important to improve performance, but to ensure that changes in GTR properties are significant enough to be quantified using the analytical techniques available. The preliminary growth phase is used to ensure sufficient biomass concentrations and good growth conditions at the point of addition of GTR, while simultaneously limiting the concentration of energy sources which could be used in preference to the sulphur in GTR. Batch growth of *At. ferrooxidans* DSMZ 14882 in modified DSMZ medium 882 with an initial pH 1.8 and 1.12 g/l of ferrous iron achieved a biomass concentration of 7.25 x 10^7 cells/ml after 36 hours of incubation. It provided the best compromise between maximising cell concentration and ensuring the final pH remained in the desired range. The culture was confirmed capable of sulphur and iron oxidation subsequent to the preliminary growth phase, although a five hour lag was observed when switching to the sulphur metabolism. The biomass concentration of biomining consortium maintained on elemental sulphur in bioreactors was acceptably high without additional batch growth, at 6 x 10^8 cells/ml. A coarse filtration step was found to remove the entrained elemental sulphur while allowing planktonic sulphur oxidising autotrophs to pass through, hence limiting energy sources which would compete with the sulphur in GTR. The filtered culture demonstrated the ability to utilise sulphur sources for cell growth, achieving a higher biomass yield per mol of sulphur than *At. ferrooxidans* DSMZ 14882, which was attributed to the growth of heterotrophs. The culture preparation stage of *At. ferrooxidans* DSMZ 14882 and the biomining consortium was shown to produce a sulphur oxidising culture with limited competitive energy sources. Hence, if incubated for a

prolonged period with GTR, both cultures are likely to oxidise the sulphur present in GTR, causing devulcanisation.

4.3 Introduction

A major challenge in assessing the devulcanisation performance of microbes is the toxicity of ground tyre rubber (GTR), which can result in cell death and limit microbial performance (22,23).Furthermore, microbial devulcanisation only occurs in the outer 2 to 4 µm of the GTR particles, resulting in a small change in properties which is difficult to accurately quantify (11,34,42). *At. ferrooxidans* DSMZ 14882 and a mesophilic sulphur oxidising acidophilic biomining consortium have been identified as microbes which are likely to be capable of GTR devulcanisation. Both cultures are comparatively toxin resistant, and have the potential to process untreated GTR, eliminating the need for a solvent detoxification step (11,18,22). Furthermore, acidophilic consortia used in biomining have been shown to achieve a greater extent of sulphur oxidation than pure cultures (25). From an analysis of the literature available, it has been hypothesised that these same benefits could be observed in GTR devulcanisation processes (Chapter 2), and have the potential to improve the overall devulcanisation performance.

Previous devulcanisation studies on *At. ferrooxidans* have included a preliminary culture preparation step, which aims to maximise the devulcanisation performance by increasing the biomass concentration and limiting the availability of alternative autotrophic energy sources at the point of GTR addition (12,24). Increasing the biomass aims to maximise the devulcanisation performance by ensuring the highest possible number of cells come into contact with the GTR surface. By limiting the energy sources usually consumed by the microbes, such as inorganic sulphur compounds and ferrous iron, the microbes are forced to consume the organic polysulphidic vulcanising bonds in the GTR, causing oxidative devulcanisation (11,18,24). However, this relies on the assumption that the medium contains the remaining nutrients required for growth, and that the pH is within the growth range.

Neither *At. ferrooxidans* DSMZ 14882 nor the biomining consortium have previously been tested for devulcanisation performance. As such, the development of a preliminary growth stage is necessary to maximise the potential impact of both microorganisms on industrial GTR that has not been detoxified. The culture preparation stage for *At. ferrooxidans* DSMZ 14882 is based on previous batch growth experiments conducted for devulcanisation, which focused on maximising biomass concentration while limiting ferric iron crystallisation at the end of the growth stage (24). However, where only the initial ferrous iron concentration was varied in literature, this study varies both ferrous iron concentration and initial pH. Since high biomass concentrations of the biomining consortium were sustained in the bioreactors which maintained the culture, an additional growth step was unnecessary. As a result, the culture preparation stage for the biomining consortium focused on the removal of entrained elemental sulphur to ensure that the competing energy sources were limited. Because the cell counting method

used could not differentiate between live and dead cells, the sulphur oxidising viability of both cultures subsequent to the culture preparation stage was tested to determine, firstly, whether the culture was active and capable of sulphur oxidation, and hence devulcanisation of GTR, and secondly, whether the concentration of competing energy sources had been limited. Additionally, the pH was monitored to determine whether growth conditions remained within the acceptable range for the duration of the experiments, and whether adequate non-energy source nutrients were present in the medium.

4.4 Materials and Methods

4.4.1 Materials

American Chemical Society (ACS) grade chemicals supplied by Merck, Sigma Aldrich and Riedel de Hahn were used in this study. A 1.7 litre, 1 litre working volume glass reactor with Teflon impellor, glass sparger, built in water jacket and temperature controlled heating bath was commissioned from Glasschem, Stellenbosch, South Africa for the study. All pH measurements were conducted using a Crison GLP 21 pH meter.

4.4.2 Microbial Cultures

Acidithiobacillus ferrooxidans DSMZ 14882 was supplied by the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ), Brunswick, Germany, while a 30 °C acidophilic consortium used in biomining was supplied by the University of Cape Town, South Africa. *At. ferrooxidans* was cultured at 30 °C and 115 rpm in an orbital shaker-incubator in 250ml baffled shake flasks containing 30 ml of DSMZ Media 882 consisting of 132 $(NH_4)_2SO_4$, 53 $MgCl_2$▪$6H_2O$, 27 KH_2PO_4, 147 $CaCl_2$▪$2H_2O$, 20 000 $FeSO_4$▪$7H_2O$, 0.062 $MnCl_2$▪$2H_2O$, 0.068 $ZnCl_2$, 0.064 $CoCl_2$▪$6H_2O$, 0.031 H_3BO_3, 0.01 Na_2MoO_4 and 0.067 mg/l $CuCl_2$▪2H2O in reverse osmosis treated water, adjusted to pH 1.8 using 98% H_2SO_4 and filter sterilised with 0.22 µm membranes. The 30 °C consortium was maintained in the glass bioreactor at 30 °C in 0K media containing 3.00 $(NH_4)_2SO_4$, 0.10 KCl, 0.50 K_2HPO_4, 0.50 $MgSO_4$▪$7H_2O$, 0.145 $Ca(NO_3)_2$▪$4H_2O$, 10.00 S in reverse osmosis treated water adjusted to pH 1.3 using H_2SO_4. When necessary the sulphur was sterilised separately using tyndallisation or radiation while the remaining media was autoclave sterilised for 15 min at 121 °C. Approximately half of the reactor volume was replaced with fresh media every three days and the pH adjusted using 9M KOH.

4.4.3 Preliminary Culturing Stage

4.4.3.1 At. ferrooxidans DSMZ 14882

In *At. ferrooxidans* preliminary culturing, biomass maximisation experiments were conducted in which the initial ferrous iron concentration ($FeSO_4.7H_2O$) and pH of DSMZ Media 882 were varied according to Table 4-1. Sterile 250 ml baffled shake flasks containing 30 ml of the media of interested were inoculated with 1 ml of culture which had been incubated for 48 h (~1.8 to 2.0 x 10^8 cells /ml). The flask was then

incubated at 30 °C and 115 rpm in an orbital shaker-incubator for seven days and cell concentration was determined every 6 to 24 hours under 1000x magnification using a binocular light microscope and a 0.01 mm depth Neubauer Improved Marienfield-Superior counting chamber. Each variation of DSMZ Media 882 was tested in duplicate. The best performing variation of DSMZ Media 882, DSMZ Media 882*, was selected for further experimentation.

Table 4-1: Variations of DSMZ Media 882 investigated for At. ferrooxidans growth

Initial Concentration Fe^{2+} (g/l)	Initial pH			
	1.6	1.8	1.9	2.0
0.56		x		
1.12	x	x	X	x
2.25		x		

4.4.3.2 Biomining Consortium

The required volume of consortium culture was drawn from the bioreactor 24 hours after feeding with fresh media, resulting in a cell concentration of ~5.5 to 6.0 x 10^8 cells/ml and pH ~ 1.2. A portion of the culture was then filtered into a sterile container using Whatman 1 filter paper to remove entrained elemental sulphur. 30 ml of the filtered culture and 30 ml of the unfiltered consortia culture was each placed in a sterile 250 ml baffled shake flask and incubated at 30 °C and 115 rpm in an orbital shaker-incubator. The pH was measured of the flasks and the bioreactor stock culture daily in order to determine stability of the culture and the efficacy of the sulphur removal step. The experiment was conducted once.

4.4.4 Culture viability

4.4.4.1 At. ferrooxidans DSMZ 14882

At. ferrooxidans was cultured in sterile 250 ml baffled shake flasks containing 60 ml of DSMZ Media 882* was inoculated with 2 ml of 48 hour old inoculum and incubated in a rotary shaker-incubator at 30 °C and 115 rpm. After 24 hours of incubation, the culture was supplemented with either ferrous iron, tetrathionate or irradiation sterilised elemental sulphur in concentrations which provided an available electron concentration approximately equal to the initial ferrous iron dose in DSMZ Media 882* (Table 4-2). The selected concentration of elemental sulphur and tetrathionate marginally exceeded the concentration of available sulphur in a 50 g/l of GTR (Theoretical Availability of Sulphur in GTR, Appendix B). Supplementary ferrous iron and tetrathionate were provided using 0.22 µm filter sterilised concentrated solutions of $FeSO_4.7H_2O$ and $K_2S_4O_6$, respectively, and acidified to pH 1.8 with 98% H_2SO_4. A control culture to which no supplementary energy source was added at 24 hours was included for comparison, and all conditions were tested in duplicate. The culture was incubated for a further four days and the cell concentration monitored every 6 to 48 hours depending on growth rate.

Table 4-2: Energy sources supplied in culture viability tests of prepared culture of At. ferrooxidans DSMZ 14882

Electron Source	Final Concentration (g/l)	Final Electron Source Concentration (mmol/l)
Initial Fe^{2+} Dosing	1.12	20.06
Supplemented Fe^{2+}	1.12	20.06
Supplemented S	0.14	4.37
Supplemented Tetrathionate	0.35	1.55

4.4.4.2 Biomining Consortium

Consortium culture was drawn from the bioreactor and filtered as described in the preliminary culturing step (Section 4.4.3). The culture was diluted using autoclave sterilised 0K media from which elemental sulphur was omitted to obtain a cell concentration approximately equivalent to *At. ferrooxidans* after 24 hours of growth in DSMZ Media 882*, ~3 x 10^7 cells/ml. 60 ml of the diluted culture was placed in autoclave sterilised 250 ml baffled shake flasks and then supplemented with either 0.10 g/l radiation sterilised elemental sulphur or 0.35 g/l tetrathionate with a concentrated solution $K_2S_4O_6$ adjusted to pH 1.3 with 98% H_2SO_4, ensuring that the final electron concentration was approximately equal to *At. ferrooxidans* cultures. The flasks were then incubated at in rotary shaker-incubators at 30 °C and 115 rpm for five days or 120 hours and cell counts were conducted every 6 to 24 hours depending on growth rate. The experiment was conducted once.

Table 4-3: Energy sources supplied in culture viability tests of prepared culture of the biomining consortium

Electron Source	Final Concentration (g/l)	Final Concentration (mmol/l)
Initial Sulphur Dosing	~0	~0
Supplemented Sulphur	0.10	3.11
Supplemented Tetrathionate	0.35	1.55

4.5 Results and Discussion

The devulcanisation potential of both *At. ferrooxidans* and the consortium was assessed according to the potential to oxidise sulphur, subsequent to a preliminary culturing stage. The preliminary culturing stage was adjusted to each culture, and developed to attain adequate biomass concentration, limit energy sources which would compete with the sulphur in GTR during devulcanisation experiments, and ensure conditions conducive to growth. The performance of the preliminary growth stage was then determined for various energy sources by determining the sulphur oxidation activity of the resultant culture.

4.5.1 *At. ferrooxidans*

4.5.1.1 Culture Preparation

The biomass maximisation method used for *At. ferrooxidans* DSMZ 14882 was based on work previously conducted on *At. ferrooxidans* sp. in which ferrous iron was used as the energy source (12,24). In this study, both initial ferrous iron concentration and initial pH were varied and performance of the preliminary growth phase assessed, according to the biomass concentration achieved, the final pH of the medium, the depletion of the ferrous iron and the availability of nutrients for further growth.

Medium with an initial ferrous iron concentration of 1.12 g/l and an initial pH of 1.8 was found perform best for *At. ferrooxidans* DSMZ 14882 (Figure 4-1). Although a higher biomass concentration was achieved for an initial concentration of 2.25 g/l of ferrous iron, the increased concentration of iron led to a pH higher than 2.1, and the formation of ferric iron crystals towards the end of the experiment, which would reduce the accuracy of cell counts after the initial growth phase (Table 4-4 and Figure 4-2). Total biomass was found to be maximised at an initial pH 1.8 (Table 4-5), and the maximum pH of 1.95 was well within the pH 1.8 to 2.5 range of optimum growth reported for *At. ferrooxidans* in literature (Table A-1, Appendix A). *At. ferrooxidans* sp., for which the preliminary growth phase was originally developed in devulcanisation literature, was also found to perform best in a medium containing 1.12 g/l ferrous iron, but with an initial pH of 2.5. Although, *At. ferrooxidans* sp. was shown to deplete the iron faster than *At. ferrooxidans* DSMZ 14882, 24 hours in the place of 36 hours, *At. ferrooxidans* sp. achieved a slightly lower maximum cell concentration, 6.8×10^7 cells/ml (12) compared to the 7.25×10^7 cells/ml achieved for *At. ferrooxidans* DSMZ 14882 (Table 4-4). Crystal formation was also described as problematic in medium containing higher concentrations of ferrous iron used for *At. ferrooxidans* sp. (24).

Ferrous iron was observed to be the limiting nutrient for biomass concentration for media with an initial of pH 1.8 (Figure 4-1 a), since variation in the initial ferrous iron concentration was directly proportional maximum biomass concentration. As such, the yield of biomass per gram ferrous iron was constant across the ferrous iron concentrations tested with an initial pH of 1.8 (Table 4-4). Furthermore, because the energy source limited growth under these conditions, the remaining nutrients are unlikely to have been depleted, and would be sufficient for further growth with the addition of a supplementary energy source. The maximum increase in pH was also found to be directly proportional to the number of protons (H^+ ions) required for ferrous iron oxidation according to Equation 2-1, hence corresponding to the theory in literature. Biomass yield was not, however, constant across pH, with notable decreases for initial pH 1.6 and pH 2.0 (Table 4-5). This suggests that biomass production is inhibited in the upper and lower end of the pH range tested.

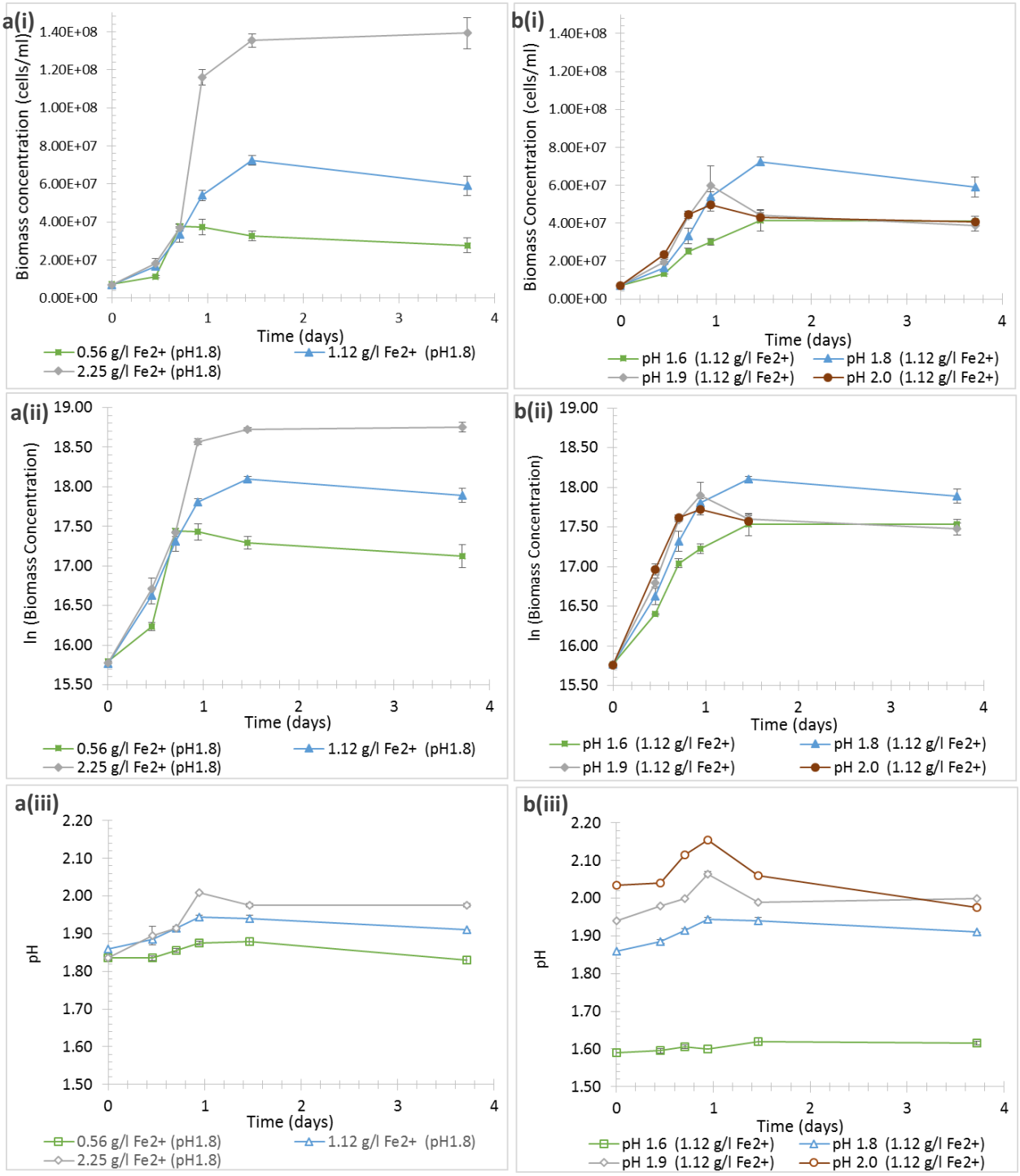

Figure 4-1: At. ferrooxidans growth maximisation experiments (a) influence of initial ferrous iron concentration on (i) biomass concentration, (ii) ln(biomass concentration) and (iii) pH; and (b) influence of initial pH variation on (i) biomass concentration, (ii) ln(biomass concentration) and (iii) pH. Error bars indicate the standard deviation of duplicate experiments.

Excessive formation of ferric iron crystals was observed in media which exceeded a maximum pH 2.0 at the end of the experiment (Table 4-4, Table 4-5 and Figure 4-2) and was accompanied by a steep drop in pH (Figure 4-1 (a)iii and (b)iii). The drop in pH for all flasks subsequent to biomass maximisation can be attributed to the chemical reactions between the oxidised ferric iron and sulphate ions, which produce ferric sulphate crystals, and result in acidification (73). However, because thermodynamic equilibrium between ferric iron and sulphate compounds in solution shifts towards crystal formation at higher pHs, this phenomenon is amplified for media with higher pH (73).

Interestingly, the maximum growth rate was also observed to be effected by initial pH (Table 4-5), however it diverged from the trends observed for biomass yield, demonstrating increased growth rate with pH. Furthermore, an increase in growth rate was also observed for the highest initial ferrous iron concentration tested, 2.25 g/l (Table 4-4), for which more than half the growth occurred at a pH in excess of 1.9 (Figure 4-1 a). Other parameters known to affect growth rate include incubation temperature and carbon dioxide mass transfer, which in turn is limited by temperature and aeration method. Since temperature and the method of aeration were constant across the flasks, the observed variation in growth rate can be attributed to pH. The maximum growth rate was observed for pH 1.9 to 2.0, but the absolute maximum growth rate may lie outside the range tested. The contradiction between the optimum initial pH for biomass yield (1.8) and the optimum pH for growth rate (>1.9) could be due to the transient growth conditions present in batch culturing (physiological constraints and play off between ferrous iron for maintenance and growth), or the competitive chemical oxidation of ferrous iron at higher pH, reducing the total ferrous iron available to the cells (non-physiological constraints).

Table 4-4: At. ferrooxidans – effect of variation in the initial ferrous iron (Fe^{2+}) concentration on the maximum biomass concentration achieved, the final pH, the maximum growth rate (μ_{max}) and ferric iron crystal formation (see Appendix B for maximum growth rate calculations)

Initial Fe^{2+} Concentration (g/l)	Initial pH	Max Biomass (cellsx10^{-7}/ml)	Yield Biomass (cellsx10^{-7}/ gFe^{2+})	Max pH	μ_{max} (h^{-1})
0.56	1.80	3.77 ±0.10	6.73 ±0.18	1.88 ±0.01	0.09 ±0.00
1.12	1.80	7.25 ±0.25	6.47 ±0.22	1.95 ±0.01	0.09 ±0.01
2.25	1.80	13.9 ±0.82	6.18 ±0.36	2.01 ±0.01	0.12 ±0.00

Table 4-5: At. ferrooxidans – effect of variation in the initial pH on the maximum biomass concentration achieved, the final pH, the maximum growth rate (μ_{max}), and ferric iron crystal formation (see Appendix B for maximum growth rate calculations)

Initial Fe^{2+} Concentration (g/l)	Initial pH	Max Biomass (cellsx10^{-7}/ml)	Yield Biomass (cellsx10^{-7}/gFe^{2+})	Max pH	μ_{max} (h^{-1})
1.12	1.60	4.14 ±0.57	3.70 ± 0.51	1.62 ±0.01	0.07 ± 0.00
1.12	1.80	7.25 ±0.25	6.47 ±0.22	1.95 ±0.01	0.09 ±0.01
1.12	1.90	6.00 ±1.00	5.36 ± 0.89	2.07 ±0.01	0.10 ± 0.01
1.12	2.00	4.98 ±0.35	4.45 ± 0.31	2.16 ±0.01	0.11 ± 0.00

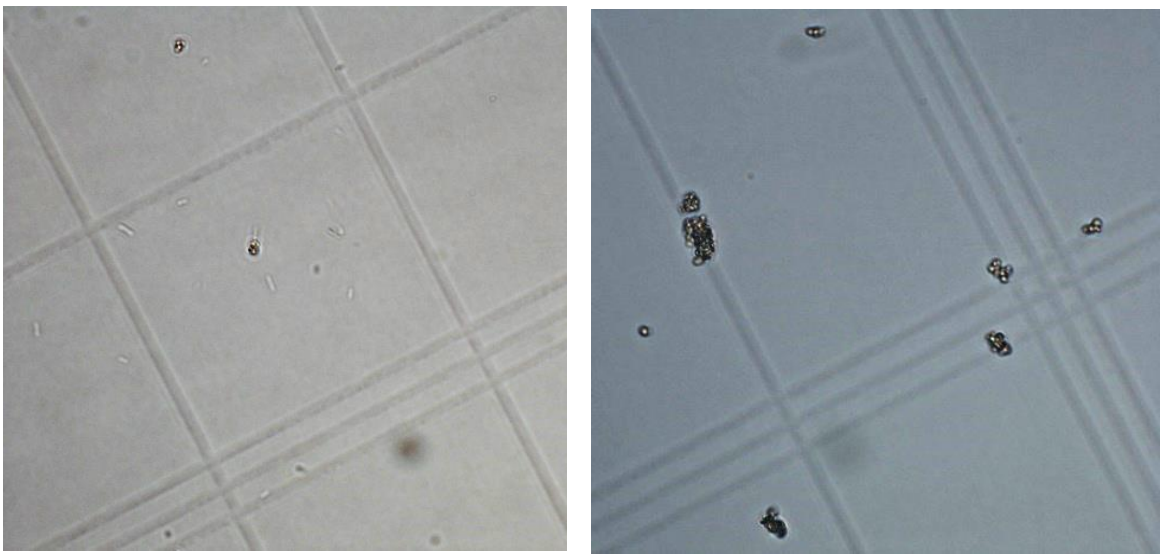

Figure 4-2: Phase contrast microscope images taken at 1000 x magnification in a Neubauer counting chamber under phase contrast microscopy of (a) At. ferrooxidans culture and (b) iron crystals

4.5.1.2 Culture Viability

Medium with an initial pH of 1.8 and ferrous iron concentration of 1.12 g/l was selected for the preliminary growth phase as it provided adequate biomass along with a final pH in the desired range. To investigate whether the culture is capable of sulphur oxidation subsequent to the preliminary growth phase, different sulphur sources were added to the medium after 24 hours. The sulphur sources investigated were elemental sulphur and tetrathionate, and the concentration added was selected to correspond to the expected sulphur available in a medium containing 50 g/l of GTR. The effect of additional ferrous iron on cell growth was also investigated to determine the potential impact of any steel or iron contained in the GTR. A control culture, to which no supplementary energy source (in the form of reduced sulphur or ferrous iron) was added, was included for comparison.

At. ferrooxidans DSMZ 14882 was shown to be capable of oxidising both elemental sulphur and tetrathionate, as evident from the equal increases in biomass concentration observed compared to the control culture (Figure 4-3 a). However, the growth rate of both elemental sulphur and tetrathionate grown cultures was very poor. A lag period of approximately 5 hours was observed between the growth on iron and the growth on sulphur sources, and can be attributed to the time taken for the microbes to switch to the new metabolic pathway. As such, the microbes will likely be able to oxidise the sulphur contained in GTR, although sufficient time should be allowed for the change in metabolism and the slow growth rate observed. Both sulphur sources resulted in a decrease in pH, as is to be expected due to the production of sulphuric acid. Although the final pH of both cultures (Table 4-6) was marginally lower than the optimal range of pH 1.8 to 2.5 reported in literature, it was well within the reported growth range (Table A-1 and Table A-2). Hence, oxidation of the sulphur concentration anticipated in GTR should not cause the pH to drop outside of the desired range for growth.

Ferrous iron was shown to be used in preference to the sulphur sources, and did not demonstrate a growth lag at any point. All ferrous iron was consumed within 36 hours. Since the concentration of ferrous iron added after 24 hours was well in excess to the concentration likely to leach from GTR, 10 mg per 100 g GTR (38), it can be assumed that any contaminant ferrous iron will be used almost immediately after leaching. As such, a switch to a sulphur oxidising metabolism can be ensured by extending the experimental period past the time required to consume any additional iron. Since most devulcanisation experiments last between 7 and 30 days (11,12,18,23,24,26,27,34,41–43), this will be unlikely to effect the overall outcome.

Table 4-6: At. ferrooxidans - summary of observed lag phase, maximum biomass, final pH and maximum growth rate (μ_{max}) subsequent to the addition of a particular energy source. The preliminary growth stage refers to the cell biomass maximisation step developed in 1.2.1, while the secondary growth stage refers to growth subsequent to the addition of energy sources added after 24 hours of incubation. Fe^{2+}- ferrous iron, S_0 – elemental sulphur and $S_4O_6^{2-}$ - tetrathionate (see Appendix B for maximum growth rate calculations)

Growth Stage	Energy Source	Conc. (mmol/l)	Lag (hours)	Max Biomass (cellsx10^{-7}/ml)	Yield Biomass (cells x10^{-7} /mmol)	Final pH	μ_{max} (h^{-1})
Preliminary	Fe^{2+}	20.05	0	5.9 ±0.2	0.30	1.78± 0.00	0.09 ±0.00
Secondary	Fe^{2+}	20.05	0	14.0 ±0.7	0.40	1.74 ± 0.00	0.12 ±0.00
Secondary	S_0	4.37	~ 5	7.3 ±0.0	0.32	1.75 ± 0.00	0.02 ±0.00
Secondary	$S_4O_6^{2-}$	1.55	~ 5	7.1 ±0.7	0.77	1.77 ± 0.00	0.01 ±0.00

4.5.2 Biomining Consortium

4.5.2.1 Preliminary Culture Preparation

The biomining consortium culture preparation presented very different challenges to the *At. ferrooxidans* DSMZ 14882. A high cell concentration was present in the bioreactor maintained culture at all times, making a preliminary growth step unnecessary. However, the culture was maintained on elemental sulphur, which would compete with the sulphur in the GTR and cause an excessive drop in pH if entrained into devulcanisation experiments. As such, the particulate sulphur had to be removed from the culture prior to incubation with GTR. Additionally, sufficient nutrients for both the heterotrophic and autotrophic microbes in the consortium needed to be provided at all times.

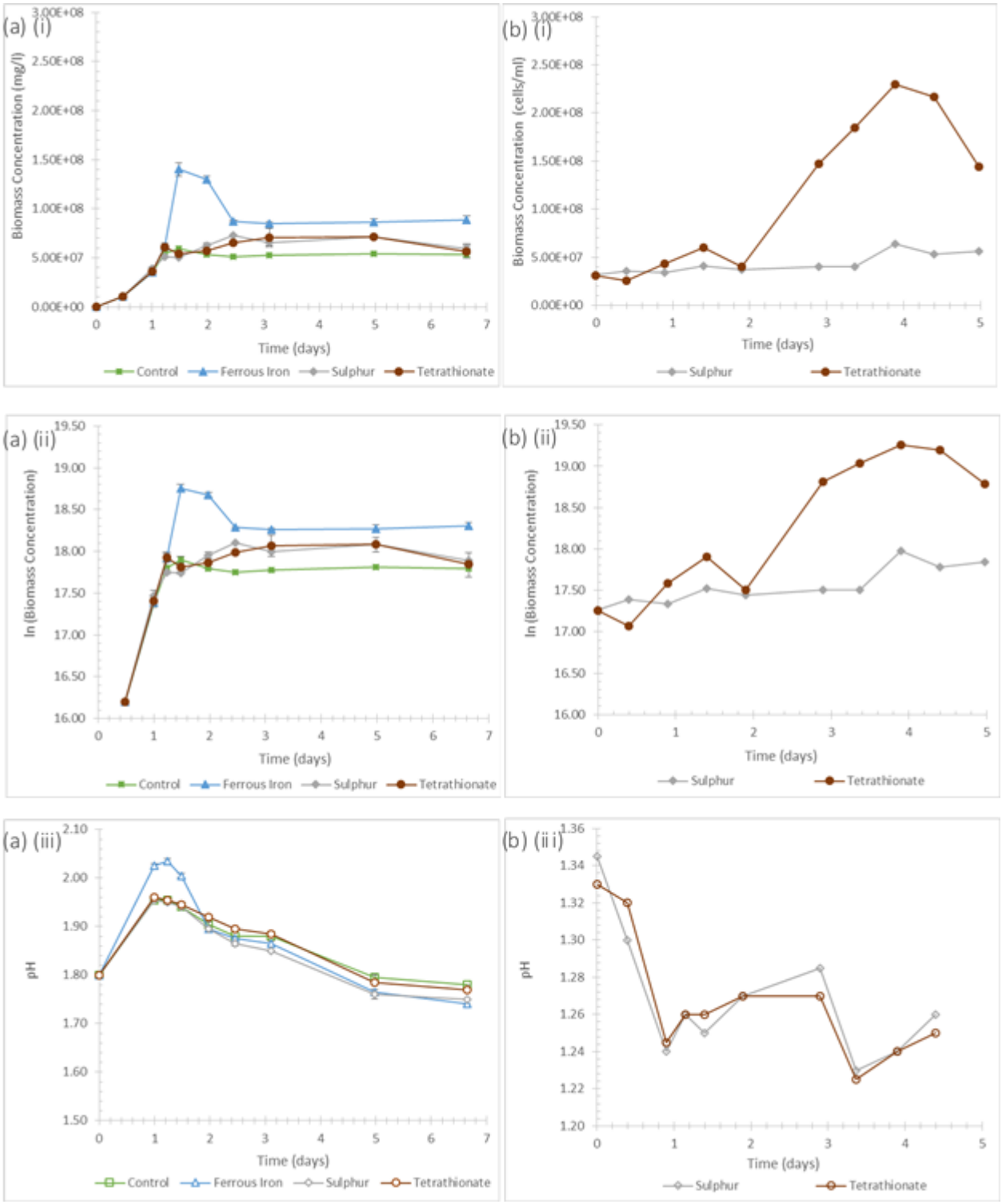

Figure 4-3: Energy variation study for (a) At. ferrooxidans (i) biomass concentration, (ii) ln(cell concentration) and (iii) pH over time and (b) consortium (i) biomass concentration, (ii) ln(biomass concentration) and (iii) pH over time. Error bars indicate standard deviation for duplicate experiments.

A decrease in pH over time was used as a crude indicator for sulphur oxidising activity, since sulphur oxidation leads to the production of sulphuric acid and the acidification of the medium. A decrease in pH of unfiltered biomining consortium incubated in shake flasks and the stock culture in the bioreactor indicated sulphur oxidation activity (Figure 4-4). In doing so, these experiments show that the biomining consortium is capable of sulphur oxidation and that there were sufficient nutrients in the medium for further growth. The accentuated decrease in pH of the bioreactor maintained culture suggests increased rate of sulphur oxidation compared to the baffled shake flask culture. Since the only difference between the two cultures was the method of agitation and aeration, it is likely that the difference in oxidation rate is due to a difference in mass transfer rate of carbon dioxide into the medium. The decreased rate of sulphur oxidation in the baffled shake flasks suggests decreased rates of carbon dioxide diffusion compared to the bioreactor, which was both sparged and agitated. The constant pH with time of flasks containing filtered biomining consortium showed that little to no sulphur oxidation occurred, indicating that Whatman 1 filter paper adequately removed the solid crystals of elemental sulphur.

4.5.2.2 Culture Viability

Filtered biomining consortium culture was supplemented with both tetrathionate and elemental sulphur to determine whether the culture was still capable of sulphur oxidation after filtration. Hence, confirming that the sulphur oxidising proponent of the biomining consortium was not removed with the elemental sulphur. The biomining consortium was diluted to correspond to the same magnitude of cell concentration as *At. ferrooxidans* DSMZ 14882 after incubation to facilitate comparison of the growth performance. The concentration of tetrathionate and elemental sulphur was selected to approximately correspond to the concentration of available sulphur in the GTR.

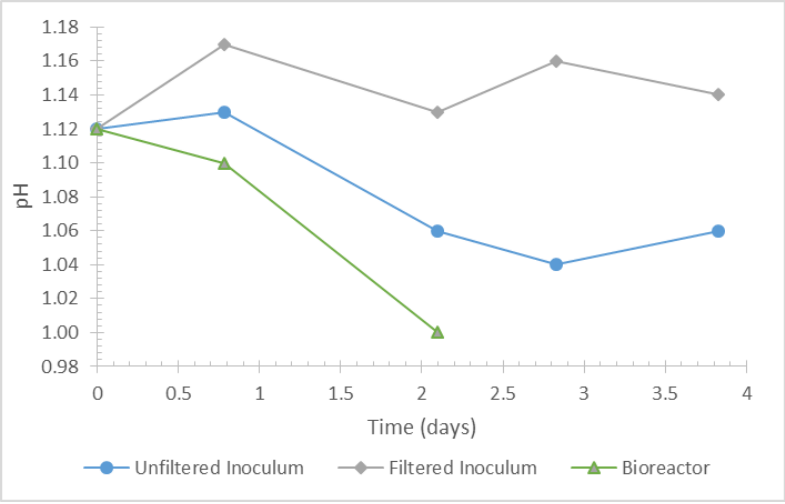

Figure 4-4: Consortium – pH over time for of the unfiltered and filtered consortium incubated in shake flasks compared to the stock consortium, contained in the bioreactor

The biomass concentration of the diluted, filtered biomining consortium was observed to increase for flasks containing both tetrathionate and elemental sulphur (Figure 4-3 b), confirming that the sulphur oxidising microbes were present, and capable of oxidising both sulphur sources. Hence, filtered biomining consortium will devulcanise GTR if the sulphur oxidising microbes are capable of metabolising the sulphide bonds present in GTR. Additionally, the minimum pH throughout the experiments was 1.23 for both sulphur sources, indicating the oxidation of the sulphur concentrations anticipated in GTR will not cause the pH drop outside the range of growth.

Table 4-7: Consortium - summary of observed lag phase, maximum biomass, final pH and maximum growth rate (μ_{max}) subsequent to the addition of a particular energy source at the given time. In the case of the consortium, the preliminary growth stage was conducted on elemental sulphur in the bioreactor and, as such, the lag and growth rate are not characterised in the shake flask experiments. The secondary growth stage refers to consortium growth subsequent to preliminary growth, including separation from entrained elemental sulphur, dilution and the addition of controlled energy sources. S_0 – elemental sulphur and $S_4O_6^{2-}$ - tetrathionate (see Appendix B for μ_{max} calculations)

Growth Stage	Energy Source	Conc. (mmol/l)	Lag (days)	Max Biomass (cellsx10^{-7}/ml)	Yield Biomass (cellsx10^{-7}/mol)	Min pH	μ_{max} (h^{-1})
Preliminary	S_0	NA	NA	3.14 x 10^7	NA	1.34	NA
Secondary	S_0	3.11	~3	6.40 x 10^7	0.75	1.23	0.04
Secondary	$S_4O_6^{2-}$	1.55	~2	23.0 x 10^7	10.70	1.23	0.03

An excessive lag of approximately three days and two days for elemental sulphur and tetrathionate, respectively, was exhibited (Table 4-7). The lag was most likely due to the dilution of the culture, since it was not exhibited in bioreactor maintained biomining consortium. Firstly, the biomass concentration indicates the sum total cells in solution, and only a fraction would be sulphur oxidising microbes. As such, the concentration of sulphur oxidising microbes would be lower than that of *At. ferrooxidans*. Secondly, dilution of the culture and replacement with fresh 0K medium would have caused a drastic decrease in the nutrients required for heterotrophic growth, inhibiting growth of heterotrophic organisms until the autotrophs had produced enough by-products to sustain them, also causing a lag. As such, the biomining consortium should not be diluted prior to devulcanisation experiments.

The yield of biomass for each sulphur source was far greater in the consortium than the pure culture of *At. ferrooxidans*, with yields of 0.32 x 10^7 cells/mmol S and 0.77 cells/mmol $S_4O_6^{2-}$ for *At. ferrooxidans* and 0.75 cells/mmol S and 10.70 cells/mmol $S_4O_6^{2-}$ for the consortium (Table 4-6 and Table 4-7). This can most likely be attributed to the concurrent growth of heterotrophs on the waste products produced by autotrophic growth. The increased yield may also have been inflated by dissolved sulphur compounds present in the consortium, such as tetrathionate, which is a proposed intermediate of elemental sulphur oxidation (Section 2.4.2.1), but will not be removed by the coarse filtration step.

4.6 Conclusions

Culture preparation methods were developed for *At. ferrooxidans* DSMZ 14882 and the biomining consortium, which facilitated devulcanisation by ensuring sufficient biomass, good growth conditions, and limited nutrients which would compete with the sulphur in the GTR at the point of GTR addition. Culture viability tests demonstrated that both cultures were capable of sulphur oxidation after the preparation stage, further confirming that the microbes will be able to utilise the sulphur in GTR, causing devulcanisation.

The *At. ferrooxidans* DSMZ 14882 culture preparation comprised of a 24 hours growth stage in energy source limited medium prior to the addition of GTR. Medium with an initial ferrous iron concentration of 1.12 g/l and an initial of pH 1.8 was selected for the preliminary growth stage as it provided the best compromise between maximum biomass, growth rate and increased pH for *At. ferrooxidans* DSMZ 14882. Both the literature and the current study achieved total biomass concentrations of approximately 7×10^7 cells/ml using an initial ferrous iron concentration of 1.12 g/l (12,24). The combination of low initial ferrous iron concentration and initial pH limited the maximum pH to below pH 2.0. As such, the maximum pH was well within the optimum growth range reported in literature and the formation of ferric iron crystals was limited. In the culture viability experiments, the preliminary growth stage with medium containing an initial ferrous iron concentration of 1.12 g/l and pH of 1.8, was shown to result in an active culture, capable of sulphur oxidation and suitable for use in devulcanisation experiments. Although ferrous iron was used preferentially to sulphur energy sources, it was depleted within 36 hours of incubation and sulphur oxidation commenced within 42 hours of inoculation (18 hours of sulphur addition). As such, the viability experiments demonstrated that not only that culture was capable of sulphur oxidation within a short period of time, but that the initial energy source was eliminated within 36 hours. Hence, in devulcanisation experiments the only energy source available after 36 hours of incubation would be sulphur in the GTR. Furthermore, the pH remained within the reported growth range for the duration of the sulphur oxidising experiments, indicating that the oxidation of the anticipated concentration of sulphur in GTR would not cause a negative impact on growth conditions.

The cell concentration of the biomining consortium maintained in the bioreactors was sufficiently high, approximately 6×10^8 cells/ml. As such, the biomining consortium did not require a preliminary growth phase, but did require the removal of entrained elemental sulphur to prevent it from being used in preference to the sulphur in GTR. Coarse filtration using Whatman 1 filter paper removed a sufficient amount of sulphur from the culture to prevent excessive sulphur oxidation, but also allowed the planktonic cells to pass through into the filtered broth. Culture viability tests demonstrated that the filtered biomining consortium was capable of utilising both elemental sulphur and tetrathionate for cell growth, confirming the presence of viable sulphur oxidising autotrophs in the filtered culture. The long lag incurred for both sulphur sources was attributed to the dilution of the filtered culture with fresh medium, suggesting that the consortium should not be diluted. Overall, the biomass yield of the biomining consortium per mole sulphur source was much

higher than the pure culture of *At. ferrooxidans*, since the biomining consortium biomass concentration reflected both heterotrophic and autotrophic cell mass. The pH of the biomining consortium remained well within the pH 1.0 to 1.3 growth range, with a minimum pH of ~1.22 suggesting that sulphur concentrations anticipated in GTR will not cause the pH to drop out of range.

5 The Devulcanisation of Industrial Waste Ground Tyre Rubber using *At. ferrooxidans* DSMZ 14882 and a Biomining Consortium

5.1 Context

The devulcanisation investigation fulfilled the main aim of the project in addition to the second and third research questions, as well as the main objectives. The culture preparation methods determined for *At. ferrooxidans* DSMZ 14882 and the biomining culture in Chapter 4 were employed to produce the active cultures used for the devulcanisation experiments. Culture performance was characterised in terms of growth performance of the culture and devulcanisation achieved. The toxicity of the acetone-leached component of ground tyre rubber (GTR) to *At. ferrooxidans* DSMZ 14882 was investigated to help identify the origin of the most toxic compounds in the GTR.

5.2 Abstract

Acidithiobacillus ferrooxidans has been identified as an effective microbial devulcaniser of ground tyre rubber (GTR) in a number of studies, while co-culturing it with *At. thiooxidans* has been found to further improve performance. In addition, *At. ferrooxidans* has been found to be comparatively resistant to some of the toxins present in GTR. However, devulcanisation studies on *At. ferrooxidans* have only investigated pure cultures or co-cultures rather than the complex acidophilic consortia commonly applied to industrial biomining. This chapter investigates the devulcanisation performance of the type culture of *At. ferrooxidans,* DSMZ 14882, and a mesophilic biomining consortium, when used to treat industrially sourced GTR which had not been detoxified. Both cultures were shown capable of devulcanisation of the untreated GTR after 30 days of incubation, despite the negative impact of the GTR on growth performance. *At. ferrooxidans* DSMZ 14882 increased the sol fraction of the GTR by 1.09 ± 0.02 % without causing any polymer degradation. The biomining consortium increased the sol fraction by 0.56 ± 0.01 %, but also caused polymer degradation at the surface of the GTR particles due to the activity of the heterotrophic microorganisms. Cell lysis in both *At. ferrooxidans* DSMZ 14882 and the biomining consortium was attributed to organic toxins in the acetone-extractable fraction of the GTR. However, non-acetone extractable toxins present in the GTR caused extensive cell death, and were therefore identified as the biggest challenge to improving growth performance. Attached cells in the non-sulphur oxidising component of the biomining consortium displayed the greatest amount of toxin resistance.

5.3 Introduction

Microbial devulcanisation of ground tyre rubber (GTR) using sulphur oxidising microbes exploits the highly selective microbial metabolism to oxidise the sulphur crosslinks near the GTR particle surface. The oxidation of the sulphur both breaks the sulphur crosslinks and creates chemically active sites on the surface of the GTR particle, improving bonding between reclaimed GTR filler particles and new rubber and plastic matrices (11). Improved bonding improves material properties and, consequently, has the potential to increase the

percentage of reclaimed GTR that can be incorporated into new tyre rubber without reducing the quality of the end product (6,11,16). However, a major challenge in microbial devulcanisation is the presence of toxins in the GTR, which can cause cell death and limit the extent of the change in surface properties (22).

At. ferrooxidans has been shown to be particularly toxin resistant compared with other devulcanising microbes, especially with regards to zinc, which is present in GTR in the acid soluble form of zinc oxide (22,23). It is hypothesised earlier in this study (Section 2.5) that biomining consortia could further improve the devulcanisation performance of sulphur oxidising autotrophs by consuming the organic toxins produced during autotrophic growth, and potentially any organic chemicals which leach out of GTR during treatment. However, the impact of untreated, industrial GTR on growth performance has been characterised for neither *At. ferrooxidans* nor any biomining consortium. With the aid of the culture preparation steps developed in Section 4, this study aims to compare the growth performance of *At. ferrooxidans* in the presence of industrial, untreated GTR to that of the biomining consortium and, furthermore, to the performance of a strain of *At. ferrooxidans* previously tested using laboratory made, detoxified GTR.

An additional challenge faced in microbial devulcanisation is a lack of standardised methods to characterise the effects of the microbial treatment, especially with regards to the determination of sol fraction, which can vary greatly between studies (12,27). This study, therefore, employed two methods of sol fraction analysis, combined with proximate analysis, FTIR-ATR, SEM-EDS and SEM imaging to both maximise its comparability with results obtained in literature and to gain as much understanding of the effect of the microbial treatment on the GTR characteristics as possible. Crosslink density and the selectivity of the devulcanisation achieved according to the Horikx model (Section 2.2.3) could not be determined due to physical constraints.

5.4 Materials and Methods

5.4.1 Ground Tyre Rubber

Mesh 40 ambient ground tyre rubber (GTR) produced by Dawhi, Germiston, South Africa was used for all experiments and was sterilised with a 25 kGray dose of gamma irradiation at HEPRO, Milnerton, Cape Town, South Africa. The particle size distribution was determined using a SaturnDigiSizer 5200 Particle Size Analyser, Micrometrics, Lincolnshire, United Kingdom. The sulphur content of the GTR was determined using a LECO SC632 Sulphur and Carbon Analyser with triplicate 0.2 g samples. Acetone leaching was conducted on a portion of the GTR using Soxhlet extraction for 16 hours, after which it was vacuum dried at 60 °C and -60 mmHg and then radiation sterilised.

5.4.2 Microbial Cultures and Growth Characterisation

At. ferrooxidans DSMZ 14882 and a biomining consortium were used in this study. Both the cultures and the culturing methods used were identical to those described in Chapter 4. Biomass concentration was determined at 1000x magnification using a counting chamber and bright field light microscope as specified in Chapter 4. Phase contrast imaging at 1000x magnification was conducted on an Eclipse E800, Nikon, Tokyo,

Japan. The concentration of soluble zinc was quantified using atomic adsorption spectroscopy (AAS) with a NovAA® 400P, Analytik Jena AG, Jena, Germany with standards from Merck, Darmstadt, Germany.

5.4.3 Microbial Devulcanisation

The culture preparation steps developed in Chapter 4 were used to prepare the devulcanisation cultures of *At. ferrooxidans* and the biomining consortium. *At. ferrooxidans* culture preparation was conducted using an initial growth step in DSMZ Medium 882*, altered to contain 1.12 g/l ferrous iron and adjusted to pH 1.8. Baffled shake flasks containing 1 ml of *At. ferrooxidans* inoculum and 30 ml of DSMZ Medium 882* were cultured at 30 °C and 115 rpm for 24 hours. For the biomining consortium preparation, active culture was drawn from a stable bioreactor culture with pH 1.24. The biomining consortium culture was then filtered using Whatman 1 filter paper to remove entrained elemental sulphur, while allowing the planktonic cells to pass through with the broth, and contained a final biomass concentration of 5.8 x 10^8 cells/ml. 30 ml of filtered biomining consortium was then placed in each 250 ml baffled shake flask. After the culture preparation step for *At. ferrooxidans* and the biomining consortium, 1.5 g of GTR was added to each flask and the devulcanisation cultures were incubated at 30 °C and 115 rpm for a further 15, 24 or 30 days. Cell counts were conducted over the duration of the experiments, and each time period was repeated in triplicate for both *At. ferrooxidans* and the biomining consortium. Control GTR experiments were included to determine the effect of the sterile medium of each culture on the GTR, and the effect of the GTR on the medium conditions (Table 5-1). For *At. ferrooxidans* control GTR experiments, sterile GTR was incubated in 250 ml baffled shake flasks with 30 ml of sterile DSMZ Media 882* at pH 1.8 for 15, 24 and 30 days with each time period conducted in triplicate. Biomining consortium control GTR was produced in the same way, except that sterile 0K medium adjusted to pH 1.24 was used in the place of DSMZ Medium 882*. Evaporative weight loss in the shake flasks was adjusted for every three days, using autoclave sterilised reverse osmosis treated water. At the end of each experiment the GTR was removed from the broth by filtration, rinsed using reverse osmosis water and dried overnight at 21 °C. The broth from each flask was collected and the pH and soluble zinc concentration measured and recorded.

Growth performance of the *At. ferrooxidans* and the biomining consortium devulcanisation cultures was compared to a corresponding control culture and a sulphur oxidising culture. Both the control culture and the sulphur oxidising culture were prepared in the same manner as the microbial devulcanisation experiments and repeated in duplicate. However, no additional energy source was added to the control culture, while the sulphur oxidising cultures were supplemented with 0.4 g/l of elemental sulphur in the place of GTR. To determine whether the cells were viable at the end of the 30 day experiments, a sample from each control culture and each devulcanisation culture was used to inoculate 25 ml of fresh medium contained in 50 ml centrifuge tubes. The cultures were then incubated at 30 °C and 115 rpm for one month to assess

whether growth occurred. *At. ferrooxidans* cultures were placed in sterile DSMZ Medium 882, while the biomining consortium cultures were placed in sterile 0K Medium containing an excess of elemental sulphur.

*Table 5-1: Overview of experiments conducted to characterise devulcanisation and cell growth performance *energy sources for At. ferrooxidans were added after 1 day of incubation in Medium DSMZ 882*, while energy sources for the biomining consortium were added at the start of the experiment.*

Experiment Name	Active Culture	Supplementary Energy Source Added*	Loading (g/l)	Parameter of Interest		
				Biomass	Medium Properties	GTR Properties
Control GTR	No - Sterile	GTR	50		X	X
Devulcanisation	Yes	GTR	50	X	X	X
Control Culture	Yes	None	NA	X		
Sulphur Oxidising Culture	Yes	Elemental Sulphur (S_0)	0.4	X		

5.4.4 Effect of Acetone Extractable Fraction of GTR on Cell Growth

At. ferrooxidans devulcanisation experiments were repeated with acetone Soxhlet extracted GTR. Cell growth was characterised for a control culture, a culture containing the untreated GTR used in previous experiments (devulcanisation), and acetone leached GTR over a period of 10 days, using the devulcanisation experimental method outlined in Section 5.4.3.

*Table 5-2: Overview of experiments to determine effect of acetone extractable fraction of GTR on cell growth of At. ferrooxidans. *Supplementary energy sources were added after 1 day of incubation in Medium DSMZ 882**

Experiment Name	Active Culture	Supplementary Energy Source Added*	Loading (g/l)	Parameter of Interest		
				Biomass	Medium Properties	GTR Properties
Control Culture	Yes	None	NA	X		
Devulcanisation	Yes	GTR	50	X		
Acetone Extracted GTR Devulcanisation	Yes	Acetone Extracted GTR	50	X		

5.4.5 Ground Tyre Rubber Characterisation

The extent of devulcanisation was measured using two soluble (sol) fraction methods. Method 1 was adapted from ASTM D6814 and D297 (32,33) and Method 2 was adapted from previous microbial devulcanisation studies (12,34,41,43). Soxhlet extraction was conducted using a glass 1000 ml round bottomed flask, sample chamber, water cooled condenser and 350W heating mantle. Each Soxhlet apparatus was loaded with 600 ml of solvent (AR Grade, KIMIX, Cape Town, South Africa) and three to four GTR samples per extraction. Ground tyre rubber samples of between 0.35 and 0.45 g were placed in 150 Mesh stainless steel mesh pockets and vacuum dried to determine the dry weight ($m_{dry,1}$). In Method 1, the samples were extracted for 16 h using acetone Soxhlet extraction, then vacuum dried and extracted with toluene at room temperature

for 72 h. The samples were vacuum dried again to determine the total extracted mass ($m_{final,1}$). Method 1 sol fraction was defined as a fraction of $m_{dry,1}$ (Equation 5-1).

$$sol\ fraction = \frac{(m_{dry,1} - m_{final,1})}{m_{dry,1}} \qquad \textit{Equation 5-1}$$

For Method 2, the dry samples ($m_{dry,1}$) were immersed in acetone at room temperature for 24 hours, vacuum dried to determine the new weight ($m_{dry,2}$), and then extracted for 72 h using toluene Soxhlet extraction. The extracted samples were vacuum dried again to determine the final extracted weight ($m_{final,2}$). The Method 2 sol fraction was defined as a fraction of $m_{dry,2}$ (Equation 5-2). All vacuum drying was conducted for 12 hours at 60 °C and -60 mmHg. Error was calculated using standard deviation of replicate measurements, and t-tests were performed to determine statistical significance of any observed change in sol fraction. Error propagation theory was used to calculate any compounded errors from mathematical operations.

$$sol\ fraction = \frac{(m_{dry,2} - m_{final,2})}{m_{dry,2}} \qquad \textit{Equation 5-2}$$

FTIR-ATR analysis of GTR was conducted using an infrared imaging microscope (Nicolet iN10, Thermo Fischer Scientific, Waltham, MA, USA) with a germanium crystal with a collection time of 56 seconds, averaging 224 scans. Samples were analysed in crumb form and held in place by a stainless steel crucible.

Proximate analysis was performed using a thermogravimetric analyser (TGA/DSC 1 Start System, Mettler Toledo, Greifensee, Switzerland) according to the method described in literature (46).

Scanning electron microscopy (SEM) with backscatter electron (BSE) imaging and energy dispersive x-ray spectroscopy (EDS) (MERLIN SEM with Gemini II Column, Zeiss, Oberkochen, Germany) was used to analyse the sulphur content at the edge of GTR particles. Measurements were conducted with a working distance of 7.5 mm for all elemental analysis. GTR samples were resin-mounted, ground, and then polished using 1 µm diamond paste to achieve a pseudo cross section of the particles which allowed for analysis of the sulphur content of the outer 4-6 µm GTR particle. Samples were gold coated (10 nm) to increase conductivity and prevent charge build up in the chamber. High resolution imaging was conducted using secondary electron imaging on gold coated carbon tape mounted GTR samples at a variety of magnifications. A variable pressure SEM was used to provide supplementary images of microbial structures (LEO 1550, Zeiss, Oberkochen, Germany). Samples spent less than 30 min in the chamber to limit the effects of the vacuum.

5.5 Results

5.5.1 GTR Feedstock Characterisation

Dawhi Ground tyre rubber (GTR) produced from waste truck tyre was selected because it has a higher content of natural rubber than passenger car tyre derived GTR (9,75). Mesh 40 (470 µm), the smallest industrially available fraction, was selected to maximise surface area to volume ratio and had a 50% cut-off of 230 µm

(Figure 5-1). The sulphur content was determined to be 2.09±0.26 mass%. The microbially available sulphur was estimated as 69±8 mg/l for a 50 g/l solids loading of GTR assuming spherical particles and an average particle penetration depth of 2 µm (calculation and method presented in Appendix B, Theoretical Availability of Sulphur in GTR). Gamma irradiation exposure of 25 kGray was selected as it meets the sterilisation requirements for medical implants and did not have any significant impact on the soluble fraction (Method 1) or proximate analysis of the GTR (Appendix C, Sterilisation of GTR Crumb).

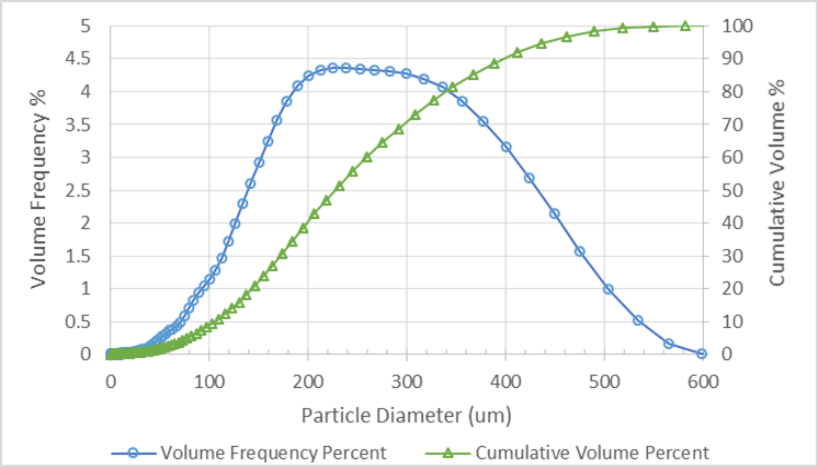

Figure 5-1: Particle size distribution of Mesh 40 Dawhi GTR feedstock

5.5.2 Microbial Growth and Medium Conditions during Devulcanisation

The devulcanisation experiment microbial growth performance of each culture was assessed by comparing the biomass concentration over time for the devulcanisation experiment to a control culture and a sulphur oxidising culture (outlined in Table 5-1). In order to assess the impact of GTR on the suitability of the growth conditions, the effect of GTR on the medium pH and zinc concentration under both sterile (control GTR) and cultured (devulcanisation) conditions was investigated (Table 5-1). Lastly, the impact of acetone extractable chemicals in GTR on growth performance of *At. ferrooxidans* was investigated by comparing growth performance a control culture, a devulcanisation culture with GTR, and a devulcanisation culture with acetone extracted GTR (Table 5-2).

Microbial growth performance of the *At. ferrooxidans* control culture, sulphur oxidising culture and devulcanisation culture is presented in Figure 5-2. Identical growth patterns were observed in the first 36 hours of the control culture and the sulphur oxidising culture growth. The close correlation suggested that the sulphur oxidising culture exclusively consumed the ferrous iron energy source provided in DSMZ Medium 882 during the first 36 hours of incubation, despite the addition of elemental sulphur after 24 hours of growth. After the exhaustion of ferrous iron at 36 hours, the control culture entered a short death phase, and stabilised at approximately 4 x 10^7 cells/ml. Conversely, the sulphur oxidising culture entered a 36 hour lag, followed by growth which could be attributed to elemental sulphur oxidation, or consumption of the

elemental sulphur energy source. Despite the presence of sulphur in GTR, an additional energy source, the devulcanisation culture biomass concentration decreased substantially after GTR addition and stabilised at approximately 3 x 10^7 cells/ml for the remainder of the experiment. The decreased final biomass concentration of the devulcanisation culture compared to the control culture suggests that GTR caused increased cell lysis, and possibly cell death, in *At. ferrooxidans*. The presence of active microbes in the control culture and the sulphur oxidation culture, but not the devulcanisation culture after 30 days of incubation (Appendix C - Figure C-8), further confirmed that GTR caused cell death.

The biomining consortium control culture, sulphur oxidising culture and devulcanisation culture growth performance is presented in Figure 5-3. The control culture biomass concentration remained relatively stable for the first five days of incubation, while the sulphur oxidising culture biomass concentration increased above the initial concentration within three days. The increase in biomass and limited lag of the sulphur oxidising culture indicated good sulphur oxidation activity in the biomining consortium. Since the biomass concentration of the biomining consortium control culture did not increase above the initial biomass concentration, the impact of entrained sulphur compounds on biomass was insignificant. Both the control culture and the sulphur oxidising culture entered the death phase after five days, stabilising at approximately 2 x 10^8 cells/ml and 4 x 10^8 cells/ml, respectively. The steady decline in biomass for the first five days of incubation of the biomining consortium devulcanisation culture indicated the occurrence of cell lysis, demonstrating the negative impact of GTR on cell growth. However, the biomass concentration of the devulcanisation culture stabilised at 3 x 10^8 cells/ml, which was higher than the control culture and suggests a net increase in biomass concentration. Active sulphur oxidising microbes were detected in both the control culture and the sulphur oxidising culture after 30 days of incubation (Appendix C - Figure C-9). The increase in biomass concentration observed in the devulcanisation culture is unlikely to be due to the growth of the sulphur oxidising proponent of the biomining consortium, since no active sulphur oxidising microbes were observed after 30 days (data not shown). However, the absence of sulphur oxidising microbes does not preclude the presence of active non-sulphur oxidising microbes at the end of the experiment.

Medium conditions for both *At. ferrooxidans* and the biomining consortium were substantially impacted by the presence of GTR (Figure 5-4). The microbial activity of both cultures had relatively little impact on the pH compared to the presence of GTR in sterile medium (Figure 5-4 (a) i and (b) i). The slight increase in pH in the *At. ferrooxidans* devulcanisation experiment compared to the control GTR experiment over the first 15 days could be attributed to ferrous iron oxidation. Most zinc leaching occurred in the first 15 days of incubation (Figure 5-4 (a) ii and (b) ii). No difference in the concentration of aqueous zinc over time was observed for the two control GTR media (Figure 5-4 (a) ii and (b) ii), suggesting that pH and medium composition did not impact zinc leaching. However, a slight decrease in the concentration of aqueous zinc

was observe for day 24 to 30 in the biomining consortium devulcanisation experiment (Figure 5-4 (b) ii) compared to the same time period in the *At. ferrooxidans* devulcanisation experiment (Figure 5-4 (a) ii).

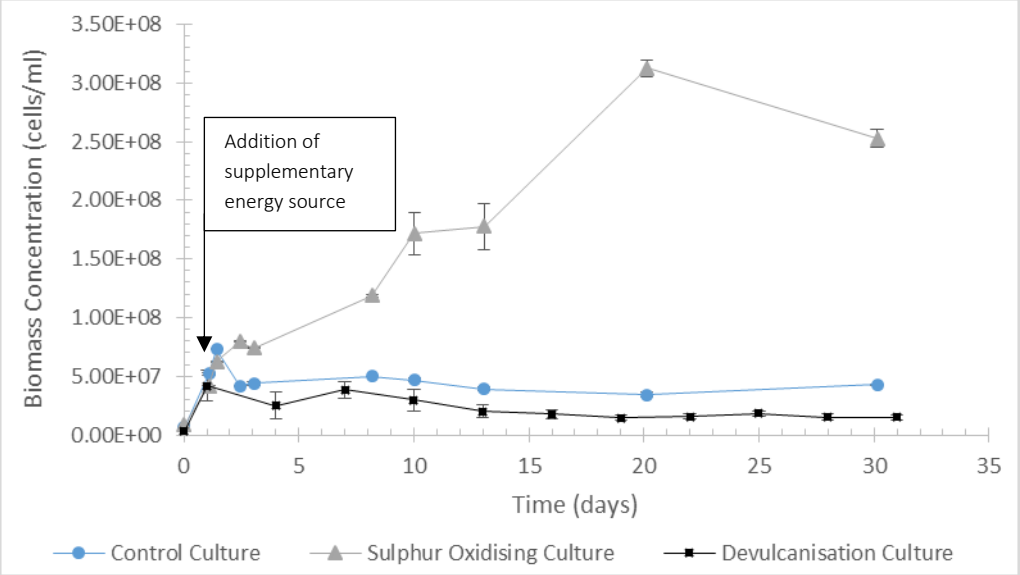

*Figure 5-2: At. ferrooxidans biomass concentration over time for (i) the control culture, (ii) the sulphur oxidising culture and (iii) the devulcanisation culture. *Supplementary energy sources added after 24 hours of incubation, as indicated by arrow.*

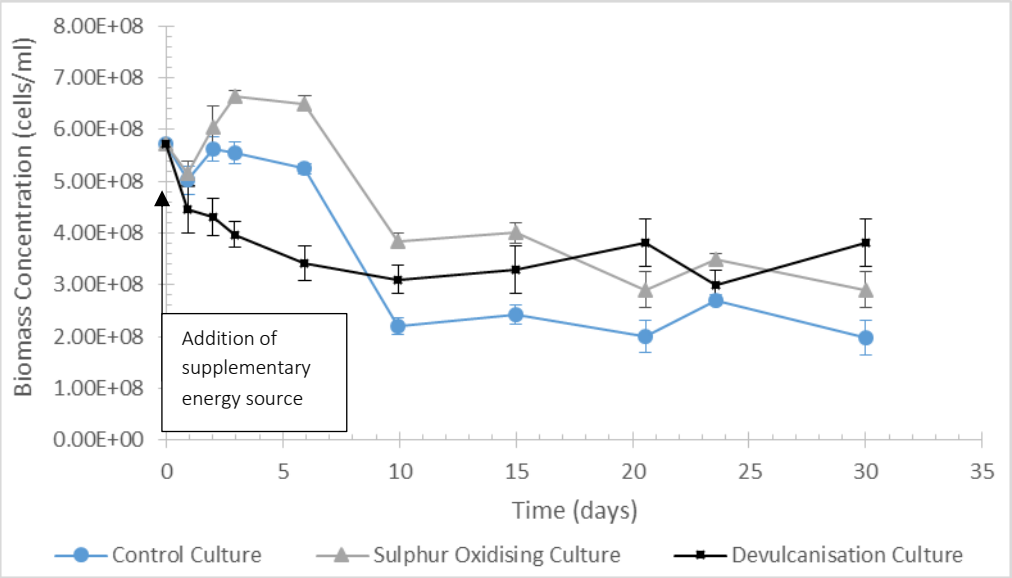

*Figure 5-3: Biomining consortium biomass concentration over time for (i) the control culture, (ii) the sulphur oxidising culture and (iii) the devulcanisation culture. *Supplementary energy sources added at beginning of incubation period, as indicated by arrow*

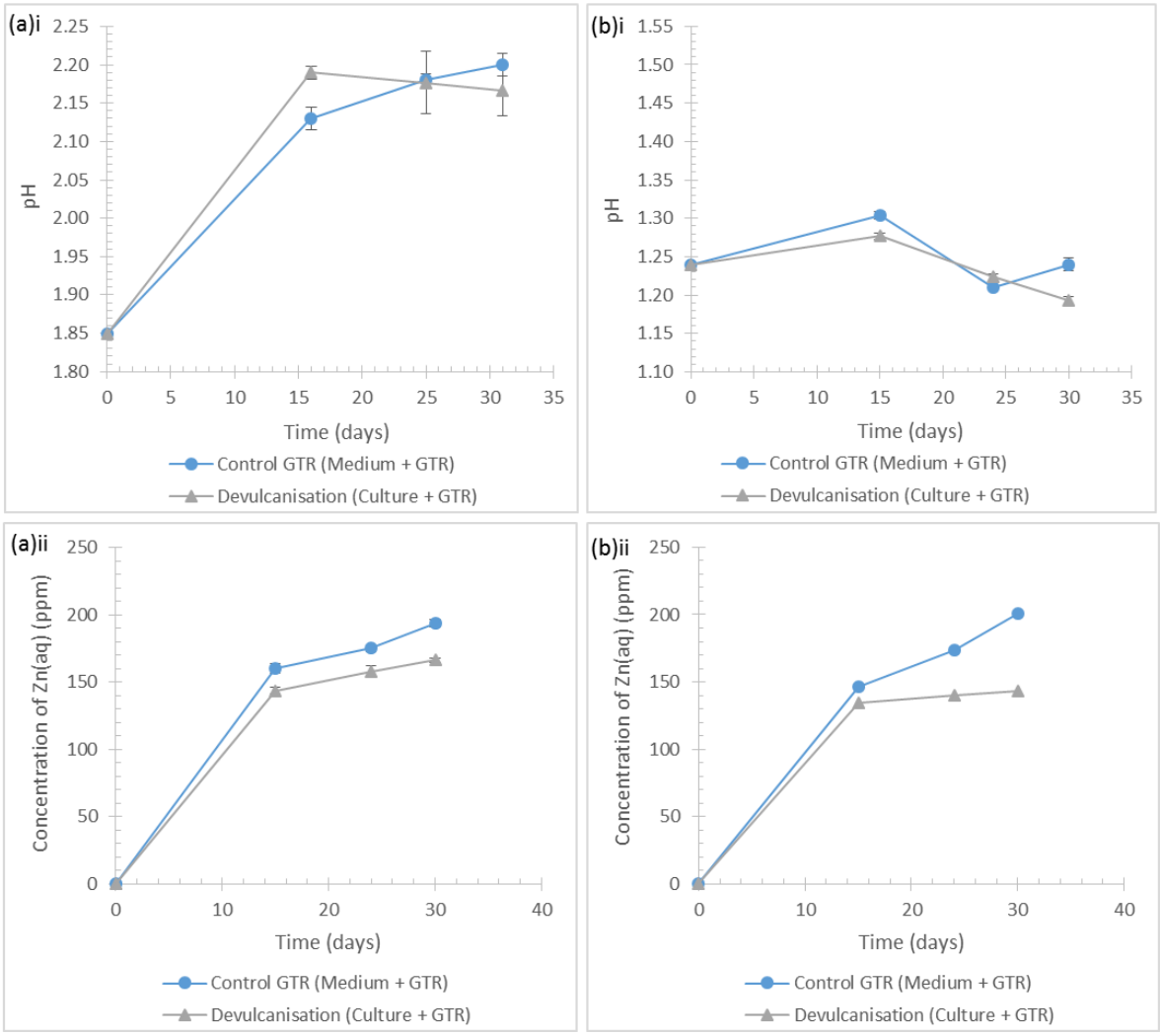

Figure 5-4: Effect of GTR on medium conditions in sterile medium (control GTR) and cultured medium (devulcanisation experiments) for (a) At. ferrooxidans and (b) the biomining consortium in terms of (i) medium pH and (ii) aqueous zinc concentration.

The effect of acetone extracted GTR on *At. ferrooxidans* growth compared to untreated GTR and a control culture is presented in Figure 5-5. The control culture biomass again exhibits uninterrupted growth for 36 hours and stabilises at approximately 6 x 10^7 cells/ml for the remainder of the experiment. Conversely, the cell growth of both the untreated GTR devulcanisation culture and the acetone-leached GTR devulcanisation culture were inhibited immediately after GTR addition at 24 hours. Thereafter, the untreated GTR caused excessive cell lysis, and the biomass concentration and stabilised at less than half its original value. The acetone leached GTR did not cause cell lysis. Instead, after a 36 hour lag the biomass concentration of the acetone leached GTR devulcanisation culture increased above the maximum concentration of the control culture, suggesting cell growth on the additional sulphur provided by the acetone leached GTR. Interestingly, neither the untreated GTR devulcanisation culture nor the acetone leached devulcanisation culture contained viable culture after 10 days of incubation (data not shown), while viable culture was again found in the control culture. The occurrence of cell death for both the untreated and acetone-leached GTR cultures

suggests that although cell lysis was caused by the acetone extractable fraction of the GTR, cell death was caused by the non-acetone-extractable components contained in the GTR.

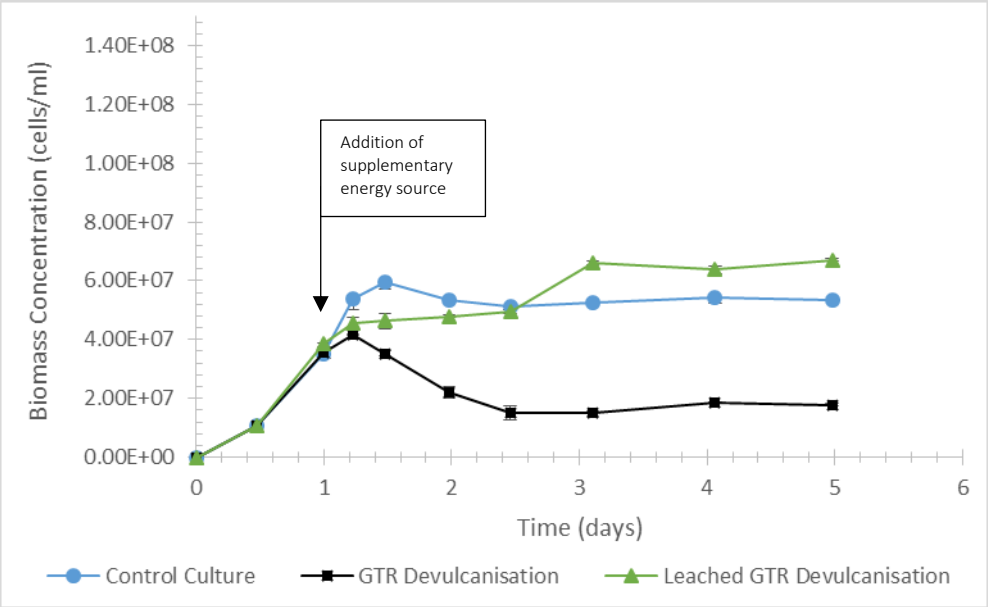

Figure 5-5: Acetone leached GTR experiment - At. ferrooxidans biomass concentration with time for a control culture, a devulcanisation culture containing untreated GTR and a devulcanisation culture containing acetone leached GTR

5.5.3 Control GTR and Devulcanised GTR Properties

The properties of medium treated GTR (control GTR, or cGTR) and microbially treated GTR (devulcanised GTR, or dGTR) of both *At. ferrooxidans* and the biomining consortium were determined. Characterisation methods included sol fraction, proximate analysis, FTIR-ATR, SEM-EDS and SEM-imaging.

5.5.3.1 Sol Fraction

The extent of devulcanisation, measured as a change in soluble (sol) fraction was determined using two different methods to improve the comparability to literature of the study. In Method 1, the sol fraction was determined as the total weight change in GTR resulting from an acetone Soxhlet extraction followed by room temperature toluene extraction (**Error! Reference source not found.** (left)). In Method 2, the GTR was first extracted with acetone at room temperature, and the sol fraction was defined as the weight change due to a subsequent toluene Soxhlet extraction (**Error! Reference source not found.** (right)). The Soxhlet extraction step removed the largest portion of extractable material in each method. As a result, Method 1 sol fraction was dominated by the acetone extractable fraction, and Method 2 sol fraction was dominated by the toluene extractable fraction (Table 5-3 and Table 5-4). Due to the difference in solvent boiling points, the acetone Soxhlet extraction used by Method 1 occurred at a lower temperature than the toluene Soxhlet extraction of Method 2. Method 2 resulted in higher values for the sol fraction than Method 1, however it also resulted in a larger amount of error (**Error! Reference source not found.**) and no statistically significant differences between control GTR (cGTR) and microbially devulcanised GTR (dGTR) for *At. ferrooxidans* or the biomining

consortium (Table 5-3 and Table 5-4). Despite the smaller values obtained for Method 1, greater accuracy was achieved and statistically significant differences between cGTR and dGTR were observed for both *At. ferrooxidans* and the biomining consortium (Table 5-3 and Table 5-4).

An equal decrease in the Method 1 sol fraction of DSMZ Medium 882* and 0K Medium treated cGTR within the first 15 days of incubation, indicates that both media caused equal leaching of a portion of the chemicals in the GTR sol fraction (Appendix C - Figure C-12). In *At. ferrooxidans* experiments, Method 1 showed a consistent, statistically significant increase of ~0.45% sol fraction with microbial treatment (**Error! Reference source not found.** & Table 5-3). In contrast, the biomining consortium Method 1 sol fraction demonstrated a consistent, statistically significant decrease of approximately 0.25% with microbial treatment (**Error! Reference source not found.** & Table 5-4). No change in cGTR and dGTR sol fraction was observed between Day 15 and Day 30 in either *At. ferrooxidans* (**Error! Reference source not found.** and Table 5-3) or the biomining consortium (**Error! Reference source not found.** and Table 5-4), indicating that the observed sol fraction changes occurred within the first 15 days of incubation.

In Method 2, the 0K Medium was observed to increase sol fraction leaching by 1.37% compared to DSMZ Medium 882* (Appendix C - Figure C-20). For *At. ferrooxidans*, the Method 2 sol fraction increased approximately 1.09% with microbial treatment, (**Error! Reference source not found.** & Table 5-3) confirming trends in Method 1. Contrary to Method 1 results for the biomining consortium, Method 2 showed an increase of approximately 0.54% sol fraction with microbial treatment (**Error! Reference source not found.**). Due to the large experimental error, Method 2 was unable to provide statistically significant results, despite indicating larger changes in sol fraction than Method 1 (Table 5-3 and Table 5-4).

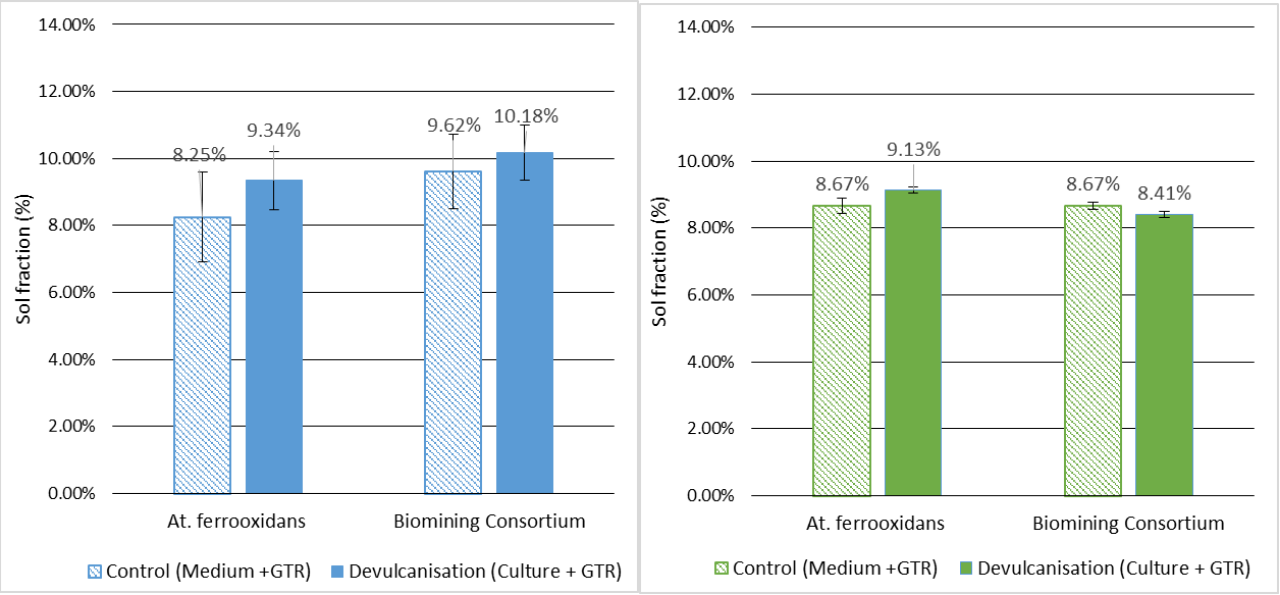

Figure 5-6: Sol fraction – comparison of Method 1 (left) and Method 2 (right) cGTR and dGTR sol fraction after 30 days of incubation

Table 5-3: At. ferrooxidans – difference in sol fraction between cGTR and dGTR. Results which are significant are indicated with an asterisk for a 95% confidence level (*) and a double asterisk (**) for a 90% confidence interval. Please see Appendix C for original charts and t-tests. Standard deviation errors indicated in tables were calculated using error propagation theory.

	15 Days	24 Days	30 Days
Acetone			
Method 1	0.14±0.00	0.47±0.00	0.58±0.00
Method 2	0.43±0.00		0.48±0.00
Toluene			
Method 1	0.19±0.00	0.01±0.00	-0.11±0.00
Method 2	1.45±0.01		1.09±0.02
Total Extract			
Method 1	0.33±0.00	0.48±0.00 *	0.46±0.00 *
Method 2	1.75±0.01		1.46±0.01

Table 5-4: Consortium – difference in sol fraction between cGTR and dGTR. Results which are significant are indicated with an asterisk for a 95% confidence level (*) and a double asterisk (**) for a 90% confidence interval. Please see Appendix C for original charts and t-tests. Standard deviation errors indicated in tables were calculated using error propagation theory.

	15 Days	24 Days	30 Days
Acetone			
Method 1	-0.17±0.00 *	-0.31±0.00 *	-0.31±0.00
Method 2			-0.19±0.00
Toluene			
Method 1	-0.08±0.00	0.06±0.00	0.06±0.00
Method 2			0.56±0.01
Total Extract			
Method 1	-0.24±0.00 *	-0.25±0.00 *	-0.26±0.00 *
Method 2			0.36±0.01

5.5.3.2 Proximate Analysis

Proximate analysis was used to determine the composition of the GTR before and after treatment, and is divided into an oil component which contains light carbon molecules; volatiles, which typically consist of the rubber portion; fixed carbon, in GTR this is mainly carbon black; and ash which contains metals and minerals (Table 5-5). Untreated GTR was treated in duplicate to give an indication of the sample and instrument error, which could be seen to be very small. The ash content of both *At. ferrooxidans* cGTR and the consortium cGTR was lower than the sterile feed GTR, indicating that both media leached ash from the GTR over the course of the experiment. Interestingly, the biomining consortium dGTR ash content was lower than the cGTR, while the *At. ferrooxidans* dGTR ash content was higher than the corresponding cGTR. These results indicate that the biomining consortium caused further leaching of ash, while *At. ferrooxidans* caused the

adsorbance of ash. Furthermore, *At. ferrooxidans* dGTR ash was the colour of ferric iron, rust orange, in contrast to the white-grey ash of the other samples (Figure 5-7).

Table 5-5: Proximate analysis results of sterile GTR (irradiated at 25 kGray) in addition to control GTR and devulcanised GTR from At. ferrooxidans and consortium devulcanisation experiments

	Sterile GTR			*At. ferrooxidans*		Consortium	
Component	**GTR A**	**GTR B**	**Average**	**cGTR**	**dGTR**	**cGTR**	**dGTR**
Oils	5.93%	5.97%	5.95%	6.08%	6.08%	6.26%	6.17%
Volatiles	56.93%	57.04%	56.99%	57.08%	56.39%	57.16%	57.45%
Fixed C	30.32%	30.38%	30.35%	30.70%	30.75%	30.56%	30.59%
Ash	6.81%	6.62%	6.72%	6.15%	6.78%	6.02%	5.79%
Total	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%

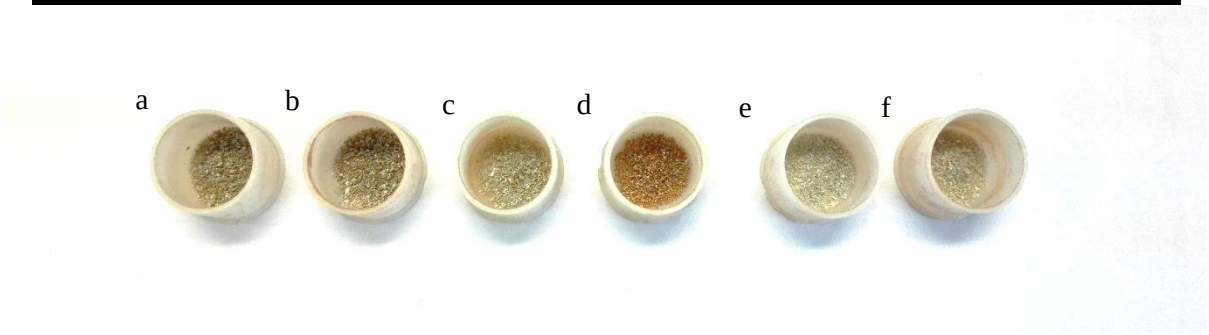

Figure 5-7: Photograph of ash of all samples analysed by TGA. From left to right (a) GTR A (b) GTR B (c) At. ferrooxidans cGTR (d) At. ferrooxidans dGTR (e) Consortium cGTR and (f) Consortium dGTR

5.5.3.3 FTIR-ATR Surface Characterisation

FTIR-ATR spectra were obtained for media treated GTR (cGTR) and microbially treated GTR (dGTR) incubated for 30 days to investigate changes in carbon and sulphur bonds at the surface of the GTR particles (Figure 5-8). Manual adjustment of the baseline was required to correct for baseline drift which resulted from the carbon black content of the GTR (76). No change in the peak at 1538 cm^{-1}, which indicates methyl-assisted conjugate double bond ($-(C=C)_n-$, n>6), was detected in *At. ferrooxidans* dGTR (Figure 5-8 (a) i) or cGTR (Figure 5-8 (a) ii), confirming that C=C bond breakage due to microbial activity did not occur. Conversely, both C=C bond breakage and methyl-group bond breakage due to microbial activity was observed in the biomining consortium (Figure 5-8(b)i and ii). The biomining consortium dGTR peaks at 1538 cm^{-1} (Figure 5-8(b) ii) are lower than the corresponding cGTR peaks (Figure 5-8 (b) i), indicating microbial breakage of C=C bonds. Furthermore, the peaks at 1446 cm^{-1} and 3020 – 2840 cm^{-1}, which indicate-CH_3 and -CH_2 symmetric and antisymmetric stretching, of the biomining consortium dGTR (Figure 5-8 (b) ii) are smaller than the cGTR (Figure 5-8(b)i), indicating that the biomining consortium caused additional methyl-group bond breakage. No peaks indicating –S=O (1070 cm^{-1}), sulfoxide (1070-1010 cm^{-1}) or sulfone (1300 – 1350 and 1100 – 1150 cm^{-}

[1]) could be distinguished due to the high prevalence of other peaks in this region – most likely -CH_n and -C-C- . The full list of peaks, and the potential chemical groups may be found in Appendix C.

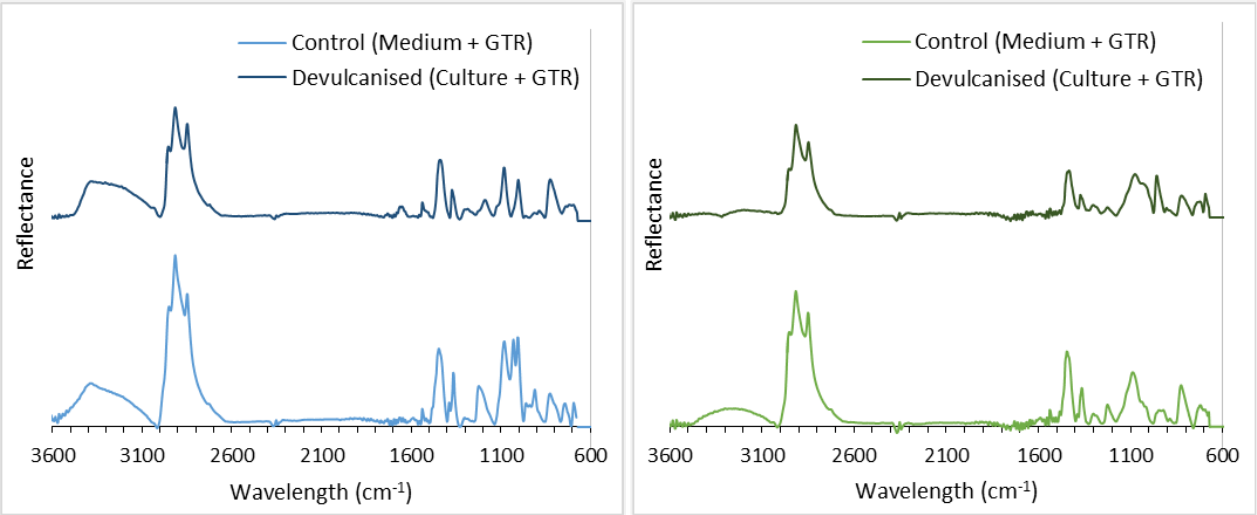

Figure 5-8: FTIR-ATR spectra for At. ferrooxidans cGTR and dGTR (left) and the biomining consortium cGTR and dGTR (right) treated for 30 days. Repeat readings in Appendix C.

5.5.3.4 SEM-EDS Analysis of Surface

SEM-EDS analysis was used to determine the qualitative change in sulphur content near the edge of the GTR particle surface of resin mounted GTR particles. Due to the varied shape and orientation of the mounted GTR particles, it was found that readings taken at the very edge of the particle often contained resin within the beam excitation region (Figure 5-9 a). Resin could be detected by increased levels of chlorine, which are not present in the GTR, (Figure 5-9). The interference caused by the resin resulted in an artificial sulphur gradient near the edge of the particle, since an increase in mass percent sulphur was observed as the fraction of GTR in the beam excitation region increased (Figure 5-9 (b) and (c)). To best approximate the conditions at the surface of the GTR, particle edges with a steep gradient were selected for analysis, and the 'edge' of the GTR was then approximated as the point at which the chlorine mass-percentage reading decreased below 0.25%. A crude estimate of sulphur variation was determined using Equation 5-3. Readings from sterile media treated GTR (cGTR) and microbially-treated GTR (dGTR) were then compared.

The ΔS of the cGTR for both the *At. ferrooxidans* cGTR and the biomining consortium cGTR is fairly constant, at 0.5 mass%, while the ΔS for the *At. ferrooxidans* dGTR and the biomining consortium more than doubles, at 1.2 mass% and 1.0 mass% respectively (Table 5-6). Expressed as relative ΔS, the *At. ferrooxidans* dGTR is more than three times larger than the corresponding cGTR, at 52.2% and 16.7% respectively (Table 5-6). The difference in relative ΔS of consortium cGTR and dGTR is not as large as *At. ferrooxidans*, at 33.3% and 44.4% respectively. The increase in ΔS of *At. ferrooxidans* treated dGTR compared to cGTR strongly suggests the occurrence of microbial desulphurisation at the surface of dGTR. Although not large as *At. ferrooxidans*, the

increased change in relative ΔS observed in biomining consortium treated dGTR suggests the occurrence of microbial desulphurisation.

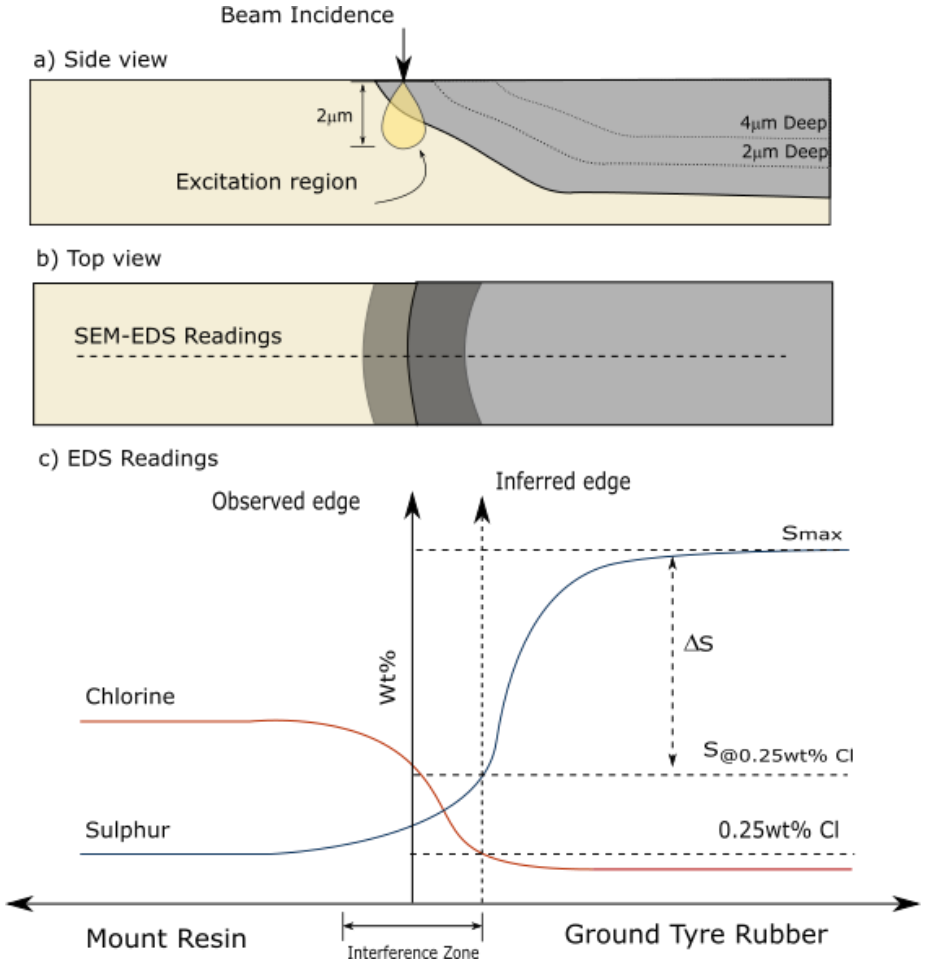

Figure 5-9: Illustration of SEM-EDS analysis of resin mounted GTR (a) as a cross-section, (b) from the top of the resin mount and (c) the corresponding EDS readings

$$\Delta S_{rel} = \frac{S_{max,interior} - S_{Cl=0.25\%}}{S_{Cl=0.25\%}} \qquad \textit{Equation 5-3}$$

Table 5-6: ΔS and relative ΔS for cGTR and dGTR samples for each microbe (please see Appendix C for raw data)

Microbe	ΔS		Relative ΔS	
	Control	Experiment	Control	Experiment
At. ferrooxidans	0.5	1.2	16.7%	52.2%
Biomining consortium	0.5	1	33.3%	44.4%

5.5.3.5 SEM IMAGING

SEM secondary electron images of a variety of irradiation sterilised GTR particles are presented to illustrate the variation in untreated GTR particle morphology (Figure 5-10), ranging from jagged edges, to polymer spheres, and smooth shearing.

In the images of *At. ferrooxidans* cGTR and dGTR treated for 30 days (Figure 5-11) the DSMZ Media 882 does not appear to cause any discernible changes in the cGTR morphology and there is no conclusive evidence of microbial effect on the dGTR due to the variability of surface morphology of the raw material. There is no conclusive evidence that the 0K* media had an impact on the surface of the consortium cGTR after incubation for 15 days or 30 days (Figure 5-13 to Figure 5-14), apart from a slight increasing of pockmarking on smooth surfaces. However, increasing microbial attachment with time was observed at the surface of the consortium dGTR (Figure 5-13 to Figure 5-14).

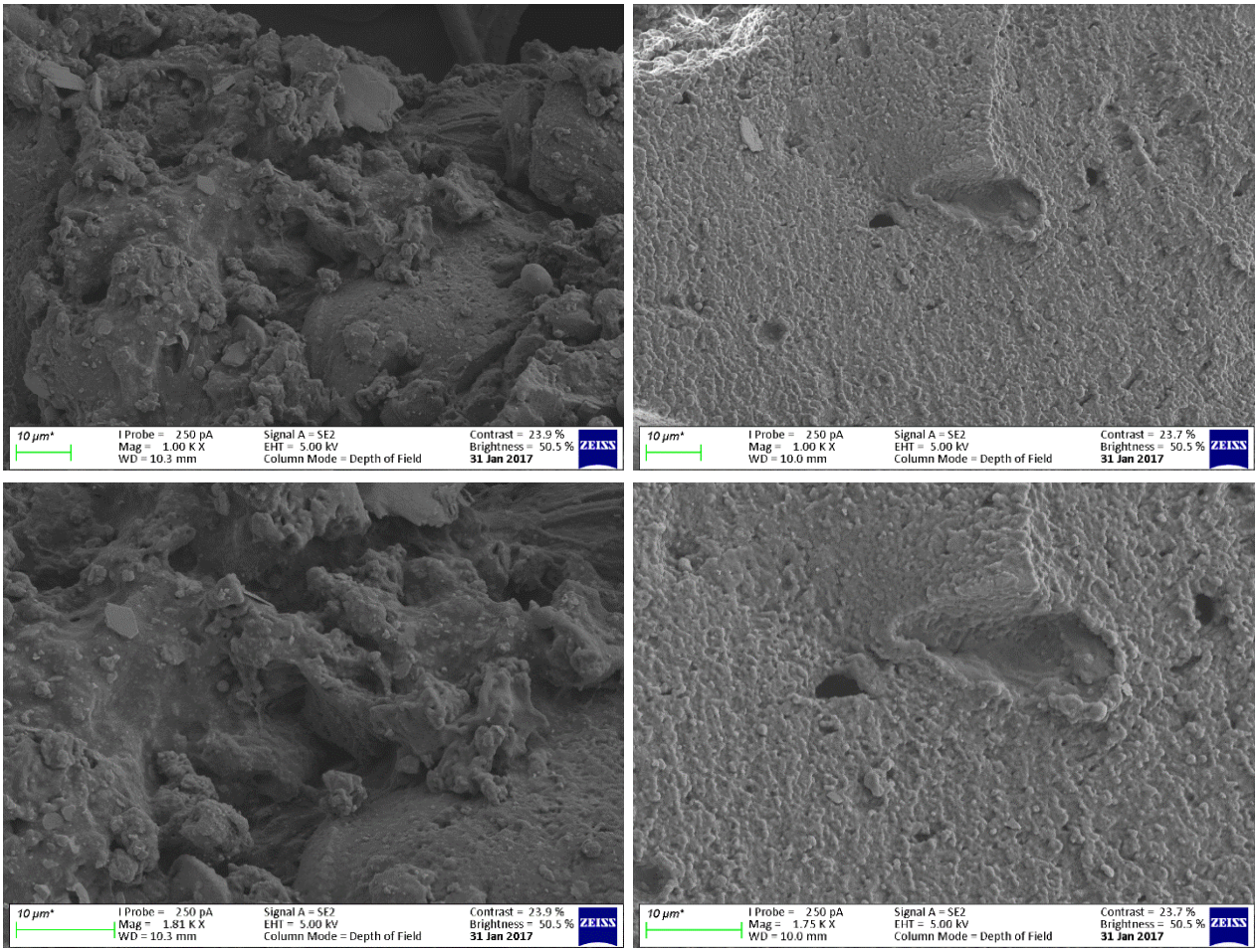

Figure 5-10: SEM secondary electron images illustrating the variation of surface morphology of 25 kG irradiated DAHWI 40 mesh crumb. Top images at 1000x magnification and bottom images at ~1800x magnification.

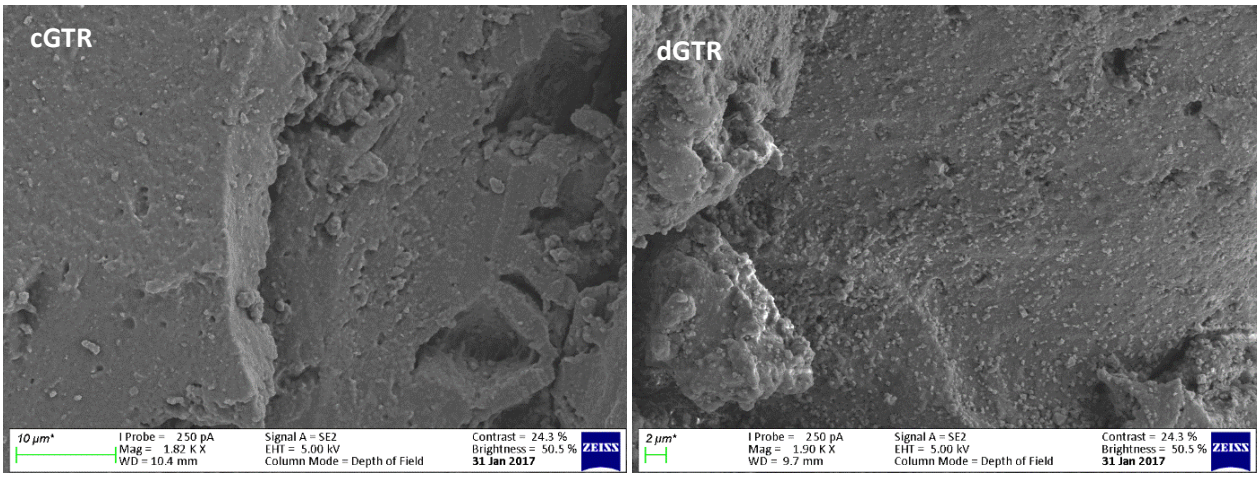

Figure 5-11: At. ferrooxidans – SEM secondary electron images of control GTR (cGTR) incubated in medium for 30 days and devulcanised GTR (dGTR) treated with At. ferrooxidans for 30 days. Images taken at 1800 – 1900x magnification.

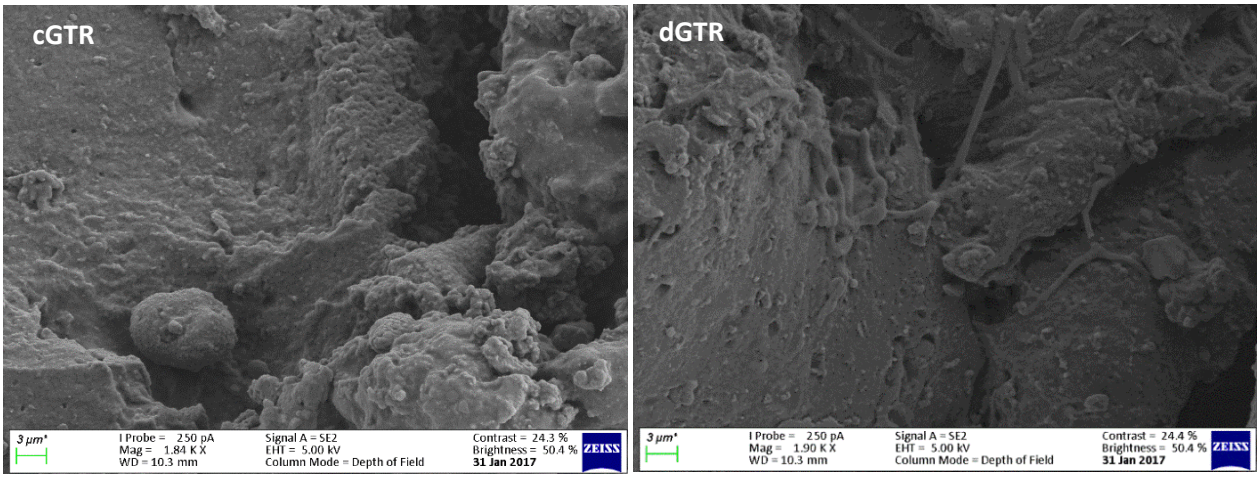

Figure 5-12: Biomining consortium after 15 days – SEM secondary electron images of control GTR (cGTR) incubated in medium for 15 days and devulcanised GTR (dGTR) treated with biomining consortium for 15 days. Images taken at 1800 – 1900x magnification.

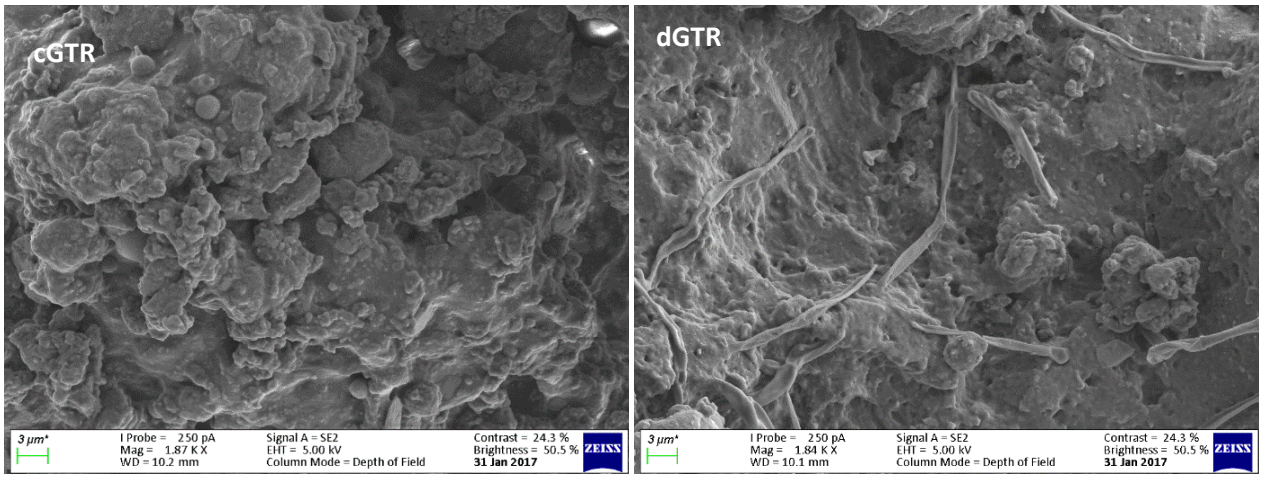

Figure 5-13: Consortium after 30 days – SEM secondary electron images of control GTR (cGTR) incubated in medium for 30 days and devulcanised GTR dGTR) treated with biomining consortium for 30 days. Images taken at 1800 – 1900x magnification.

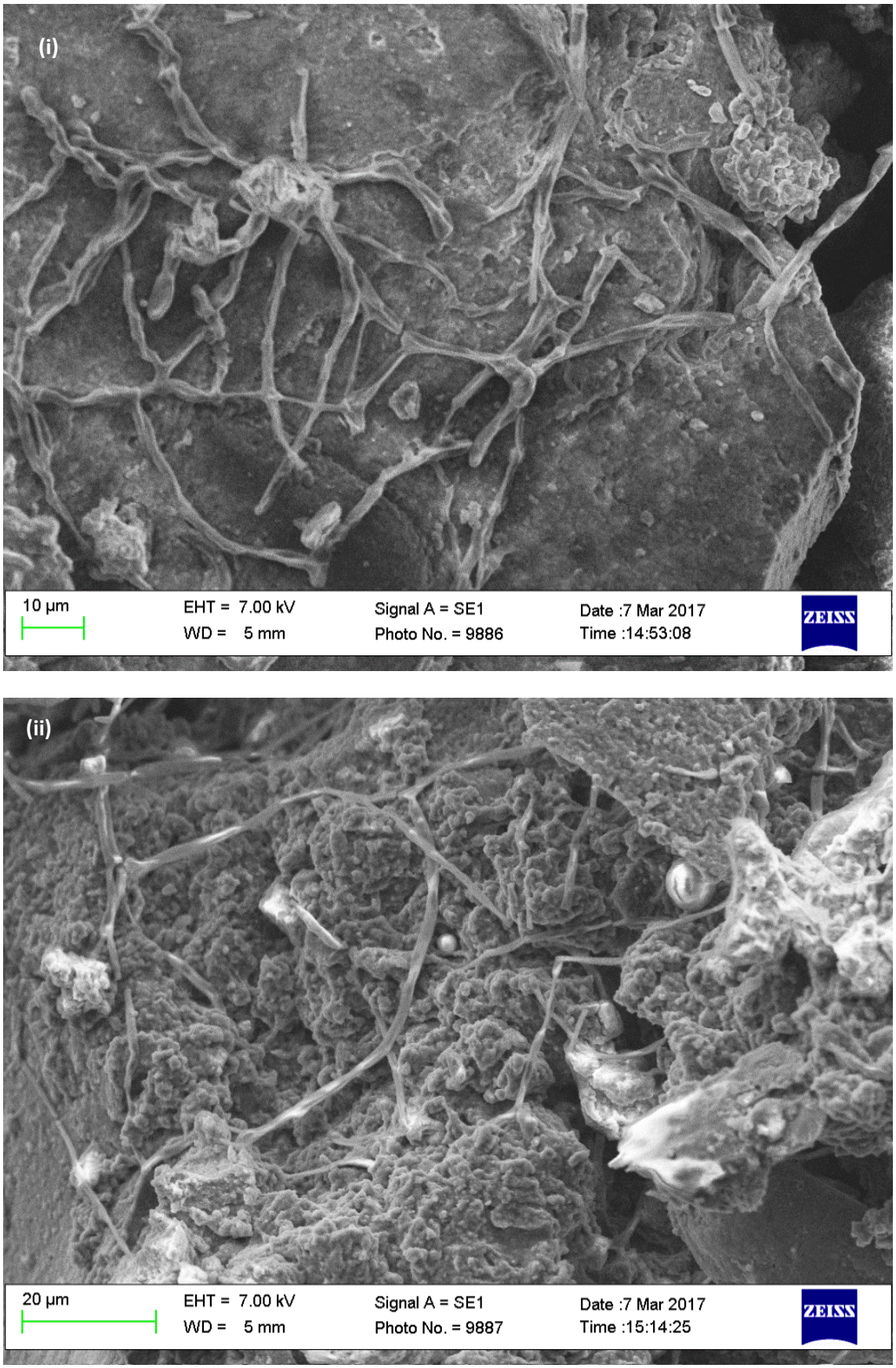

Figure 5-14: Variable pressure SEM images of microbial attachment on 30 °C consortium dGTR after 30 days of incubation (taken using LEO SEM to reduce effects of vacuum on cell structures)

5.6 Discussion

5.6.1 Sulphur Oxidation Activity of Cultures

The sulphur oxidation activity of *At. ferrooxidans* and the biomining consortium in this chapter corresponded well to the findings presented in Chapter 4. The control culture biomass concentration over time of *At. ferrooxidans* (Figure 5-2) and the biomining consortium (Figure 5-3) indicated that limited amounts of competitive energy sources, in the form of ferrous iron or entrained reduced inorganic sulphur, were present during the devulcanisation experiments. As such, any competitive energy sources were consumed within the first two days of incubation, and the only available energy source for the remainder of the devulcanisation experiments was the sulphur in the GTR. Furthermore, the sulphur oxidising cultures showed that both *At. ferrooxidans* (Figure 5-2) and the biomining consortium (Figure 5-3) oxidised elemental sulphur within the first few days of addition. Consequently, it can be concluded that both cultures could adapt to the sulphur oxidation metabolisms necessary to devulcanise GTR within the 30 day incubation period provided. The sulphur oxidation activity findings correspond to reports for *At. ferrooxidans* sp. (12,24) and YT-1 (23), which achieved devulcanisation using a similar growth method to that used for *At. ferrooxidans* in this study.

5.6.2 Microbial Growth Performance during Devulcanisation

The untreated GTR used in the 30 day devulcanisation experiments adversely affected the growth performance of devulcanisation cultures of *At. ferrooxidans* (Figure 5-2) and the biomining consortium (Figure 5-3). Growth inhibition, cell lysis and cell death after 10 to 30 days of incubation due to the addition of untreated GTR was observed for both cultures (Figure 5-2, Figure 5-3 and Figure 5-5).

Increased cell lysis in the *At. ferrooxidans* devulcanisation culture was observed compared to literature reports of *At. ferrooxidans* sp. (12), which suffered initial cell lysis but then recovered. Poorer performance in the current study was expected due to the omission of solvent leaching GTR detoxification, and the use of an industrial GTR which contains SBR, a known toxin for some strains of *At. ferrooxidans* (22). *At. ferrooxidans* sp. devulcanisation was carried out on ethanol leached, laboratory manufactured GTR which contained only GTR (12), reducing both the toxicity (12,13,22) and the complexity of the feed material. The <10 day active period of the *At. ferrooxidans* strain tested matched reports on *At. ferrooxidans* ATCC 13661 which produced sulphate for up to 7 days (11), but was shorter than other strains tested in literature, including DSMZ 583 (18), active for 10 to 20 days, *At. ferrooxidans* spp. (12) approximately 18 days and *At. ferrooxidans* YT-1 (23), active for 30 days. Variation in strain performance was likely caused by variation in strain toxin resistance, GTR composition, and the detoxification procedures used (Appendix A, Table A-1 and Table A-2).

Although cell lysis and cell death were observed in the sulphur oxidising component of the biomining consortium, a net increase in planktonic cells (Figure 5-3) and attached cells (Figure 5-13 to Figure 5-14) indicated that the non-sulphur oxidising component of the consortium continued to grow for the duration of

the experiment. Since no sulphur oxidising microbes were observed at the end of the experiment, and the sulphur and trace amounts of iron contained in the GTR were the only autotrophic energy sources available (Chapter 2.3), it can be concluded that the observed cell growth was heterotrophic. These microbes could have sourced carbon from simple organics produced by autotrophs (53), organic chemicals in the sol fraction, carbon black, or rubber polymer in the GTR to grow. It could not be determined whether the attached growth (Figure 5-13 to Figure 5-14) was prokaryotic or eukaryotic (77,78). *Acidophilum* spp. were identified as the prokaryotic heterotrophs most likely to be dominant, but *Alicyclobacillus* spp. and eukaryotic organisms may also have occurred (Chapter 2.3, Table A-1 and Table A-2). No reports on biomining consortium devulcanisation are available for comparison in literature.

Cell lysis in the devulcanisation cultures was caused by the presence of acetone-extractable compounds in the GTR (Figure 5-5). However, cell death was observed in devulcanisation cultures containing both acetone-leached GTR and untreated GTR within 10 days (Figure 5-5), despite sufficient nutrients in the growth medium and pH remaining within the acceptable growth range for both cultures (Figure 5-4 (a)i and (b)i), Table A-1 and Table A-2). These findings suggest that although cell lysis due to the acetone-extractable component of GTR is problematic to cell growth performance, non-acetone extractable compounds in GTR presented an even greater challenge to this study.

Potential toxins include the ash component of the GTR (13,22,23), SBR (22) and remaining extractable chemicals with a lower polarity than acetone (32). Non-polar chemicals are least likely to have caused cell death, since the difference in polarity with water makes them unlikely to leach into the aqueous solution, and no leaching of non-polar chemicals was observed during the acetone-extracted GTR devulcanisation experiments (Appendix C, Images of Experiments). SBR could, however, have contributed to GTR toxicity as it is known to account for approximately 22% of the rubber polymer in the Dawhi GTR used in the experiment (75) and is toxic to some strains of *At. ferrooxidans* (22). The ash content of the GTR is also likely to have contributed to the toxicity of GTR, as a portion of the ash was observed to leach into the medium (Table 5-5) and can include zinc and barium (32). Zinc, a heavy metal toxin, was observed to reach aqueous concentrations exceeding 150 ppm (2.3 mM) over the course of the devulcanisation experiments (Figure 5-4 (a)ii and (b)ii).

Zinc can cause cell inhibition at concentrations below 2.3 mM, as a strain of *At. ferrooxidans* has been reported which is susceptible to only 0.092 mM of zinc when metabolising RISCs (71). However, zinc tolerant strains of *At. ferrooxidans* can be resistant to over 1000 mM and *At. caldus* is only inhibited at 200 mM of zinc (73), far in excess of the 2.3 mM measured during devulcanisation experiments. Therefore, if zinc was the main cause of cell death, it indicates poor zinc tolerance in the *At. ferrooxidans* strain and the sulphur oxidising component of the biomining consortium used. Microbial attachment and the resultant formation of biofilms leads to increased toxin resistance (79), and could explain the notable increase biomass

concentration at the surface of the biomining consortium GTR compared (Figure 5-13 to Figure 5-12) to the slight changes observed in the planktonic biomass (Figure 5-3). Further investigation into the benefits of biofilm assisted devulcanisation processes using heavy metal resistant microbial cultures could improve performance. Alternatively, the use of a dilute acid leaching to target the removal of the GTR ash prior to microbial treatment could be investigated.

5.6.3 GTR Devulcanisation Performance

GTR properties indicating devulcanisation and polymer degradation were investigated. Devulcanisation was measured using sol fraction of the GTR and the SEM-EDS detection of sulphur removal near the GTR particle surface. Polymer degradation was investigated by FTIR-ATR analysis and SEM imaging of the GTR particle surface. Two methods of sol fraction measurement were used to improve comparability to literature. Since the experimentally measured sol fraction consists of both solvent extractable chemical additives and the soluble portion of the rubber polymer, it was observed to vary depending on the extraction method and solvents used. The Method 1 sol fraction was dominated by acetone extractable compounds, while the Method 2 sol fraction was dominated by toluene extractable compounds (Table 5-3 and Table 5-4). To control for any leaching of chemical additives or devulcanisation due to the sterile medium, the change in sol fraction due to microbial devulcanisation activity was measured as the difference between the medium treated GTR (cGTR) and microbially treated GTR (dGTR). Although the toluene Soxhlet extraction used in Method 2 achieved more complete removal of the soluble portion of the rubber polymer in GTR (32), it also resulted in increased error. The error was most likely due to the high temperature of toluene Soxhlet extraction, which exceeds 100 °C and can cause changes to the GTR properties (33,44).

At. ferrooxidans was conclusively found to cause devulcanisation of the industrial Dawhi GTR tested without causing carbon bond degradation. Both sol fraction methods indicated an increase in sol fraction with microbial treatment compared to medium treatment (**Error! Reference source not found.** and Table 5-3). An increase in sol fraction corresponds to an increase in free rubber polymer, which results either from the breakage of vulcanising sulphur crosslinks, or the breakage of carbon bonds in the rubber polymer. Since no breakage of double carbon bonds (1538 cm^{-1}) or methyl carbon bonds (3020 – 2840 and 1446 cm^{-1}) was detected at the surface of the GTR during FTIR-ATR analysis (Figure 5-8(a)i & ii), it can be concluded that the increase in sol fraction was due to devulcanisation. The large variation in GTR particle morphology meant that no conclusive changes in surface characteristics could be detected using SEM imaging (Figure 5-10 and Figure 5-11). No carbon bond degradation was anticipated, since previous devulcanisation studies using *At. ferrooxidans* have not detected carbon bond breakage (12,24), and *At. ferrooxidans* is an autotroph and not known to be capable of complex organic carbon degradation (53). The change in Method 1 sol fraction due to devulcanisation (**Error! Reference source not found.**and Table 5-3), 0.46 to 0.48%, was small, but within the ranges reported for *Gordonia amicalisa*, the only other microbe for which devulcanisation performance

has been characterised according to ASTM D6814 (27,33). The change in Method 2 sol fraction (**Error! Reference source not found.** and Table 5-3), 1.09% to 1.45%, was approximately half of the 2.74% increase reported in literature for *At. ferrooxidans* sp. using the same sol fraction method with ethanol leached, laboratory manufactured GTR (12). The increased devulcanisation performance of *At. ferrooxidans* sp. (12) is most likely due the reduced toxicity and increased NR content of the laboratory manufactured, ethanol leached GTR used, compared to the untreated Dawhi GTR used in the present study. Ethanol leaching would have removed a variety of toxic, polar compounds from the GTR (13,22,23). SBR, present in the Dawhi crumb (75) but not the *At. ferrooxidans* sp. crumb (12), is also known to be toxic to some strains of *At. ferrooxidans* (22) and may have further reduced performance of *At. ferrooxidans* DSMZ 14882. Additionally, increased toxin resistance in *At. ferrooxidans* sp. compared to *At. ferrooxidans* DSMZ 14882 may have contributed to the reduced performance observed in this study (23).

The biomining consortium exhibited poorer devulcanisation performance than *At. ferrooxidans*, and resulted in polymer degradation due to carbon bond breakage of the rubber polymer in the GTR. Devulcanisation was suggested by the observation of reduced concentrations of sulphur near the surface of microbially treated GTR particle, albeit to a lesser extent than *At. ferrooxidans* (Table 5-6). Devulcanisation is further supported by an increase in the Method 2 sol fraction due to microbial treatment of approximately half the magnitude of the *At. ferrooxidans* change was observed (**Error! Reference source not found.**). However, the change in Method 2 sol fraction was statistically insignificant due to the small change and large experimental error (Table 5-4). FTIR-ATR analysis indicated that carbon degradation occurred as a result of microbial activity of the biomining consortium. Polymer degradation due to biomining consortium activity was indicated by the breakage of C=C and -CH_2 and -CH_3 bonds, demonstrated by the reduced size of the peaks at 1538 cm^{-1}, 3020 – 2480 cm^{-1} and 1446 cm^{-1} in dGTR samples (Figure 5-8 (b)ii) compared to the biomining consortium cGTR (Figure 5-8 (b)i). The occurrence of carbon bond breakage corresponds to the hypothesis that the microbial attachment observed at the surface of biomining consortium dGTR (Figure 5-13 to Figure 5-14)) consisted of organic carbon metabolising, heterotrophic microorganisms. However, no conclusive evidence of carbon degradation could be seen in the SEM images (Figure 5-13 to Figure 5-14) due to the variation in the particle morphology of the Dawhi GTR feedstock (Figure 5-10). The statistically significant decrease in Method 1 sol fraction with biomining consortium treatment was unexpected (**Error! Reference source not found.** and Table 5-4), as no similar trends in literature have been reported in literature. Furthermore, the carbon bond breakage observed in FTIR-ATR analysis (Figure 5-8(b)i and ii) would be expected to increase the sol fraction of the biomining consortium treated GTR, since polymer degradation releases segments of carbon chains from the rubber matrix. However, the measured sol fraction is the sum of both the extractable chemical additives and the soluble portion of rubber in GTR. Because the feed GTR was not solvent leached prior to treatment, a portion of the chemical additives leached out of the GTR during the experiments; evident from the reduced sol fraction of cGTR and dGTR compared to the feed material at Day 0 (Appendix

C - Figure C-12 and Figure C-20). The decrease in sol fraction of the biomining consortium with microbial treatment in Method 1 is also displayed in the preliminary, room temperature acetone extraction step of Method 2 (Table 5-4), strongly suggesting that the biomining consortium increased the extent of leaching of acetone soluble chemical additives from the GTR. An increase in leaching of acetone soluble chemical additives during treatment will have obscured any increases in sol fraction due to devulcanisation and carbon bond breakage in acetone extractions. The increase in sol fraction reported by Method 2 suggests that the more polar, toluene extractable chemical additives in GTR were not leached by the microbes.

No conclusive evidence of devulcanisation due to the biomining consortium microbial activity could be determined using sol fraction analysis. However, both sol fraction methods indicated increased leaching of acetone extractable compounds from the GTR in the presence of the biomining consortium. Sol fraction results were inconclusive because the two methods contradicted one another: Method 1 indicated a -0.25% statistically significant decrease in the sol fraction of dGTR (**Error! Reference source not found.** (left)) and Method 2 indicated a statistically insignificant +0.56% increase in the sol fraction of dGTR (**Error! Reference source not found.** (right)). The decrease in sol fraction observed in Method 1 biomining consortium dGTR compared to the cGTR is unexpected as no similar trends have been reported in microbial devulcanisation literature (12,27). Furthermore, the carbon bond breakage observed in FTIR-ATR analysis (Figure 5-8(b)i and ii) would be expected to increase the sol fraction of the biomining consortium treated dGTR since polymer degradation would normally release segments of carbon chains from the rubber matrix and increase the sol fraction more rapidly than devulcanisation.

The poorer devulcanising performance of the biomining consortium suggested that the dominant sulphur oxidisers were either more susceptible to toxins in the GTR, or less effective than *At. ferrooxidans.* A previous study found that *At. thiooxidans*, which is one of the two additional sulphur oxidising microbes likely to be dominant in the consortium (Section 2.3), was not able to desulphurise GTR as effectively as *At. ferrooxidans* (11). This may be due to differences between the sulphur oxidation metabolisms used by the two microbes (Section 2.3). *At. caldus*, the other sulphur oxidising microbe likely to be dominant, has not previously been tested in literature but has a sulphur metabolism which is most similar to that of *At. thiooxidans* (Section 2.3) and may result in similar GTR devulcanisation performance. Some acidophilic heterotrophs have been shown to be capable of metabolising complex organic molecules, including as naphthalene (*Acidocella aromatica*), and could be responsible for the polymer degradation observed at the surface of the GTR particle.

5.7 Conclusions

At. ferrooxidans was conclusively found to cause devulcanisation without causing carbon bond degradation, which corresponded to previous studies on other *At. ferrooxidans* strains. The increase in sol fraction with microbial treatment could be entirely attributed to sulphur bond breakage (devulcanisation) because no carbon bond breakage occurred. Devulcanisation near the surface of the GTR particle was confirmed by SEM-

EDS analysis, which demonstrated a decrease in sulphur concentration near the surface of the GTR particles. Due to increased toxicity of the untreated Dawhi GTR used in this study, the change in sol fraction in this study (1.09 to 1.45%) was approximately half the value reported for ethanol leached GTR literature (2.74%). The morphology of the GTR was not found to change conclusively with medium treatment or microbial treatment.

The biomining consortium devulcanisation performance was poorer and less conclusive than *At. ferrooxidans*. Additionally, the biomining consortium removed a portion of the acetone extractable compounds from the GTR and caused carbon degradation at the surface of the GTR particle. Devulcanisation was indicated by a decrease in sulphur concentration near the edge of the GTR particles and an increase in the Method 2 sol fraction with microbial treatment. However, the Method 2 sol fraction increase, was only half the change observed for *At. ferrooxidans*, and was statistically insignificant. Increased removal of acetone extractable compounds with microbial treatment was observed in the Method 1 and Method 2 acetone extraction steps, and could have been the result of the activity of heterotrophic microorganisms. Carbon bond degradation due to heterotrophic microbial activity was observed on the surface of the GTR particle using FTIR, however physical degradation could not be conclusively observed with SEM imaging.

This study confirms that the properties of untreated GTR can be altered by treatment with both *At. ferrooxidans* and the biomining consortium. However, the poor devulcanisation performance of both cultures suggests that the process needs to be improved, either through increased toxin resistance of the microbial cultures or through pretreatment of the GTR crumb. Although the acetone-extractable toxins in the GTR caused substantial lysis, it was found that non-acetone-extractable constituents caused cell death in *At. ferrooxidans* and the sulphur oxidising component of the biomining consortium within 10 to 30 days of incubation. As such, the toxicity of the non-solvent-extractable portion of the GTR presents the largest challenge to improving devulcanisation performance of GTR. The non-acetone-extractable constituents in the Dawhi GTR identified as most likely to cause cell death were the SBR and heavy metals in the ash, particularly zinc. However, the soluble zinc concentration measured were relatively low (<3 mM), suggesting poor zinc resistance in *At. ferrooxidans* DSMZ 14882 and the sulphur oxidising component of the biomining consortium, compared to other strains of acidophiles tested in literature. SBR is part of the GTR rubber polymer and therefore cannot be removed using standard leaching methods. Ash is unlikely to be removed by ethanol leaching, however dilute acid leaching may aid in its removal from the GTR prior to microbial treatment. Consequently, the selection of microbial cultures and culturing methods which improve resistance to heavy metals, especially to zinc, and SBR could improve performance.

6 Concluding Remarks and Recommendations

The microbial devulcanisation performance of the type strain *At. ferrooxidans* DSMZ 14882 and a 30 °C acidophilic biomining consortium on industrial ground tyre rubber (GTR) was investigated. Culture preparation steps were developed for *At. ferrooxidans* DSMZ 14882 and the biomining consortium to ensure the cultures were sufficiently active at the point of GTR addition (Chapter 4). In the main devulcanisation experiments prepared cultures of *At. ferrooxidans* DSMZ 14882 and the biomining consortium were incubated with industrial Dawhi Mesh 40 GTR for 30 days. The microbial growth performance in the presence of GTR and the devulcanisation performance were characterised for each culture. Lastly, the toxicity of the acetone-extractable components in the GTR was investigated.

Culture preparation steps were developed for *At. ferrooxidans* DSMZ 14882 and the biomining consortium according to the nature of the cultures. The culture preparation step for both cultures ensured adequate cell concentration compared to literature, good sulphur oxidation activity, limitation of energy sources which could compete with the sulphur in GTR, and an acceptable pH for the duration of the experiment. The culture preparation step developed for *At. ferrooxidans* DSMZ 14882 consisted of incubation in ferrous iron limited DSMZ Medium 882* in shake flasks for 24 hours prior to the addition of GTR. The selected medium ensured a maximum biomass concentration similar to devulcanisation studies in literature (12,24). Furthermore, the ferrous iron in DSMZ Medium 882 was depleted within 36 hours of incubation, and therefore would not provide an alternative energy source to the sulphur in GTR. The biomining consortium was maintained at high biomass concentrations in bioreactors, and, therefore, did not require the preliminary growth stage used for *At. ferrooxidans* DSMZ 14882. However, the biomining consortium contained excess elemental sulphur which needed to be removed prior to devulcanisation experiments to ensure the sulphur in GTR was utilised. Coarse filtration with Whatman 1 filter paper was found to adequately remove entrained elemental sulphur while allowing planktonic cultures to pass through. Both *At. ferrooxidans* DSMZ 14882 and the biomining consortium were shown capable of sulphur oxidation within 48 hours of the culture preparation step and the pH remained in the desired range for the duration of the trial experiments.

The devulcanisation experiments confirmed the sulphur oxidation activity of both *At. ferrooxidans* DSMZ 14882 and the biomining consortium, through the increase in sol fraction and removal of surface sulphur. The type strain *At. ferrooxidans* DSMZ 14882 was conclusively found to be capable of devulcanisation of untreated Dawhi Mesh 40 GTR without causing polymer degradation. The reduced devulcanisation performance compared to literature, Δsol 1.45% compared to Δsol 2.74% (12), could be attributed to the increased toxicity of the untreated Dawhi GTR compared to ethanol leached, laboratory manufactured GTR used in the previous study, and the reduced toxin resistance of *At. ferrooxidans* DSMZ 14882. The biomining consortium devulcanisation performance was less conclusive, but suggested a small amount of devulcanisation, Δsol 0.56%, in addition to carbon bond degradation at the surface. Biomining consortium

was also observed to increase the extent of leaching of chemical additives in the GTR. The extent of devulcanisation and carbon degradation of the biomining consortium GTR could not be fully quantified due to feedstock and analytical limitations.

Dawhi ground tyre rubber (GTR) adversely affected growth performance of both cultures, resulting in cell lysis and the death of *At. ferrooxidans* DSMZ 14882 and the sulphur oxidising microbes in the biomining consortium. Although substantial cell lysis could be attributed to components in the acetone-extractable fraction of the GTR, the non-acetone-extractable fraction led to extensive cell death and, therefore, presented the biggest challenge to cell growth performance. It was hypothesised that SBR, as well as, zinc and other heavy metals contained in the GTR ash, were responsible for cell death. If soluble zinc was the primary toxin, the relatively low concentration observed (<3 mM) indicated very low zinc resistance of *At. ferrooxidans* DSMZ 14882 and the biomining consortium compared to literature, which has reported zinc tolerance of 200 to 1000 mM for similar, toxin resistant acidophiles. Interestingly, a portion of the non-sulphur oxidising microbes in the biomining consortium was observed to attach to the GTR and continued to grow throughout the incubation period. Since carbon bond degradation was observed at the surface of the biomining consortium treated GTR, the attached growth was hypothesised to be heterotrophic. The microbial population of the biomining consortium, however, was not fully characterised and it could not be ascertained whether the growth was prokaryotic or eukaryotic. The improved toxin resistance of the attached cells compared to the sulphur-oxidising cells was most likely due to a combination of increased toxin resistance of the heterotrophs and increased toxin resistance due to biofilm formation. These results suggest that devulcanisation performance of Dawhi GTR could best be improved by increasing strain resistance to non-acetone extractable compounds such as SBR and heavy metals. Alternatively, soluble ash could be targeted for removal with dilute acid leaching.

RECOMMENDATIONS

Future experiments should focus on further optimising microbial devulcanisation for maximum microbial growth and impact on industrial GTR, as well as, improving the toxin resistance of the microbes. Selection of strains which are already heavy metal and SBR resistant would accelerate and improve the process, however adaptation to the toxins may also be attempted. Alternatively, an investigation of a system which encourages biofilm growth could be pursued, since biofilm colonies have been reported to be more toxin resistant than planktonic cells. A full study on a variety of leaching methods and agents would be recommended to improve the microbial performance while limiting the extent of leaching required. The effect of GTR detoxification leaching is poorly understood, and as a result there is a large scope for further investigation. Screening of leaching agents could be accelerated and optimised for the best detoxification results using analytical techniques to characterise the soluble metals and organics contained in GTR leachate.

A number of analytical techniques require more refinement before they can be applied to microbial devulcanisation experiments. Development of methods which improve the accuracy of cross-link density measurement would allow for devulcanisation to be characterised using Horikx plots, and would improve understanding of the selectivity of the devulcanisation process. Additionally, measurement of sulphate production would help to accurately track the desulphurisation activity of the acidophiles. However, to ensure both accuracy and sensitivity of the sulphate measurements, pH adjustment using an acid other than sulphuric acid would be required to reduce the baseline concentration of sulphate. Although the measurement of acidophilic sulphate production has been reported using hydrochloric acid to adjust pH, the increased concentration of chlorine ions can negatively affect culture growth and any impact should be closely monitored.

To fully understand the impact of the biomining consortium on the GTR properties, the use additional characterisation techniques would be necessary. Devulcanisation experiments on uniform GTR flakes would allow for the changes in surface morphology to be properly investigated and would improve the quality of the FTIR-ATR readings. Additionally, GTR flakes would allow for the more accurate analysis of sulphur concentration near the surface of the GTR using SEM-EDS. The use of laboratory manufactured GTR would further aid initial characterisation tests and comparisons by providing a greater level of homogeneity in the samples. However, tests using laboratory manufactured GTR should be supplemented by tests on industrial GTR to assess the effectiveness of the process. Further characterisation of the microbial population of the consortium throughout devulcanisation experiments using fluorescence in-situ hybridisation (FISH) would also be recommended to improve understanding of the impact of GTR on consortium ecology.

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Appendix A. Literature Review

a. Common Prokaryotes in Mesophilic and Moderately thermophilic Acidophilic Consortia

Table A-1: Iron and sulphur oxidising prokaryotic organisms present in biomining consortia (RS – reduced sulphur)

Microbe	Temperature	pH Range	Electron Receptor	Carbon Metabolism	Electron Donor	References
Eubacteria						
Acidimicrobium ferrooxidans	Moderate Thermophile (45 – 50°C)	Opt 2	Facultative anaerobe	Autotroph, Mixotroph or Heterotroph	Fe^{2+}	(56,80)
Acidiphilium acidophilum (previously Thiobacillus acidophilus)	Mesophile (<25 – 30 °C Opt 25 – 30 °C)	1.5 – 6.5 (Opt 2.5 - 3)	Obligate aerobe	Autotroph, Mixotroph or Heterotroph	RS	(56,70,81,82)
Acidithiobacillus caldus	Moderate Thermophile (20 – 55°C, Opt 45°C)	1 – 3.5	Obligate Aerobe	Autotroph or Mixotroph	RS	(56,69,81,83)
Acidithiobacillus albertensis	Mesophile	2 – 4.5 (Opt 3.5 - 4)	Aerobe	Autotroph	RS	(56,84,85)
Acidithiobacillus ferrooxidans	Mesophile (10 – 37°C, Opt 25 – 30)	1 – 4.5 (Opt 1.8 – 2.5)	Facultative Anaerobe (O or Fe^{3+})	Autotroph	Fe^{2+}, RS, H_2, Formic Acid	(56,69,86–88)
Acidithiobacillus ferrivorans	Psycrotolerent Mesophile (4 – 37°C, Opt 28-33°C)	1.9 - 3.4	Facultative Anaeobe (uses Fe^{3+})	Obligate Autotroph	RS	(69,88,89)
Acidiferrobacter thiooxydans	Mesophile (Opt 38°C)	Min 1.2 (Opt 2)	Facultative Anaeobe (uses Fe^{3+})	Obligate Autotroph	Fe^{2+}, RS	(69,90)
Acidithiobacillus thiooxidans	Mesophile (10 – 37°C, Opt 28-30 °C)	0.5 – 5.5 (Opt 2.0 – 3.0)	Obligate Aerobe	Autotroph	RS	(56,69,81,88)
Ferromicrobium acidophilus	Mesophile (<20 – 40, Opt 37°C)	1.3 – 4.8 (Opt 2-2.5)	Aerobe	Heterotroph	Fe^{2+}	(56,70)
Leptospirillum ferrooxidans	Mesophile	Min 0.7 (Opt ~1.6-2)	Obligate aerobes (O)	Autotroph	Fe^{2+} only	(56,81,91)
Leptospirillum thermoferrooxidans	Moderate Thermophile	(Opt ~1.5-1.8)	Obligate aerobes (O)	Autotroph	Fe^{2+} Only	(56,81)

Table A-1 Continued: Iron and sulphur oxidising prokaryotic organisms present in biomining consortia (RS – reduced sulphur)

Microbe	Temperature	pH Range	Electron Receptor	Carbon Metabolism	Electron Donor	References
Leptospirillum ferriphilum	Mesophile (Opt 30 – 37°C)	1 – 2 (Opt ~1.5-1.8)	Obligate aerobes (O)	Autotroph	Fe^{2+} Only	(81,83,91)
Sulfobacillus acidophilus	Moderate Thermophile (45 – 50°C)	Opt 1.8	Facultative anaerobe	Autotroph, Mixotroph or Heterotroph	Fe^{2+}	(56,80,92)
Sulfobacillus disulfidooxidans	Psycrotolerant Mesophile (4 – 40°C, Opt 35°C)	0.5 – 6.0 (Opt 1.5 – 2.5)	Anaerobe	Mixotroph	RS	(56,93)
Sulfobacillus thermosulfidooxidans	Moderate Thermophile (20 – 60°C, Opt 37 – 50°C)	1 – 5.5 (Opt 1.6 – 2.7)	Facultative anaerobe	Autotroph, Mixotroph or Heterotroph	Fe^{2+}	(56,80,83,93)
Thiomonas cuprinus	Mesophile (Opt 30 – 36°C)	(Opt 3 – 4)	Aerobe	Mixotroph	RS	(56,94)

Table A-2: Non-iron and sulphur oxidising heterotrophic prokaryotes found in biomining consortia

Microbe	Temperature	pH Range	Electron Receptor	Carbon Metabolism	Electron Donor	References
			Archaea			
Thermoplasma acidophilum	Moderate thermophile (45 – 63°C, Opt 59°C)	0.5 – 4 (Opt 1-2)	Facultative anaerobe	Heterotroph	Archaea	(56,70)
Thermoplasma volcanium	Moderate thermophile (<35 – 55°C, Opt 59°C)	1 – 4 (Opt 2)	Facultative anaerobe	Heterotroph	Archaea	(56,70)
Picrophilus oshimae	Moderate thermophile (45 – 60°C, Opt 60)	-0.2 – 3.5 (Opt 0.7)	Obligate aerobe	Heterotroph	Archaea	(56,57,70)
Picrophillus torridus	Moderate thermophile (Opt 60)	-0.2 – 3.5 (Opt 0.7)	Obligate aerobe	Heterotroph	Archaea	(56,57,70)

Table A-2 continued: Non-iron and sulphur oxidising heterotrophic prokaryotes found in biomining consortia

Microbe	Temperature	pH Range	Electron Receptor	Carbon Metabolism	Electron Donor	References
			Eubacteria			
Acidiphilium cryptum	Mesophile (20 – 41°C, Opt 35 – 41°C)	1.9 – 5.9 (Opt 3)	Aerobic	Heterotroph	Organotroph (?) gets electrons from organics, also oligotrophy,S	(56,70,95)
Acidiphilium symbioticum	Mesophile (Opt 37°C)	1.5-5 (Opt 3-4)	Aerobic	Heterotroph	Organotrophic, not can deal with higher concentration of sugar	(56,70,96)
Acidiphilium rubrum	Mesophile	2.5 – 6	Aerobic	Heterotroph	Citric acid and Tween 80	(56,70,97)
Acidiphilium angustum	Mesophile	2.5 – 6	Aerobic	Heterotroph	Citric acid and Tween 80	(56,70,97)
Acidiphilium organovorum	Mesophile (20 – 45°C, Opt 37°C)	2 – 5.5 (Opt 3)	Aerobic	Heterotroph	Organic carbon	(56,70,98)
Acidiphilium multivorum	Mesophile (17 – 42°C, Opt 27 – 35°C)	1.9 – 5.6 (Opt ~ 3.5)	Aerobic	Heterotroph	Organic compounds	(56,70,99)
Acidocella facilis	Mesophile (25 – 37°C)	2.5 - 6	Aerobic	Heterotroph	Citric acid and Tween 80	(56,70,97,100)
Acidocella aminolytica	Mesophile (20 -37°C)	3 - 6	Aerobic	Heterotroph	Organic Carbon	(56,70,100,101)
Acidomonas methanolica	Mesophile (30 – 42°C)	2 - 5.5	Aerobic	Facultative Methalytroph	Organic carbon	(56,70,102)
Acidobacterium capsulatum	Mesophile (20 – 37)	3 - 6	Aerobic	Heterotroph	Organic carbon	(56,70,103)
Alicyclobacillus acidocaldarius	Thermophile (45 – 70°C, Opt 60 – 65°C)	2 – 6 (Opt 3-4)	Aerobic	Heterotroph	Organic carbon	(56,70,104,105)
Alicyclobacillus acidoterrestris	Moderate thermophile (<35 – 55°C, Opt 42 – 53°C)	2.2 – 5.8	Aerobic	Heterotroph	Organic carbon	(56,70,105)
Alicyclobacillus cycloheptanicus	Moderate thermophile (40 – 53°C, Opt 48°C)	3 – 5.5 (Opt 3.5 – 5.5)	Aerobic	Heterotroph	Organic carbon	(56,70,93,105)

b. Calculation of Gibbs Energy of At. Ferrooxidans Iron Metabolism

The difference in electrode potential for iron oxidation coupled to oxygen reduction at pH 2 is calculated using Equation A-1 to Equation A-3. $E_o{}'$ is used to denote standard electrode potential at pH 2 and $\Delta E_o{}'$ denotes the difference. It is shown that $\Delta E_o{}'$ is 400 mV.

$$\Delta E_o{}' = E_o{}'(red) + E_o'(ox) \quad \text{Equation A-1}$$

$$\therefore \Delta E_o{}'(FeOx) = E_o{}'\left(\tfrac{1}{2}O_2 \rightarrow H_2O\right) + E_o{}'(Fe^{2+} \rightarrow Fe^{3+}) \quad \text{Equation A-2}$$

$$\therefore \Delta E_o{}'(FeOx) = 1120 + (-700) = 400\ mV \quad \text{Equation A-3}$$

The Gibbs free energy ($\Delta G^{o\prime}$) released by a redox reaction is a function of both the number of electrons per mole of iron (n) and the $\Delta E_o{}'$, as demonstrated by Equation A-4.

$$\Delta G^{o\prime} = -nF\Delta E_o{}' \quad \text{Equation A-4}$$

$$\Delta G^{o\prime} = -(1)(96.49)(0.4)\frac{kJ}{mol.V}V = -38.60\frac{kJ}{mol} \quad \text{Equation A-5}$$

Where $\Delta G^{o\prime}$ is the Gibbs free energy at pH 2, n is the number of electrons released per mole of iron oxidised and F is Faraday's constant. Since only 1 mole of electrons is released per mole iron oxidised, the Gibbs free energy for this reaction is -38.60 kJ/mol. All values for calculations based on (62).

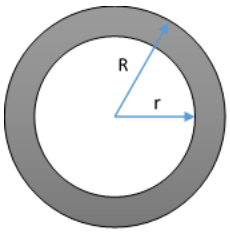
Appendix B. Development of Preliminary Culturing Step

a. Theoretical Availability of Sulphur in GTR

i. Overview

Surface phenomenon

- Limitation on total change in properties
- Particle size will impact the total changes detected
- From literature it is suggested that a maximum penetration of 4µm is achieved, and that the average penetration is ~2 µm



0 < R-r <4µm

R-r ~ 2 µm

Volume fraction affected: $\frac{V_R - V_r}{V_R}$

For estimate of volume fraction effected:

- Assume spherical particles
- Assume penetration of 2 µm
- Use mean particle size if particle size distribution unavailable

R↓ , $\frac{V_R - V_r}{V_R}$ ↑

Maximum changes recorded in literature

- ~16% desulphurisation (ambient, <400 µm)[1]
  - Min volume fraction effected ~ 3%
- ~14% desulphurisation (74 µm)[2]
  - Volume fraction effected ~ 15%
- 2.94% change in soluble fraction (<200 µm, mostly 50 µm)[10]
  - Volume fraction effected ~ 22%

ii. Method

Calculation of volume penetrated for a single particle size:

Since $V_R = \frac{4}{3}\pi R^3$ and $V_r = \frac{4}{3}\pi r^3$, and $\frac{V_R - V_r}{V_R}$ is the volume fraction of the particle effected by the microbes, V_{effect} can also be expressed as follows:

$$\frac{V_R - V_r}{V_R} = \frac{R^3 - r^3}{R^3}$$

Since R- r is assumed to be approximately 2µm, the effected volume of any given particle size can be determined.

Calculation of volume penetrated for a particle size distribution:

Let $y_i = mass\ fraction\ of\ particle\ size$ "i"

Let $\overline{\frac{V_R - V_r}{V_r}} = the\ average\ accessible\ volume\ for\ a\ particle\ distribution$

Therefore,

$$\overline{\frac{V_R - V_r}{V_r}} = \sum_i^n \left(y_i \times \frac{R_i^3 - r_i^3}{R_i^3} \right)$$

Calculation of available sulphur:

If it is assumed that all the sulphur in the accessible volume of the particle (the outer 2µm layer) can be oxidised by the microbe, and that there is homogenous distribution of sulphur, the concentration of available sulphur can be calculated as follows:

$$C_{available\ Sulphur} = \left(\frac{V_R - V_r}{V_r}\right) x_{sulphur} C_{rubber}$$

Where $x_{sulphur}$ is the fraction of sulphur in the rubber and C_{rubber} is the concentration of rubber.

iii. CALCULATIONS

Table B-1 demonstrates the calculation of available sulphur for a relatively simple particle distribution, determined using a set of sieves. This method found an average effected volume of 5.3% for DAHWI 40 Mesh GTR.

Table B-1: Table demonstrating calculation of volume fraction effected for a simple particle size distribution.

Interval (µm)	Midpoint		Retention			Volume Fraction Effected	
	Diameter (µm)	Radius (µm)	Weight (g)	Fraction	Cum%	For Size Interval	In Particle Size Distribution
x > 850			0	0	0	0	0
600 < x < 850	725	362.5	0.8	0.014286	0.014286	0.016461	0.000235
425 < x < 600	512.5	256.25	9.6	0.171429	0.185714	0.023232	0.003983
250< x < 425	337.5	168.75	26.2	0.467857	0.653571	0.035136	0.016439
x < 250	125	62.5	19.4	0.346429	1	0.092961	0.032204
Total			56	1			0.052861

In order to achieve more accurate results, the effected volume calculations were repeated using size distribution data obtained from a Saturn DigiSizer 5200 V1.12, shown in Figure B-1.Using this method the accessible volume was calculated to be 6.62% of the total sample volume. Figure B-2 shows accessible volume of each sample interval as a percent of the total sample volume as well as the cumulative accessible volume. Assuming that there is uniform distribution of sulphur throughout the particle volume, the average accessible volume obtained from the Saturn DigiSizer particle distribution, 6.62%, the sulphur mass% from the LECO analysis, 2.09±0.26 mass%, and a concentration of 50g/l of GTR, the concentration of available sulphur is determined to be approximately 0.07 g/l (see Table B-2).

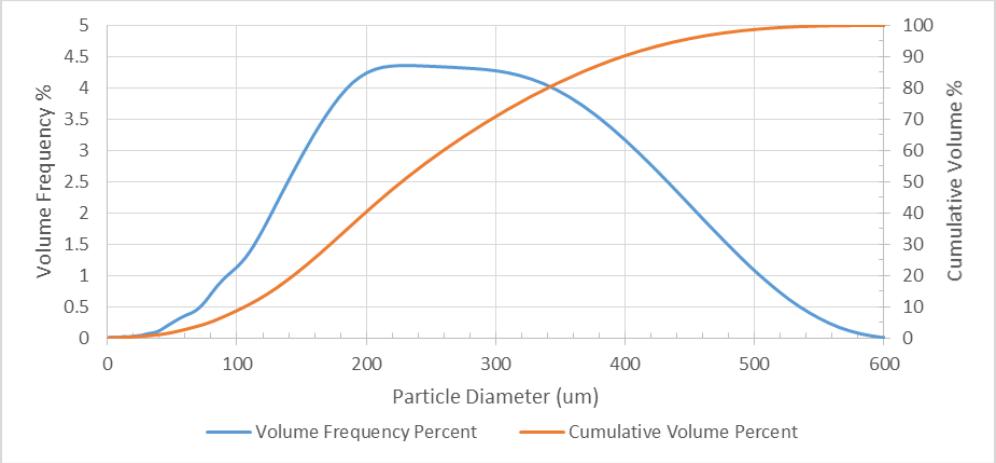


Figure B-1: Particle size distribution of DAHWI Mesh 40 ground truck tyre rubber

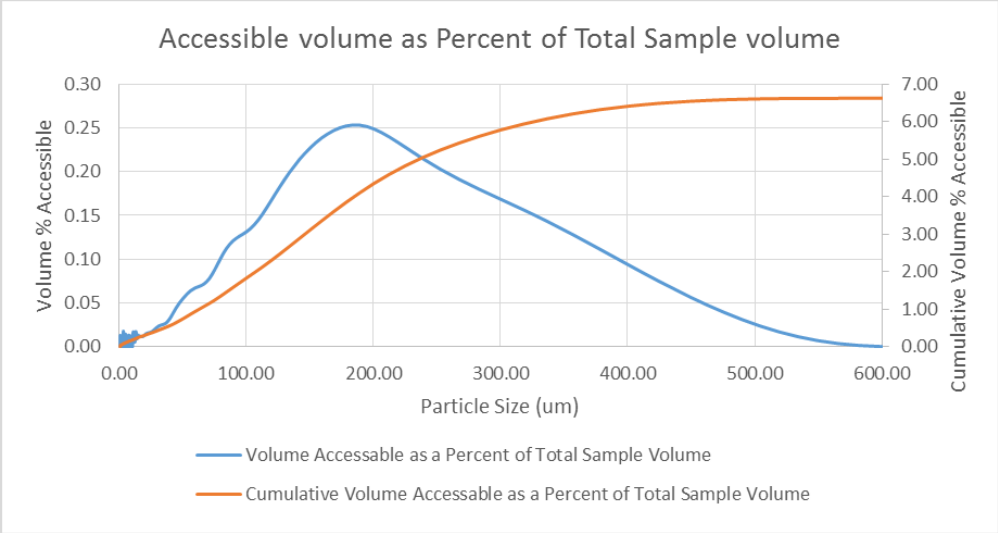


Figure B-2: Accessible volume of rubber as a percent of total sample volume

Table B-2: Determination of concentration of accessible sulphur in a 50 g/l slurry of GTR

Parameter	Amount	Unit
Concentration Rubber	50	g/l
Accessible volume	6.62%	volume%
%Sulphur in GTR(avg)	2.09%	mass%
%Sulphur in GTR (error)	0.26%	mass%
Total Sulphur in GTR (avg)	1.0433	g/l
Total Sulphur in GTR (error)	0.1280	g/l
Sulphur Accessible (avg)	0.06907	g/l
Sulphur Accessible (error)	0.00847	g/l

b. Images of Experiments

i. Experimental Set-Up

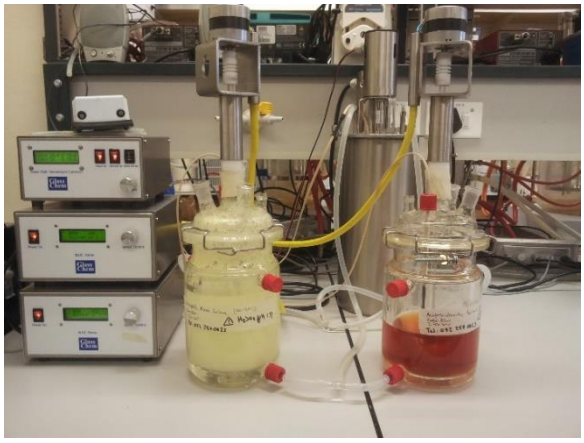


Figure B-3: Trial run in 1 litre bioreactors with sulphur based consortia (left) and iron based consortia (right)

Figure B-4: First day of liquid storage of pure cultures of At. ferrooxidans stored on pyrite (left) and At thiooxidans stored on sulphur (right)

ii. Images of Cells Under Light Microscopy

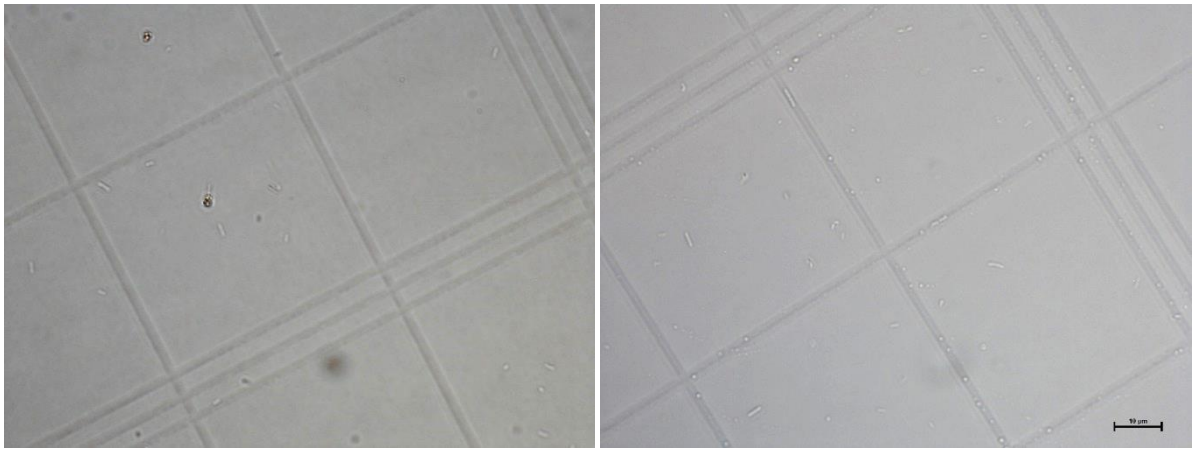


Figure B-5: Image of cultures in Petroff-Hausser counting chamber under 1000x magnification in a phase contrast microscope. Left - At. ferrooxidans and right – biomining consortium.

iii. Images of Media Selection Experiments

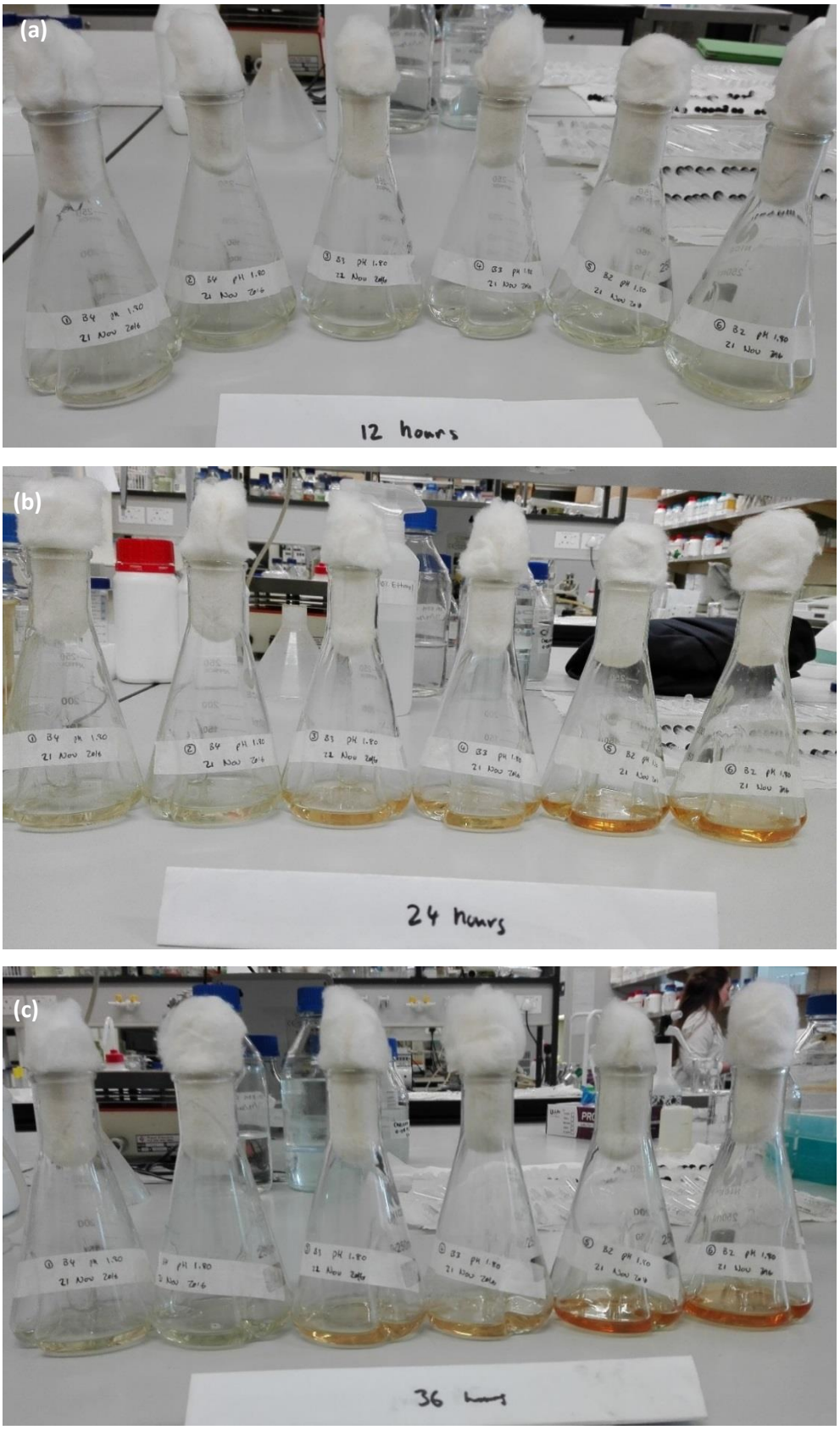


Figure B-6: At. ferrooxidans media selection - Initial Fe^{2+} concentration variation with initial pH 1.8 (a) after 12 hours, (b) after 24 hours and (c) after 36 hours of incubation. From left Fe^{2+} 0.56 g/l, 1.12 g/l, 2.25 g/l.

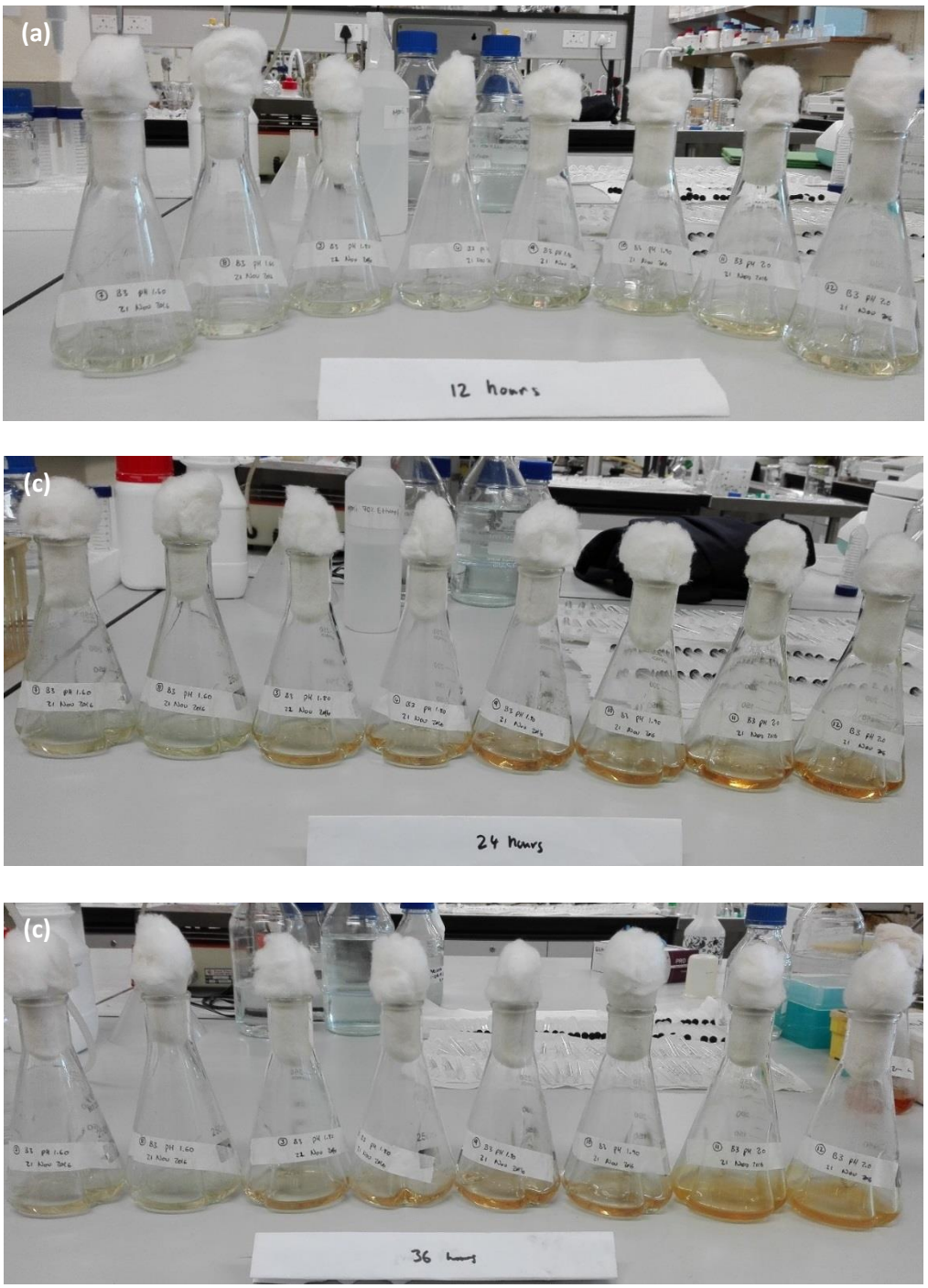


Figure B-7: At. ferrooxidans – initial pH variation with Fe^{2+} concentration of 1.12 g/l in duplicate after (a) 12 hours, (b) 24 hours and (c) 36 hours. From left: initial pH 1.6, 1.8, 1.9, and 2.0.

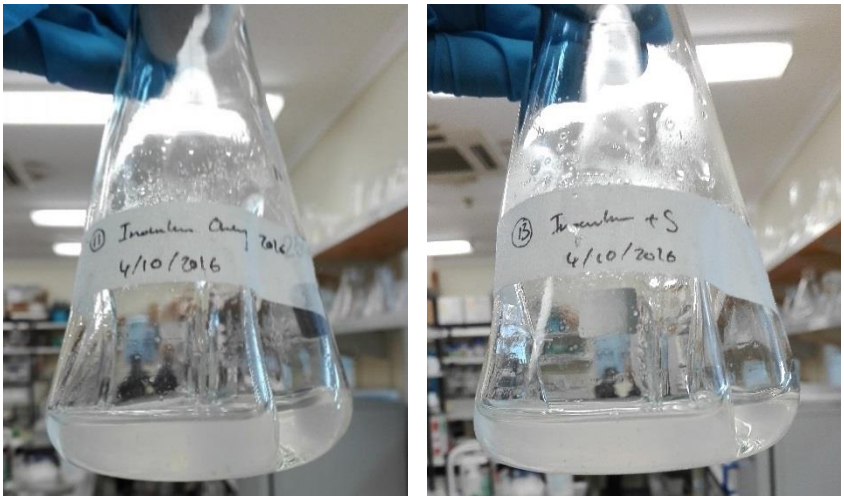


Figure B-8: Consortium – filtered inoculum only (left) and filtered inoculum with additional elemental sulphur (right), which is in too low a concentration to be visible

c. Maximum Growth Rate Calculations

i. At. ferrooxidans

Table B-3: Calculation of required concentration of various electron sources based on constant electron concentration

Electron Source	**Concentration Added (g/l)**	**Mass Total (g)**	**M (g/mol)**	**Moles Electron Source Added**	**Moles e^-** per Mole added	**Moles e- in solution**	**Final Electron Concentration (mol/l)**
Initial Fe^{2+} Dosing	1.12	0.0672	55.845	0.001203	1	0.001203	0.02006
Additional Fe^{2+}	1.12	0.0672	55.845	0.001203	1	0.001203	0.02006
Additional S	0.14	0.00835	32.065	0.000260	6	0.001562	0.02604
Additional Tetrathionate	0.47	0.0282	302.45	0.000093	14	0.001305	0.02176

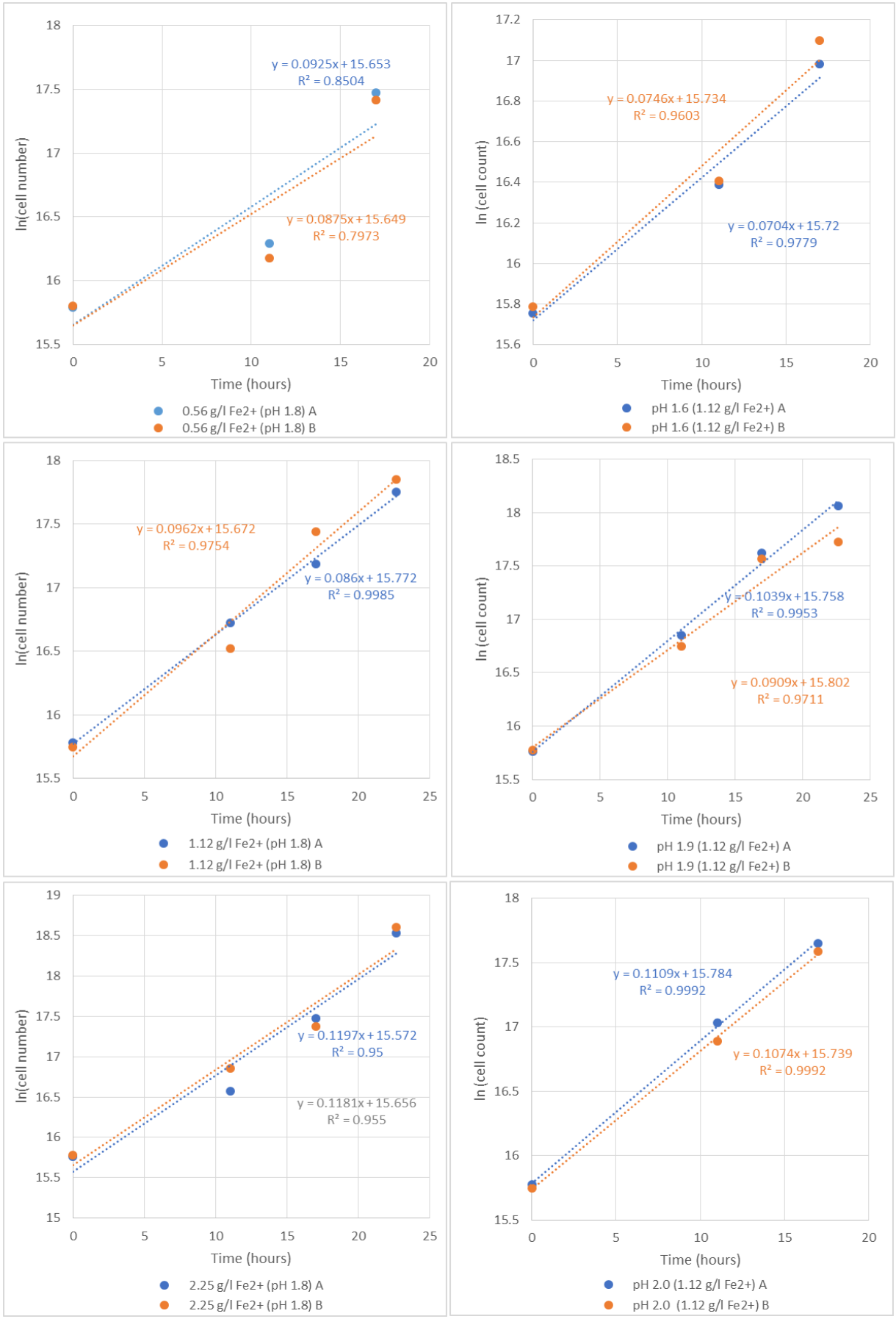


Figure B-9: At. ferrooxidans – determination of maximum growth rate for varying initial concentrations of ferrous iron and initial pH

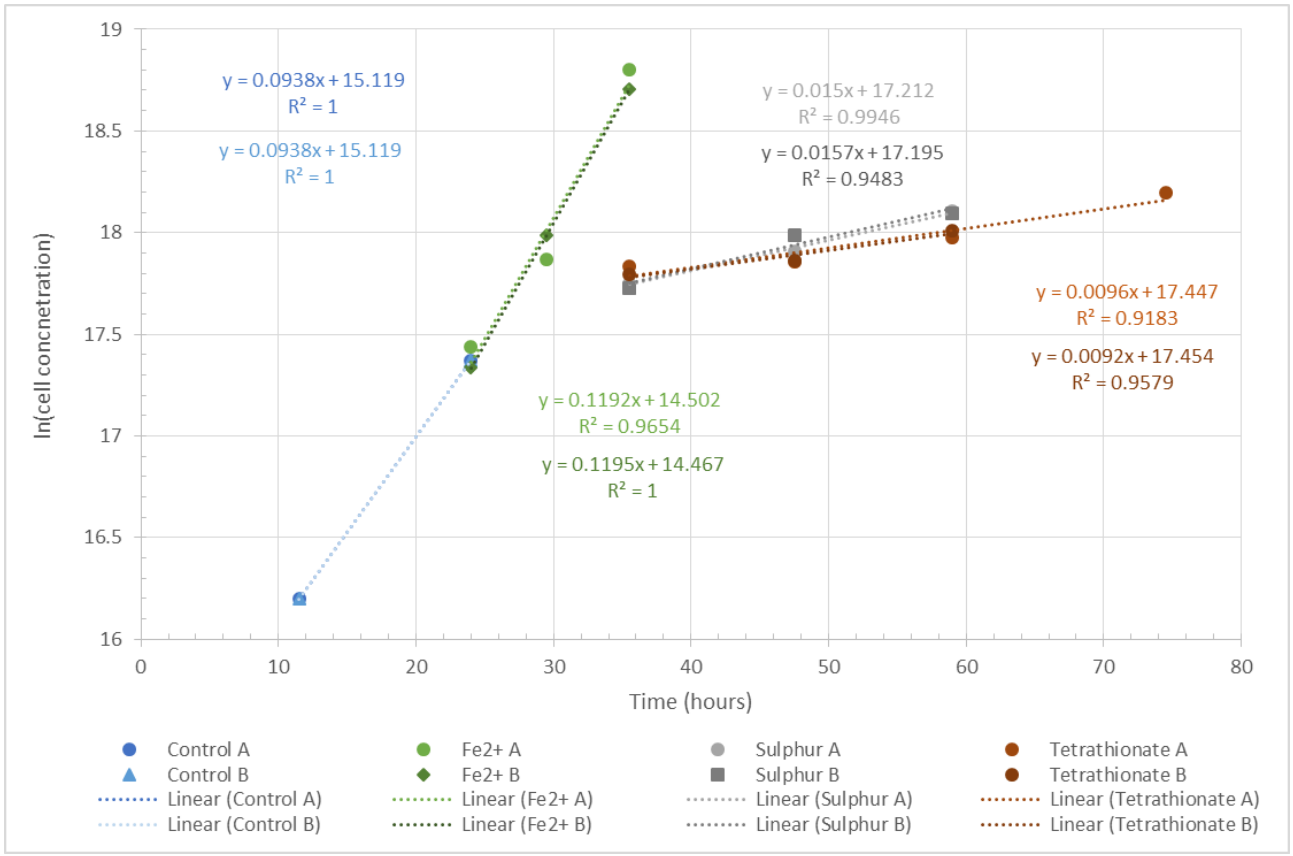


Figure B-10: At. ferrooxidans – determination of maximum growth rate for different electron sources in the culture viability tests

ii. Biomining Consortium

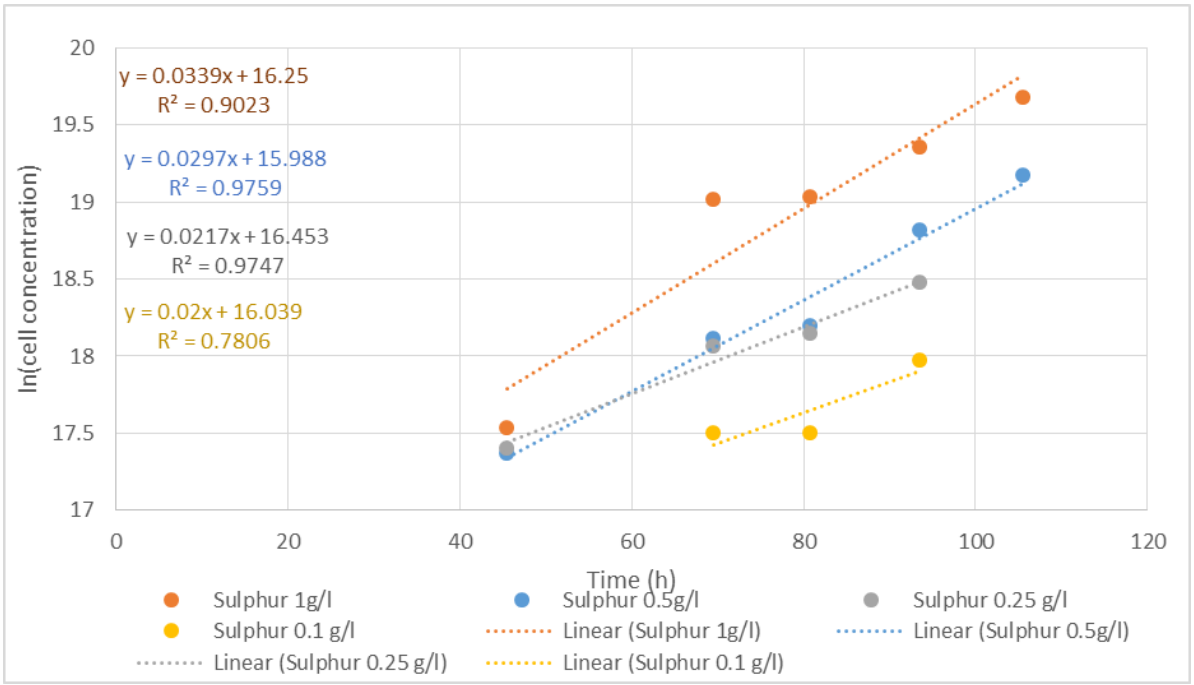


Figure B-11: Consortium – determination of maximum growth rate for varying initial concentrations of elemental sulphur

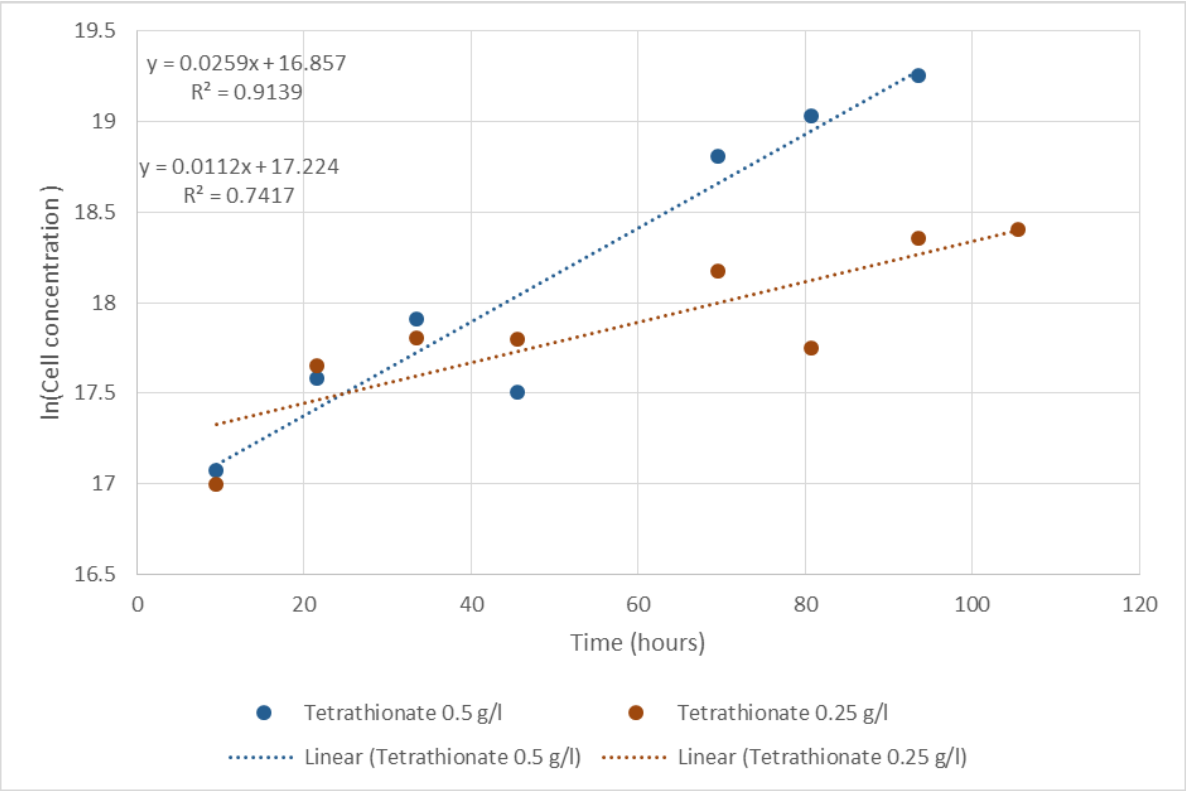


Figure B-12: Consortium – determination of maximum growth rate for varying initial concentrations of tetrathionate

Appendix C. Microbial Devulcanisation

a. Sterilisation of GTR Crumb

In order to characterise the potential effect of high levels of gamma irradiation on GTR, soluble percentage and proximate analysis were conducted for unirradiated (0kG), medium irradiation (15 kG) and high irradiation (25 kG) GTR. 25 kG was selected as the upper limit as this is the level used to irradiated plastic and rubber medical implants (106).

A weak upwards trend in soluble percentage of the rubber with increasing exposure to gamma irradiation can be observed in Figure C-1. However, these changes were found to be statistically insignificant when analysed using t-test statistics (see Table C-1).

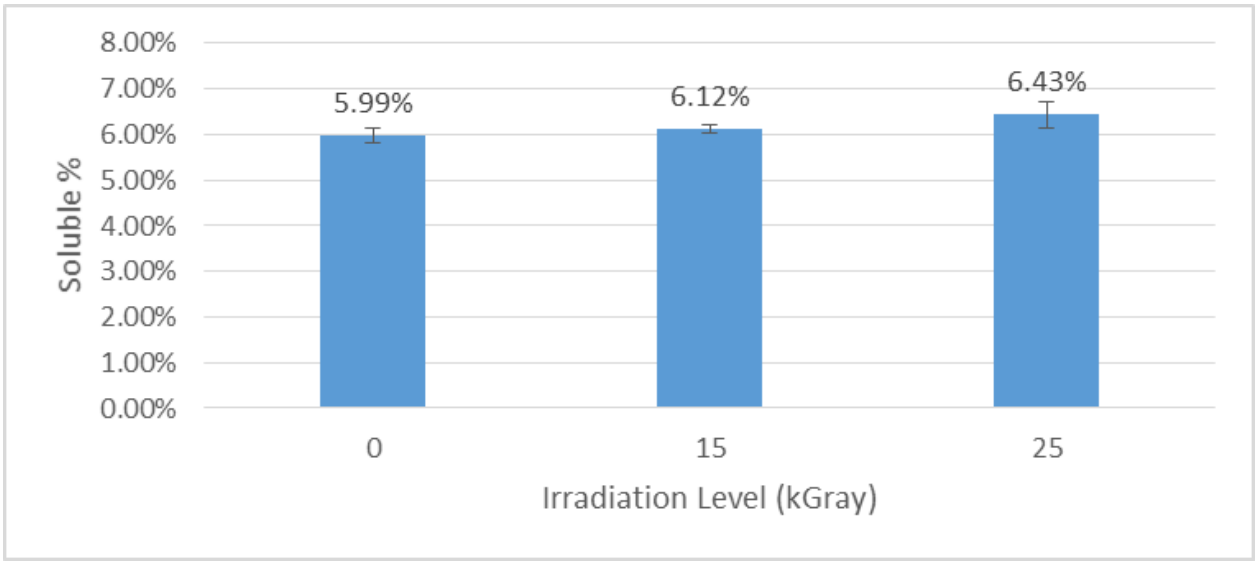


Figure C-1: Effect of gamma irradiation on soluble fraction of DAWHI 40 mesh ground tyre rubber

The proximate analysis results (Table C-2) indicate that there are no major effects on the rubber properties, apart from a minor increase in water content 0.05% and a minor decrease in both oils content (0.32%) and volatiles content (0.08%) for the irradiated rubber.

Table C-1: T-tests for rubber irradiated at 0, 15 and 25 kG. Tests shown determine whether differences between 0-15, 0-25 and 15-25 are significant.

F-Test Two-Sample for Variances		
	0	*15*
Mean	-0.09086	-0.09159
Variance	1.42E-06	3.16E-07
Observations	3	3
df	2	2
F	4.482944	
P(F<=f) one-tail	0.182384	
F Critical one-tail	19	

F-Test Two-Sample for Variances		
	25	*0*
Mean	-0.0934	-0.09086
Variance	4E-06	1.42E-06
Observations	3	3
df	2	2
F	2.825663	
P(F<=f) one-tail	0.261393	
F Critical one-tail	19	

F-Test Two-Sample for Variances		
	25	*15*
Mean	-0.0934	-0.09159
Variance	4E-06	3.16E-07
Observations	3	3
df	2	2
F	12.66729	
P(F<=f) one-tail	0.073167	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	0	*15*
Mean	-0.09086	-0.09159
Variance	1.42E-06	3.16E-07
Observations	3	3
Pooled Variance	8.67E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.961803	
P(T<=t) one-tail	0.195307	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.390615	
t Critical two-tail	2.776445	

Not significant

t-Test: Two-Sample Assuming Equal Variances		
	25	*0*
Mean	-0.0934	-0.09086
Variance	4E-06	1.42E-06
Observations	3	3
Pooled Variance	2.71E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	-1.89204	
P(T<=t) one-tail	0.065721	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.131441	
t Critical two-tail	2.776445	

Not significant

t-Test: Two-Sample Assuming Equal Variances		
	25	*15*
Mean	-0.0934	-0.09159
Variance	4E-06	3.16E-07
Observations	3	3
Pooled Variance	2.16E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	-1.51026	
P(T<=t) one-tail	0.102747	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.205494	
t Critical two-tail	2.776445	

Not Significant

Table C-2: Comparison of proximate analysis results for irradiated and untreated rubber both with the water fraction included and the water fraction excluded

Variable	With Water		Without Water	
	0 kGray	25 kGray	0 kGray	25 kGray
Water	0.50%	0.55%		
Oils	5.80%	5.72%	5.83%	5.75%
Volatiles	57.20%	56.80%	57.45%	57.13%
Fixed carbon	30.30%	30.40%	30.43%	30.58%
Ash	6.26%	6.50%	6.29%	6.54%
Total	100.06%	99.97%	100.00%	100.00%

It was found that gamma irradiation up to and including 25 kGray could be used to sterilise the GTR without statistically significant impact on the soluble fraction of the rubber (see Figure C-1) or the proximate analysis properties.

b. Images of Experiments

i. Experimental Set-Up for Soxhlet Extraction

Figure C-2: Soxhlet extraction experimental set-up

ii. Devulcanisation Experiments

Figure C-3: At. ferrooxidans – devulcanisation experiment after 30 days of incubation, sterile control (left) inoculated flask (right)

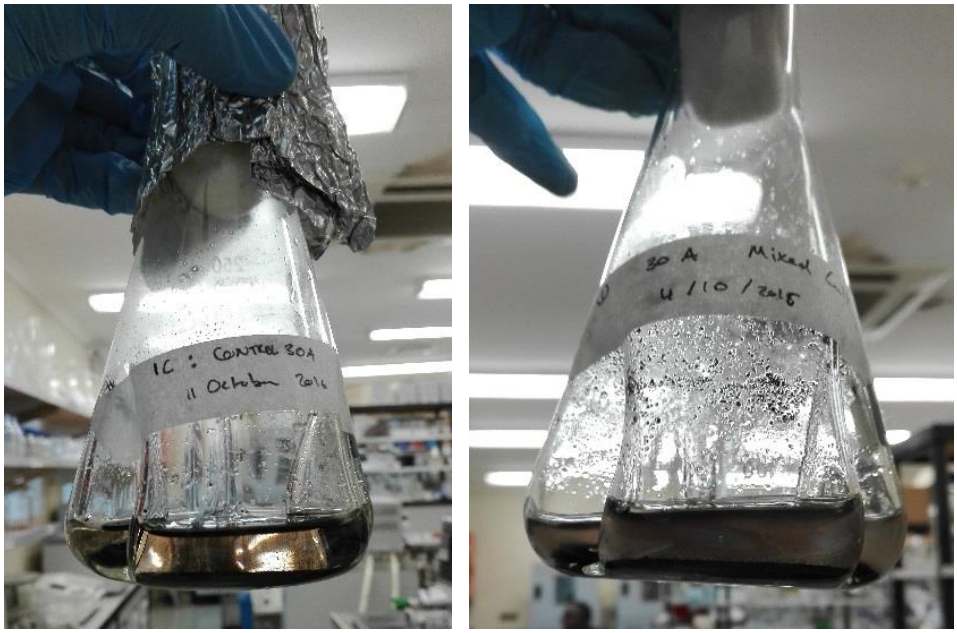


Figure C-4: Consortia – devulcanisation experiment after 30 days of incubation, sterile control (left) inoculated flask (right)

iii. Acetone Leached GTR Experiments

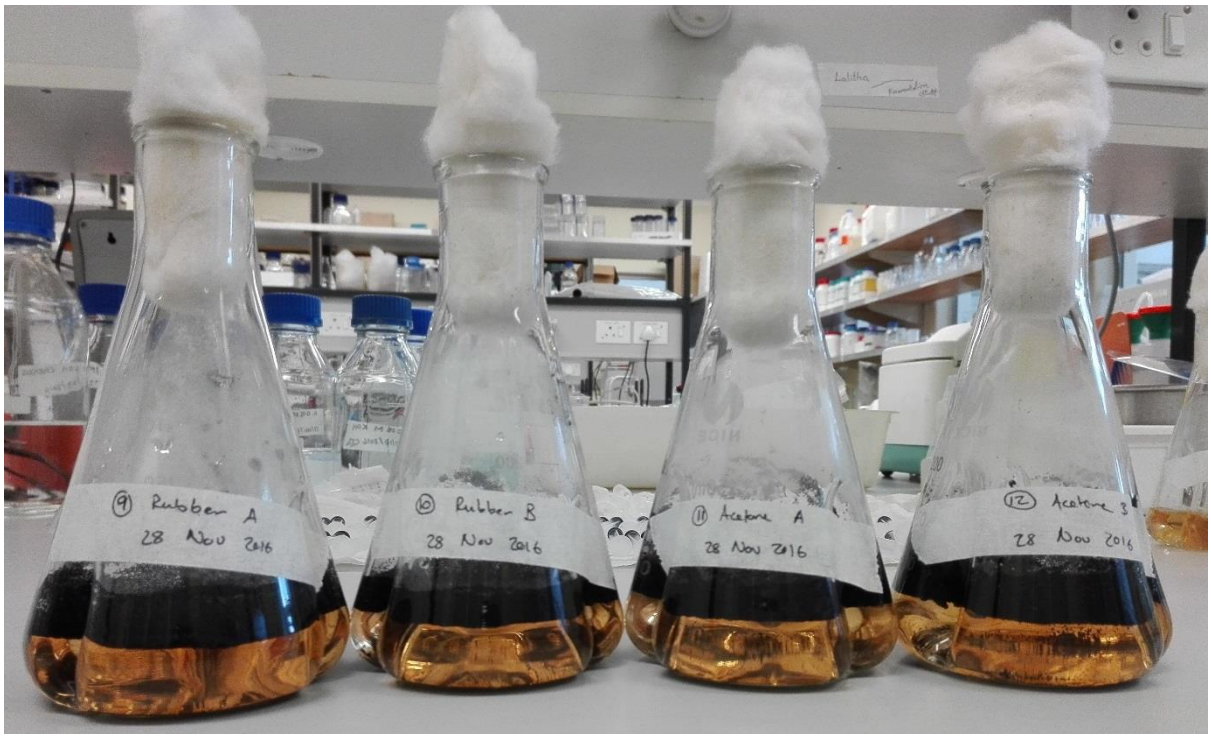


Figure C-5: At. ferrooxidans toxicity investigation – at point of addition of untreated GTR (left duplicate) and acetone extracted GTR (right duplicate), total incubation time 36 hours

Figure C-6: At. ferrooxidans toxicity investigation – 24 hours after addition of untreated GTR (left duplicate) and acetone extracted GTR (right duplicate), total incubation time 60 hours

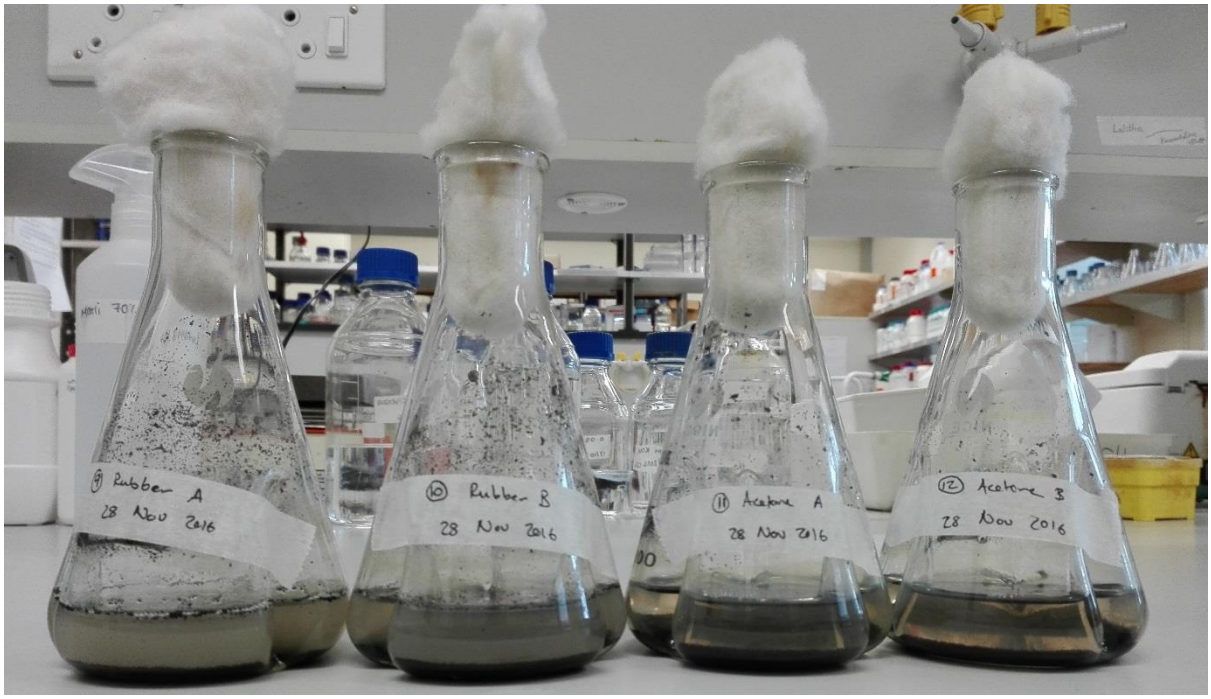


Figure C-7: At. ferrooxidans toxicity investigation – 84 hours after addition of untreated GTR (left duplicate) and acetone extracted GTR (right duplicate), total incubation time 120 hours. Murky leachate clearly visible in GTR samples.

iv. VIABILITY

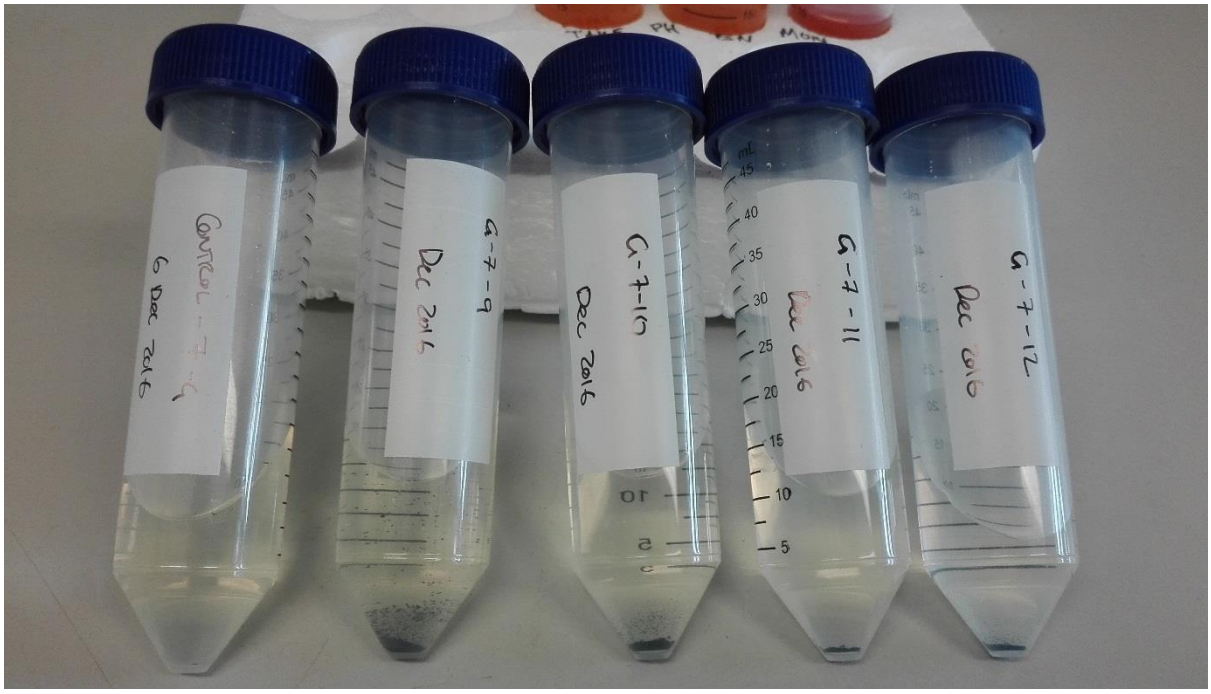


Figure C-8: At. ferrooxidans viability of culture – samples incubated for 48+ hours which are, from left, sterile control of DSMZ 882 media, media inoculated with culture incubated with untreated GTR for 10 days (duplicate), media inoculated with culture incubated with acetone leached GTR for 10 days (duplicate). As can be seen both the untreated GTR and acetone leached GTR have caused the cultures to no longer be viable.

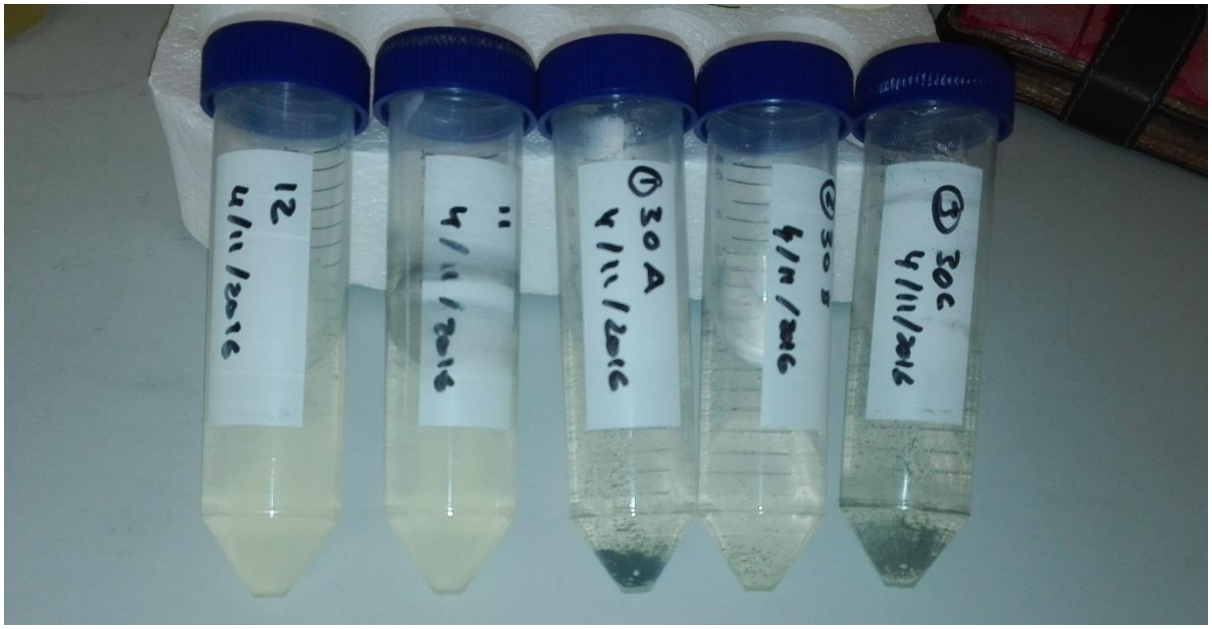


Figure C-9: Consortium viability test after 30 days of incubation– from left, control consortium samples to which no GTR was added (duplicate), consortium incubated in presence of GTR (triplicate). Opaqueness of control samples indicates viable sulphur growth, while clarity of GTR samples indicates no growth.

c. Soluble Fraction Method 1

i. Acetone, Toluene and Total Extraction Graphs

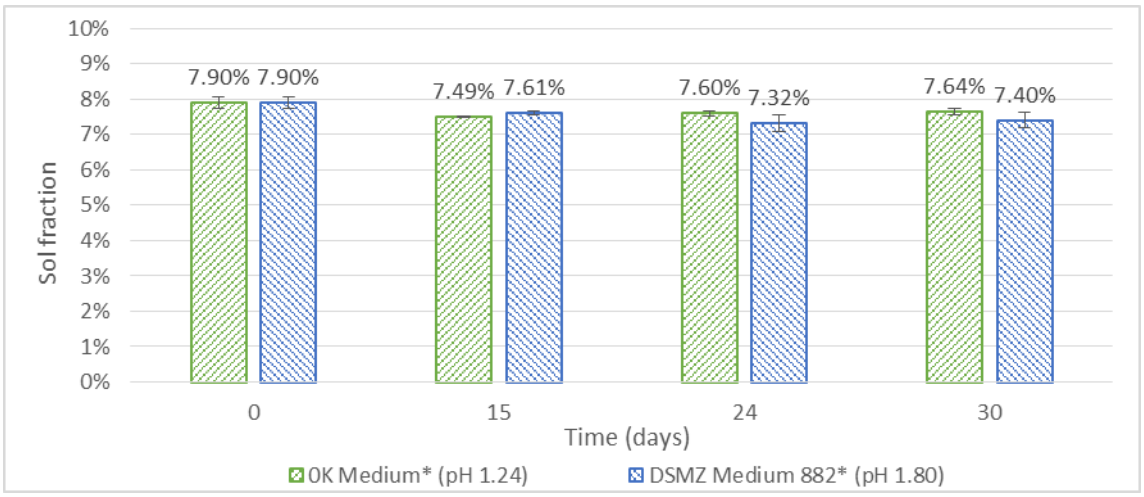


Figure C-10: Method 1 Sol Fraction - Effect of media and media pH on acetone extractables

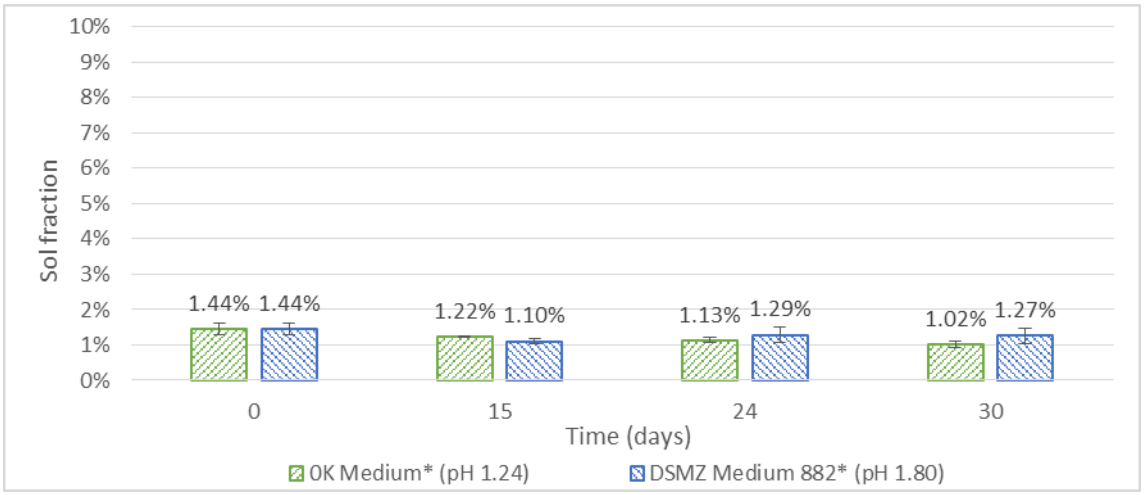


Figure C-11: Method 1 Sol Fraction - Effect of media and media pH on toluene extractables

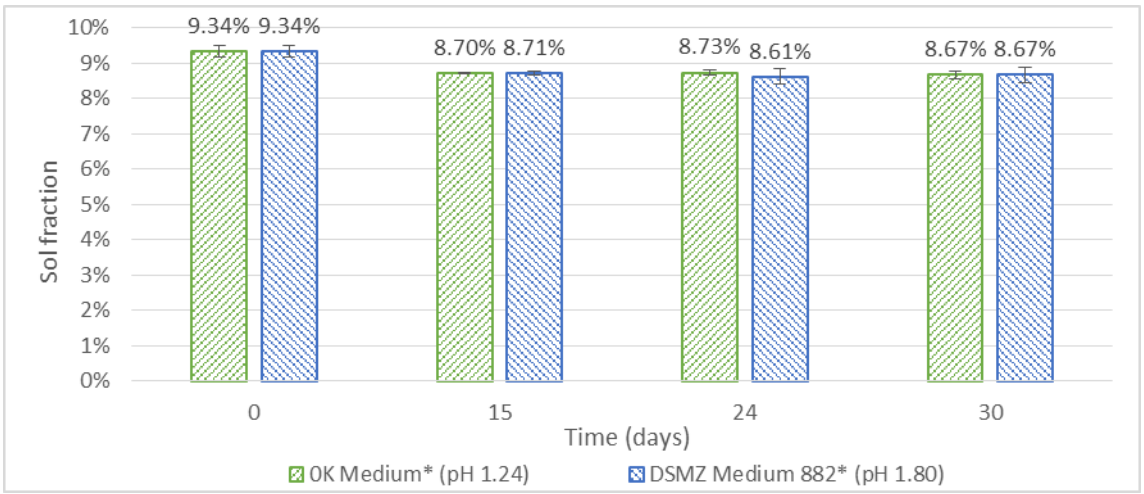


Figure C-12: Method 1 Sol Fraction - Effect of media and media pH on total extractables

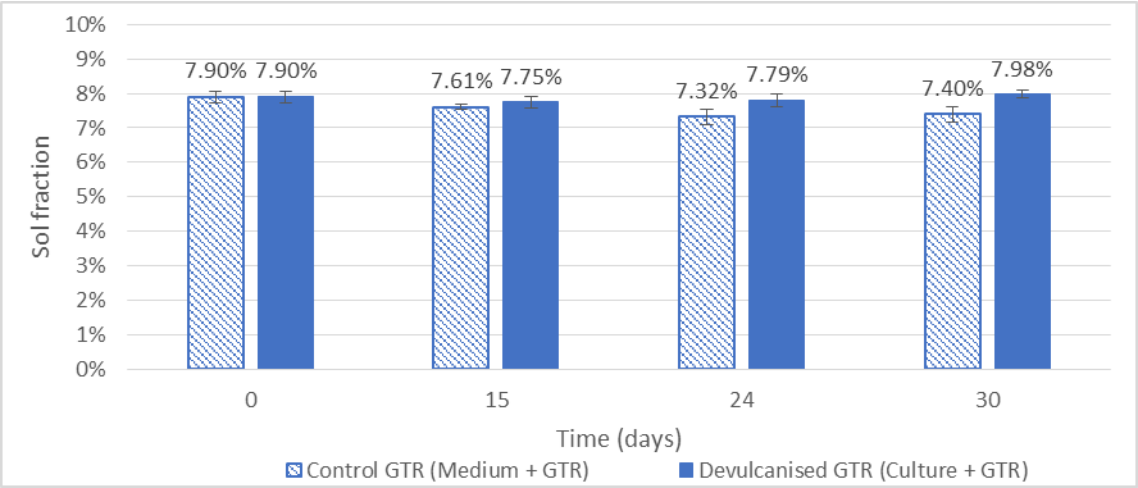


Figure C-13: Method 1 Sol Fraction - At. ferrooxidans acetone extracted sol fraction

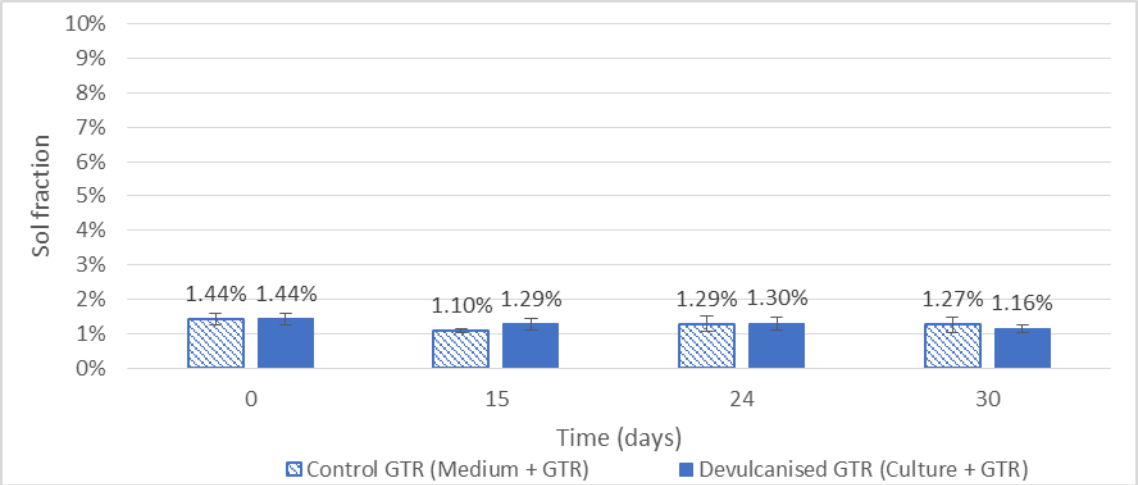


Figure C-14: Method 1 Sol Fraction - At. ferrooxidans toluene extracted sol fraction

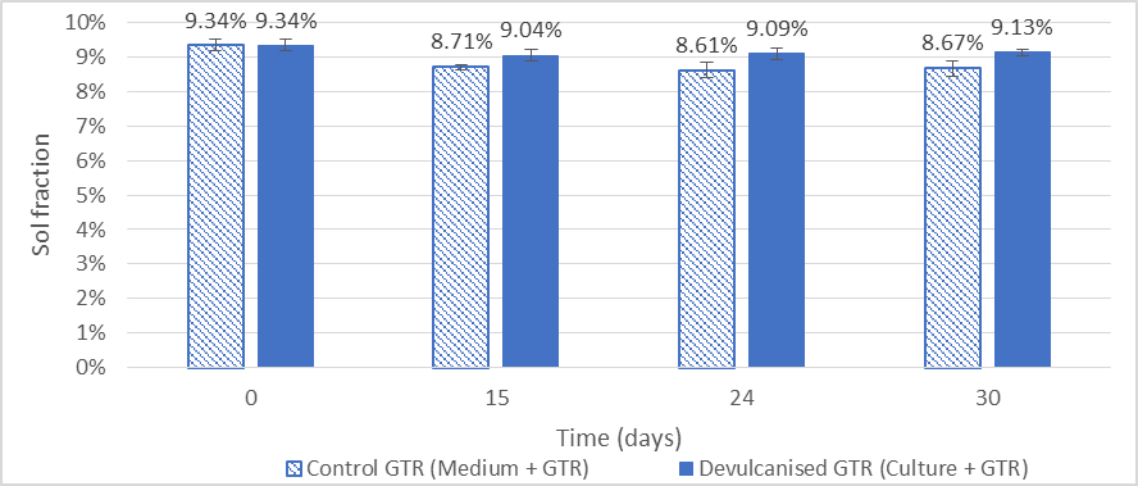


Figure C-15: Method 1 Sol Fraction - At. ferrooxidans total extracted sol fraction

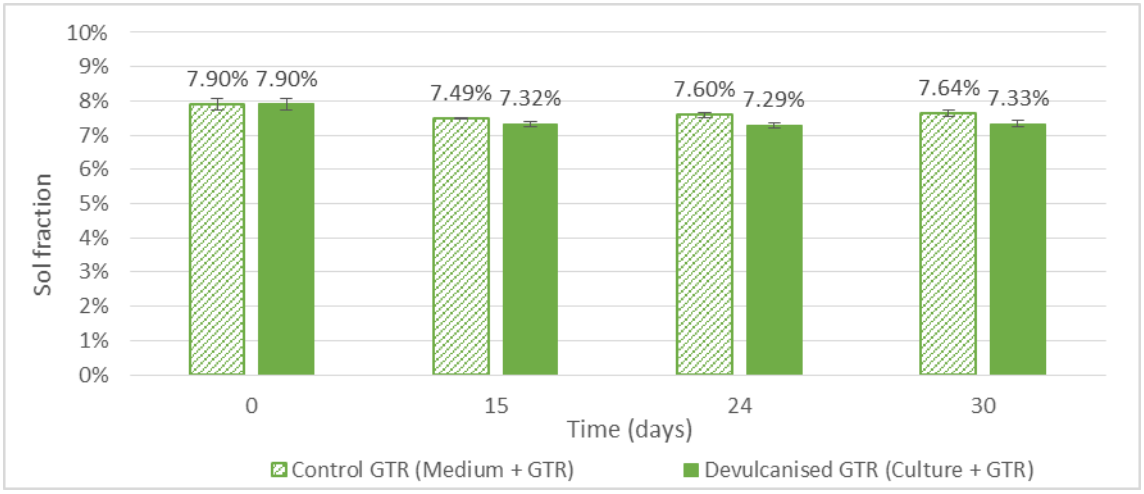


Figure C-16: Method 1 Sol Fraction - biomining consortium acetone extracted sol fraction

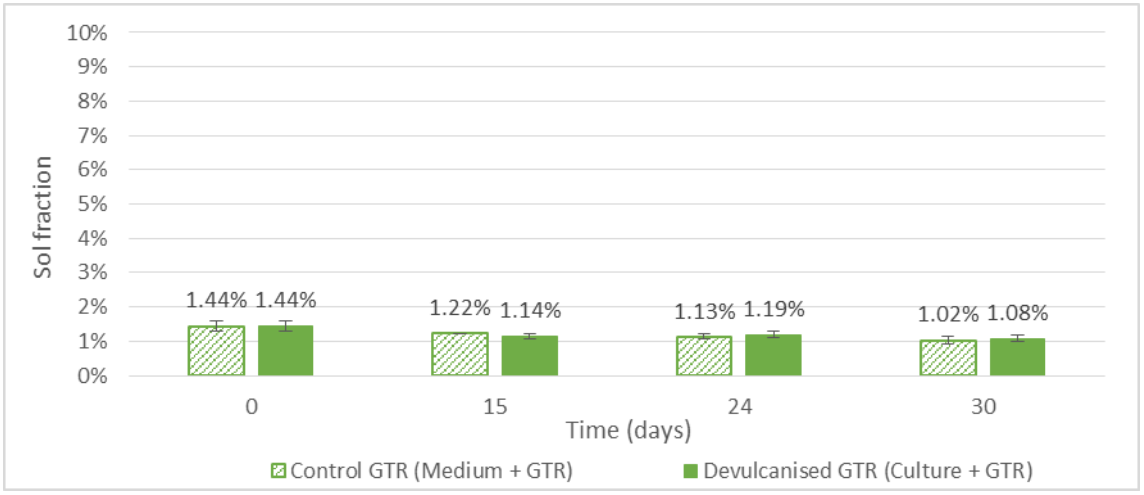


Figure C-17: Method 1 Sol Fraction - biomining consortium toluene extracted sol fraction

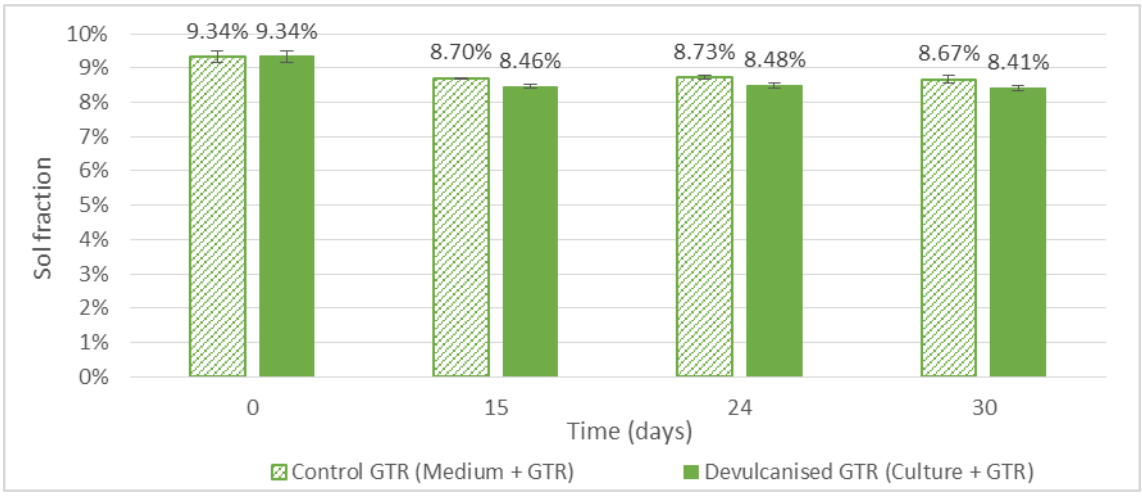


Figure C-18: Method 1 Sol Fraction - biomining consortium total extracted sol fraction

ii. T-TESTS TO DETERMINE STATISTICAL SIGNIFICANCE AND ELIMINATION OF OUTLIERS AND:

As can be seen in Table C-3 to Table C-5, Flask 3 for Day 15 lay 2.79% away from the average of the triplicate. This was in excess of two standard deviations of all the other triplicate samples analysed, and as a result was omitted from analysis.

Table C-3: Method 1 sol fraction - At. ferrooxidans acetone sol fraction triplicate results

Flask	Day 15		Day 24		Day 30	
	cGTR	dGTR	cGTR	dGTR	cGTR	dGTR
1	7.61%	7.51%	7.15%	7.33%	7.39%	7.57%
2	7.55%	7.99%	7.36%	8.14%	7.41%	7.91%
3	7.67%	5.48%	7.46%	7.91%	7.41%	8.16%
Avg	7.6%	7.0%	7.3%	7.8%	7.4%	7.9%
StDev	0.1%	1.1%	0.1%	0.3%	0.0%	0.2%
RSD	0.66%	15.58%	1.78%	4.38%	0.16%	3.06%

Table C-4: Method 1 sol fraction - At. ferrooxidans toluene sol fraction triplicate results

Flask	Day 15		Day 24		Day 30	
	cGTR	dGTR	cGTR	dGTR	cGTR	dGTR
1	1.15%	1.37%	1.20%	1.55%	1.55%	1.23%
2	1.07%	1.21%	1.24%	1.18%	1.26%	1.21%
3	1.09%	0.98%	1.42%	1.16%	0.98%	1.10%
Avg	1.1%	1.2%	1.3%	1.3%	1.3%	1.2%
StDev	0.0%	0.2%	0.1%	0.2%	0.2%	0.1%
RSD	3.19%	13.43%	7.61%	13.92%	18.31%	4.94%

Table C-5: Method 1 sol fraction - At. ferrooxidans total sol fraction triplicate results

Flask	Day 15		Day 24		Day 30	
	cGTR	dGTR	cGTR	dGTR	cGTR	dGTR
1	16.01%	15.58%	16.37%	15.23%	16.16%	15.38%
2	16.26%	15.46%	15.92%	14.74%	15.60%	15.60%
3	15.69%	11.34%	15.10%	14.65%	15.37%	15.82%
Avg	16.0%	14.1%	15.8%	14.9%	15.7%	15.6%
StDev	0.2%	2.0%	0.5%	0.3%	0.3%	0.2%
RSD	1.46%	13.97%	3.33%	1.71%	2.10%	1.17%

There were no outliers in the consortium triplicates, as can be seen in Table C-6 to Table C-8.

Table C-6: Consortium acetone sol fraction triplicate results using Method 1 Sol Fraction

Flask	Day 15		Day 24		Day 30	
	cGTR	dGTR	cGTR	dGTR	cGTR	dGTR
1	7.53%	7.38%	7.51%	7.46%	7.69%	7.40%
2	7.50%	7.24%	7.50%	7.15%	7.76%	7.43%
3	7.43%	7.36%	7.78%	7.25%	7.48%	7.16%
Average	7.49%	7.32%	7.60%	7.29%	7.64%	7.33%
St.Dev	0.04%	0.06%	0.13%	0.13%	0.12%	0.12%
RSD	0.60%	0.85%	1.74%	1.77%	1.58%	1.68%

Table C-7: Consortium toluene sol fraction triplicate results using Method 1 Sol Fraction

Flask	Day 15		Day 24		Day 30	
	cGTR	dGTR	cGTR	dGTR	cGTR	dGTR
1	1.15%	1.18%	1.23%	1.12%	1.08%	0.96%
2	1.20%	1.20%	1.14%	1.22%	0.93%	1.10%
3	1.30%	1.04%	1.03%	1.24%	1.06%	1.18%
Average	1.22%	1.14%	1.13%	1.19%	1.02%	1.08%
St.Dev	0.06%	0.07%	0.08%	0.06%	0.07%	0.09%
RSD	5.10%	6.09%	7.12%	4.62%	6.48%	8.56%

Table C-8: Consortium total sol fraction triplicate results using Method 1 Sol Fraction

Flask	Day 15		Day 24		Day 30	
	cGTR	dGTR	cGTR	dGTR	cGTR	dGTR
1	8.68%	8.56%	8.74%	8.58%	8.78%	8.36%
2	8.70%	8.44%	8.64%	8.37%	8.69%	8.53%
3	8.73%	8.40%	8.82%	8.49%	8.53%	8.34%
Average	8.70%	8.46%	8.73%	8.48%	8.67%	8.41%
St.Dev	0.02%	0.07%	0.07%	0.08%	0.10%	0.09%
RSD	0.20%	0.78%	0.84%	0.98%	1.17%	1.03%

Table C-9: At. ferrooxidans t-test to determine statistical significance between cGTR and dGTR for Method 1 Sol Fraction of acetone sol fraction determination

15 Days

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	0.0699	0.0761
Variance	1.78E-04	3.84E-07
Observations	3	3
df	2	2
F	464.03	
P(F<=f) one-tail	0.0022	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	dGTR	*cGTR*
Mean	0.0699	0.0761
Variance	1.78E-04	3.84E-07
Observations	3	3
Pooled Variance	8.928E-05	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.7981	
P(T<=t) one-tail	0.2348	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.4695	
t Critical two-tail	2.7764	

Not significant

24 Days

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	0.0779	0.0732
Variance	1.75E-05	2.55E-06
Observations	3	3
df	2	2
F	6.85	
P(F<=f) one-tail	0.1275	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	dGTR	*cGTR*
Mean	0.0779	0.0732
Variance	1.75E-05	2.55E-06
Observations	3	3
Pooled Variance	1.002E-05	
Hypothesized Mean Difference	0	
df	4	
t Stat	1.8135	
P(T<=t) one-tail	0.0720	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.1440	
t Critical two-tail	2.7764	

Not significant

30 Days

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	0.0788	0.0740
Variance	8.72E-06	2.18E-08
Observations	3	3
df	2	2
F	400.79	
P(F<=f) one-tail	0.0025	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Unequal Variances

	dGTR	*cGTR*
Mean	0.0788	0.0740
Variance	8.72E-06	2.18E-08
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	2.7943	
P(T<=t) one-tail	0.0539	
t Critical one-tail	2.9200	
P(T<=t) two-tail	0.1078	
t Critical two-tail	4.3027	

Not significant

Table C-10: At. ferrooxidans t-test to determine statistical significance between cGTR and dGTR for Method 1 Sol Fraction of toluene sol fraction determination

15 Days

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	0.0118	0.0110
Variance	3.79E-06	1.85E-07
Observations	3	3
df	2	2
F	20.52	
P(F<=f) one-tail	0.0465	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Unequal Variances

	dGTR	*cGTR*
Mean	0.011841 8	0.011008 9
Variance	3.79E-06	1.85E-07
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	0.7235	
P(T<=t) one-tail	0.2723	
t Critical one-tail	2.9200	
P(T<=t) two-tail	0.5446	
t Critical two-tail	4.3027	

Not significant

24 Days

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	0.0130	0.0129
Variance	4.90E-06	1.44E-06
Observations	3	3
df	2	2
F	3.41	
P(F<=f) one-tail	0.2269	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	dGTR	*cGTR*
Mean	0.01299 3	0.012876 7
Variance	4.90E-06	1.44E-06
Observations	3	3
Pooled Variance	3.17E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.0800	
P(T<=t) one-tail	0.4701	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.9401	
t Critical two-tail	2.7764	

Not significant

30 Days

F-Test Two-Sample for Variances

	cGTR	*dGTR*
Mean	0.0127	0.0118
Variance	8.06E-06	5.12E-07
Observations	3	3
df	2	2
F	15.73	
P(F<=f) one-tail	0.0598	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	cGTR	*dGTR*
Mean	0.012657 5	0.011829 1
Variance	8.06E-06	5.12E-07
Observations	3	3
Pooled Variance	4.28E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.4902	
P(T<=t) one-tail	0.3248	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.6497	
t Critical two-tail	2.7764	

Not significant

Table C-11: At. ferrooxidans T-test to determine statistical significance between cGTR and dGTR for Method 1 Sol Fraction of total sol fraction determination

15 Days			15 Days without Outlier			24 Days			30 Days		
F-Test Two-Sample for Variances			F-Test Two-Sample for Variances			F-Test Two-Sample for Variances			F-Test Two-Sample for Variances		
	dGTR15	*cGTR15*		*cGTR15*	*dGTR15*		*cGTR24*	*dGTR24*		*cGTR30*	*dGTR30*
Mean	0.1413	0.1599	Mean	0.1599	0.1552	Mean	0.1512	0.1596	Mean	0.1522	0.1603
Variance	5.84E-04	8.15E-06	Variance	8.15E-06	7.25E-07	Variance	2.25E-05	1.50E-05	Variance	2.28E-05	4.85E-06
Observations	3	3	Observations	3	2	Observations	3	3	Observations	3	3
df	2	2	df	2	1	df	2	2	df	2	2
F	71.71		F	11.24		F	1.50		F	4.70	
P(F<=f) one-tail	0.0138		P(F<=f) one-tail	0.2064		P(F<=f) one-tail	0.4006		P(F<=f) one-tail	0.1756	
F Critical one-tail	19		F Critical one-tail	199.5		F Critical one-tail	19		F Critical one-tail	19	
t-Test: Two-Sample Assuming Unequal Variances			t-Test: Two-Sample Assuming Equal Variances			t-Test: Two-Sample Assuming Equal Variances			t-Test: Two-Sample Assuming Equal Variances		
	dGTR15	*cGTR15*		*cGTR15*	*dGTR15*		*cGTR24*	*dGTR24*		*cGTR30*	*dGTR30*
Mean	0.1413	0.1599	Mean	0.1599	0.1552	Mean	0.1512	0.1596	Mean	0.1522	0.1603
Variance	5.84E-04	8.15E-06	Variance	8.15E-06	7.25E-07	Variance	2.25E-05	1.50E-05	Variance	2.28E-05	4.85E-06
Observations	3	3	Observations	3	2	Observations	3	3	Observations	3	3
			Pooled Variance	5.67E-06		Pooled Variance	1.87E-05		Pooled Variance	1.38E-05	
Hypothesized Mean Difference	0		Hypothesized Mean Difference	0		Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
df	2		df	3		df	4		df	4	
t Stat	-1.3260		t Stat	2.1536		t Stat	-2.3858		t Stat	-2.6798	
P(T<=t) one-tail	0.1580		P(T<=t) one-tail	0.0602		P(T<=t) one-tail	0.0378		P(T<=t) one-tail	0.0276	
t Critical one-tail	2.9200		t Critical one-tail	2.3534		t Critical one-tail	2.1318		t Critical one-tail	2.1318	
P(T<=t) two-tail	0.3160		P(T<=t) two-tail	0.1203		P(T<=t) two-tail	0.0755		P(T<=t) two-tail	0.0552	
t Critical two-tail	4.3027		t Critical two-tail	3.1824		t Critical two-tail	2.7764		t Critical two-tail	2.7764	
Not statistically significant			Therfore on the verge of being statistically significant			Statistically significant			Statistically significant		

Table C-12: Consortium t-test to determine statistical significance between cGTR and dGTR for Method 1 Sol Fraction of acetone sol fraction determination

Day 15

F-Test Two-Sample for Variances		
	dGTR	*cGTR*
Mean	0.0732	0.0749
Variance	5.859E-07	2.997E-07
Observations	3	3
df	2	2
F	1.9548	
P(F<=f) one-tail	0.3384	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	dGTR	*cGTR*
Mean	0.0732	0.0749
Variance	5.859E-07	2.997E-07
Observations	3	3
Pooled Variance	4.428E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	-3.0020	
P(T<=t) one-tail	0.0199	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0399	
t Critical two-tail	2.7764	

Significant

Day 24

F-Test Two-Sample for Variances		
	cGTR	*dGTR*
Mean	0.0760	0.0729
Variance	2.613E-06	2.484E-06
Observations	3	3
df	2	2
F	1.0519	
P(F<=f) one-tail	0.4873	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	cGTR	*dGTR*
Mean	0.0760	0.0729
Variance	2.613E-06	2.484E-06
Observations	3	3
Pooled Variance	2.549E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.3784	
P(T<=t) one-tail	0.0381	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0761	
t Critical two-tail	2.7764	

Significant

Day 30

F-Test Two-Sample for Variances		
	dGTR	*cGTR*
Mean	0.0733	0.0764
Variance	2.265E-06	2.187E-06
Observations	3	3
df	2	2
F	1.0357	
P(F<=f) one-tail	0.4912	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	dGTR	*cGTR*
Mean	0.0733	0.0764
Variance	2.265E-06	2.187E-06
Observations	3	3
Pooled Variance	2.226E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	-2.5636	
P(T<=t) one-tail	0.0312	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0624	
t Critical two-tail	2.7764	

Significant

Table C-13: Consortium t-test to determine statistical significance between cGTR and dGTR for Method 1 Sol Fraction of toluene sol fraction determination

Day 15

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	0.0114	0.0122
Variance	7.248E-07	5.778E-07
Observations	3	3
df	2	2
F	1.2545	
P(F<=f) one-tail	0.4436	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	dGTR	*cGTR*
Mean	0.0114	0.0122
Variance	7.248E-07	5.778E-07
Observations	3	3
Pooled Variance	6.513E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	-1.1611	
P(T<=t) one-tail	0.1551	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.3102	
t Critical two-tail	2.7764	

Not significant

Day 24

F-Test Two-Sample for Variances

	cGTR	*dGTR*
Mean	0.0113	0.0119
Variance	9.758E-07	4.567E-07
Observations	3	3
df	2	2
F	2.1364	
P(F<=f) one-tail	0.3188	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	cGTR	*dGTR*
Mean	0.0113	0.0119
Variance	9.758E-07	4.567E-07
Observations	3	3
Pooled Variance	7.163E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.8749	
P(T<=t) one-tail	0.2155	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.4310	
t Critical two-tail	2.7764	

Not significant

Day 30

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	0.0108	0.0102
Variance	1.28E-06	6.622E-07
Observations	3	3
df	2	2
F	1.9325	
P(F<=f) one-tail	0.3410	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	dGTR	*cGTR*
Mean	0.0108	0.0102
Variance	1.28E-06	6.622E-07
Observations	3	3
Pooled Variance	9.71E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.6800	
P(T<=t) one-tail	0.2669	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.5338	
t Critical two-tail	2.7764	

Not significant

Table C-14: Consortium t-test to determine statistical significance between cGTR and dGTR for Method 1 Sol Fraction of total sol fraction determination

Day 15		
F-Test Two-Sample for Variances		
	dGTR15	*cGTR15*
Mean	0.0846	0.0870
Variance	6.457E-07	4.699E-08
Observations	3	3
df	2	2
F	13.7413	
P(F<=f) one-tail	0.0678	
F Critical one-tail	19	
t-Test: Two-Sample Assuming Equal Variances		
	dGTR15	*cGTR15*
Mean	0.0846	0.0870
Variance	6.46E-07	4.70E-08
Observations	3	3
Pooled Variance	3.464E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	-4.9866	
P(T<=t) one-tail	0.0038	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0076	
t Critical two-tail	2.7764	
Statistically significant		

Day 24		
F-Test Two-Sample for Variances		
	dGTR24	*cGTR24*
Mean	0.0848	0.0873
Variance	1.037E-06	8.118E-07
Observations	3	3
df	2	2
F	1.2777	
P(F<=f) one-tail	0.4390	
F Critical one-tail	19	
t-Test: Two-Sample Assuming Equal Variances		
	dGTR24	*cGTR24*
Mean	0.0848	0.0873
Variance	1.04E-06	8.12E-07
Observations	3	3
Pooled Variance	9.245E-07	
Hypothesized Mean Difference	0	
df	4	
t Stat	-3.1788	
P(T<=t) one-tail	0.0168	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0336	
t Critical two-tail	2.7764	
Statistically significant		

Day 30		
F-Test Two-Sample for Variances		
	cGTR30	*dGTR30*
Mean	0.0867	0.0841
Variance	1.55E-06	1.121E-06
Observations	3	3
df	2	2
F	1.3831	
P(F<=f) one-tail	0.4196	
F Critical one-tail	19	
t-Test: Two-Sample Assuming Equal Variances		
	cGTR30	*dGTR30*
Mean	0.0867	0.0841
Variance	1.55E-06	1.12E-06
Observations	3	3
Pooled Variance	1.336E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.7296	
P(T<=t) one-tail	0.0262	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.0525	
t Critical two-tail	2.7764	
Statistically significant		

d. Soluble Fraction Method 2

i. Acetone, Toluene and Total Extraction Graphs

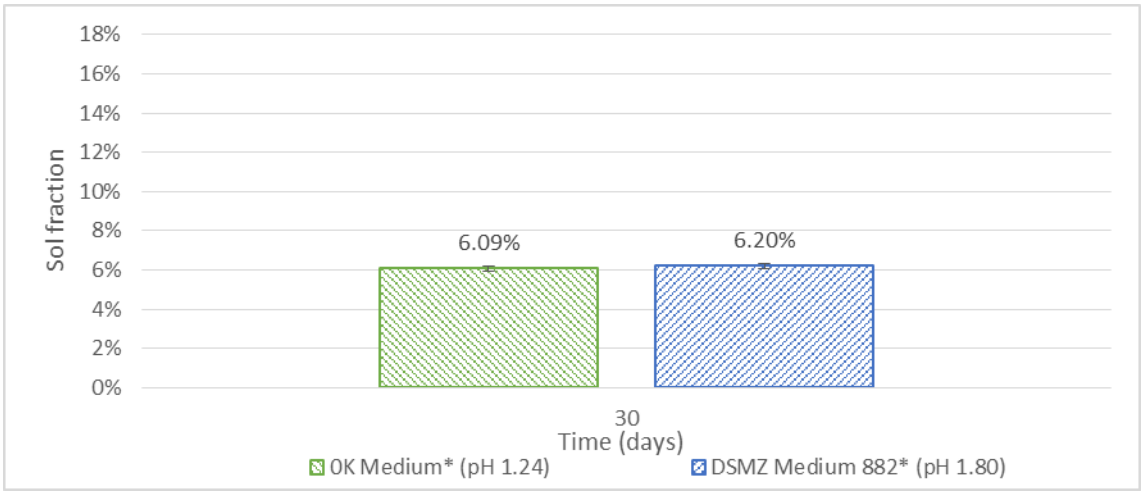


Figure C-19: Method 2 Sol Fraction - Influence of media pH on acetone sol fraction after 30 days

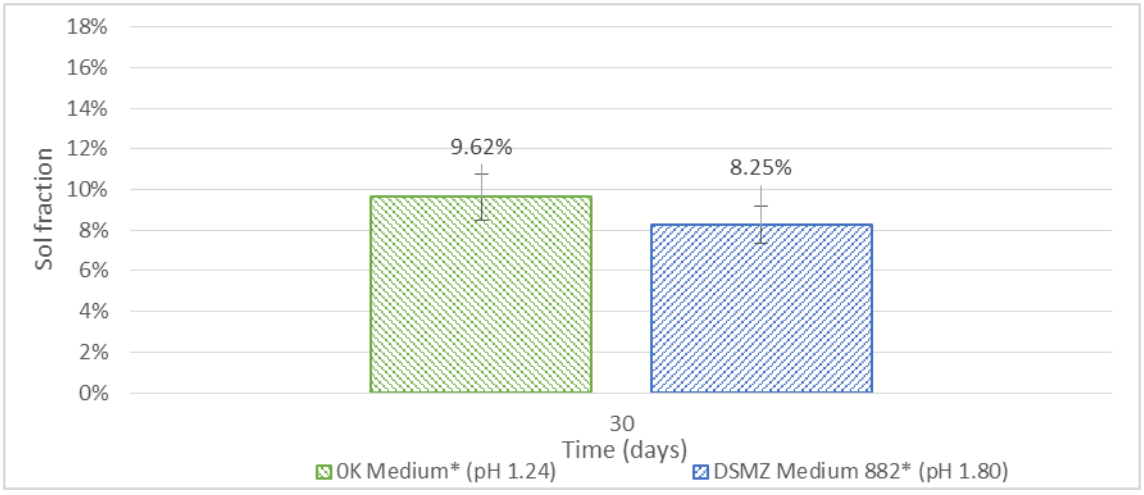


Figure C-20: Method 2 Sol Fraction - Influence of media pH on toluene sol fraction after 30 days

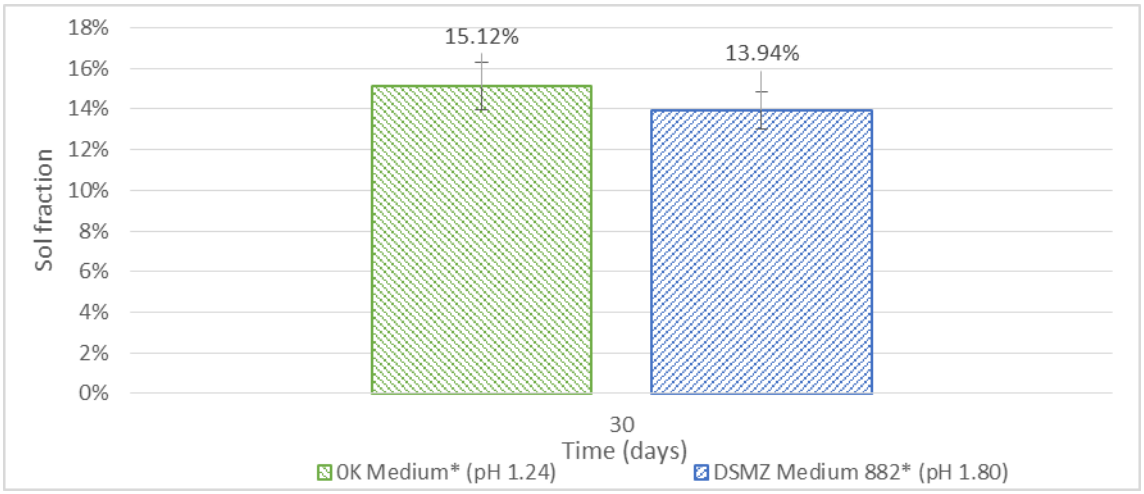


Figure C-21: Method 2 Sol Fraction - Influence of media pH on total sol fraction after 30 days

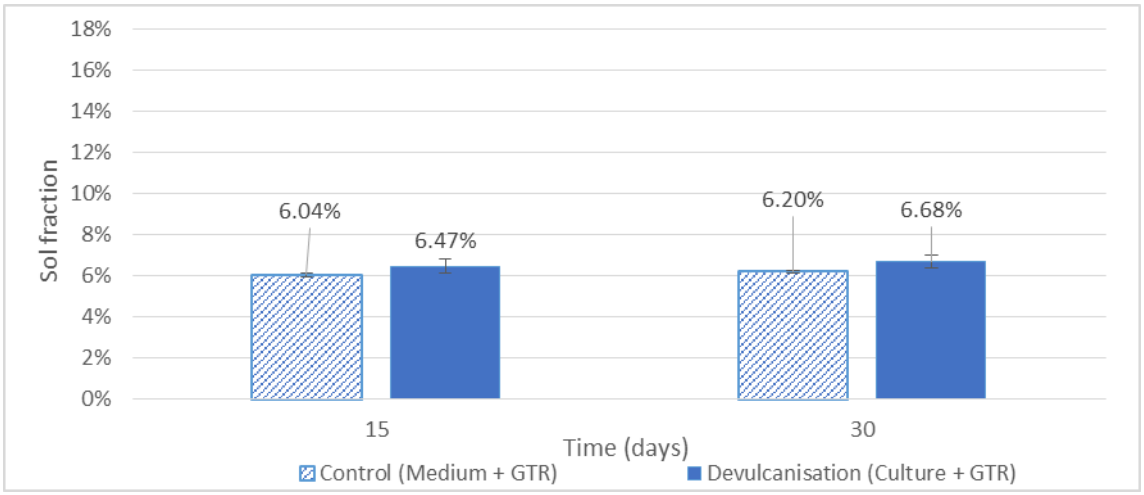


Figure C-22: Method 2 Sol Fraction - At. ferrooxidans acetone sol fraction over time for (a) control – GTR treated by sterile media (b) experiment – GTR treated by At. ferrooxidans culture

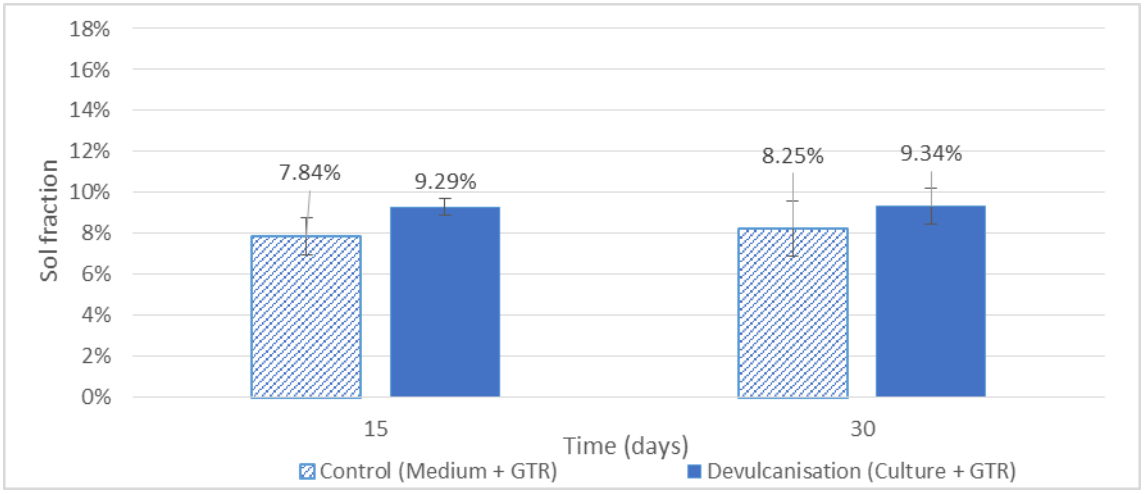


Figure C-23: Method 2 Sol Fraction - At. ferrooxidans toluene sol fraction over time for (a) control – GTR treated by sterile media (b) experiment – GTR treated by At. ferrooxidans culture

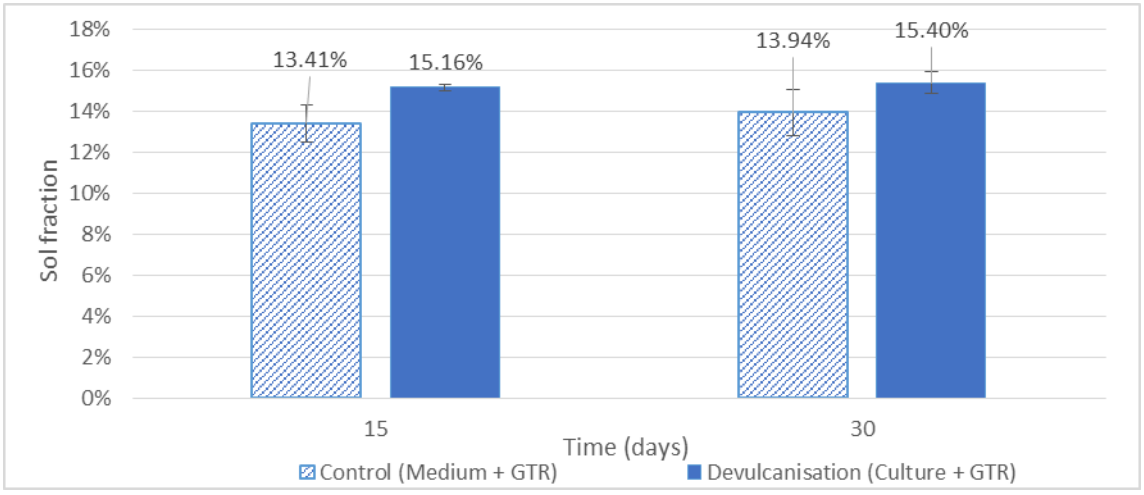


Figure C-24: Method 2 Sol Fraction - At. ferrooxidans total sol fraction over time for (a) control – GTR treated by sterile media (b) experiment – GTR treated by At. ferrooxidans culture

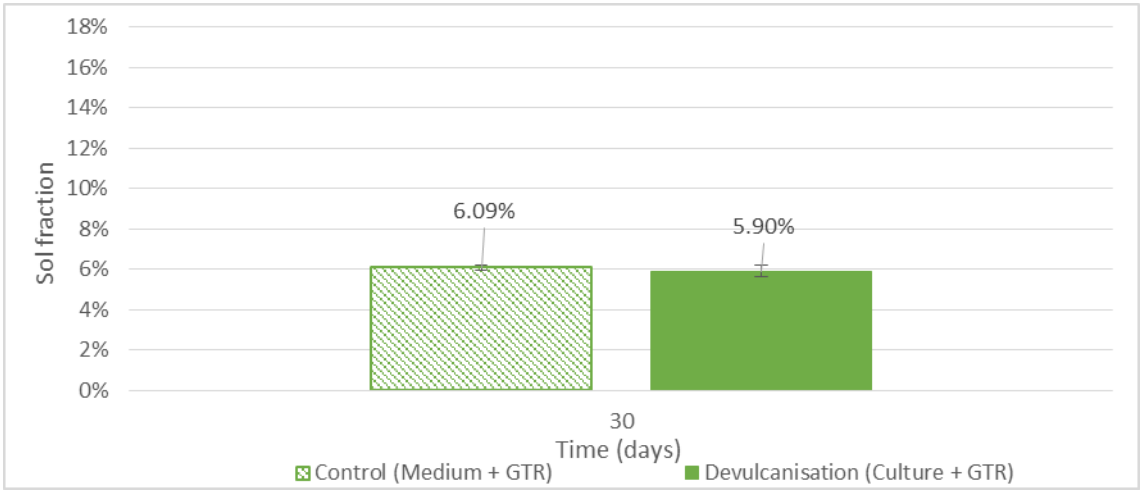


Figure C-25: Method 2 Sol Fraction – consortium acetone sol fraction after 30 days for (a) control – GTR treated by sterile media and (b) experiment – GTR treated by mesophilic consortium

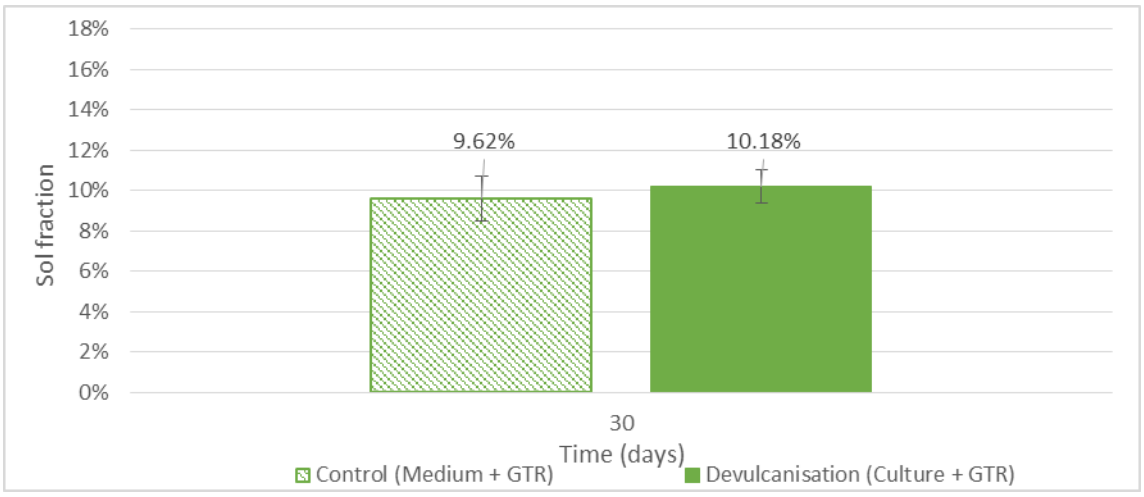


Figure C-26: Method 2 Sol Fraction - consortium toluene sol fraction after 30 days for (a) control – GTR treated by sterile media and (b) experiment – GTR treated by mesophilic consortium

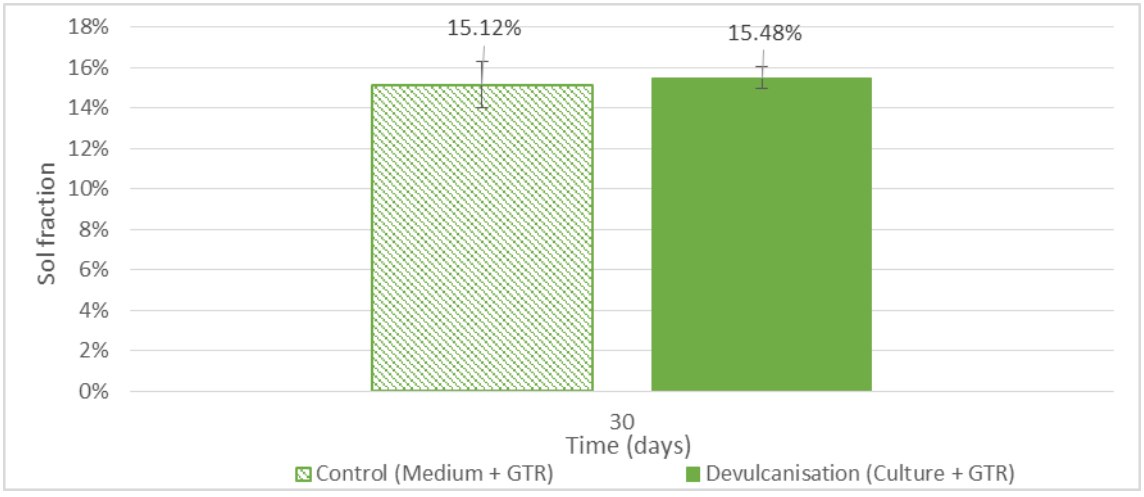


Figure C-27: Method 2 Sol Fraction - consortium total sol fraction after 30 days for (a) control – GTR treated by sterile media and (b) experiment – GTR treated by mesophilic consortium

ii. T-TESTS TO DETERMINE STATISTICAL SIGNIFICANCE

Table C-15: At. ferrooxidans - T-test analysis of significance of differences between acetone extractable fractions for Method 2 sol fraction determination

At F 15 Days

F-Test Two-Sample for Variances

	cGTR	*dGTR*
Mean	-0.0647	-0.0604
Variance	1.59E-05	1.82E-06
Observations	3	3
df	2	2
F	8.7627	
P(F<=f) one-tail	0.1024	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	cGTR	*dGTR*
Mean	-0.0604	-0.0647
Variance	1.818E-06	1.593E-05
Observations	3	3
Pooled Variance	8.87E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	1.7520	
P(T<=t) one-tail	0.0773	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.1547	
t Critical two-tail	2.7764	

Not significant

AtF 30 Days

F-Test Two-Sample for Variances

	dGTR	*cGTR*
Mean	-0.0668	-0.0620
Variance	1.36E-05	6.18E-07
Observations	3	3
df	2	2
F	21.9435	
P(F<=f) one-tail	0.0436	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Unequal Variances

	dGTR	*cGTR*
Mean	0.0668	0.0620
Variance	1.357E-05	6.183E-07
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-2.2267	
P(T<=t) one-tail	0.0779	
t Critical one-tail	2.9200	
P(T<=t) two-tail	0.1559	
t Critical two-tail	4.3027	

Not significant

AtF Controls across Time

F-Test Two-Sample for Variances

	cGTR15	*cGTR30*
Mean	-0.0604	-0.0620
Variance	1.82E-06	6.18E-07
Observations	3	3
df	2	2
F	2.9398	
P(F<=f) one-tail	0.2538	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	cGTR15	*cGTR30*
Mean	0.0604	0.0620
Variance	1.818E-06	6.183E-07
Observations	3	3
Pooled Variance	1.22E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	1.7030	
P(T<=t) one-tail	0.0819	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.1638	
t Critical two-tail	2.7764	

Not significant

AtF Experiments across Time

F-Test Two-Sample for Variances

	dGTR15	*dGTR30*
Mean	0.0647	0.0668
Variance	1.59E-05	1.36E-05
Observations	3	3
df	2	2
F	1.1739	
P(F<=f) one-tail	0.4600	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	dGTR15	*dGTR30*
Mean	0.0647	0.0668
Variance	1.593E-05	1.357E-05
Observations	3	3
Pooled Variance	1.47E-05	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.6747	
P(T<=t) one-tail	0.2684	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.5368	
t Critical two-tail	2.7764	

Not significant

Table C-16: At. ferrooxidans - T-test analysis of significance of differences between toluene extractable fractions for Method 2 sol fraction determination

15 Days		
F-Test Two-Sample for Variances		
	C15	*E15*
Mean	7.84E-02	9.29E-02
Variance	1.23E-04	2.67E-05
Observations	3	3
df	2	2
F	4.6055	
P(F<=f) one-tail	0.1784	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	C15	*E15*
Mean	7.84E-02	9.29E-02
Variance	1.23E-04	2.67E-05
Observations	3	3
Pooled Variance	7.49E-05	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.0532	
P(T<=t) one-tail	0.0546	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.1093	
t Critical two-tail	2.7764	

Therefore on the verge of being statistically significant

30 Days		
F-Test Two-Sample for Variances		
	C30	*E30*
Mean	8.25E-02	9.34E-02
Variance	2.69E-04	1.14E-04
Observations	3	3
df	2	2
F	2.3537	
P(F<=f) one-tail	0.2982	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	C30	*E30*
Mean	8.25E-02	9.34E-02
Variance	2.69E-04	1.14E-04
Observations	3	3
Pooled Variance	1.91E-04	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.9660	
P(T<=t) one-tail	0.1944	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.3887	
t Critical two-tail	2.7764	

Therefore not significant

Controls across Time		
F-Test Two-Sample for Variances		
	C30	*C15*
Mean	8.25E-02	7.84E-02
Variance	2.69E-04	1.23E-04
Observations	3	3
df	2	2
F	2.1818	
P(F<=f) one-tail	0.3143	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	C30	*C15*
Mean	8.25E-02	7.84E-02
Variance	2.69E-04	1.23E-04
Observations	3	3
Pooled Variance	1.96E-04	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.3568	
P(T<=t) one-tail	0.3696	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.7393	
t Critical two-tail	2.7764	

Therefore not significant

Experiments across Time		
F-Test Two-Sample for Variances		
	E30	*E15*
Mean	9.34E-02	9.29E-02
Variance	1.14E-04	2.67E-05
Observations	3	3
df	2	2
F	4.2691	
P(F<=f) one-tail	0.1898	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances		
	E30	*E15*
Mean	9.34E-02	9.29E-02
Variance	1.14E-04	2.67E-05
Observations	3	3
Pooled Variance	7.04E-05	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.0696	
P(T<=t) one-tail	0.4739	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.9478	
t Critical two-tail	2.7764	

Therefore not significant

Table C-17: At. ferrooxidans - T-test analysis of significance of differences between total extractable fractions for Method 2 sol fraction determination

15 Days

F-Test Two-Sample for Variances

	C15	*E15*
Mean	1.34E-01	1.52E-01
Variance	1.31E-04	3.26E-06
Observations	3	3
df	2	2
F	40.2008	
P(F<=f) one-tail	0.0243	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Unequal Variances

	C15	*E15*
Mean	1.34E-01	1.52E-01
Variance	1.31E-04	3.26E-06
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	2.6167	
P(T<=t) one-tail	0.0601	
t Critical one-tail	2.9200	
P(T<=t) two-tail	0.1203	
t Critical two-tail	4.3027	

On verge of being statistically significant

30 Days

F-Test Two-Sample for Variances

	C30	*E30*
Mean	1.39E-01	1.54E-01
Variance	2.17E-04	4.46E-05
Observations	3	3
df	2	2
F	4.8633	
P(F<=f) one-tail	0.1706	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	C30	*E30*
Mean	1.39E-01	1.54E-01
Variance	2.17E-04	4.46E-05
Observations	3	3
Pooled Variance	1.31E-04	
Hypothesized Mean Difference	0	
df	4	
t Stat	1.5683	
P(T<=t) one-tail	0.0959	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.1919	
t Critical two-tail	2.7764	

Therefore not significant

Controls across Time

F-Test Two-Sample for Variances

	C30	*C15*
Mean	1.39E-01	1.34E-01
Variance	2.17E-04	1.31E-04
Observations	3	3
df	2	2
F	1.6530	
P(F<=f) one-tail	0.3769	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	C30	*C15*
Mean	1.39E-01	1.34E-01
Variance	2.17E-04	1.31E-04
Observations	3	3
Pooled Variance	1.74E-04	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.4878	
P(T<=t) one-tail	0.3256	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.6512	
t Critical two-tail	2.7764	

Therefore not significant

Experiments across Time

F-Test Two-Sample for Variances

	E30	*E15*
Mean	1.54E-01	1.52E-01
Variance	4.46E-05	3.26E-06
Observations	3	3
df	2	2
F	13.6643	
P(F<=f) one-tail	0.0682	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	E30	*E15*
Mean	1.54E-01	1.52E-01
Variance	4.46E-05	3.26E-06
Observations	3	3
Pooled Variance	2.39E-05	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.60	
P(T<=t) one-tail	0.291922	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.583844	
t Critical two-tail	2.776445	

Therefore not significant

Table C-18: Biomining consortium - T-test analysis of significance of differences between acetone, toluene and total extractable fractions for Method 2 sol fraction determination

Acetone

F-Test Two-Sample for Variances

	E30	*C30*
Mean	5.90E-02	6.09E-02
Variance	1.34E-05	2.79E-06
Observations	3	3
df	2	2
F	4.8083	
P(F<=f) one-tail	0.1722	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	E30	*C30*
Mean	5.90E-02	6.09E-02
Variance	1.34E-05	2.79E-06
Observations	3	3
Pooled Variance	8.09E-06	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.8051	
P(T<=t) one-tail	0.2330	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.4659	
t Critical two-tail	2.7764	

Not significant

Toluene

F-Test Two-Sample for Variances

	C30	*E30*
Mean	9.62E-02	1.02E-01
Variance	1.85E-04	1.01E-04
Observations	3	3
df	2	2
F	1.8261	
P(F<=f) one-tail	0.3538	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	C30	*E30*
Mean	9.62E-02	1.02E-01
Variance	1.85E-04	1.01E-04
Observations	3	3
Pooled Variance	1.43E-04	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.5720	
P(T<=t) one-tail	0.2990	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.5979	
t Critical two-tail	2.7764	

Not significant

Total

F-Test Two-Sample for Variances

	C30	*E30*
Mean	1.51E-01	1.55E-01
Variance	1.96E-04	4.35E-05
Observations	3	3
df	2	2
F	4.5097	
P(F<=f) one-tail	0.1815	
F Critical one-tail	19	

t-Test: Two-Sample Assuming Equal Variances

	C30	*E30*
Mean	1.51E-01	1.55E-01
Variance	1.96E-04	4.35E-05
Observations	3	3
Pooled Variance	1.20E-04	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.4026	
P(T<=t) one-tail	0.3539	
t Critical one-tail	2.1318	
P(T<=t) two-tail	0.7079	
t Critical two-tail	2.7764	

Not significant

e. TGA

Table C-19: Determination of combined instrument error and GTR variation for 25 kG irradiated rubber

Component	25 kGray Sterilised GTR		Statistics		
	GTR A	GTR B	$\bar{x}$	σ	RSD
Water					
Oils	5.93%	5.97%	5.95%	0.017%	0.012%
Volatiles	56.93%	57.04%	56.99%	0.051%	0.036%
Fixed C	30.32%	30.38%	30.35%	0.029%	0.021%
Ash (SE_{max})	6.81%	6.62%	6.72%	0.098%	0.069%
Total	100.00%	100.00%	100.00%		

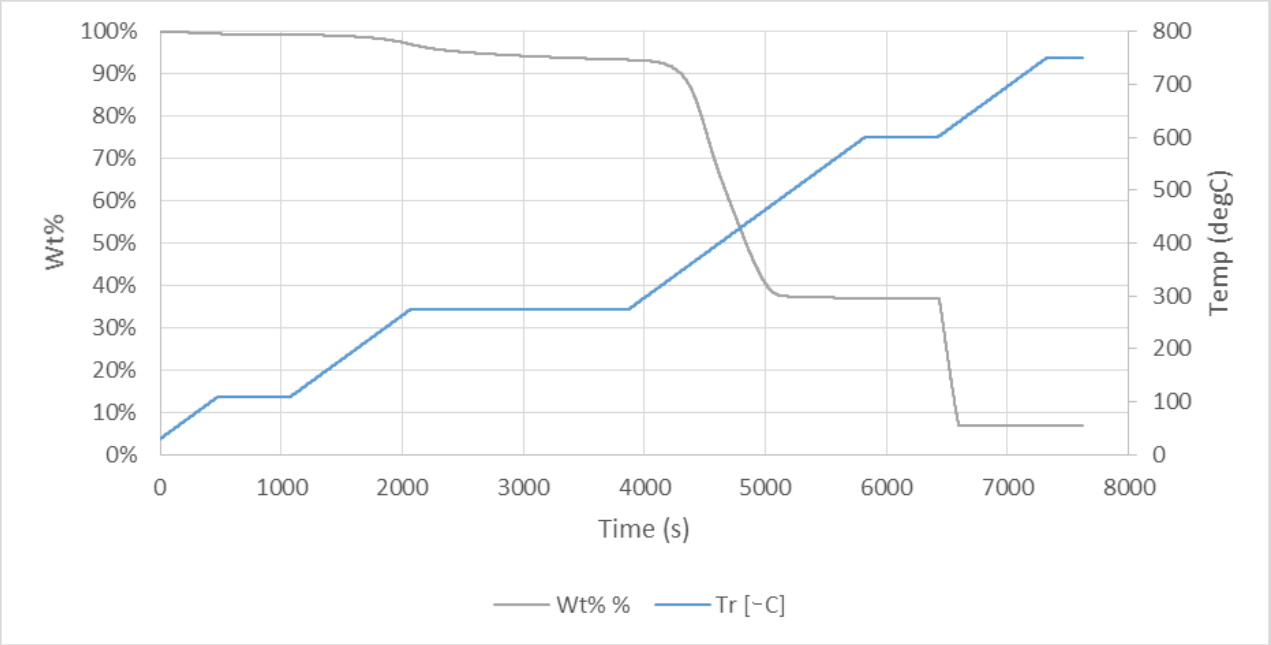


Figure C-28: Temperature and mass profile for proximate analysis of 25 kG irradiated GTR

f. FTIR- ATR

Table C-20: Summary of peaks of interest and corresponding chemical compounds (107)

Peak	Chemical Group	*At. ferrooxidans*				Consortium		
		C30		E30		C30		E30
2942 2916 2850	Light Atoms C-H Stretching (2800 – 3300) O-H and N-H Stretching (3700 – 2500)	✔	>	✔	>	✔	>	✔
1538	-(C=C)n- for n>6 (methyl assisted conjugated double bonds) (12)	✔	~	✔	>	✔	>	✔
1446	CH_2 Sym. & Asym deformation (1470-1440) CH_3 Asym deformation (1470 – 1440)	✔	~	✔	~	✔	>	✔
1365	-S=O_2- Asym stretching (1400 – 1310) -CF_3 Stretching (1380 – 1300)	✔	=	✔	>	✔	>	✔
1307	-CH Deformation (1330 – 1250) -CO-NH Amide 3 (1250- 1340) -CF_3 Stretching (1380 – 1300)	✔	=	✔	=	✔	=	✔
1224-1180	-CF_2 Stretching (1300 – 1120) -S=O_2- Sym stretching (1200-1120) -C-O- Stretching (1300 – 1100)	✔	>2x	✔	=	✔	>2x	✔
1084	-C-C- Stretching (1150 – 950)	✔	>	✔	>	✔	=	✔
1000	-C-C- Stretching (1150 – 950)? -C_6H_5 In plane deformation of benzene ring (1040 – 980)	✔	>	✔	>	✔	=	✔
960	Unknown	✔	>	✔	<	x		✔
900	-CCH_{aromat} Aromatic C-H out of plane bend (900 – 670) -COC- Sym Stretching in Ethers (930 – 830)	✔	>	x	>	✔	>	x
830	-CCH_{aromat} Aromatic C-H out of plane bend (900 – 670) -COC- Sym Stretching in Ethers (930 – 830)	✔	>	✔		✔	>	✔

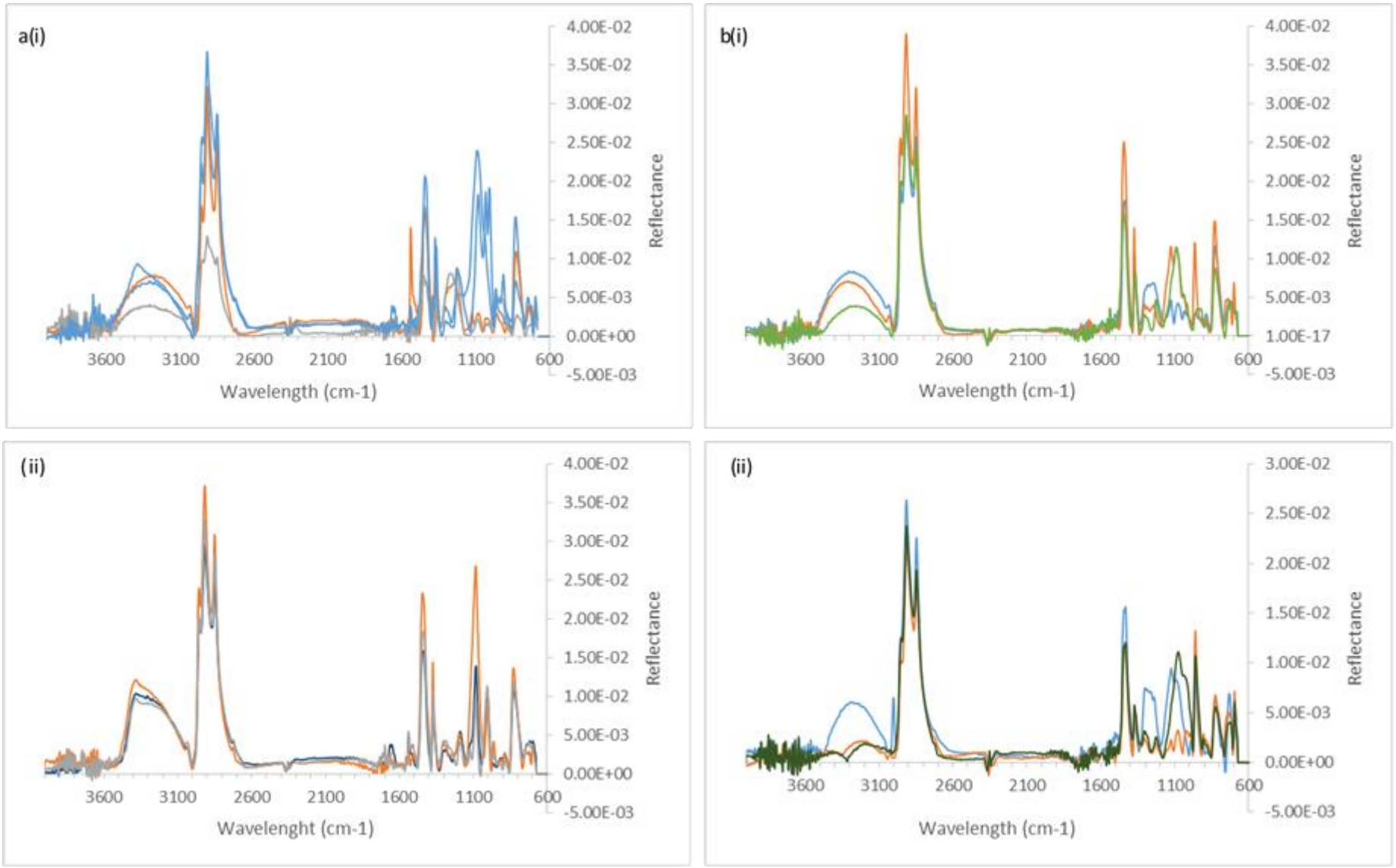


Figure C-29: All baseline adjusted FTIR-ATR spectra for (a) At. ferrooxidans (i) cGTR and (ii) and dGTR; and the consortium (i) cGTR and (ii) dGTR after 30 days of treatment

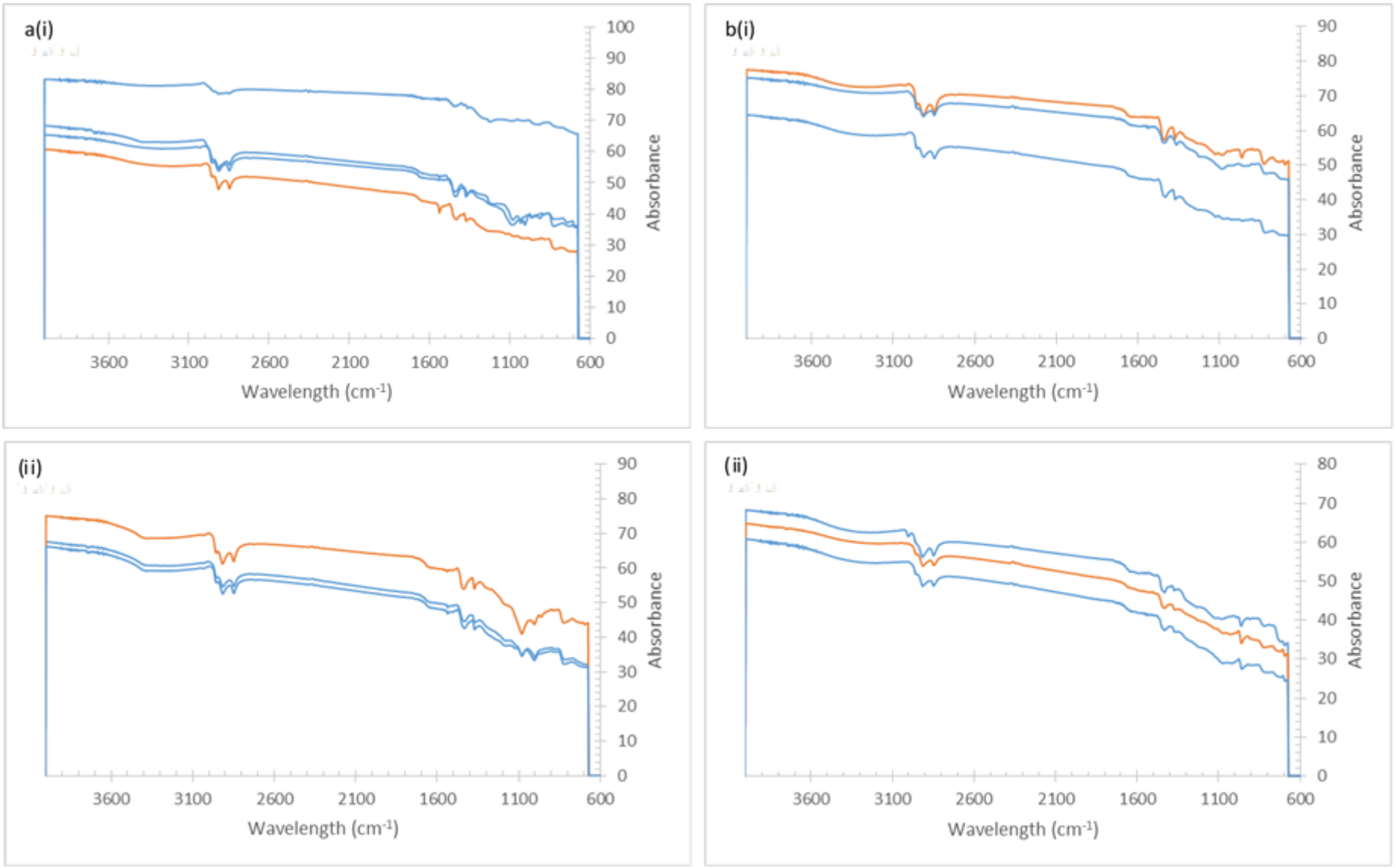


Figure C-30: All unadjusted FTIR-ATR spectra for (a) At. ferrooxidans (i) cGTR and (ii) and dGTR; and the consortium (i) cGTR and (ii) dGTR after 30 days of treatment

g. SEM-EDS

All SEM-EDS graphs shown below. Some of these were omitted from the main body of the text as the chlorine gradient indicated that the GTR was too shallow near the edge of the particle.

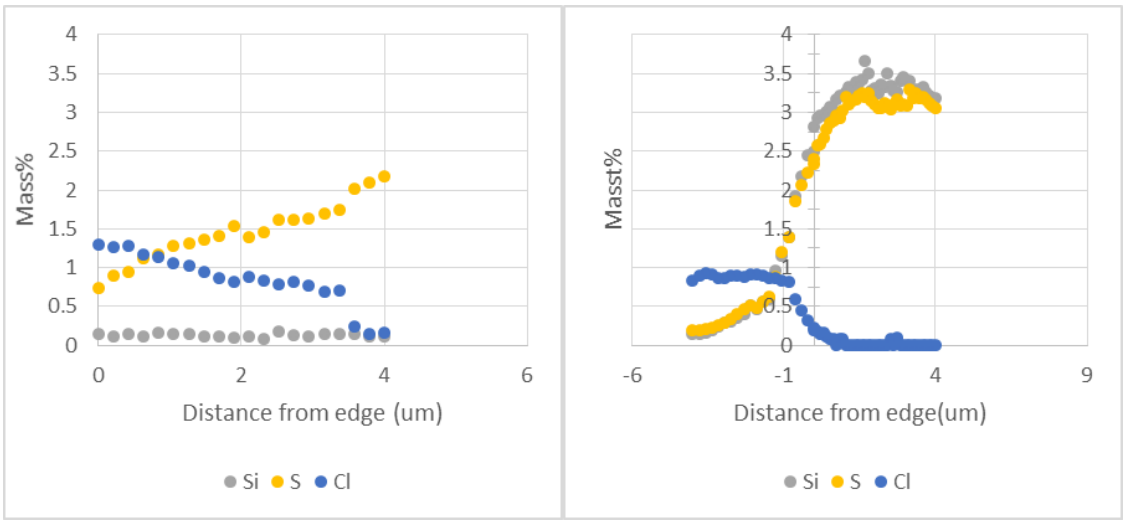


Figure C-31: At. ferrooxidans - cGTR (left) and dGTR (right) after 30 days of incubation

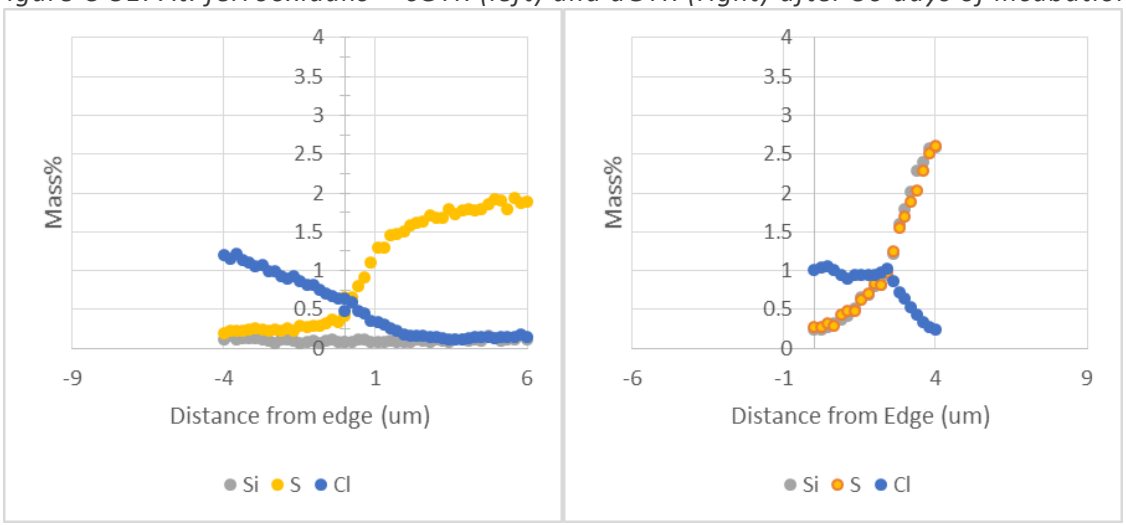


Figure C-32: At. ferrooxidans - cGTR (left) and dGTR (right) after 30 days of incubation

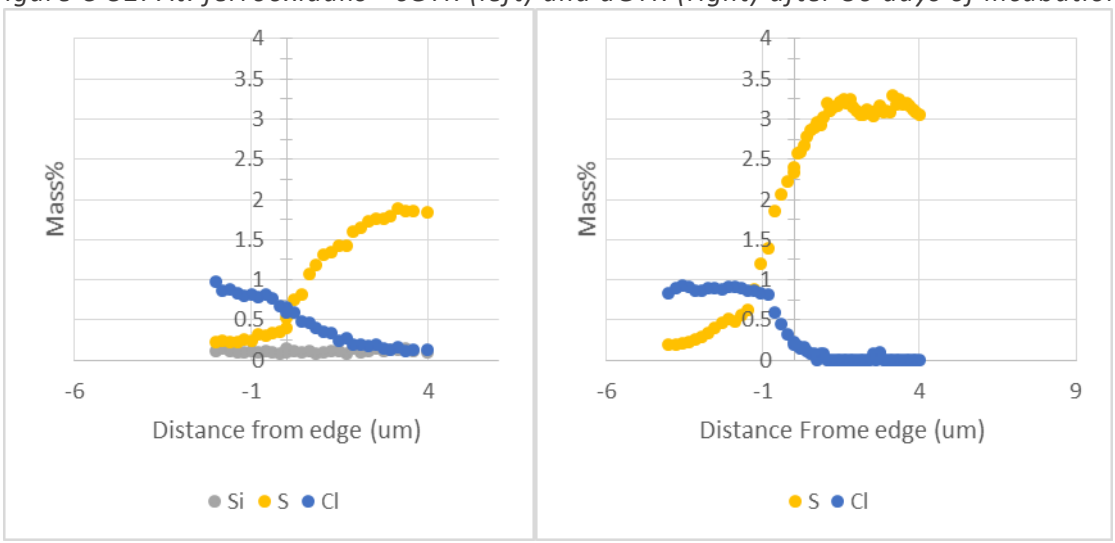


Figure C-33: Consortium cGTR (left) and dGTR (right) after 30 days of incubation

h. Additional SEM Images

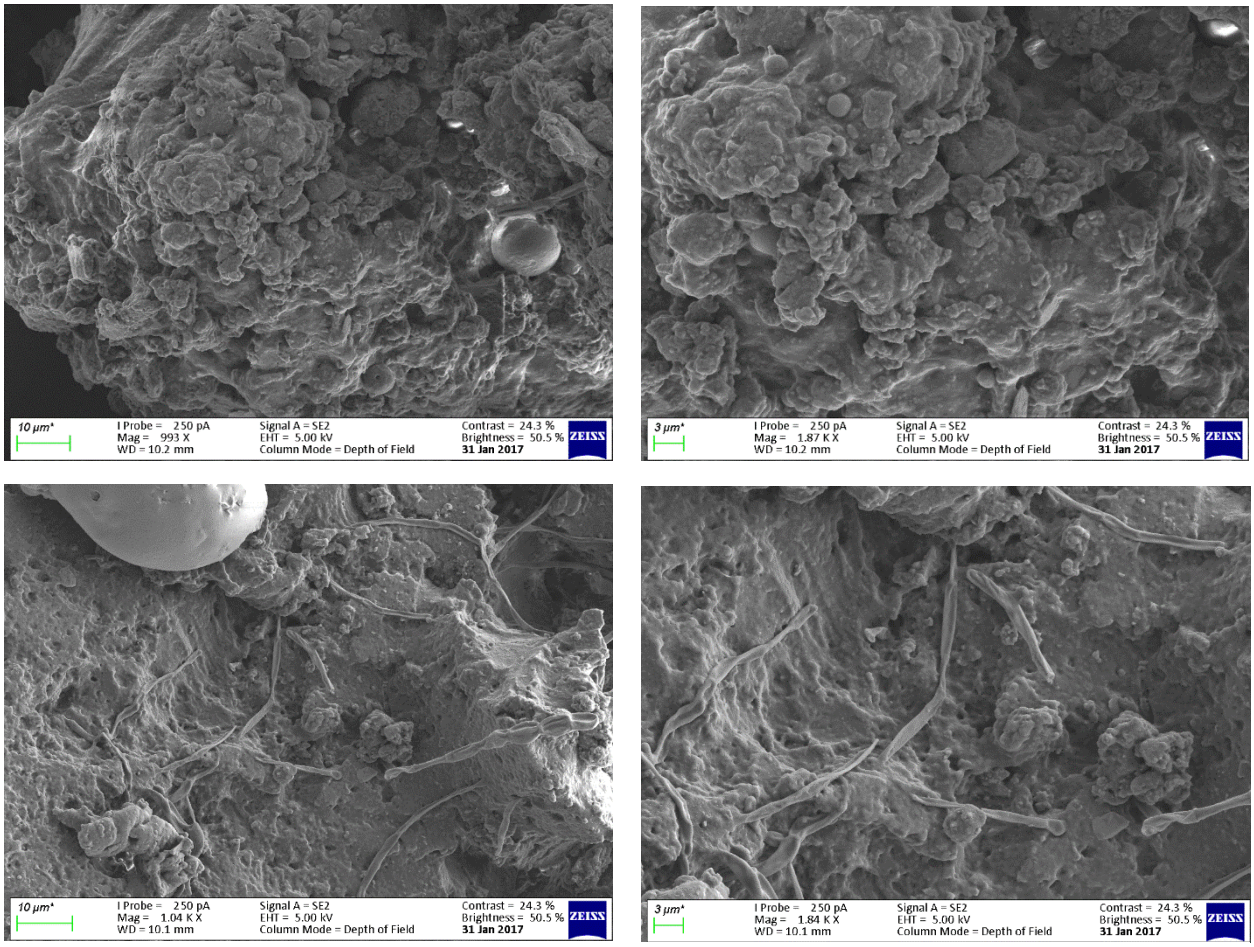


Figure C-34: Biomining consortium – microbial growth at surface of GTR particles after 30 days of incubation

i. Sulphate Analysis in Media

i. Challenges

Since sulphate is the end product of microbial desulphurisation of GTR, the change in sulphate concentration in the media can be used to indicate the extent of sulphur removal from the devulcanised GTR. It has been used by a number of studies in literature and can indicate the desulphurisation activity of the microbes over time (11,18,24).

Two methods of sulphate analysis were investigated (1) ion chromatography using a DIONEX Series 4500i IC with a Star-Ion-A300 Anion Peek column (100 x 4.60 mm) and (2) turbidometric analysis of sulphate concentration according to ASTM D516-16.

Three general difficulties were faced by both techniques in this study, which attempted to detect the change in sulphate concentration over time of acidophiles cultured in the presence of industrial GTR. Firstly, since sulphuric acid was used to adjust the pH to 1.3 to 1.8, the baseline concentration of the sulphate was very high compared to the anticipated change. Since the analytical techniques used to quantify sulphate

concentrations require low concentrations, samples had to be extensively diluted, and precision and accuracy of the results were limited, resulting in the anticipated changes lying within the error of the techniques. Secondly, zinc oxide present in the GTR can leach out, forming Zn^{2+} ions in solution, which combine with the sulphate to form zinc sulphate and precipitate out of solution if the pH exceeds 5. If this occurs during dilution, it causes a decrease in observed sulphate concentration, potentially obscuring any increases due to microbial devulcanisation. Lastly, barium compounds are present in some tyre rubber, and may react with the sulphate in solution to form barium sulphate, which is insoluble in aqueous solutions, and would also cause an artificial decrease in the observed sulphate concentration.

IC analysis was further complicated since the column was damaged by the media which had come into contact with GTR. It was hypothesised that this may have been due to water-soluble plasticisers and other organics leaching out of the GTR over the duration of the experiment, which then interacted with, and damaged, the plastic column. Turbidometric analysis, although more labour intensive, was more robust than IC analysis. Neither analytical technique was able to detect any meaningful changes in sulphate concentration, most likely due to a combination of side reactions and the high sulphate baseline concentration.

ii. Recommendations

Future studies which aim to monitor the change in sulphate concentration due to microbial devulcanisation can improve their accuracy in three ways. Firstly, the baseline sulphate concentration should be reduced. This has been done in previous studies by either increasing the pH as much as possible, and by using hydrochloric acid to adjust the pH of the solution instead of sulphuric acid. However, the success of this method depends on the tolerance of the microbe to a higher pH and concentration of chloride ions. Secondly, the number of unknown chemical compounds in solution should be reduced to reduce the number of unknown side reactions, which could possibly be achieved by using a laboratory made tyre rubber. This could eliminate compounds such as barium. Lastly, since zinc oxide is an integral component in tyre rubber and cannot be excluded even under laboratory condition, the pH of the solution and samples should be kept below 5 at all times to ensure that any zinc sulphate remains in solution.