INVESTIGATING THE EFFECT OF ACID STRESS ON SELECTED MESOPHILIC BIOLEACHING MICROORGANISMS

By Research Candidate:

NGOMA I-MUAKA EMMANUEL

Student No: 209239352

Supervisors:

Ass Prof. T.V. Ojumu Prof. S.T.L. Harrison

Thesis submitted in accomplishment of the requirements of the

Magister Technologiae in Engineering degree,

Department of Chemical Engineering,

Cape Peninsula University of Technology

February 2015

DECLARATION

FEBRUARY 2015

CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

DEPARTMENT OF CHEMICAL ENGINEERING

FOR COMPLETION BY MASTERS DEGREE CANDIDATE

Title:	Mr	**Student Nº:**	209239352
Name, Surname:	I-Muaka Emmanuel, Ngoma		
Postal address: (to which result can be posted)	Centre for Bioprocessing Engineering		
	Department of Chemical Engineering, University of Cape Town		
	P.Bag X3, Rondebosch 7701		
Telephone Nº:	083 496 9248		
Email address:	Emmanuel.Ngoma@uct.ac.za		
Dissertation Title:	An investigation of the effect of acid stress on selected acidophilic		
	bioleaching micro-organisms.		

DECLARATIONS:

(i). I hereby grant the Cape Peninsula University of technology free licence to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever of the above dissertation. I am presenting this dissertation in FULL/PARTIAL fulfilment of the requirements for my degree.

(ii). I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own.

Signature:		**Date:**	24 February 2015

DEDICATION

"I dedicate in the 1st instance this thesis to the almighty GOD who gave me the strength, the courage and the patience to complete this study. I live to give HIM praise.

**Joshua 1:9* "Have I not commanded you? Be strong and of good courage; do not be afraid, nor be dismayed, for the LORD your GOD is with you wherever you go." – The Holy Bible*

I thank HIM also for my family; my wife Karyn, my son Angelo and my daughter Imelda, my late father Emmanuel, my mother Anne, my sisters and my brothers who together with HIS mercies have been the driving force behind my life's successes. All these sources have been the pillars upon which I am able to stand. Helping Words and Words of Wisdom have encouraged and assisted greatly in facing the challenges that I met. Such words I share below:

"A family that prays together stays together." – Al Scalpone

Words cannot express my grateful thanks to those above who have made me who I am"

SYNOPSIS

Heap bioleaching is a microbially-assisted hydrometallurgical metal extraction process in which metals are solubilised from low grade ore by ferric iron and acid leach agents. Particularly for low grade ores, heap bioleaching provides several advantages over conventional technologies, it is simpler and safer to operate with low capital and operation cost and acceptable recoveries. Key challenges with heap bioleaching systems include the need to minimise leach durations and optimise both rate and extent of metal recovery.

There is limited understanding of the sub-processes involved in microbially assisted bioheap leaching (nutrient transport, microorganisms attachment to mineral, effects of metal concentration on microorganisms viability, …). Mineral ore agglomeration is a pre-treatment process typically carried out in the setup of the bioheap operation to dissolve metal oxide residue, neutralise acid consuming gangue, prepare agglomerates of the mixed particle size fraction to optimise heap permeability, prepare the ore surface for microbial attachment and optimise moisture content and mineral exposure to leaching reactions. Most agglomeration processes are carried out with an acidic solution. This may create an acid stress condition for the bioleaching microorganisms when inoculated into the operation. This is particularly relevant when the inoculum is introduced during the agglomeration process and may remain under the highly acidic conditions for a protracted time. However, quantitative data on the recommended acid concentration during agglomeration processes is very limited and is dependent on the ore treated. Similarly little is reported on the response of the acidophilic bioleaching microorganisms to acid stress.

This research project contributes to the bioleaching knowledge base by providing an understanding of the effect that acid stress has on the mesophilic species typically implicated in mineral sulphide bioleaching as a function of acid concentration (and resulting acidity) and duration of exposure. The study addresses the following specific key factors:

- The effect of acid stress due to acid concentration and exposure time on performance on mesophile microorganisms in terms of the microbial and ferrous iron oxidation.
- The interaction of acidity and exposure time with respect to microbial stress on the mesophilic bioleaching system performance.
- The nature of the stress response observed i.e. only the lag period or also the rate of ferrous iron and sulphur oxidation on the initiation of the leaching process.
- The observed effects on microbial activity mediated through the number of active cells or through the activity of these cells.

Quick fit stirred tank reactors (STR) containing 3% pyrite concentrate and 1 litre Norris media (Norris, 1983), aerated with 2 $L.min^{-1}$ compressed air and stirred at 550 rpm were inoculated with a mixed mesophilic culture mainly *Acidithiobacillus ferrooxidans*, *Acidiplasma cupricumulans*, *Ferroplasma acidiphilum*, and predominantly *Leptospirillum ferriphilum* following its pre-stress at 0.34M, 0.51M and 0.68M acid (H_2SO_4), whilst operating as a batch system. A Control, inoculated with an un-stressed culture, was run concurrently. The cultures were subjected to these acid stresses for a period of one hour, three hours and 24 hour and assessed for microbial growth and activity, leaching performance and microbial speciation.

Findings showed an increasing period necessary for microbial recovery with increased acid stress and increased time exposure. A similar leaching performance to the Control were recorded soon after the cultures recovered from the stress when the acid concentrations used were low, but the highest acid concentration (0.68M) combined with the longer exposure time (24 h) compromised the overall leaching performance and the required time of recovery was extended to as high as 200 h. Equally the microbial growth rates were similar to that of the Control culture following the recovery period. The yield in terms of microbial cells produced per kg iron oxidised decreased with increased acid stress but not necessarily with increased exposure time. The extent of iron solubilisation, at the time the Control achieved its highest solubilisation, decreased with both increases in acid stress concentrations and in exposure time. Microbial speciation indicated that four of the initial six species in the mixed culture were sensitive to acid stress. Only three species survived the stress in the early stages of the experiment and one specie disappeared during the course of the leaching experiment leaving just two species surviving. Of the two surviving species, *Fe. acidiphilum* and *L. ferriphilum,* the latter dominated to a final ratio of 99% to 1%.

Some recommendations have been made for future studies, namely:

- Acid stress effects should be tested on simulated heap leaching experiments using agglomerated ore.
- Acid concentration and exposure time should be increased to assess the extent of microbial recovery and acid tolerance levels.
- Similar experiment should be conducted using moderate thermophile and thermophile cultures.
- A conglomerate of a more defined mixed culture should be used to assess the acid resistant species.
- Physico-chemical conditions resulting from the acid agglomeration, such as shear stress, increase temperature, radiation should be considered to be assessed further.

ACKNOWLEDGEMENTS

I am sincerely grateful to all those who contributed to fulfilment of my thesis:

- Firstly and foremost, I will honour the Lord for keeping me strong and helping me to persevere.
- Thank you to my supervisors, Ass Prof T.V. Ojumu and Prof S.T.L. Harrison, for their input and guidance along the way. It was greatly appreciated.
- A huge thank you to Prof S.T.L. Harrison from the bottom of my heart for always making me aspire for greatness, for always landing the helping hand and for being a great person.
- A heartfelt thank you to the Centre for Bioprocess Engineering Research (CeBER - UCT) staff: Sue Jobson for taking time to cover all administrative requirements, Lesley Mostert for taking time in assisting in the formatting, Nangamso Cawe for her technical assistance, Marijke Fagan for taking time to advise and read my thesis, Elaine Govender and Alex Opitz for their assistance in the analysis of the results and for their guidance in the write-up, Nathan van Wyk for all the analysis and friendship.
- A big thank you goes out to the rest of the CeBER members for their support and friendship. Wynand, Theo, Qubekani, Sharon, Madelyn, Caryn, your input has been of great value.
- For their financial support in my scholastic development, I would like to thank the Centre for Bioprocess Engineering Research (CeBER). You are a family to me.
- Thank you to my friends and family for keeping me motivated. Thanks, Adhemar, Bolha, Teddy, Alain, Dr Bruno, Jean-Jacques, Nanou, Eva, Jannot, Dr Roland, Thierry, Kasongo, Mume, Ditend, Christian, ...
- Last but definitely not least, to my family for your prayers, love and encouragement; all of which will forever be reciprocated. I am blessed to have you.

LIST OF PUBLICATIONS AND PRESENTATIONS

1. Tupikina, O.V., Ngoma, I.E., Minnaar, S., Harrison, S.T.L. (2011). Some aspects of the effect of pH and acid stress in heap bioleaching. Minerals Engineering, **24**, 1209-1214.
2. Chiume, R., Minnaar, S.H., Ngoma, I.E., Bryan, C.G., Harrison, S.T.L. (2012). Microbial colonisation in heaps for mineral bioleaching and the influence of irrigation rate. *Minerals Engineering*, **39**, 156-164.
3. Ngoma, I.E., Ojumu, T.V., Harrison, S.T.L. (2013). Investigating the effect of acid stress on selected mesophilic bioleaching microorganisms. Presented at Mineral Processing Conference 2013 (MinProc 2013). Cape Town 7 – 8 August 2013.
4. Ngoma, I.E., Harrison, S.T.L. (2014). Investigating the effect of acid stress on selected mesophilic microorganisms implicated in bioleaching. Presented at Biohydrometallurgy 2014. Falmouth, Cornwall, UK. 9 – 11 June 2014.
5. Fagan, M.A., Ngoma, I.E., Chiume, R.A., Minnaar, S.H., Sederman, A.J., Johns, M.L., Harrison, S.T.L. (2014). MRI and gravimetric studies of hydrology in drip irrigated heaps and its effect on the propagation of bioleaching microorganisms. *Hydrometallurgy*, in press.
6. Ngoma, I.E., Ojumu, T.V., Harrison, S.T.L. (2015). Investigating the effect of acid stress on selected mesophilic bioleaching microorganisms. *Minerals Engineering* (Accepted).

NOMENCLATURE

Abbreviations

- CeBER Centre for Bioprocess Engineering Research
- DNA Deoxyribonucleic acid
- EOM Extracellular organic material
- EPS Extracellular polymeric substances
- gDNA Genomic deoxyribonucleic acid
- PCR Polymerase chain reaction
- PLS Pregnant leach solution
- PSD Particle size distribution
- qPCR Quantitative real-time polymerase chain reaction
- R&D Research and development
- rDNA Ribosomal deoxyribonucleic acid
- RNA Ribonucleic acid
- ROM Run-off mine
- rRNA Ribosomal ribonucleic acid
- STR Stirred tank reactor

Chemical Formula

- $(NH_4)_2SO_4$ Ammonium sulphate
- Ag Silver
- AgCl Silver chloride
- Al^{3+} Aluminium ion
- $C_{12}H_8N_2.H_2O$ 1-10 Phenantroline
- CH_3COOH Acetic acid
- CO_2 Carbon dioxide
- Cu Copper
- Cu_2S Chalcocite
- Cu_3AsS_4 Enargite
- $CuFeS_2$ Chalcopyrite
- CuO Copper (II) oxide
- CuS Covellite
- Fe^{2+} Ferrous iron
- Fe Iron
- Fe^{3+} Ferric iron
- FeS_2 Pyrite
- $FeSO_4.7H_2O$ Ferrous sulphate hepta-hydrate
- H_2O Water
- H_2S Hydrogen sulphide
- H_2SO_4 Sulphuric acid
- K_2SO_4 Potassium sulphate
- KCl Potassium chloride
- KH_2PO_4 Di-hydrogen potassium phosphate
- Mg^{2+} Magnesium ion
- $MgSO_4.7H_2O$ Magnesium sulphate hepta-hydrated
- Na_2CO_3 Sodium carbonate
- $NH_2OH.HCl$ Hydroxylamine (Hydroxylammonium Chloride)
- $NH_4C_2H_3O_2$ Ammonium acetate

- $NH_4H_2PO_4$ Ammonium di-hydrogen orthophosphate
- O_2 Oxygen
- Pt Platinum
- S Elemental sulphur

Symbols	Description	Units
• μ	Specific growth rate	h^{-1}
• μ_{max}	Maximum specific growth rate	h^{-1}
• A_o	Arrhenius constant	h^{-1}
• d	dilution ratio	
• E_a	Activation energy	kJ mol^{-1}
• K_s	Half saturation constant	g L^{-1}
• R	Universal gas constant	J K^{-1} mol^{-1}
• r_{Fe}	Rate of iron oxidation	g Fe m^{-3} h^{-1}
• r_x	Rate of growth of microorganisms	g L^{-1} h^{-1}
• S	Concentration of limiting substrate	g L^{-1}
• T	Growth temperature	K
• t	Time	h
• X	Concentration of microorganisms	cell mL^{-1}
• $Cx\ max$	Maximum cell concentration	cell mL^{-1}

GLOSSARY

- **Acidophile**: Microorganism which thrives in acid environment, typically at pH of 3 and below.
- **Archaea**: Grouping of unicellular microorganisms that are genetically distinct from bacteria and eukaryotes, often thriving in extreme environmental conditions.
- **Autotroph**: Microorganisms that utilise carbon dioxide (CO_2) as their only carbon source.
- **Chemotaxis**: Active movement of bacterium toward or away from a chemical stimulus.
- **Heterogeneous**: Different in nature.
- **Heterotroph**: Microorganism that utilises organic material as its only carbon source.
- **Homogeneous**: Uniform; consistent.
- **Inhibition**: Prevention or slowing of microbial functions in response to a chemical or physical stimulus.
- **Inoculum**: Initial microbial cells added to the heap system from which microbial growth proceeds.
- **Mesophile**: Microorganism that can grow in the temperature range 20-45°C.
- **Metabolism**: The system of chemical reactions occurring within the microbial cells, including both the breakdown of compounds via catabolism and the synthesis of compounds through anabolism.
- **Planktonic**: Freely suspended.
- **Raffinate**: Acidified leach solution used to irrigate ore bed.
- **Thermophile**: Microorganism that can grow at elevated temperatures i.e. above 40°C.
- **Biotic** Relating to or resulting from living organisms
- **Abiotic** Physical rather than biological; not derived from living organisms

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

1.1 Background

Pyrometallurgical and hydrometallurgical processes have been used widely in the extraction and refining of precious, semi-precious and base metals, such as iron, copper, gold and platinum, from mineral ore. Conventionally the pyrometallurgical methods employed require crushing, milling and concentration by flotation, followed by smelting, whilst typical hydrometallurgical approaches use acid and/or ferric iron leaching in tanks or heaps and pressure oxidation. Tank leaching and pressure oxidation also require the liberation and preparation of a concentrate, whereas heap leach can be used for whole, low grade ore.

Copper is easily recovered from the available high grade concentrates via smelting and this technique has previously dominated the industry. As the demand on copper metal has increased in the last twenty years while grades have decreased, the interest has also turned to the available copper reserves contained in low grade, complex and refractory ores (Watling, 2006). For low grade ore, the use of conventional extraction methods (*e.g.* crushing and milling for liberation, concentration by flotation followed by smelting) is less economically viable option due to its high energy requirement and associated environmental burden (Viera *et al*., 2007). The development of heap bioleaching as an alternative technology provides high metal recoveries within a reasonable timeframe, at low cost and low processing energies. It is a hydrometallurgical process which involves the microbially-assisted leaching of sulphide minerals, building on acid heap leaching which has been applied since the 1960's (Petersen and Dixon, 2007a). It is commonly employed when dealing with low grade and run-of-mine ores as it allows processing without energy intensive milling (Rawlings and Johnson, 2007). Heap bioleaching provides several advantages over other technologies, such as the use of simple equipment and operation, short construction times, low capital investment and operational costs (particularly due to the cycling of reagents, acceptable yields and reduced environmental burden (Watling, 2006; Pradhan *et al.*, 2008). A key challenge is in ensuring that the leach times required are minimised to minimise hold-up of inventory.

Heap bioleaching operations have been employed in Australia, Brazil, Chile, China and Peru (Petersen and Dixon, 2007a). The mineral reserves in the African continent will likely put the continent in a leading position in the production of metal to supply the world demand (InfoMine.com, 2014). South Africa is likely to play a leading strategic role as industry and science councils active in South Africa such as Mintek, Goldfields, Anglo American Corporation, BioMin and BHP Billiton have already contributed substantially to bioleaching research and development (R&D) over the years. Furthermore, extensive research on bioleaching technology has been conducted by various academic

research groups, making the present-day bioleaching process a considerably developed technology. However, there are still limitations to this technology, particularly in heap bioleaching, as only partial process control can be exerted due to the numerous parameters which influence the component sub-processes of the process (Petersen and Dixon, 2007b).

1.2 Problem Statement and Research Objectives

As seen in Figure 1.1 the global trend for the copper price has shown a constant increase since the year 2003, except for the effect of the world economic crisis in 2009. It has reached its record highest price of US$ 10,000 per metric ton in 2010 and it is currently sitting at US$ 7,000 per metric ton (Info Mine, 2013).

Figure 1-1: Copper price in US$ per metric ton for the past 24 years (InfoMine.com, 2014)

The recovery of the available mineral reserves is predicted to play a major role in the socio-economic development of the African continent (Ndlovu, 2008). Therefore, now that the copper price is high, it is imperative to develop technologies that are less susceptible to the fluctuations in the mineral price, cost effective, environmentally responsible, simple to apply and which improve resource productivity (Watling, 2006; Afewu and Dixon, 2006; Pradhan *et al.*, 2008), bearing in mind that the depletion of the high-grade mineral reserves have required development of alternative biotechnological processes to harness the remaining low-grade deposits (Rawlings *et al.*, 2003).

Heap bioleaching has been demonstrated to be one such alternative technology. It works by using microorganisms to generate the leach agents (Fe^{3+} and H^{+}) to leach sulphide minerals, solubilising base metal sulphides and allowing precious metal to be amenable to leaching. A desire to extend its usage has driven the need to improve the current limited knowledge of the bioleaching sub-processes in order to reduce leaching times and enhance the extent of extraction.

In heap bioleaching, microorganisms facilitate the dissolution of sulphide metals by making available ferric iron and protons responsible for the metal attack, by oxidising the ferrous iron and reduced sulphur species present in the ore, thereby providing a continuous and potentially well-distributed source of leach agents. Ideally, the rate of microbial regeneration of these leach agents ensures their plentiful supply such that availability of mineral to leach is rate limiting. To achieve this, effective microbial colonisation is required. The microbial colonisation of heaps is very much dependent on the effective introduction of microorganisms, their adaptation to the new conditions (humidity, acidity, aeration, energy and carbon source) and maintenance of metabolic activity. Heaps well colonised by leaching microorganisms are expected to produce high metal yield in a reduced timeframe with respect to un-colonised heaps since these microorganisms are responsible for the oxidation of the ferrous iron to ferric iron responsible for the metal attack and subsequent dissolution. In addition, they solubilise the sulphur species formed, generating acidity and preventing passivation of the mineral surface. However, there is minimal understanding of the different phenomena involved in a microbially operated bioheap (Pradhan *et al.*, 2008). Watling (2006) put into perspective different contributors to this lack of deep understanding of bioleaching. She highlighted the need to investigate different aspects of the bioleaching of low grade ore, such as chemistry, microbiology and hydrodynamics. Harrison (2012) further illustrated the need for a cross-disciplinary and integrated understanding of the sub processes and the interactions between these different aspects.

Microbial communities are exposed to various process stresses during bioleaching, either in tank or heap operation. These may be physical (temperature elevation or osmotic stress) (Stott *et al*., 2003; Franzmann *et al*., 2005; Plumb *et al*., 2008a), chemical (increase or decrease in pH, salinity, oxygen concentration, metal or reactive oxygen species) (Bailey and Hansford, 1993; Macario *et al*., 1999; Hawkes *et al*., 2004; Plumb *et al*., 2008b) or mechanical (increased levels of hydrodynamic stress) or a combination thereof (Blancarte-Zurita *et al*., 1986; Bailey and Hansford, 1993; Han and Kelly, 1998; Nemati and Harrison, 2000; Nemati *et al*., 2000; Harrison *et al*., 2007). Previous studies on microbial stress have focused on physiological and hydrodynamic stresses (Acevedo, 2000; Nemati and Harrison, 2000; Nemati *et al*., 2000; Harrison *et al*., 2003b; Sissing and Harrison, 2003; Raja, 2005; Harrison *et al*., 2007) and oxidative stress (Jones *et al*., 2009). There is limited information on microbial performance with respect to acid stress condition, with some recent performance data presented from the complex heap leaching system (Tupikina *et al*., 2011, 2013a).

Acid agglomeration when preparing ore bodies for bioleaching has been reported to be beneficial for microbial attachment, neutralisation of acid consuming gangue, dissolution of oxide metal residue, the optimisation of moisture content and mineral exposure to leaching reactions (Brierley, 2001; Watling, 2006) as well as manipulating permeability. However, no quantitative data have been presented on the recommended acid concentration during the agglomeration processes, nor on the response of the acidophilic bioleaching microorganisms to acid stress. These data could be used in the management of the acid stress condition. To address this, this research focuses on the effect of acid stress on the mesophilic species typically implicated in mineral sulphide bioleaching as a function of acid concentration (and resulting acidity) and duration of exposure. The investigation herein intends to address part of this, focusing specifically on:

- The effect of acid stress due to acid concentration and exposure time on performance in terms of the microbial and ferrous iron oxidation.
- The interaction of acidity and exposure time with respect to microbial stress on the bioleaching system performance.
- The nature of the stress response observed i.e. only the lag period or also the rate of microbial growth and leaching on the initiation of the leaching process.
- The observed effects on microbial activity i.e. effects brought about by the number of active cells or through the activity of these cells.

1.3 Scope and Limitations

The aim of this study is to add to the bioleaching knowledge base by providing research data on microbial stress aspects of heap bioleaching. This investigation focuses on the effect of acid stress on the mesophilic species implicated in sulphide mineral leaching as a function of acid concentration (and the resulting acidity) and the duration of exposure to it; with the leaching response being monitored at a bulk community performance level.

1.4 Thesis Structure

This introduction highlights the research objectives and defines the scope and the limitations of the study. It is followed by a review of the available relevant literature, presented in Chapter 2. This provides an overview of the heap bioleaching technology, with an emphasis on the key role played by microorganisms, as well as mineral ore agglomeration as a pre-treatment step required in the construction of a heap bioleaching operation. The review also identifies some factors capable of affecting heap bioleaching performances mainly due to stress encountered by the microorganisms during the pre-treatment stage of the operation. The scope of the study is focused on responses to acid

stress at varying duration. To analyse the response, cell growth and microbial leaching profiles are recorded and analysed after the initial microbial inocula are subjected to varying acid conditions for different times. Further the effect these subjected stresses on the microbial speciation is determined.

The experimental programme adopted to test the hypotheses, is presented in Chapter 3. The reactor systems and microbial consortium used are described. Sampling approaches are described. Further, the methodologies used, the experimental approach and the analysis adopted are detailed.

Intra and inter-experiment reproducibility was analysed to validate the study. Different concentrations of acid were used to stress the inocula for various exposure times to assess the recovery period and the subsequent performances. The stress response was assessed in terms of microbial growth and iron leaching. These results were analysed in terms of time to onset of activity, maximum rates and extent of reaction. Microbial speciation was analysed at completion of the experiment and at different stages of the experiment post inoculum stress. These results are presented and discussed in Chapter 4.

In related Chapter 5, the conclusions from the findings are presented. The constraints of the study are indicated and its potential impact on improving the bioleaching process described. Recommendations for further investigations within this research field are made.

CHAPTER 2: LITERATURE REVIEW

2.1 Heap Bioleaching – A Complex Operation

2.1.1 *Description of Heap Bioleaching Operations*

Heap bioleaching is a biotechnological process that enables extraction of semi-precious, precious and base metals from low grade mineral ore with the assistance of microorganisms. The bioleaching of base metal sulphides by ferric iron and acid leach agents is a typical example. To construct a heap, the run-of-mine (ROM) ore or a blend of ore mixed to control the grade is crushed to particle sizes, typically < 25 mm (Petersen and Dixon, 2007a), that could provide suitable mineral liberation for significant extraction within a suitable leaching time (Bartlett, 1992) while ensuring sufficient ore bed permeability. The ore is then agglomerated with acidified solution prior to heap stacking (Petersen and Dixon, 2007a). Agglomeration is a process in which fine-grained particles and clays (~1 mm) within the ore are attached to larger particles (10 to 20 mm) (O'Kane Consultants Inc, 2000). In copper-ore leaching, sulphuric acid solution is frequently used to bind the fine and large particles together by a liquid bridge; water alone does not achieve it (Kodali *et al*., 2011). This beneficial process promotes the formation of a more homogeneous heap for uniform percolation and thus efficient metal extraction (Bartlett, 1992; Petersen and Dixon, 2007b).

The heap is stacked on an impermeable layer connected to a drainage system (Petersen and Dixon, 2007a). An aeration system is inserted at the base of the heap. After construction, the heap is aerated from the bottom via the pipeline network (Petersen and Dixon, 2007a). Aeration rates range from 0.08 to 2 $m^3.m^{-2}.h^{-1}$ (Petersen and Dixon, 2007a). The heap is irrigated from above using an acidic feed solution containing ferrous iron and nutrients such as nitrogen, phosphorus, magnesium and potassium, which is applied over the heap surface at irrigation rates ranging from 5 to 20 L m^{-2} h^{-1} using drippers typically spaced 30 to 100 cm apart (Petersen and Dixon, 2007a). Solution distribution through the heap is governed by the combined effects of gravity and capillary action (Petersen and Dixon, 2003). As solution percolates through the ore bed whilst influencing solution-ore and microorganism-ore contact, the minerals dissolve into solution.

The solution flow and aeration play an important role in facilitating the transfer of heat within the heap (Petersen and Dixon, 2007a). The heap is allowed to develop the naturally-occuring microorganisms, and at times is inoculated with different types of microorganisms (Brierley, 2001), which are able to catalyse the iron and sulphur oxidation reactions that occur during the bioleaching process. Efficient irrigation is necessary for the transport of microorganisms, reactant solutes and leaching products into and out of the heap, and maintenance of temperatures within the limits of

activity of the microorganisms (Petersen and Dixon, 2007a). Figure 2.1 illustrates a typical heap bioleaching operation for low grade sulphidic minerals.

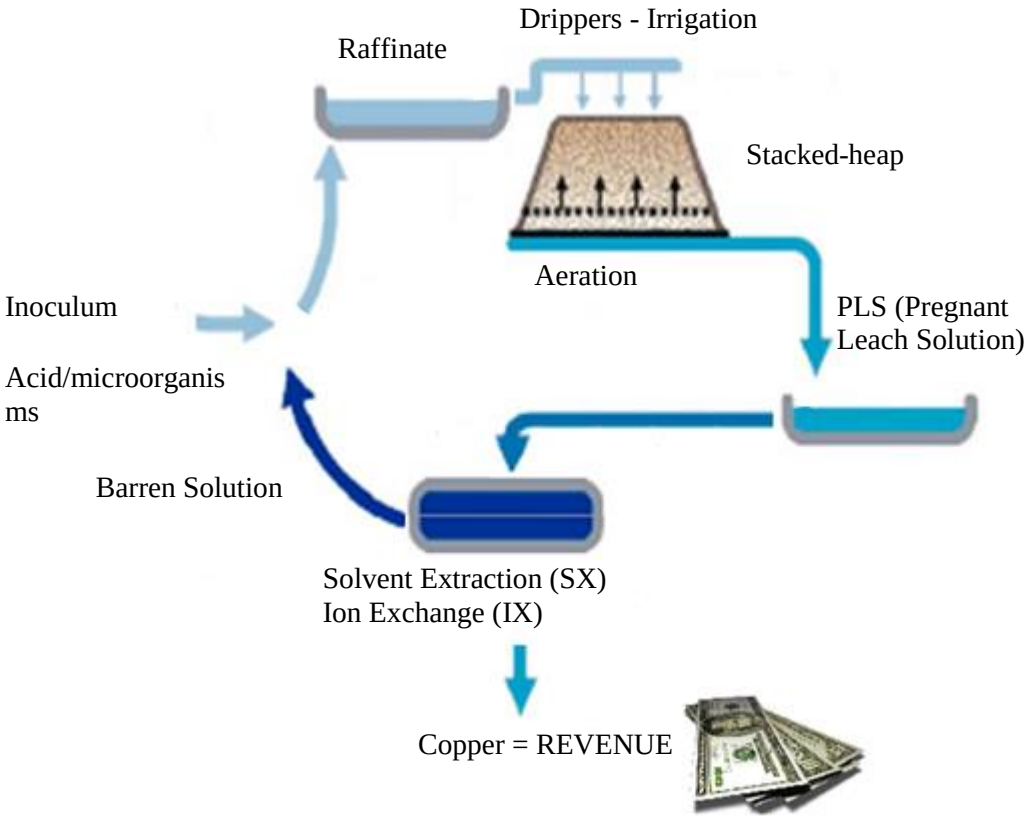


Figure 2-1: Illustration of a heap leaching operation: Solution is trickled on top of the ore bed, the bed is aerated from the bottom to promote bioleaching of sulphide minerals, recovery of copper metal and recycling of barren solution to minimise water use (Adapted from Watling, 2006).

There is a lag time before the microorganisms attach to the mineral, grow and begin to contribute to sulphide oxidation. This lag time can be reduced by utilising leach solutions containing active microbial communities already acclimatised to leaching environments (Watling, 2006) or by introducing these active communities during the agglomeration process (Brierley, 2001).

The drainage system collects the pregnant leach solution (PLS) (Petersen and Dixon, 2007a). This undergoes further processing to recover the metal via solvent extraction and electro-winning or ion exchange using a resin bed. The effluent, i.e. barren solution, is recycled for re-irrigating the heap. Organic matter remaining in the recycled effluent can cause microbial inhibition (Brierley, 2001; Rawlings *et al.,* 2003; Pradhan *et al.,* 2008). Pradhan *et al.* (2008) reported that metal oxidation mediated by microorganisms can be inhibited by accumulation of heavy metals such as copper, zinc, arsenic and iron. Escobar and Lazo (2003) suggested purge and dilution of irrigation solution to avoid the build-up of constituents inhibiting microbial growth and activity. Alternatively, flushing out the

heap from time to time could be considered. However, this may not be practical on a large industrial heap scale. In both cases, impact on the water balance must be considered.

2.1.2 *Leaching Chemistry*

The reaction pathways are complex and have been outlined by Sand and Gehrke (2006) and Rohwerder and Sand (2007). The leaching process involves mineral oxidation by ferric iron (Fe^{3+}) which releases the metal ions into solution (Equation 2.1). The non-oxidative dissolution of the mineral by sulphuric acid (H_2SO_4) occurs simultaneously (Equation 2.2). Microbial ferrous iron oxidation reactions also occur (Equation 2.3). The oxidation of ferrous iron to ferric iron regenerates the ferric irons required in the metal dissolution reaction. This only applies to non-acid soluble minerals.

$$MFeS_2 + 4Fe^{3+} \rightarrow 5Fe^{2+} + M^{2+} + 2S^0 \qquad \text{Equation 2.1}$$

$$MFeS_2 + 4H^{+} \rightarrow Fe^{2+} + M^{2+} + 2H_2S \qquad \text{Equation 2.2}$$

$$2Fe^{2+} + 2H^{+} + 0.5O_2 \rightarrow 2Fe^{3+} + H_2O \qquad \text{Equation 2.3}$$

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \qquad \text{Equation 2.4}$$

$$4FeS_2 + 15O_2 + 2H_2O \rightarrow 4Fe^{3+} + 8SO_4^{2-} + 4H^{+} \qquad \text{Equation 2.5}$$

During the mineral dissolution, the sulphur component of the sulphide mineral undergoes two different oxidation mechanisms (Schippers *et al*., 1996; Schippers, 2007; Rohwerder and Sand, 2007). Acid-insoluble metal sulphides, *e.g.* pyrite (FeS_2), proceed via the thiosulphate pathway in which thiosulphate is oxidised to sulphate and elemental sulphur (Schippers *et al*., 1996; Schippers, 2007; Rohwerder and Sand, 2007). Acid-soluble sulphide minerals, *e.g.* chalcopyrite ($CuFeS_2$), are transformed into elemental sulphur in the absence of sulphur oxidising microorganisms through the polysulphide pathway (Schippers *et al*., 1996; Schippers, 2007; Rohwerder and Sand, 2007). The oxidation of sulphur to sulphate (Equation 2.4) generates acid required to leach the mineral and maintain a low pH environment. Maintenance of preferred pH range of 1 to 2 is important for both microbial iron and sulphur oxidation, leading to ferric iron and acid regeneration by the microbial communities (Watling, 2006). Low pH is also important to maximise the solubility of iron as it is a key leach agent.

The extracellular organic materials (EOM) and the extracellular polymeric substances (EPS) produced by the autotroph microorganisms together with oxygen are used by the heterotroph microorganisms to oxidise the ferrous iron to ferric iron which in turn attacks the metal in the mineral. The metal is then

released in solution and the thiosulphate produced is oxidised to sulphate in presence of hydrogen ion and form sulphuric acid necessary for the acidity required. Typical leaching reactions that occur in the heap are illustrated in Figure 2.2.

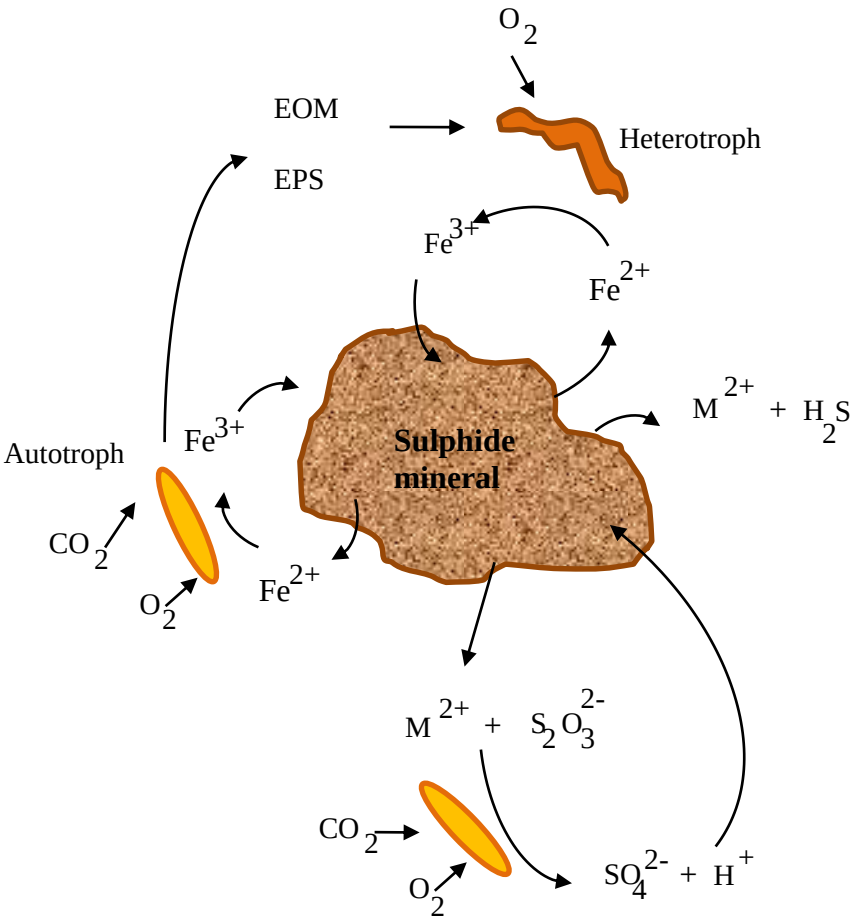


Figure 2-2: Illustration of heap leaching reactions catalysed by microorganisms (adapted from Schippers *et al.*, 1996; Rohwerder and Sand, 2007)

2.2 Function of Microorganisms in the Leaching Process

2.2.1 *Bioleaching Mechanisms*

The involvement of microorganisms in the leaching reactions has been the subject of much debate. Direct leaching was one of the main mechanisms proposed initially (Devasia *et al.*, 1993). In this mechanism it was theorised that microorganisms attached to the mineral surface to oxidise the sulphide phase by enzymatic attack, without any requirement for ferric or ferrous irons. Tributsch (2001) reported that observations from electron microscopy images contradicted this mechanism. To date, the key leaching mechanisms postulated to contribute to bioleaching of mineral sulphides as

highlighted in previous studies (Devasia *et al*., 1993; Tributsch, 2001; Sand *et al*., 2001; Rawlings, 2002; Sand and Gehrke, 2006; Watling, 2006) are:

- *Indirect non-contact mechanism:* Microorganisms oxidise ferrous irons to ferric irons in the bulk solution, and sulphides or intermediates to sulphate and acid. The sulphide mineral is either oxidised by ferric irons or dissolved in the acidic solution (Tributsch, 2001). No adhesion of microorganisms to the mineral surface is required *i.e.* oxidation may occur by planktonic microorganisms (Devasia *et al*., 1993; Tributsch, 2001; Sand and Gehrke, 2006).

- *Indirect contact mechanism:* Microorganisms attached to the mineral surface oxidise ferrous iron to ferric iron within a biofilm comprised of microorganisms and extracellular polymeric substances (EPS). The EPS acts as a reaction medium in which the ferric iron and protons generated induce thiosulphate and sulphate formation (Tributsch, 2001) and may be concentrated to produce an optimised leach environment.

- *Cooperative leaching mechanism:* This is the result of symbiotic activity between the freely suspended microorganisms and the attached cells. The waste ferrous and sulphur species released during the sulphide mineral leaching are used as an energy source by the suspended cells (Tributsch, 2001).

These mechanisms indicate the importance of both microbial retention within the leaching environment and attachment to mineral surfaces.

2.2.2 ***Microbial Characteristics and Diversity***

Both bacteria and archaea are found in the natural leaching environment and grow in inorganic, aerobic and low pH environments (Rawlings and Johnson, 2007). Generally all microorganisms require nutrients such as carbon, oxygen, nitrogen, sulphur, and phosphorus for growth, cell maintenance and metabolic activities (Bailey and Ollis, 1986). These autotrophic microorganisms use CO_2 sourced from the atmosphere as a carbon source (Rawlings and Johnson, 2007; Pradhan *et al.*, 2008). Bioleaching microorganisms typically oxidise iron and/or sulphur to provide a metabolic energy i.e. they are chemolithotrophs (Rodriguez *et al*., 2003). Autotrophic microorganisms may produce extracellular organic material (EOM) and extracellular polymeric substances (EPS) providing a carbon source for the heterotrophic microorganisms present in the heaps (Pradhan *et al*., 2008). Metabolising waste organic products is important to maintain a suitable leaching environment and avoid inhibition of microbial growth and biooxidation (Rawlings and Johnson, 2007).

The bioleaching microorganisms are subjected to further classification because they function at different temperatures. The optimal temperature for effective microbial activity are: 20 to 40°C for

mesophilic microorganisms (predominantly bacteria); 40 to 60°C for moderately thermophilic microorganisms (bacteria and archaea); and greater than 65°C for extreme thermophilic microorganisms (predominantly archaea) (Bailey and Ollis, 1986; Rawlings and Johnson, 2007). When higher heap temperatures develop, typically due to the exothermic oxidation reactions, the microbial community present is modified to favour the development of thermophilic microorganisms (Pradhan *et al*., 2008). These higher temperatures of 60°C and above are recognised to enhance chalcopyrite leaching and to be necessary to achieve desired copper recoveries.

2.2.3 ***The Major Species of Microorganisms implicated in Leaching***

The microbial communities in mineral-processing bioreactors and in bioleach heaps are diverse. Many of the important organisms in these systems are well known. Generally, two or three species specific to a particular temperature range dominate in the various mixed culture communities that exist in the bioreactors (Norris, 2007). Acidophiles have been reviewed widely and their diversity has been widely noted (Norris and Johnson, 1998; Hallberg and Johnson, 2001), as well as their temperature ranges for growth (Norris, 1990; Franzmann *et al*., 2005), their taxonomy (Goebel *et al*., 2000), and their ecology and community structures (Johnson, 1998; Baker and Banfield, 2003). Most of the familiar acidophiles involved in mineral sulphide oxidation can be placed in one of three broad groups on the basis of their temperature ranges for growth as mentioned in Section 2.3.2 and their evolutionary relationships. These are the mesophilic proteobacteria, Gram-positive moderate thermophiles and the thermophilic archaea (Norris, 2007).

2.2.3.1 *Mesophiles*

Proteobacteria are generally the most active in mineral sulphide oxidation below 45°C, particularly *Acidithiobacillus* and *Leptospirillum* (Table 2.1). Several subgroups of strains within the species *Acidithiobacillus ferrooxidans* have been reported by Karavaiko *et al*. (2003). Other species of *Acidithiobacillus* oxidise sulphur but not iron. The main sulphur-oxidising species are *At. thiooxidans* and *At. caldus*. *At. caldus* has greater thermotolerance than *At. thiooxidans* (Norris, 2007). Some other sulphur-oxidising *Acidithiobacilli* have yet to be named, including those found in marine environments that tolerate higher levels of sodium chloride than the familiar species (Simmons and Norris, 2002). The *Leptospirillum* genus is also very diverse. Markosyan (1972) named the original isolate *Leptospirillum ferrooxidans*. As more strains were isolated and referred to as *Leptospirillum*-like bacteria, their variety became evident (Harrison and Norris, 1985). Coram and Rawlings (2002) subsequently distinguished *Leptospirillum ferriphilum* from *Leptospirillum ferrooxidans* on the basis of the ribosomal RNA (rRNA) gene sequences and copy numbers (two and three copies respectively). Another species, *L. ferrodiazotrophum* was proposed for nitrogen-fixing representative of a third rRNA sequence cluster of strains by Tyson *et al*. (2005). Some species of *Leptospirillum* grow up to

about 45°C (Norris, 1983; Franzmann *et al*., 2005) and even higher (49°C) (van Hille *et al*., 2013). Another example is a strain of the species *L. thermoferrooxidans* (Golovacheva *et al*., 1993), which was subsequently lost (Rawlings, 2002).

2.2.3.2 *Moderate Thermophiles*

Norris (2007) reported that there is not a precise temperature that divides mesophile activity from moderate thermophile activity. Some acidophiles, for example, *At. caldus* and *Acidimicrobium ferrooxidans*, are quite active from about 25°C to almost 55°C. However, for most of the iron- and/or sulphur-oxidising acidophiles studied thus far, mesophiles have been reported to have an optimum temperature of about or often below 40°C while moderate thermophiles have an optimum temperature of or above 45°C. Golovacheva and Karavaiko (1979) established the *Sulfobacillus* genus for a moderate thermophile, *Sb. thermosulfidooxidans*. Recent isolates of these Gram-positive spore-forming bacteria include two species from natural geothermal sites that show some growth at almost 65°C and have optimum temperatures about 10°C higher than the 45-50°C optimum temperature of *Sb. thermosulfidooxidans* (Norris, unpublished data). A second genus of moderately thermophilic Gram-positive, iron-oxidising bacteria, not related to *Sulfobacillus*, contain a single species at present, *Acidimicrobium ferrooxidans*. It is widespread in acidic natural geothermal sites and mineral sulphide mine environments (Clark and Norris, 1996). It is suggested that the microbial diversity of the acidophilic, moderately thermophilic cultures is much greater than currently identified; this remains an area of study.

2.2.3.3 *Thermophiles*

There is an overlap of temperature ranges for growth of moderately thermophilic iron-oxidising bacteria with those of some thermoacidophilic archaea; however, the lowest optimum temperatures among the thermophilic studies, for example about 68°C for *Sulfolobus metallicus*, are well above those of most thermotolerant *Sulfobacillus* species (Norris, 2007). *S. metallicus* has been used in most mineral sulphide oxidation work at about 70°C, although it was not identified as such earlier (Norris, 1997). Gericke and Pinches (1999) reported that it may well be the dominant strain in most studies of mineral sulphide oxidation at 65-70°C where the species were not identified. Later work has shown that, in continuous or semi-continuous culture, *Metallosphaera* frequently outcompetes *S. metallicus* (Harrison, unpublished data). At 75°C it is replaced by other organisms such as *Metallosphaera sedula, Metallosphaera hakonensis* and various unnamed isolates. The number of species known to be directly involved in mineral sulphide oxidation at high temperature appears to be rivalling that of mesophiles and moderate thermophiles at lower temperatures, even though not much documented (Norris, 2007).

2.2.4 *Microbial Consortium*

Rawlings and Johnson (2007) have reviewed some of the microbial aspects of bioleaching and found that the current focus is on the selection of microbial communities which provide enough biodiversity for optimal heap leaching performance. Johnson's research group is conducting work on designing combinations of microbial consortia to endorse effective microbial activity and adaptability to the changing leaching environment. Identification of all microorganisms present in leaching environments and extensive research on their specific contributions to the biooxidation process enables separation of microorganisms with significant effects from those with a lesser impact. This will contribute to the design of appropriate inoculum compositions specific to the different bioleaching environments. The utilisation of only the relevant microbial populations could potentially reduce the operational cost associated with aeration and nutrient supply that would otherwise be incurred when undesired microbial communities are present in the heap (Rawlings and Johnson, 2007). More importantly, it is necessary to ensure that the diversity of species is present to allow transition into new operation regimes.

The research of Minnaar *et al* (2010) and Tupikina et al (2011, 2013, 2014) has demonstrated the progression of dominant organisms following colonisation, demonstrating lead and follower organisms at a particular temperature, as well as a progression through the temperature profile. So far, Johnson's work has shown that mixed cultures are more efficient in the bioleaching process than pure cultures. Further, it is well known that mixed cultures enhance process robustness and resilience to perturbations (Roychoudhury, 2004; Miura *et al*., 2007; Oyekola *et al*., 2007). This is a common biological trait demonstrated with plants, for example, robustness of pastures (Masel and Siegal, 2009; Whitacre, 2012; Lempe *et al*., 2013) and also corresponds to typical industrial practice, leading to the choice of a mixed culture of mesophilic microorganisms to inoculate the systems utilised in this investigation.

2.2.5 *Microbial Growth Dynamics in Heap Colonisation*

Attachment of the microorganisms to the ore surface is influenced by the microbial culture history, physicochemical environment, ore surface and contacting mechanism (Bromfield *et al*., 2011; Chiume *et al*., 2012; Africa *et al*., 2013; Tupikina *et al*., 2013, 2014). Following this, the overall accumulation of microbial cells within the porous matrix environment is controlled by the multiplication of the microorganisms (van Loosdrecht *et al*., 1990; Rockhold *et al*., 2002; Lizama *et al*., 2005) and the fluid flow environment (Chiume *et al*., 2012; Govender *et al*., 2013; Fagan *et al*., 2014).

A typical microbial growth curve is illustrated in Figure 2.3. Once the microorganisms are introduced into the heap leaching systems via inoculation, they may experience a lag phase in which their growth

is restricted due to their slow adaptation from the conditions in the previous environment to the new leaching environment (Shuler and Kargi, 1992). To minimise the lag phase, a highly active and concentrated microbial population is used in the inoculum (Bailey and Ollis, 1986; Shuler and Kargi, 1992). Furthermore, the conditions of inoculum development should match those present in the heap to minimise the adaptation time required.

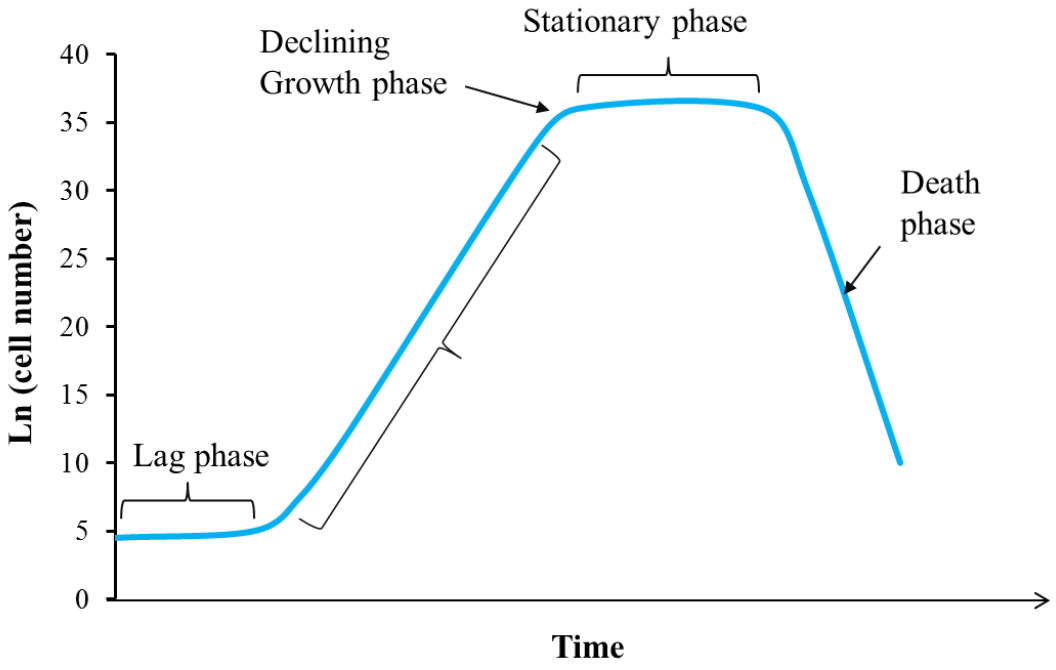


Figure 2-3: A typical microbial growth curve (adapted from Bailey and Ollis, 1986)

Once the cells have adapted to the leaching environment, the microorganisms use the nutrients available for assimilation and energy provision to enable multiplication under balanced growth conditions during the exponential phase (Shuler and Kargi, 1992). Bioleaching microorganisms gain energy for growth through the oxidation of ferrous iron and inorganic sulphur compounds (Devasia *et al*., 1993) to regenerate ferric iron and sulphuric acid.

The rate at which the microorganisms multiply, r_x, is a first order function of the microbial species concentration, X, where μ is the specific growth rate [h^{-1}] (Pirt, 1975):

$$r_x = \frac{dX}{dt} = \mu X \qquad \textbf{Equation 2.6}$$

Substrate-limited growth during the exponential phase can be described by Monod kinetics (Monod, 1949), in which the specific growth rate is given by:

$$\mu = \frac{\mu_{\max} S}{K_s + S} = \frac{\mu_{\max} Fe^{2+}}{K_s + Fe^{2+}}$$

Equation 2.7

S is the concentration of the limiting substrate. For bioleaching, the limiting substrates that the microorganisms rely on are typically the reduced ferrous and sulphur compounds. Equation 2.7 is shown for ferrous iron as the limiting substrate. Here K_s is the ferrous iron concentration corresponding to the specific growth rate at half its maximum value (Bailey and Ollis, 1986) and is a measure of the microorganism's ability to scavenge ferrous iron. Additional limiting substrates *e.g.* CO_2 or O_2 can be included using a multiplicative term as expressed in Equation 2.8. Alternatively, the dominating effect can be determined and used alone. The Monod kinetics approach is simple and effective when determining growth rates for continuous culture.

$$\mu = \mu_{\max}\left(\frac{S_1}{K_{S1} + S_1}\right)\left(\frac{S_2}{K_{S2} + S_2}\right)$$

Equation 2.8

$$\mu = A_o e^{-\frac{E_a}{RT}}$$

Equation 2.9

Aside from microbial growth being a function of the growth substrate available, microbial growth is also known to be pH dependent and has an Arrhenius dependence on temperature (Equation 2.9), where E_a is the activation energy, *R* is the Universal gas constant, A_o is the Arrhenius constant and *T* is the growth temperature). Typically, the growth rate increases with increasing temperature until optimum growth conditions are reached. Some growth rates determined in previous studies (Breed and Hansford, 1999; Dempers *et al.*, 2003) are presented in Table 2.1.

Investigations into the growth kinetics of bioleaching microorganisms have been conducted mostly in continuous culture systems (Breed and Hansford, 1999; Dempers *et al.*, 2003; Petersen and Dixon, 2007a; Minnaar *et al.*, 2010). Dempers *et al.* (2003) reported growth rates of a mixed mesophilic consortium under conditions typical of a *Leptospirillum* dominated culture, based on their ferrous iron bioenergetics. These are displayed in Table 2.1 for a range of mesophilic temperatures (30 to 40°C) and low pH values (pH 1.1 to 1.7). Growth rates of *At. ferrooxidans* on various substrates in batch shake flasks under optimal growth conditions have also been documented by Karavaiko *et al*. (2006). Plumb *et al*. (2008a) monitored the growth of pure and mixed bioleaching strains on low grade chalcopyrite ore in batch shake flask experiments. All strains showed an increase in cell number with time. Furthermore, a slower increase in cell number was observed with decreasing temperature (Franzmann *et al*., 2005).

After the exponential growth phase, a decline in growth rate is experienced when key nutrients in the leaching environment become depleted (Shuler and Kargi, 1992) or conditions become inhibitory for the microorganisms due to the accumulation of dissolved salts, metals and hydroxyl-iron precipitates. In the stationary phase, the growth rate and death rate are balanced while metabolism continues (Bailey and Ollis, 1986; Shuler and Kargi, 1992). Thereafter, an increasing death rate results in the declining cell numbers (Bailey and Ollis, 1986). To maintain a healthy and active microbial community within the heap leaching system, a need to understand the nature of the bioleaching microorganisms and factors controlling their growth and metabolism is crucial.

Table 2-1: Growth rates of mesophilic microorganisms in various systems

Microorganism	Substrate	System	Temperature (°C)	pH	Growth rate (μ, h^{-1})	Reference
At. ferrooxidans	Low grade chalcopyrite containing ore	Glass column (0.4 m height, 160 mm inner diameter)	RT ± 23	1.7	0.0364 from day 16 to 22, 0.0005 from day 23 to 50	Minnaar *et al.*, 2010
At. ferrooxidans	Ferrous iron	Batch shake flask	Optimal	Optimal	0.19	Karavaiko *et al.*, 2006
At. ferrooxidans	Sulphur	″	″	″	0.069	″
At. ferrooxidans	Chalcopyrite	″	″	″	0.05	″
At. thiooxidans	Sulphur	″	″	″	0.046	″
At. thiooxidans	Low grade chalcopyrite containing ore	Glass column (0.4 m height, 160 mm inner diameter)	RT ± 23	1.7	0.083 from day 16 to 22, 0.0008 from day 23 to 50	Minnaar *et al.*, 2010
At.caldus	″	″	″	″	0.0045, 0.0038	″
L. ferriphilum	″	″	″	″	0.0343, 0.0051	″
Predominantly L. ferrooxidans	Ferrous iron media	Continuous flow bioreactor (Height: Diameter ratio of 1.32, working volume of 1 L)	30	1.7	0.079	Dempers *et al.*, 2003
″	″	″	35	1.7	0.119	″
″	″	″	40	1.7	0.038	″
″	″	″	″	1.5	0.077	″
″	″	″	″	1.3	0.087	″
″	″	″	″	1.1	0.089	″
L. ferriphilum	″	″	40	1.1	0.1024	Breed and Hansford, 1999
″	″	″	″	1.3	0.1043	″
″	″	″	″	1.5	0.1227	″
″	″	″	″	1.7	0.0952	″
L. ferrooxidans	Ferrous iron	Batch shake flask	Optimal	Optimal	0.069	Karavaiko *et al.*, 2006

Table 2-2: Operating ranges for important bioleaching bacteria operating in the mesophilic or early thermophilic temperature range (Rossi (1990), Watling (2006) and Schippers (2007))

Microbial Species	Carbon source	Energy Oxidiser source	pH optimum	pH operational growth range	Temperature optimum (°C)	Temperature operational growth range (°C)
At. ferrooxidans	Autotrophic	Iron and sulphur	2.5	1.3 to 4.5	30 to 35	10 to 37
At. thiooxidans	Autotrophic	Sulphur	2.0 to 3.0	0.5 to 5.5	28 to 30	10 to 37
At. acidophilus	Autotrophic	Sulphur	~ 3	~ 3	25 to 30	25 to 30
At. caldus	Autotrophic	Sulphur	2.0 to 2.5	1.0 to 3.5	45	32 to 52
L. ferrooxidans	Autotrophic	Iron	1.5 to 3.0	1.3 to 4.0	28 to 30	~ 30
L. ferriphilum	Autotrophic	Iron	1.3 to 1.8	-	30 to 37	-
Fm. acidiphilum	Heterotrophic	Iron	2 to 2.5	1.3 to 4.8	37	< 10 to 45
Fp. acidiphilum	Autotrophic Heterotrophic	Iron	1.7	1.3 to 2.2	35	15 to 45
S. thermosulfidooxidans	Autotrophic Heterotrophic	Iron and sulphur	~ 2	1.5 to 5.5	45 to 48	20 to 60

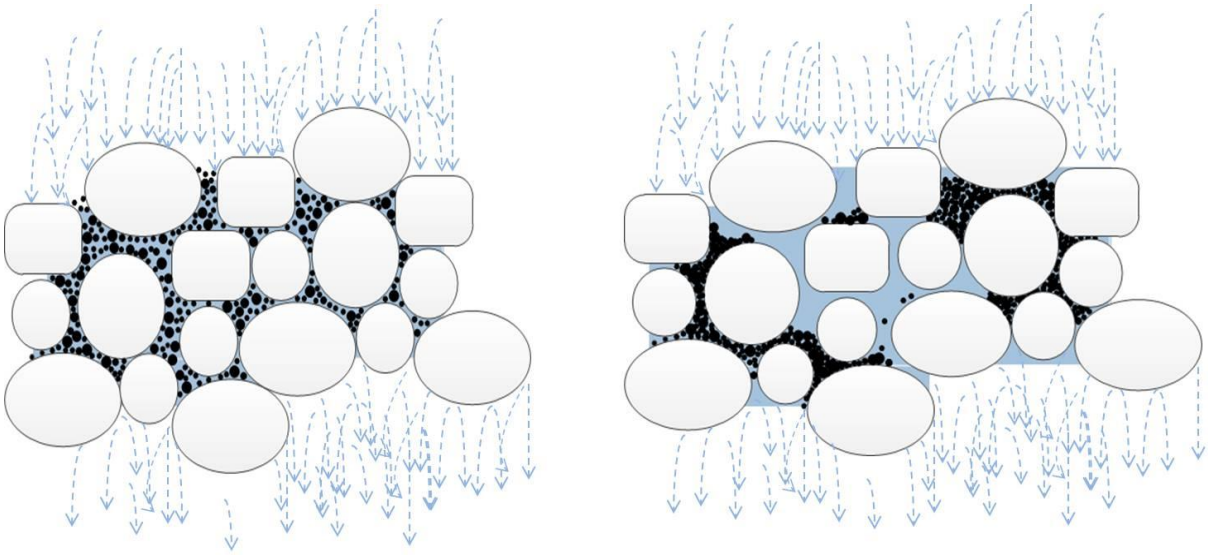
2.3 Heap Preparation – Crushed Ore Agglomeration

2.3.1 *Benefits of Agglomeration*

Several heap leach operations have experienced problems associated with poor recovery due to percolation issues caused by low-grade complex ores, tailings and clayey deposits. Poor percolation can lead to low metal extraction due to solution channelling or the development of impermeable (dead) zones within the heap (Kappes, 2005; Schlitt, 1992). The ore body is crushed in heap preparation to expose the mineral of interest and increase the liberation of the metal, enhancing its extraction during the leaching step and reducing extraction time. This however, increases the amount of fine material present containing the mineral. The fine materials need to be controlled so that the percolation of the constructed heap and the metal leaching are optimal, thus the agglomeration process is introduced to handle this fine. Agglomeration improves the uniform percolation of solution through the heaps of ore and is applicable to many ores, wastes and milled tailings (Dhawan *et al*., 2012; Bouffard, 2005).

The term agglomeration is a descriptive term in particle technology. In the case of fine powders (less than 10 μm), particle adhesion and agglomeration may occur due to attractive surface forces; whereas, in the case of larger particles, adhesion forces must be produced by the addition of liquid or binders or both to obtain stable and strong agglomerates as is the case in heap leaching operations (McClelland, 1988; Kodali *et al*., 2011). During the agglomeration process, fine-grained particles and clays (~1 mm) within the ore are attached to larger particles (10 to 20 mm) using liquid or binders (O'Kane Consultants Inc., 2000) and in copper leaching, sulphuric acid solution is frequently used to bind the fine and large particles together by a liquid bridge; water alone will not achieve it (Kodali *et al*., 2011). This beneficial process promotes the formation of a more homogeneous heap for uniform percolation and efficient metal extraction (Bartlett, 1992; Petersen and Dixon, 2007b).

Crushed ore agglomeration has two major aims. Firstly, it is the best opportunity for the thorough application of the leaching solution prior to building the heap to initiate the leaching process itself (Dhawan *et al*., 2012; Bouffard, 2005; Purkiss and Anthony, 2004). Sulphuric acid, for metals such as copper, nickel and uranium and cyanide solution for metals such as gold and silver, are used as the agglomeration solution to improve the leaching rate from low grade ores (Bouffard, 2005). Secondly, the addition of the leaching solution facilitates agglomeration by coalescing fine particles onto larger rock particles via liquid bridges, leading to more uniformly permeable heaps as shown on Fig 2-4 (Dhawan *et al*., 2012; Kodali *et al*., 2011; Bouffard, 2005). Agglomeration also combats the acid neutralising capacity of the gangue mineral and prepares the ore for the microbial attachment.



Agglomerated ore **Non-agglomerated ore**

Figure 2-4: Comparison of solution percolation in agglomerated ore favouring liquid retention necessary for microorganism transport and attachment versus non-agglomerated ore favouring preferential channelling resulting in microorganism wash-out (adapted from Chamberlin, 1980)

2.3.2 *Mechanism of Agglomeration*

The binding mechanisms of agglomeration can be classified into five categories: (1) solid bridges, (2) adhesion and cohesion forces, (3) surface tension and capillary pressure, (4) attraction forces between solids and (5) interlocking bonds (Lewandowski and Kawatra, 2009; Moats and Janwong, 2008). Kodali *et al*. (2011) reported that in crushed ore agglomeration, adhesion and cohesion forces are dominant for binder-assisted agglomeration, whereas surface tension and capillary forces dominate the system for non-binder or wet agglomeration.

Crushed ore agglomerates can take two forms: fine particles adhering to coarse particles and fine particles adhering to each other. On the basis of microscopic investigations, Tibbals (1987) reported two types of agglomerates: first, one in which particles of 1 mm or larger in size formed the core and were surrounded by a shell of finely divided material (rim agglomerates); and second, one in which particles were composed of granular members having no clearly defined core (nucleated/conglomerate). The rim agglomerates were mechanically more stable and were preferred for leaching (Tibbals, 1987).

2.4 Operating Factors Affecting The Leaching Performance

2.4.1 *Factors Affecting Heap Bioleaching*

The performance of a heap operation is affected by several environmental, biological and physicochemical factors. Operating temperature, acidity and heap aeration are the most important factors which have the potential to cause the most stress on the bioleaching microorganisms and negatively affect the leaching operation. These factors are now examined in more detail.

The temperature of the heap determines which microorganisms influence the heap during the course of the bioleaching process. Microbial communities are dynamic, adapting to the changing heap leaching environment. To be cost effective, heaps are usually initiated at ambient temperature. However, as the microbial colonisation moves through the heap and the exothermic biochemical and leaching reactions within the heap take place, the temperature increases. This, in turn, causes the microbial community to vary from the mesophiles to the moderate thermophiles to the extreme thermophiles in the deepest core of the heap.

Various researches have looked at the effect of different temperatures on the performance of bioleaching microorganisms. Different microbial species are most active within a narrow range of temperatures (Table 2-2). They become inactive when optimal temperatures deviate with a few degrees, displaying reduced activity (Franzmann *et al*., 2005; Halinen *et al*., 2009; Ongendangenda and Ojumu, 2011; Watling *et al*., 2013) and lower attachment efficiency (Bromfield *et al*., 2011).

Watling *et al*. (2013) observed a decrease in the microbial complexity with increasingly harsh conditions. From an initial 16 strains in the inoculum at 35°C, a deviation to 38.6°C in combination with various other factors such as pH and solid loading changes reduced the strain count to 12. This was mainly due to the ability of the surviving strain to oxidise iron and to be tolerant to ferric iron with the surviving strain out-competing non-surviving strains, indicating that a slight change in temperature is capable of favouring the growth of a species while inhibiting another. Van Hille *et al*. (2013) also observed a change in microbial community on a 2°C temperature increase in a reactor inoculated with a *L. ferriphilum* dominated BIOX culture. An increase from 48°C to 50°C resulted in the loss of *L. ferriphilum* and a decrease in leaching performance. The surviving community was dominated by *A. cupricumulans* and *At caldus* (van Hille *et al*., 2013). Similarly, the culture composition changed in response to changing pH. Tupikina *et al*. (2011) also studied the effect the change in temperature from 50°C to 60°C had on the bioleaching performance and she concluded that effective thermophilic colonisation occurs in heaps without impacting the bioleaching performance when the irrigation solution is maintained between pH 1.1 and 2.0.

Many more factors have been identified to be important to the integral bioleaching process of constructed heap and are required to be controlled sufficiently to increase the performance of the system. Pradhan *et al*. (2008) have identified some of these factors summarised in Table 2.3.

Table 2-3: Heap bioleaching parameters (adapted from Pradhan *et al.*, 2008)

Parameter	Description
Ore mineralogy:	The ore needs to contain sulphide minerals susceptible to ferric and acidic leaching and access to the mineral must be assured by suitable particle sizes or porosity. The gangue material influences acid consumption and may liberate additional ions into solution *e.g.* Al^{3+}, Mg^{2+}, some of which may be inhibitory.
Aeration:	The supply of O_2 and CO_2 to the leaching system provides components required for microbial growth and ferrous iron oxidation.
Irrigation:	The irrigant supplies reactant solutes *e.g.* H_2SO_4 and controls precipitation of salts that might block the percolation channels, pH and, to an extent, temperature via heat transfer within the heap. Note that acid consumption is a major contributor to processing costs (Watling, 2006).
Temperature:	The temperature of the heap determines which microorganisms govern the heap during the course of the bioleaching process. Microbial communities are dynamic, adapting to the changing heap leaching environment.
Biology:	The microbial diversity and adaptation abilities of microorganisms, population density and spatial distribution of microorganisms, location with respect to mineral grains, and microbial activity play significant roles in the leaching process.
pH:	Control of the leaching environment for optimal microbial activity.
Redox potential:	The rate of mineral dissolution is a function of the ferric to ferrous iron ratio (Fe^{3+}/Fe^{2+}). High redox potentials tend to inhibit the bioleaching of chalcopyrite (Córdoba *et al*., 2008)

2.4.2 *Effect of Temperature*

Franzmann *et al*. (2005) used the Ratkowsky equation to describe the effect of temperature on the rate of ferrous iron oxidation or the time required to oxidise a specified amount of sulphur by a range of selected acidophilic bioleaching microorganisms. The application of the equation to iron oxidation data produced estimates of cardinal temperature for activity (T_{MIN}, T_{OPT} and T_{MAX}) to a greater precision than previously available. It was observed that sulphur oxidation rate increased with increasing temperature above the T_{OPT} for growth until the increasing temperature affected the biomass to such an extent that it was reduced to below the threshold.

Breed *et al*. (1999) reported on the effect of temperature on the continuous ferrous iron oxidation kinetics of a *Leptospirillum ferrooxidans* dominated culture. A continuous bacterial culture with a feed concentration of 12 $g.L^{-1}$ ferrous iron at a dilution rate between 0.01 to 0.06 $L.h^{-1}$ and temperature between 30°C and 40°C showed that an increase in the temperature from 30°C to 40°C did not affect either the maximum yield or the maintenance coefficient on ferrous iron. The maximum specific growth rate increased from 0.046 h^{-1} to 0.077 h^{-1} and the maximum specific ferrous iron utilisation rate increased from 8.65 to 13.58 mmol Fe^{2+} per mmol C per hour across the range from 30 to 40°C. The kinetic constant in bacterial ferrous iron oxidation increased linearly with increasing temperature. Furthermore, at each steady state, the total genomic DNA extracted from the biomass and analysed using the restriction enzyme analysis PCR amplified 16S rDNA indicated that *L. ferrooxidans* was the only ferrous iron oxidising species to be detected.

2.4.3 *Effect of Acidity*

An important parameter for successful microbial colonisation and active metabolism in a heap bioleaching operation is a suitable pH. Heaps are tens of metres in height and the pH of the irrigating solution travelling through the heap varies significantly (Tupikina *et al*., 2011). The solution pH of a given bioleaching operation is usually determined by the balance between the acid-producing and the acid-consuming reactions and also by the input of acid or alkali into the system (Plumb *et al*., 2008).

These solution pHs have considerable effect on the microbial growth and the Fe^{2+} and S^0 oxidation during the leaching process. The variation in the effect of pH on the growth and activity of bioleaching strains also has huge implications for the selection of strains to use for a specified bioleaching operation. Bioleach heaps have a wide pH gradient between the feed solution and the effluent (PLS), typically pH 1.0 – 2.5. Studies on heap bioleach operations in South America have shown that while the fresh feed contained 8 $g.L^{-1}$ of sulphuric acid as free acid (~pH 0.9), the effluent stream gave a high pH value of between 2.2 and 2.4 (Ojumu *et al*., 2006). The solution pH is critical to the availability of the ferric reagent for the leaching of most sulphide minerals (Ingledew, 1982). In

bioleaching it is crucial to keep the iron in solution by preventing the precipitation of ferric iron to hydroxyl and sulphate complexes which will reduce the amount of ferric iron in the leaching medium.

Solution pH also affects the ferrous iron and sulphur oxidation of the leaching organisms. du Plessis *et al*. (2007) and van Aswegen *et al*. (2007) reported that a pH greater than 2.0 has a negative effect on the microbial community in a heap operation system. Meruane and Vargas (2003) established that, on oxidation of ferrous iron by *Acidithiobacillus ferrooxidans* in the pH range 2.5 – 7.0, the inhibition of ferrous iron oxidation activity was observed at pH values above 3.0 and was partially linked to the formation of ferric iron precipitates, which hinder the transport process on the cell surface. Ferric iron precipitates, such as jarosite, represent one of the great pH related challenges in heap bioleach operations: "it occupies space on the ore surface creating diffusion barriers" (Ojumu and Petersen, 2011). Further, the precipitate can clog the heap bed, thus reducing its permeability (Ojumu and Petersen, 2011).

Nemati *et al*. (1998), Breed and Hansford (1999) and Özkaya *et al*. (2007) studied the effect of pH on the microbial ferrous iron oxidation and although a wide range of optimum pH (1.5 – 3.5) was reported, recent studies have indicated that solution pH greater than 2.0 can lead to a high risk of bacterial de-activation, resulting in loss of the microbial culture (van Aswegen *et al*., 2007; Plumb *et al*., 2008; Penev and Karamanev, 2010).

Tupikina *et al*. (2011, 2013a) investigated the effect of pH and acid stress on moderately thermophilic and thermophilic mixed cultures in a heap bioleaching system. The microbial oxidation rate of ferrous iron was reported to exceed the mineral leaching rate across the pH range 1.1 – 2.0. Culture acclimatisation was required when conditions became more acidic. Below the pH value of 1.1 a reduction in the microbial community was observed, both in concentration and diversity. It was also reported that iron precipitation occurred in the columns at pH greater than 1.7 and affected the leaching performance of the system. When the irrigation feed pH was decreased to 1.4 and lower, a net increase in the cumulative total soluble iron was observed. It was concluded that effective thermophilic colonisation was possible in heaps without impacting the bioleach performance when the irrigation feed is maintained between pH 1.2 – 1.7.

2.4.4 *Effect of Aeration*

Most leaching bacteria are aerobic and chemolithotrophic. Aeration takes care of the supply of both oxygen and carbon dioxide to the bioleaching system. Sufficient carbon dioxide serves as a source of carbon needed for biomass generation. At the bottom of the pile, where air is forced into the heap, oxygen is close to saturation, but as the air flows upwards, the microorganisms catalysing the oxidation of sulphide consume oxygen and, as a result, a degree of oxygen depletion near the top of

the heap prevails (Pradhan *et al*., 2008). The oxygen levels throughout the heap therefore vary widely based on permeability, temperature, oxidation rates, sulphide-sulphur content, microbial community and other factors that are not well understood yet. Brierley (2001) reported on that the possibility of having microorganisms capable of using alternative electron acceptors to colonise portions of the bioheap that have limited oxygen supply should be considered for the optimisation of a heap bioleaching operation.

Similarly, CO_2 from air is consumed by the microorganisms as a carbon source; hence care must be taken to avoid CO_2 limitation. This is addressed by Bryan *et al* (2012) and Petersen *et al* (2010). Lizama (2001) reported on the copper bioleaching behaviour in an aerated heap, after correlating heap aeration with copper bioleaching by monitoring oxygen levels, copper content and microbial activities at different heights in the heap during a bioleaching process. They found that the degree of oxygen depletion near the top of the heap was in direct relation to the microbial oxygen consumption below. Oxygen content by itself was not a good indicator of heap leaching performance because its concentration varied with depth. Heap oxidative capacity was a better indicator of leaching. Copper leaching was directly related to oxygen consumption in the heap and oxygen consumption, in turn, was related to microbial activity and the rate of forced aeration. It was reported that the increase in the aeration rate improved the copper leaching.

2.5 Review of Investigation Conducted

Literature has described the work done in the pre-treatment of mineral ore for the bioleaching operation, specifically the agglomeration step. It has highlighted the importance of agglomeration in the process to increase the permeability of the packed material with adequate percolation thereby leading to adequate metal extraction. Agglomeration in the metal extraction of copper, nickel and uranium is done with sulphuric acid but the level of acid concentration varies from one operation to another. The concentrations generally applied in industry and the basis on which is determined are not widely available in the literature. These acid concentrations reduce the pH considerably in the packed bed and generally create additional stresses on the microorganisms when they are introduced in the operation.

Three major stress factors have been identified as being able to influence the leaching performance in a heap system: temperature, acidity (pH) and aeration (O_2 and CO_2). The change in temperature has the potential to force the colonisation of a heap to adapt from the top of the heap where ambient temperature affects the natural colony. However as the microbial colony moves deeper into the heap and due to the exothermic leaching reactions, the temperature inside the heap increases, inhibiting the initial community that started the operation and forcing a different community of microbial species to

take over the operation. Control of the pH in the heap bioleaching operation has been identified as a very important parameter because it has a considerable influence on the microbial growth and the Fe^{2+} and S^0 oxidation. However, it is difficult to control in the heap and is rather maintained in an acceptable operating range. A variety of pH values have been studied to establish the ranges at which different microorganisms perform at their optimal leaching rate and the ranges in which inhibition occurs. A general observation revealed that leaching microorganisms operate best in the pH range of 1.0 – 2.5, a very acidic medium. But this is not usually the case for all the operations. Some mineral ores, such as carbonate, oxide and copper mineral, are acid consuming which could increase the pH inside the heap and affect the leaching performance of the microorganisms. Others, such as sulphide minerals are acid forming.

Finally, aeration is the main supply of carbon, in the form of CO_2, and O_2 required for microbial metabolism. It is supplied from the bottom of the heap and a gradient of air is created from the source point at the bottom of the heap where the leaching bacteria are supplied with a huge amount of air to the top of the heap where the amount of O_2 and CO_2 is reduced. This balance of air flow and irrigation needs to be achieved optimally to increase the activity of the microorganisms in the system.

This review has highlighted work done on the heap leaching and an overview of the elements to take into account during the operation of a heap bioleaching system. It is noted that there has not been much investigation into the initial stress encountered by microorganisms when introduced into the acidic environment of freshly agglomerated materials in the operation. This mainly because most copper heap operation do not inoculate with microorganisms, but rather allow the naturally-occurring microorganisms to develop in heap. In some cases raffinate or intermediate leach solution (ILS), which contains microorganisms, will be used for agglomeration of the ore. Hence, this is the scope of this study as detailed in Section 1.3.

2.6 Research Motivation, Hypotheses and Key Questions

The literature review has highlighted the gap in the understanding of the effect of acid agglomeration as a pre-treatment for ore in heap bioleaching on the performance of leaching microorganisms. Few researchers have paid attention to this area and most known acid concentrations used in the industry are not published in the public domain. Understanding and quantifying the acid concentration effect on the microbial community is important to optimise the leaching performance of any given heap operation. This highlights the need to gain a better understanding of (i) the acid concentration used in the agglomeration process, (ii) the effect the acid stress has on the performance of the leaching by the stressed microorganisms, and (iii) microbial recovery after subjection to high acid concentration. This

knowledge is required to optimise the agglomeration process by understanding the recovery and the subsequent leaching performance of the selected consortium of microorganisms.

To date, the literature has shown:

- The necessity of performing agglomeration in the pre-treatment of mineral ore in the heap bioleaching operation to increase the solution percolation, with emphasis on acid agglomeration (Dhawan *et al*., 2012; Kodali *et al*., 2011; Bouffard, 2005).
- The growth rates of bioleaching microorganisms on different substrates under varied leaching conditions (Breed and Hansford, 1999; Dempers *et al*., 2003; Karavaiko *et al*., 2006; Petersen and Dixon, 2007a; Minnaar *et al*., 2010).
- The effect of temperature on the bioleaching performance and microbial community. Temperature deviation affects the activity of the leaching microorganisms and could favour the production of ferric complex precipitates that effect the leaching performance (Franzmann *et al*., 2005; Halinen *et al*., 2009; Ongendangenda and Ojumu, 2011; Watling *et al*., 2013) and reduces microbial attachment (Bromfield *et al*., 2011). The rate of sulphur oxidation increases with temperature to an optimum temperature (Franzmann *et al*., 2005). Temperature progression within the heap requires a succession of microbial communities.
- The effect of acid concentration on the ferrous iron and sulphur oxidation, on the microbial community and on the overall performance of the heap system. The ferrous iron oxidation in bioleaching is inhibited greatly by low pH due to ferric iron precipitation (Meruane and Vargas, 2003; Ojumu and Petersen, 2011) and the bioleaching microbial community is affected due to change in acidity (van Aswegen *et al*., 2007; Plumb *et al*., 2008; Penev and Karamanev, 2010).
- Lizama (2001), Brierley (2001) and Pradhan *et al*. (2008) reported on the aeration levels and the impact of these levels on the microbial community and metal recovery.

The review of several studies has indicated key gaps in the understanding of the impact of acid agglomeration on the microbial community, its subsequent recovery and overall performance. More specifically, there is a need to quantify the acid concentration acceptable to use in the agglomeration process without compromising the leaching process by the microorganisms. Quantitative studies of the effect of exposure to acid (extent and duration) on a mixed mesophilic culture in batch agitated systems need to be conducted. Knowledge of the recovery time and performance of the microbial activities following acid stress will inform the tolerance of acceptable acid concentrations that the microorganisms can support during acid agglomeration in heap bioleaching. The hypotheses formulated and the key questions derived are as follows:

Hypothesis 1 and key questions:

A mixed mesophilic culture subjected to acid stress during agglomeration will experience a temporary decrease in growth and oxidation rate with an associated delay of the microorganisms' recovery and subsequently will delay the leaching performance proportionally to the acid stress level imposed.

- What is the effect of acid concentration on microbial growth and ferrous iron oxidation?
- What is the effect of acid exposure time on microbial growth and ferrous iron oxidation?
- Does the stress response observed affect only the lag period before onset of growth and oxidation or also the rate of ferrous iron and sulphur oxidation on the initiation of leaching?
- Is the effect mediated through the number of active cells or relative activity of these cells?
- Do acidity and exposure time interact with respect to their role in microbial stress in the bioleaching system?

Hypothesis 2 and key questions:

A mixed mesophilic culture subjected to acid stress during agglomeration will undergo a reduction in species diversity to favour the development of the most acid resilient members in the community.

- What is the effect of acid concentration level on the microbial community dynamics?
- What is the effect of acid exposure time on the microbial community dynamics?
- Does the species' distribution change at initial introduction of the acid stresses exposure or during recovery?

CHAPTER 3: MATERIALS AND EXPERIMENTAL METHODOLOGY

In this chapter, the detailed experimental approach adopted in order to achieve the study's objectives and elucidate the hypotheses is presented. The aim of the study herein was to assess the effect of acid stress on the mesophilic microbial species typically implicated in mineral sulphide bioleaching as a function of acid concentration (and resulting acidity) and duration of exposure. Section 3.1 provides information on microbial cultures utilised, Section 3.2 the mineral composition and Section 3.3 the experimental media. A clear description of the apparatus selected to run the experiment is described in Section 3.4 and the analysis conducted to validate the hypothesis and answer the key questions is described in Section 3.5. Sample collection and data handling are discussed in Section 3.6 and the research strategy is then summarised in Section 3.7. The raw data are detailed in Appendix F.

3.1 Microbial Cultures Analysis

Mesophilic microorganisms were employed in the investigations. The mixed mesophiles used were obtained from a stock culture containing these microorganisms: *At. ferrooxidans*, *A. cupricumulans*, *Archaea* (*JTC Archaea 1*), *F. acidiphilum*, and predominantly *L. ferriphilum*, confirmed by qPCR (described in Section 3.5.3) and displayed in Figure 3.1. The microorganisms were grown on pyrite concentrate in a 1 L batch stirred tank reactor at 35°C and agitated at 550 rpm. The stock was sub-cultured on a weekly basis to allow the microorganisms to remain active. This was achieved by the removal of approximately 150 mL slurry and re-filling the volume up to the litre mark with fresh media and adding 3.5 g of fresh pyrite concentrate. The cell concentration was maintained at 1×10^{9} – 4×10^{9} cells.mL^{-1}.

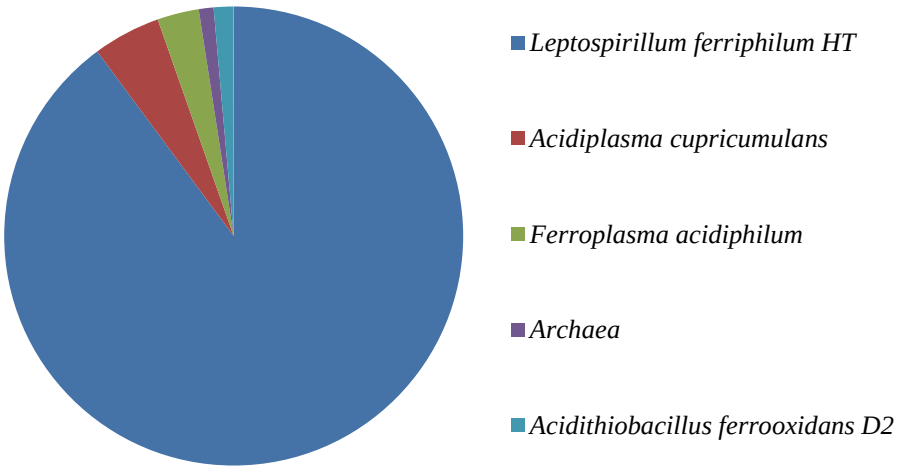


Figure 3-1: Microbial distribution of the stock culture.

3.2 Mineral Analysis

The mineral used for the energy source throughout the experiment was pyrite concentrate provided by BHP Billiton (Randburg, South Africa). The milled concentrate was wet sieved through 75 and 38 µm mesh to obtain a 38-75 µm size fraction. Size analysis of the fraction was performed using a Malvern Particle Size Analyser and 90 % of the sample was less than 53.18 µm. The composition of the pyrite was 41.3 % sulphur and 50.0 % iron. The relative density of the pyrite was 3.49 $kg.dm^{-3}$ (measured). Detailed results of the size analysis are depicted in Appendix B.

3.3 Experimental Media

For the source of micro-nutrients required for microbial growth, Norris medium (Clark and Norris, 1996) was prepared in 5 L batches with the following composition: 0.4 $kg.m^{-3}$ $(NH_4)_2SO_4$, 0.5 $kg.m^{-3}$ $MgSO_4.7H_2O$, 0.2 $kg.m^{-3}$ KH_2PO_4 and 0.1 $kg.m^{-3}$ KCl, supplemented with 150 $mg.L^{-1}$ of yeast extract. The pH was adjusted to pH 2.5 using 98% H_2SO_4. This is detailed in Appendix A. The media was then autoclaved in a Hirayama HG50 autoclave at 121°C for 25 minutes. All reagents were of analytical grade. A fresh batch of newly prepared feed was used for each experiment.

3.4 Equipment

3.4.1 *Stirred Tank Reactor*

Representations of the closed stirred tank reactor (STR) and the experimental set-up used are shown in Figure 3.2 and Figure 3.3. Identical small scale STRs of 100 mm internal diameter and 180 mm height were utilised with 1 litre of experimental solution at a height around 100 mm, leaving 80 mm headspace as specified in Table 3.1. The reactor was fitted with four baffles and agitated with a 57 mm diameter impeller situated at a clearance of 10 mm at a speed of 550 rpm (tip speed of 1.64 $m.s^{-1}$). The lid was fitted with 3 ports for condensation control, the sparger and the sampling point. The STR was jacketed to control the temperature.

Table 3-1: Stirred tank reactor specifications

Parameter	Specification
Total volume	1300 mL
Working volume	1000 mL
Total height	200 mm
Vessel diameter	100 mm
Impeller design	Axial 4 pitched blades up-pumping propeller
Impeller diameter	57 mm
Impeller clearance	10 mm
Operating Conditions	0.7 L of media and 3% Pyrite concentrate

3.4.2 *Experiment Procedure*

A set of four reactors were run at any given time with one reactor for the Control and three for stress experiments. The study was carried out in submerged culture to ensure consistent conditions and to avoid the interacting effects of mineral solubilisation. Acid concentrations of 0.34, 0.51 and 0.68M H_2SO_4 were used to stress the mixed mesophilic culture over time periods of one, three and 24 hours to simulate the acid stress during start-up of the bioleaching operation. The inocula for these Control cultures, like the stressed culture collected from stock reactors, were left outside optimal conditions for the chosen exposure time before being re-introduced into the reactor and subjected to optimal operation conditions. Thereafter, the acid stressed inocula and Control cultures of selected mesophilic bioleaching microorganisms were returned to standard operating conditions of pH 1.4 in fresh Norris media and 3% pyrite concentrate, aerated with compressed air at 2 $L.min^{-1}$ and their performance assessed on cultivation in a batch stirred tank at 35°C until complete pyrite leaching was achieved. The rates of ferrous iron oxidation and microbial growth were investigated as proxy measures of microbial performance. The pH was adjusted to pH 1.4 for all reactors on inoculation using 1.13 mL H_2SO_4 and 5.60, 10.40 and 15.40 mL Na_2CO_3 for the Control and 0.34, 0.51 and 0.68M cultures respectively.

Condensers, connected to a chiller (MRC, Model BL-30) containing an anti-freeze coolant and operated at 2°C, were connected to the open port on the lid of each experimental reactor to allow air escape and condense back to the reactor the hot evaporated liquid solution. With this, daily moisture loss was less than 5% of the total volume in the reactor. This was replaced with de-ionised water before sampling.

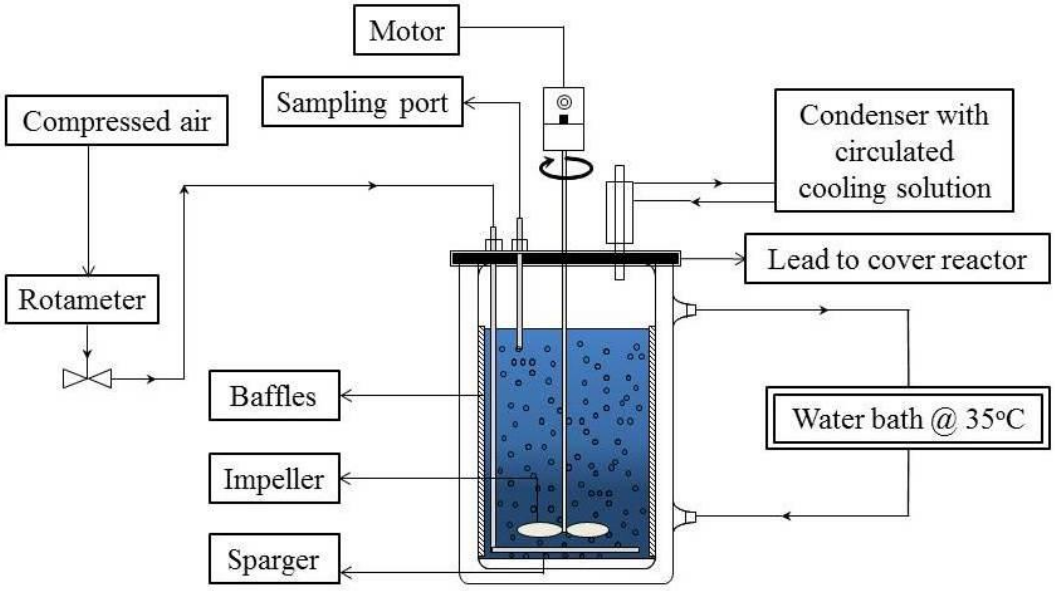
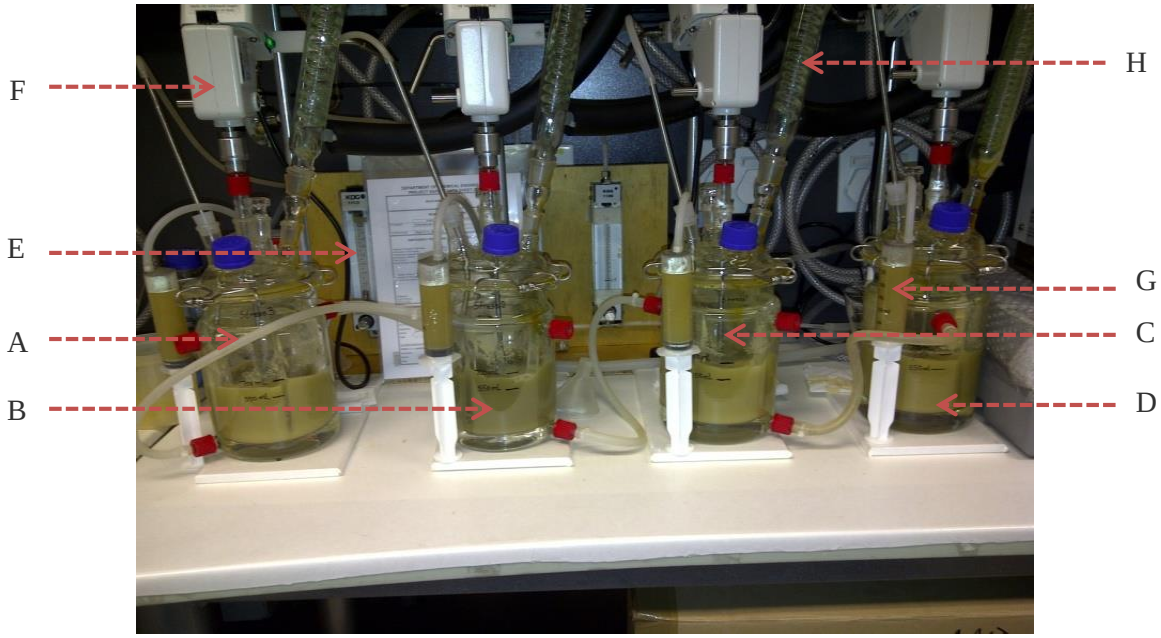


Figure 3-2: Schematic of the stirred tank reactor



KEY:

A – Reactor 1: Control

B – Reactor 2: 0.34M

C – Reactor 3: 0.51M

D – Reactor 4: 0.68M

E – Rotameter

F – Overhead Stirrer

G – Sampling Syringe

H – Condenser

Figure 3-3: Bioleaching stirred tank reactor experimental set-up

3.5 Analysis

Samples were collected daily and measured for mass balancing purposes. The pH was measured using a Metrohm 691 pH meter to ensure that an acidic environment was maintained for efficient biooxidation. The probe was calibrated for the pH range 1 to 4 (error < 1%). Redox potential was measured with reference to a saturated Ag/AgCl electrode using a Pt electrode attached to a calibrated Metrohm 827 Redox meter, and the accuracy of the probe reading was tested against a standard redox buffer solution (potential of 468 mV at 25°C) with an error < 1%. Cell growth was monitored by total cell counts (Section 3.5.1) and total iron and ferrous iron concentration were measured using spectrophotometric iron assays (Section 3.5.2) as detailed in Appendix C. When the experiment was completed, samples of the culture were collected and microbial speciation was performed using qPCR to quantify species present.

3.5.1 *Determination of Bacterial Concentration by Direct Microscopic Counting*

Cell counts were done daily on the collected samples using a Thoma Counting Chamber with well dimensions of 0.02 mm in depth and a 1/400 mm^2 area under an Olympus BX40 Microscope at 1000× magnification (oil phase, phase contrast optics). Direct microscope counting has been successfully employed by many researchers (Konishi *et al.*, 1995; Nemati and Harrison, 2000; Sissing, 2002; Harrison *et al.*, 2003a; Raja, 2005; Ngoma *et al.*, 2014). Although Konishi *et al*. (1995) reported an inherent error of this method to be as high as 11%, the reproducibility was found to be acceptable. Only the planktonic cell concentration was measured and the free cell count was postulated to be indicative of the total cell count in the reactor based on the bioleaching studies by Nemati and Harrison (2000), Nemati *et al*. (2000), Sissing (2002) and Raja (2005).

The collected aliquot was allowed to settle for 2 hours in a 2 mL Eppendorf microfuge tube to remove all residual precipitate particles. A 40 µL aliquot of the settled sample was diluted as required. A 10 µL aliquot was placed into the well of the counting chamber and coverslip placed over it. A direct count was made at a 100× magnification using an Olympus BX40 Microscope. The detection limit of the direct counting method using this counting chamber is 3×10^5 cells.mL^{-1}, with < 25% error. It is optimal to count between 30 and 300 cells to reduce the error. Dilutions of the sample of 1 – 60 × were required using Norris media (pH 1.7). The cell concentration (cells.mL^{-1}) was calculated using Equations 3.1 and 3.2.

$$Volume\ of\ 1\ small\ square\ [mm^3] = depth \times area \qquad \textbf{Equation 3.1}$$

$$Cell\ \ conc\ \ [cells/ml] = \frac{cells\ \ counted \times \frac{total\ \ no\ \ of\ \ big\ \ squares\ \ (16)}{no\ \ of\ \ squares\ \ counted\ \ (4)}}{volume\ \ of\ \ 1\ \ small\ \ square \times total\ \ no\ \ of\ \ small\ \ squares} * \frac{1}{d}$$ **Equation 3.2**

d = dilution ratio

3.5.2 ***Spectrophotometric Iron Assay***

3.5.2.1 *Spectrophotometric Ferrous Iron Assay*

The ferrous iron concentration was quantified spectrophotometrically using the colorimetric 1-10 phenanthroline method described by Komadel and Stucki (1988). The detailed protocol is given in Appendix C. This analysis is based on the formation of a range of red complexes when Fe^{2+} is mixed with 1-10 phenanthroline. Three molecules of 1-10 phenanthroline chelate with one Fe^{2+} ion. The absorbance of the coloured complex is proportional to the Fe^{2+} concentration.

Ammonium acetate buffer solution (2 mL) and 1-10 phenanthroline indicator solution (2 mL) were added to 1 mL of the sample (diluted appropriately). The reaction mixtures were vortexed to ensure good mixing. An orange-red complex formed when ferrous ions were present. The absorbance was read at a wavelength of 510 nm with a Thermo Scientific Helios α UV Visible Spectrophotometer. The absorbance was correlated with the ferrous iron concentration using a standard curve generated between 0 to 50 ppm ferrous iron for the relationship between the two parameters (standard curve and reagent preparation found in Appendix C). The correlation curve breaks down at an absorbance greater than 2.

This method has been tested for possible interference from ferric iron, with a deviation of below 5% from the standard. The exception was for certain samples exceeding the ferric iron concentration of 5000 $mg.L^{-1}$. For such samples dilution was recommended. Additionally, low concentration samples introduced greater relative uncertainty. Due to the conversion of ferrous to ferric iron by both biotic and abiotic reactions, the analysis was conducted immediately after sample collection.

3.5.2.2 *Spectrophotometric Total Iron Assay*

To quantify the total soluble iron concentration, the reduction of Fe^{3+} by hydroxylamine is achieved according to the stoichiometry in Equation 3.3.

$$\mathbf{2NH_2OH + 4Fe^{3+} \rightarrow N_2O + 4Fe^{2+} + 2.5H_2O}$$ **Equation 3.3**

Following this, the total iron can be quantified as Fe^{2+}. After the ferrous absorbance was read, an excess amount of hydroxylamine (as per detailed method in Appendix C) was added to the samples to convert all the iron present to the ferrous form. Following conversion, further absorbance values were

measured and compared to the ferrous standard curve to obtain the total iron concentrations. Ferric iron was determined by difference. For bioleaching samples the ferrous iron is often low (< 500 $mg.L^{-1}$), while the total iron can be very high (> 20 $g.L^{-1}$). In such cases ferrous and total iron should be measured separately, with a pre-dilution necessary for total iron.

3.5.2.3 *Precipitated Iron Acid Digestion*

Quantification of the total solubilised iron concentration (precipitated iron) was achieved by measuring using the colorimetric 1-10 phenanthroline method following acid digestion of the collected sample. 1 mL hydrochloric acid (32 % HCl) was added to 1mL sample and boiled on a hot plate. The digested sample was allowed to cool before 1 mL was mixed to ammonium acetate buffer solution (2 mL) and 1-10 phenanthroline indicator solution (2 mL) and absorbance read on the spectrophotometer.

3.5.3 ***Microbial Speciation Using qPCR***

Prior to exposure to the stress condition, at the end of the exposure to stress and on completion of the cultivation, culture samples were collected and centrifuged for 60 second at 4000 *g* in an Eppendorf® Mini-Spin Plus centrifuge to precipitate all fine particles (minerals, mineral precipitation and any other particles able to interfere with the microorganism recovery). The pellet was discarded and the remaining suspension was recovered and centrifuged again for 5 minutes at 14000 *g* to recover all microorganisms to the second pellet. The pellets formed were recovered by carefully removing the supernatant from the Eppendorf tubes. The recovered pellets were re-suspended in 200 µL lysing buffer (High Pure PCR Template Preparation from ROCHE, Ref 11 796 828 001), vortexed and stored at -80°C for qPCR analysis.

Using the in-house molecular biologist, the composition of the microbial community was assayed using qPCR analysis. This method is highly sensitivity, with a detection limit that theoretically allows the detection of a single DNA molecule (Hallberg and Johnson, 2001; Schippers, 2007) provided the component species or genera are known. Zhang *et al*. (2009) confirmed the reliability of real-time PCR analysis when they analysed the community dynamics of complex consortia containing chemoautotrophic and chemomixotrophic moderate thermophiles in bioleaching systems. Their real-time PCR analysis results were consistent with physiological characteristics of these strains. Similar studies have been reported from the Centre for Bioprocess Engineering (CeBER) at the University of Cape Town (Dew *et al.*, 2011; Tupikina *et al*., 2011; van Hille *et al*., 2010, 2013; Tupikina *et al*., 2013a).

3.5.3.1 *Genomic Deoxyribonucleic Acid (gDNA) Extraction*

The gDNA was extracted from microbial cells using the High Pure PCR Template Preparation Kit™ (Roche, South Africa) as per the manufacturer's guidelines detailed below.

Sample lysis and DNA binding: Proteinase k (50 µL) and Binding buffer (250 µL) were mixed with the cell suspension by vortexing. The mixture was incubated at 70°C for 5 minutes. Isopropanol (125 µL) was added and the mixture centrifuged at 14000 *g* for 60 seconds to settle the fines. The sample was pipetted into the upper reservoir of a High Filter Tube inserted into a Collection Tube, and centrifuged at 8000 *g* for 60 seconds.

Washing and elution: The Filter Tube was transferred into another Collection Tube and 500 µL Inhibitor-removal buffer added into the upper reservoir of the Filter Tube, and centrifuged at 8000 *g* for 60 seconds. The Filter Tube was further transferred into another Collection Tube and 500 µL wash buffer added into the upper reservoir of the Filter Tube, and centrifuged at 8000 *g* for 60 seconds. The washing step was repeated once. The Filter Tube was transferred into another Collection Tube and centrifuged at 14000 *g* for 30 seconds to remove the residual Wash buffer. The Filter Tube was transferred into a 1.5 mL micro-centrifuge tube, 75 µL Elution buffer added into the upper reservoir of the Filter Tube, and incubated at 70°C for 30 minutes. The tube assembly was centrifuged at 14000 *g* for 60 seconds to elute DNA into the micro-centrifuge tube. Again, 75 µL Elution buffer was added into the Filter Tube, the tube assembly incubated at 70°C for 30 minutes and centrifuged at 14000 *g* for 60 seconds. The micro-centrifuge tube now contained the extracted gDNA and the Filter Tube was discarded. The gDNA concentration was measured by absorbance at 260 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA). The gDNA was analysed using qPCR immediately or stored at -20°C to be analysed later.

3.5.3.2 *qPCR Analysis*

The microbial composition of the samples was determined by analysing the extracted gDNA using qPCR according to the CeBER protocol (Tupikina *et al*., 2013a). The samples, where applicable, were diluted to 10 ng × μL^{-1} for qPCR. A 5-point serial dilution standard curve of the plasmid DNA containing about 600 base pairs of the 16S rRNA gene sequence of the target microbial species ligated into pGEM-T Easy (Promega) was prepared. The dilution series was prepared in triplicate to span 5 orders of magnitude (2.5×10^7 to 2.5×10^3 copies.μL^{-1}) per quantification. Ten nanograms (1 µL of a 10 ng × μL^{-1} dilution) of gDNA used as a template for each sample and a no-template-control were analysed in triplicate using the Rotor-Gene 6000 qPCR machine with version 1.7 software (Corbett Research). The cycling conditions for the reaction were: 1 × (95°C, 6 min), 40 × (95°C, 10 s; 60°C, 15 s; 72°C, 20 s) and a melt curve analysis from 72°C to 95°C in 0.2 °C increments using 14

µL SYBR®. Fast. Mastermix. (KAPA Biosystems), with universal and species-specific primers shown in Table 3.2.

Table 3-2: PCR primers used to assay microbial composition in the inocula and cultivated stressed and un-stressed cultures (Tupikina *et al.*, 2013a)

Primer title	Microbial group/ species	Sequence (5' – 3')
Universal primers		
UniBactF335	Universal bacteria	GAC TCC TAC GGG AGG CAG CA
UniBactR937	Universal bacteria	TTG TGC GGG CCC CCG TCA AT
UniArchF343	Universal archaea	ACG GGG IGC AIC AGG CG
UniArchR932	Universal archaea	TGC TCC CCC GCC AAT TCC
Archaeal primers		
Ferro	*F. acidiphilum*	GAA GCT TAA CTC CAG AAA GTC TG
JTC3	*A. cupricumulans*	AAG CCT AAC TTC AGA AGG CCT G
Bacterial primers		
ATT	*At. thiooxidans.*	GGG TGC TAA TAN CGC CTG CT
At.c	*At. caldus*	CGG ATC CGA ATA CGG TCT G
At.f	*At. ferrooxidans*	AGG TGG GTT CTA ATA CAA TCT GCT
At.f D2	*At. ferrooxidans strain D2*	CGG GTC CTA ATA CGA TCT GCT
L.ferri LH	*L. ferriphilum strain LH*	GGG GGC CTG AAT AAG GTC A
SG2/STO	*S. thermosulfidooxidans*	ACG AAG ACC GGC CCG GAA GG
SDO	*S. disulfidooxidans*	GAG AAT GCC TTG GAA ACT GCA A

3.6 Sample Collection and Data Handling

Samples were collected daily using a clean syringe. Collected samples were adjusted to room temperature prior to pH and redox potential analysis. Collected samples were also allowed a two hour particle settling time before microbial counting. All iron assays were done immediately upon sample collection.

The total iron concentration released, accounting for iron in solution as well as precipitated iron was spectrophometrically measured following acid digestion using HCl as detailed in Section 3.5.2.3. The collected samples were allowed to settle for a period of time, and the method has been approved by in-house analytical laboratory.

The percentage of iron that was oxidised in solution was calculated according to Equation 3.4 accounting for the iron carried over with the inoculum.

$$\%\ \ Fe\ \ oxidised\ \ [\%] = \frac{Fe\ \ concentration\ \ [g/l].\text{Re}actor\ \ volume\ \ [l]}{Total\ \ mass\ \ Fe\ \ in\ \ Pyrite} \times 100\% \qquad \textbf{Equation 3.4}$$

The rate at which the iron was oxidised, r_{Fe}, is calculated from the concentration of the oxidised iron in solution, $[Fe^{3+}]$, as a function of time, t.

$$r_{Fe} = \frac{d[Fe^{3+}]}{dt} = \Delta[Fe^{3+}] \qquad \textbf{Equation 3.5}$$

The rate at which the bacterial concentration increased, r_x, is a first order function of the available microbial cell number, X, at a particular time, t.

$$r_x = \frac{dX}{dt} = \mu X \qquad \textbf{Equation 3.6}$$

The specific growth rate, μ, was calculated by plotting the natural logarithm of the concentration, X, as a function of time and taking the slope or from the division of the linear growth rate dX/dt by the average cell concentration.

The yield was calculated across the time interval of the experiment from inoculation until the Control culture reached maximum cell concentration as demonstrated in Figure 3.4.

$$Yield = \frac{X\,(cells.L^{-1})}{Fe^{tot}(Kg.L^{-1})} \qquad \textbf{Equation 3.7}$$

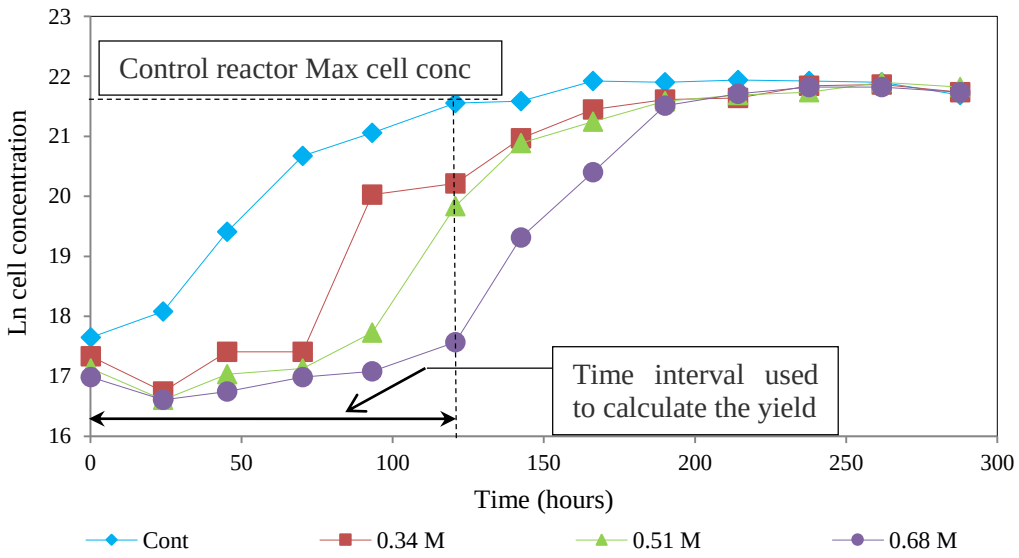


Figure 3-4: Time intervals used to calculated the Yield in terms of microbial cells produced per kg iron oxidised for the Control and stressed cultures.

The maximum leach rates were estimated assuming a linear relationship between iron concentration and time over the steepest gradient, given a correlation coefficient greater than 0.95. Initial iron dissolution concentrations were determined over the lag phase of microbial growth and estimated using similar linear trends for the change in iron concentration with time (R values). The intersection of this baseline curve and the maximum leach rate curve was used to estimate the lag period preceding the microbial ferrous iron oxidation, as demonstrated in Figure 3.5.

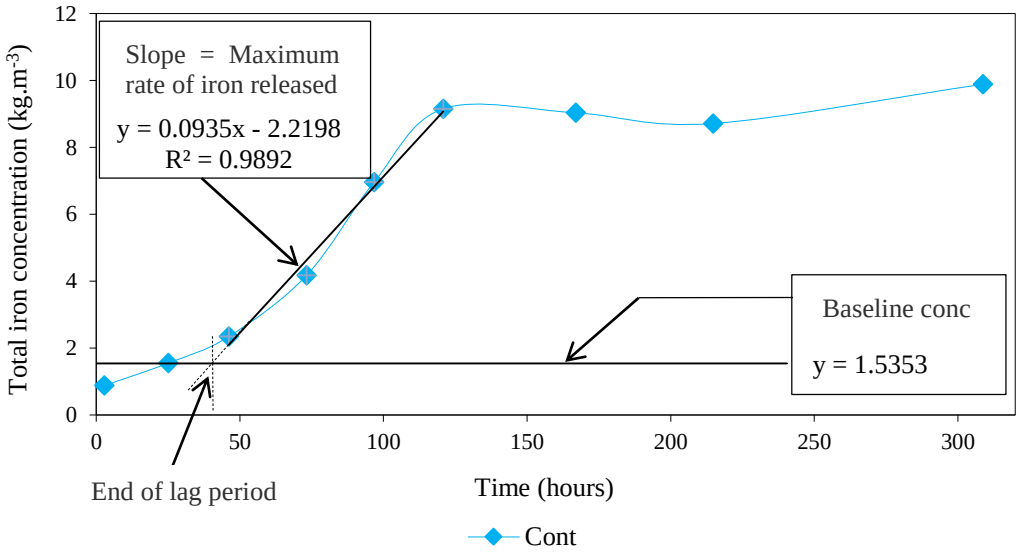


Figure 3-5: Maximum leach rate calculated from the Control total iron released in solution as a function of time – baseline generated to estimate the lag period of the microbial ferrous iron kinetics.

The maximum specific growth rates were estimated from the steepest linear gradient of the natural logarithm of cell concentration as a function of time, given correlation coefficient is greater than 0.95. Growth rates (dX/dt) were estimated using linear trends for the change in cell concentration with time (R values). The intersection of the baseline curve (cell concentration on inoculation and prior to growth) and the specific growth rate curve was used to estimate the lag period for the microbial growth curve as demonstrated in Figure 3.6.

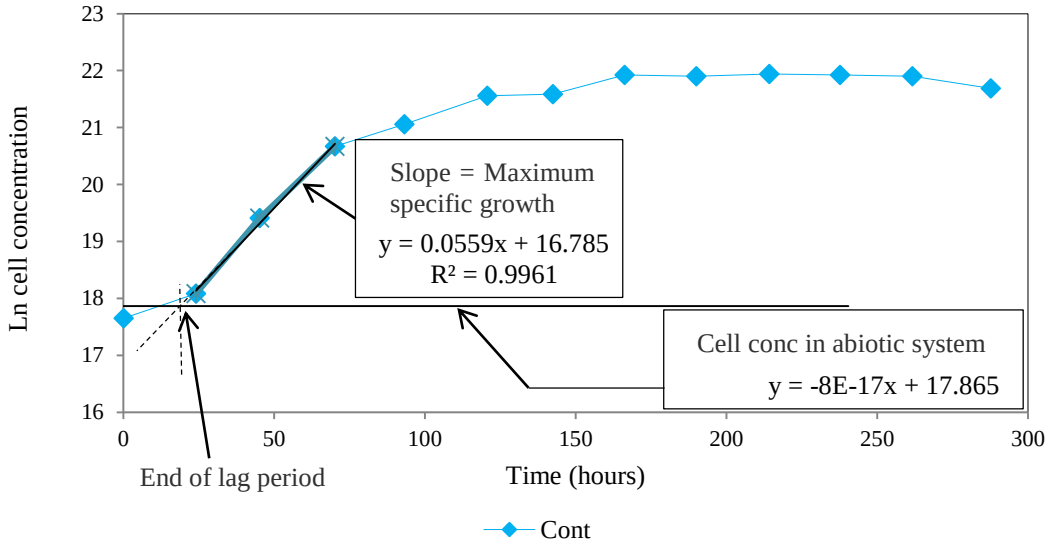


Figure 3-6: Specific growth rate calculated from the natural logarithm cell concentration as a function of time – baseline generated to estimate the lag period of the microbial growth

3.7 Research Strategy

The experiment presented herein was formulated to provide comparison of the effect of acid stress on the microbial activities and consortia. This was achieved by monitoring the growth and ferrous oxidation rate through the change of the microbial density, the change in ferrous and ferric concentrations and the microbial speciation, following exposure to this stress.

Three acid concentrations (0.34M, 0.51M and 0.68M H_2SO_4) were selected as stress factors. These include concentrations typical in acid agglomeration of low grade ore. The acid was mixed with 150 mL of the mixed mesophilic culture, stirred briefly and left static at room temperature for 1, 3 and 24 hours before the suspension was inoculated to 550 mL of Norris media containing 21 g (3%) of pyrite concentrate as specified in Table 3-3. Before aeration was commenced, the pH values of the inoculated reactors were adjusted to pH 1.40 using concentrated H_2SO_4 and 4 M Na_2CO_3 for the Control and stressed cultures respectively.

The stress response experiments were conducted in 1 L stirred tank reactors. The initial agitation rate of 350 rpm was ramped up to 550 rpm after 24 hours. This was a pre-caution to allow the unstressed cultures time to be metabolically active before exposing to the stress imposed by solids suspension; however, it is well accepted that the microorganisms are not impacted by the solids suspension under these conditions at a solids loading of 3% (Nemati *et al*., 2000; Sissing and Harrison, 2003). The agitation rate was kept at a constant speed for the rest of the experiment. Aeration was achieved with

compressed air delivered through a sparger fitted with 1 mm holes at an air flow rate of 2 $L.h^{-1}$ for the duration of the experiment.

Table 3-3: Experiments conducted in sets of four, varying time exposure and the volume required to adjust the initial pH

Test conditions				Volume of pH adjusting agent upon addition to reactor post stress to adjust pH to pH 1.4
Acid concentration [M]		**Exposure time [h]**		**[mL]**
Control	0	0	0	1.13 mL (H_2SO_4 conc)
0.34	1	3	24	5.60 mL (4M Na_2CO_3)
0.51	1	3	24	10.40 mL (4M Na_2CO_3)
0.68	1	3	24	15.40 mL (4M Na_2CO_3)

To determine the reproducibility of the experiment, a set of four reactors were inoculated with the same inocula but with three of them stressed with a 0.51M H_2SO_4 concentration in the 150 mL inocula for 3 hours and the fourth kept as the Control as described in Table 3.4. These were then re-suspended under optimal conditions and assessed for microbial growth and leaching performance. Reproducibility analyses were done on the intra-experiment reproducibility using this set of experiments and on the inter-experiment reproducibility using the Control experiment of all runs conducted throughout the research.

Table 3-4: Experiments conducted for reproducibility purposes and the volume required to adjust the initial pH

Test conditions		Volume of pH adjusting agent upon addition to reactor post stress
Acid concentration [M]	**Exposure time [h]**	**[mL]**
Control	0	1.13 mL (H_2SO_4 conc)
0.51	3	10.40 mL (4M Na_2CO_3)
0.51	3	10.40 mL (4M Na_2CO_3)
0.51	3	10.40 mL (4M Na_2CO_3)

The following assumptions and limitations were taken into consideration during the course of the experiments:

- Microorganisms used to inoculate the system were in an active growth state prior to transfer.
- Effects of shear forces and hydrodynamic conditions were constant throughout the experiments.
- Negligible cell loss through attachment to reactor walls occurred.
- Negligible temperature gradient between different sets of experiments occurred.
- The ferrous iron and total iron concentrations were attained using the spectrophotometric phenanthroline assay. The ferric iron concentration was determined by difference from $[Fe^{tot}] - [Fe^{2+}] = [Fe^{3+}]$.
- The acid stress conditions selected were selected to simulate the stress to which the microbial cultures are subjected during acid agglomeration. However, it is recognised that a variety of conditions are used, especially in terms of duration prior to commencement of irrigation and the added stress of increasing ionic strength and metal concentrations associated with acid leaching of the gangue and valued minerals.

The analysis presented in this study is based on the extrinsic factors influencing the leaching performance as presented in the methodology. While the methodology is extended to consider the sensitivity of particular species to acid stress, no attempt has been made to consider stress response at the signalling or molecular level.

The experimental work was structured such that the hypotheses presented in Section 2.6 were interrogated. The schematic in Figure 3.7 outlines how each of the key questions coincides with an aspect of the experimental data attained.

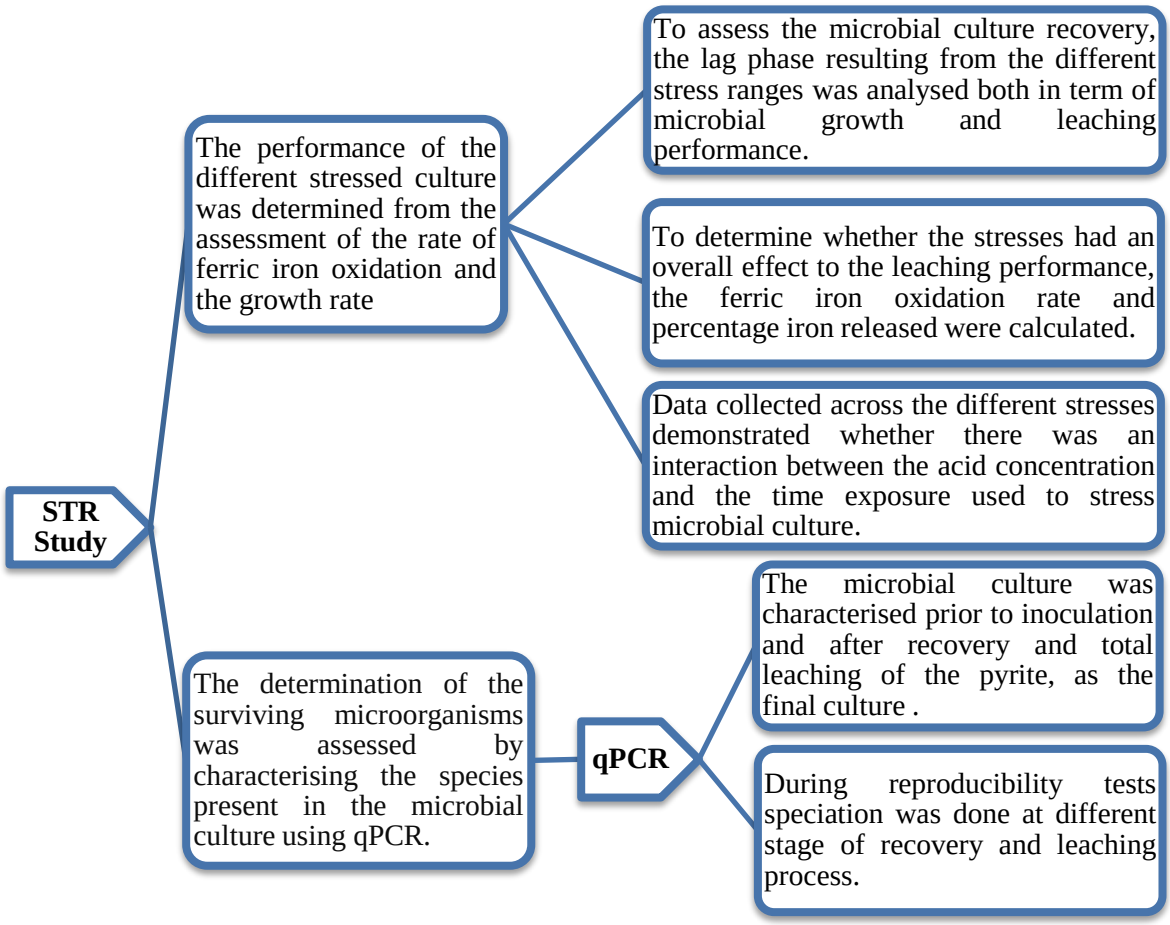


Figure 3-7: Outline of the research approach.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

The majority of operations for the heap leaching of low grade crushed mineral sulphide ores have carried out their agglomeration by mixing the ore with concentrated sulphuric acid and water. The pre-treatment of these mineral ores of low grade for bioheap leaching purposes using acidified water has proven important for preparation of microbial attachment to the mineral ore and for combating the acid neutralising capacity of the gangue material. Kodali *et al.* (2011) reported that effective agglomeration for heap leaching operations offers numerous benefits such as better heap structure by minimizing channelling and improving permeability, increasing metal recovery from low grade ores and improving availability of reactants. The poor percolation may be caused by fine particles deposit accumulated and channelled through the spaces between the coarse ore as described in Figure 2.4. Dhawan *et al*. (2012) reported that agglomeration improves the uniform percolation of solution through the heap of ore because the fines stick to the agglomerate, causing a more regular agglomerate size distribution.

Various agglomeration procedures have been tested with the use of binders to improve the stability of the agglomerate has also been reported (McClelland, 1988; Bouffard, 2005). It is desirable that the reagent used as a binder does not affect the leach chemistry and the subsequent process for metal recovery. Heap leaching of copper from low grade mineral sulphides has proven to be economically viable using sulphuric acid without an additional binder and the subsequent metal recovery also occurs in acid solution. Hence very few, if any, copper heap leaching operations add any binder to the sulphuric acid solution, owing to the binder cost, large consumption and curing issues, and limited selection of acid tolerant and microbial resistant binders (McClelland, 1988; Bouffard, 2005). Therefore acid agglomeration remains the only widely used agglomeration method in copper heap bioleaching.

The nature of the ore usually defines the acidity needed in the agglomeration process as it needs to balance the acid neutralising capacity of the gangue material. It is necessary to quantify the amount of acidity that could be used in the agglomeration process without compromising the efficiency of the microbial activity. Ensuring efficient inoculation with the rapid onset of metabolic activity will shorten the start-up phase and thereby the leaching operation and improve the economic viability of the process. The aim of this study is to determine the effect of acid concentration and time exposure on selected mixed mesophilic cultures implicated in mineral bioleaching in terms of their recovery time, their metabolic activity (impacting regeneration of leach agent), growth rate and ability to survive.

4.2 Experimental Reproducibility

4.2.1 *Intra-Experiment Reproducibility*

Both intra-experiment and inter-experiment reproducibility was analysed to provide a framework for appropriate data analysis across this study, enabling the testing of the hypothesis. For the intra-experiment reproducibility, three cultures were assessed using the same inoculum, stressed with the same concentration of sulphuric acid, exposed for the same time period, re-suspended under identical optimal conditions and run for 360 h. In this experiment 450 mL of a mesophilic culture containing 4 × 10^9 cells.mL^{-1} was split into three 150 mL inocula and stressed with 0.51M sulphuric acid for three hours before re-suspension in Norris media (Section 3.3) containing 3% m.v^{-1} (21 g) pyrite concentrate (Section 3.2). Before air was bubbled into the reactors, the pH was adjusted to pH 1.40 using 10.40 mL of Na_2CO_3. The reactors were monitored for pH, redox potential, [Fe^{2+}], [Fe^{tot}] and microbial cell count.

Averages of the three data sets and their standard deviation were calculated. The pH and redox potential as function of time is reported in Figure 4.1. Upon aeration, the pH was reduced from 1.40 to 1.30. The pH drop to 1.30 is attributed to the acidification of the medium due to the release of sulphur present in the pyrite after attack by the ferric iron present in the inoculum. The average pH gradually dropped to pH 1.00 after 261 h and to pH 0.98 after 360 h of leaching, displaying a classic pH leaching profile defined by a sharp drop in pH representing acidification of the medium due to the release of the sulphur present in the mineral in the presence of oxygen and water forming sulphuric acid. The standard deviation was ± 0.02 and the reproducibility of the pH was demonstrated during the intra-experiment reproducibility.

A similar trend in pH was observed across the three reactors with a mean value varying by 0.02 between reactors. At time 119 h the deviation was ± 0.021 and Reactor 1 and Reactor 3 had pH values of pH 1.27 and pH 1.28 with Reactor 2 at pH 1.24. Acidification occurred earlier in Reactor 2 as the onset of microbial activity in this reactor occurred earlier than Reactor 1 and Reactor 3. The microbial count for this reactor at time 119 h was 0.90 × 10^9 cells.mL^{-1} compared with 0.68 × 10^9 cells.mL^{-1} and 0.70 × 10^9 cells.mL^{-1} for Reactor 1 and Reactor 3 respectively. The pH observation also showed that in Reactor 3 the acidification was delayed compared to Reactor 1 and Reactor 2. At time 214 h its pH was at pH 1.10 when Reactor 1 and Reactor 2 were respectively at pH 1.06 and pH 1.07.

The redox potential provides a measure of the ferric to ferrous iron ratio according to the Nernst equation.

$$E' + E = \frac{RT}{nF}\ln(\frac{[Fe^{3+}]}{[Fe^{2+}]}) \qquad \text{Equation 4.1}$$

E= Redox potential $[mV]$

E'= Standard potential $[mV]$

R = Universal gas constant (8.134J mol^{-1} K^{-1})

T = Absolute temperature $[K]$

F = Faraday's constant (96485 C mol^{-1})

n = Number of electrons exchanged in reaction

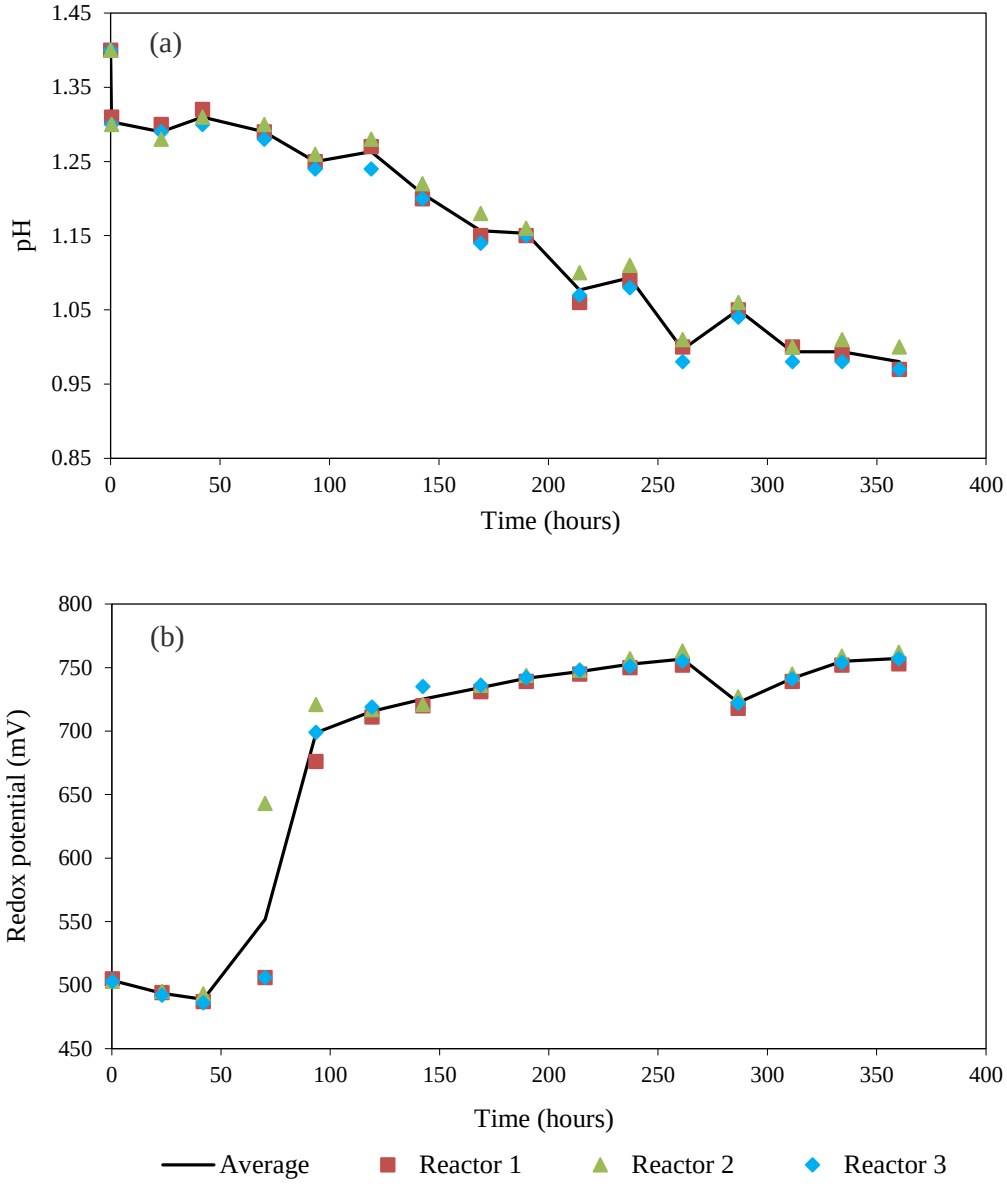


Figure 4-1: Trend in pH (a) and redox potential (b) during the leaching experiment following acid stress at 0.51M H_2SO_4 for 3 h, with triplicate experiments (shaded symbols) and the average pH and redox potential values (straight line) to demonstrate intra-experiment reproducibility.

A lower redox potential suggests a lower ferric iron concentration as compared to the ferrous iron concentration. The bioleaching mechanism indicates that the ferric iron attacks the mineral sulphide releasing the ferrous iron in solution, while the microorganisms convert the released ferrous iron to ferric iron. The relative error in redox potential was 0.77 across most of the profile. A high relative error of 14.3 % was, however, recorded at 70 h, as the lag period ended. At the beginning of the exponential phase, the average redox potential of 489 mV increased to 506 mV at 70 h for the first two reactors and to a high of 543 mV for the third reactor. The ferric to ferrous iron ratio during that time was recorded to be 1/0.37 for Reactor 1 and Reactor 2 and 1/0.007 for Reactor 3. Thereafter the redox potential across the three reactors increased to the same value and maintained this value of 741 mV varied by 4.08 mV. This indicated that the onset of microbial activity was slightly earlier in Reactor 3.

Cell counts were determined by direct microscopic counting using a Thoma counting chamber as specified in Section 3.5.1. The reproducibility results of the microbial cell count in term of the average across the three reactors and the resulting standard deviation are given as a function of time in Figure 4.2. The relative errors between samples over the duration of the experiment varied between 7 and 26 %, which are within acceptable limits given that cell quantification of bioleaching bacteria via direct microscopic counting has a relative error of 10% at cell concentrations greater than 1×10^9 cells.mL^{-1} (Bryan et al., unpublished data). The microbial lag time required for stress recovery was recorded to have lasted for just over 93.5 h. The average microbial growth across the three reactors was 0.0109 h^{-1} with a relative error of 2.6 %.

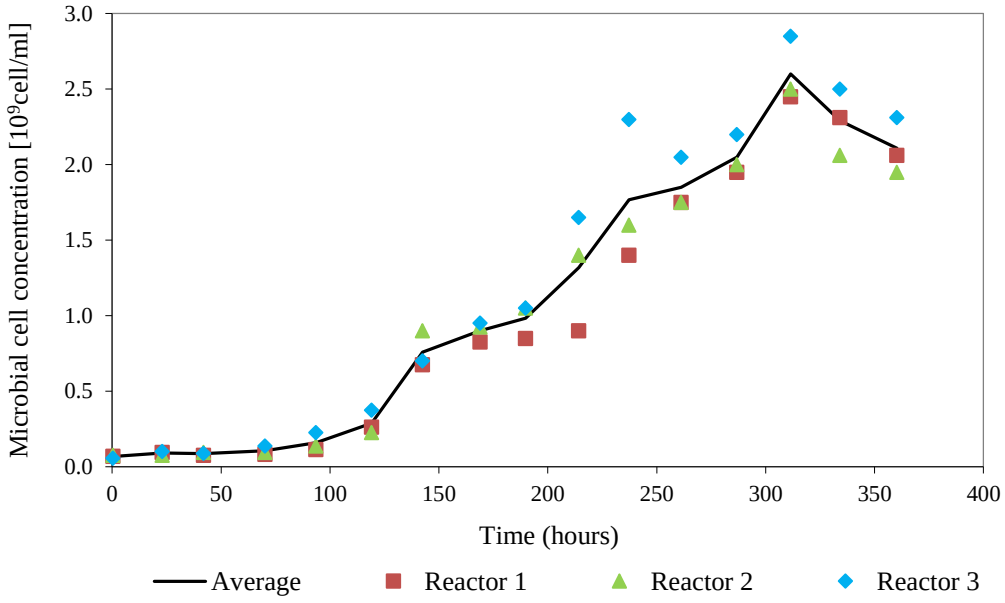


Figure 4-2: Trend in microbial cell counts during the leaching experiment following acid stress at 0.51M H_2SO_4 for 3 h, with triplicate experiments (shaded symbols) and the average cell concentration values (straight line) to demonstrate intra-experiment reproducibility.

The concentrate used in the experiments contained 96% FeS_2 and 4% other minerals. The iron released in solution was calculated from the total iron concentration measured in the collected sample and subtracted from the total initial iron in the pyrite loaded (Equation 3.4). Figure 4.3 shows the reproducibility results of the % Fe extraction as a function of time. The initial increase in percentage iron release was observed within the 0.40 h, owing to the initial chemical leaching of pyrite by ferric iron present in the inocula. The iron release subsequently lagged for a period of nearly 119 h before gradually increasing, depicting the recovery of stressed microorganisms, the oxidation of ferrous iron and the subsequent ferric leaching of the pyrite. The average leaching rate across the three reactors was calculated to be 0.028 kg iron $m^{-3}h^{-1}$ with a standard deviation of ± 0.002.

The calculated percentage iron released (both in solution and precipitated) showed consistency in the initial phases of leaching across the three reactors with a mean and standard deviation of 27.3 ± 2.1 % until 286 h was. This deviation increased at 300 h owing to an increased deviation in one of the three experiments. At 311 h the released iron for Reactor 2 was 95 % and only 60 % and 58 % for Reactor 1 and Reactor 3 respectively. This trend continued for an additional 21 h and the calculated iron extraction during that period was 59.7 ± 16.2 %. It was then reduced when Reactor 1 and Reactor 3 caught-up with Reactor 2 at 360 h. At this time, their respective iron release percentages were 88 %, 95 % and 85 % for Reactor 1, Reactor 2 and Reactor 3 with a relative error of 5.7 %.

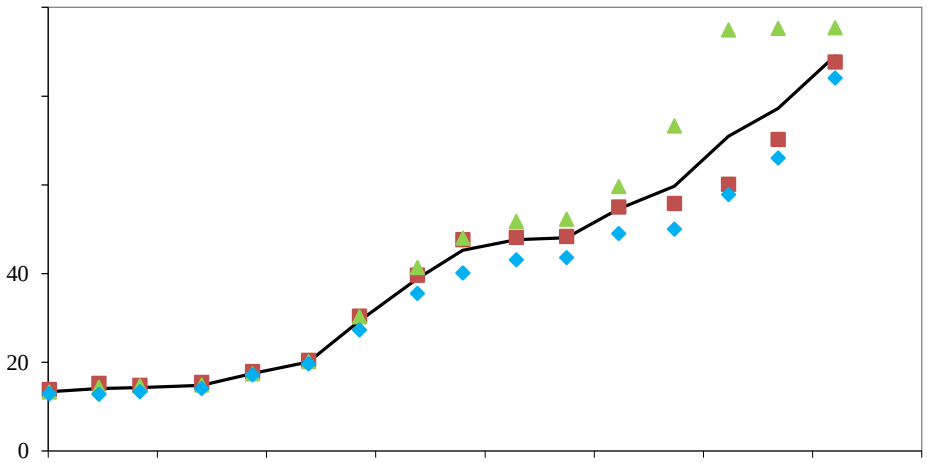


Figure 4-3: Trend of the percentage iron released (in solution and precipitated) during the leaching experiment following acid stress at 0.51M H_2SO_4 for three hours, with triplicate experiments (shaded symbols) and the average values (straight line) to demonstrate intra-experiment reproducibility.

The average relative error calculated for the redox potential was 2.5 % with a maximum of 12 % recorded at time 45.25 h, as microbial activity and leaching were initiated following the lag period. The lag period was recorded to be less than 40 h post inoculation for all three Control cultures. Following the lag phase, the increase in microbial concentration was consistent over 80 h with a relative error between 4 % and 15 %. The Control 3 culture took longer to achieve the highest microbial count, resulting in a relative error as high as 30 % at 142 h. In Figure 4.5, the microbial cell count across the three Control reactors, and its reproducibility, is shown as a function of time.

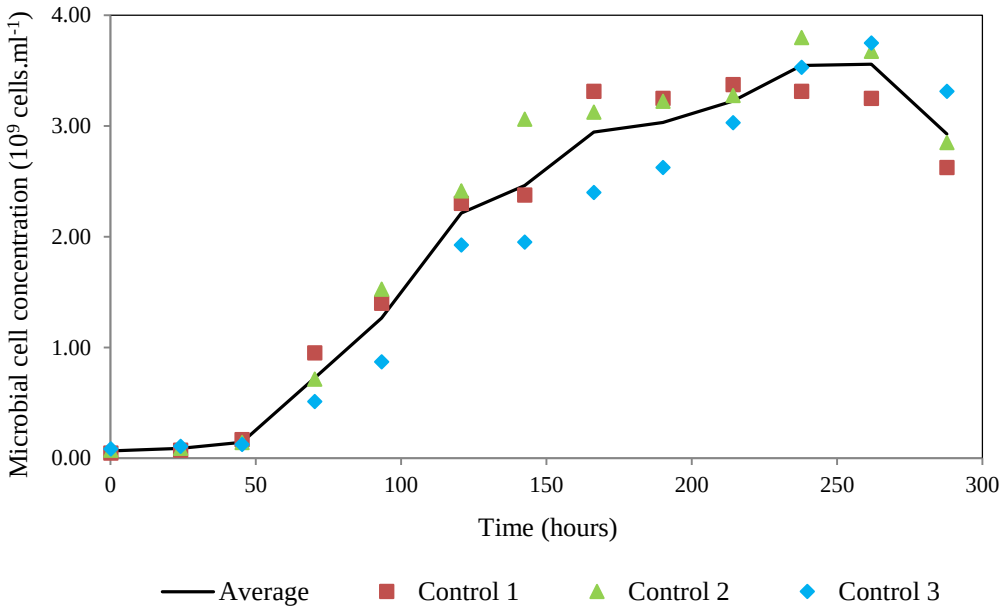


Figure 4-5: Trend in microbial cell counts during the leaching experiment of the Control cultures of the one hour, three hours and 24 hours exposure time, with triplicate Control experiments (shaded symbols) and the average values (straight line) to demonstrate inter-experiment reproducibility.

Figure 4.6 shows the % Fe extraction as a function of time across the three Control experiments. The initial increase in percentage iron release, observed within the 0.17 hour owing to the initial chemical leaching of pyrite by ferric iron present in the inocula, corresponded to a 15 % iron release. After the lag phase in microbial activity of 40 h, the iron release increased rapidly to over 70 % within a period of 141 h, and then gradually increased to 97 % at the final 287 h. The standard deviation calculated was in the range of ± 5.17 to ± 11.44. The average leaching rate across the three reactors was calculated to be 0.044 kg iron $m^{-3}h^{-1}$ with a standard deviation of ± 0.01 kg $m^{-3}h^{-1}$.

The percentage iron released (both in solution and precipitated) showed gradual increase through the leaching experiment. The standard deviation increased to a high ± 20.6 at time 93 h when the Control 1 culture was at 71 % iron release with the Control 2 and Control 3 trailing at an iron release of 35 %. It was then reduced to ± 19.5 at time 120 h, then at below ± 10.1 at time 190 h. This trend was carried

over reducing the deviation to a low ± 3.83 at time 237 h. This reduction in deviation indicated that Control 2 and Control 3 eventually caught-up with Control 1 in the later stage of the experiment. Their respective final iron release percentages at completion of the experiment were 99 %, 97 % and 87 % for Control 1, Control 2 and Control 3.

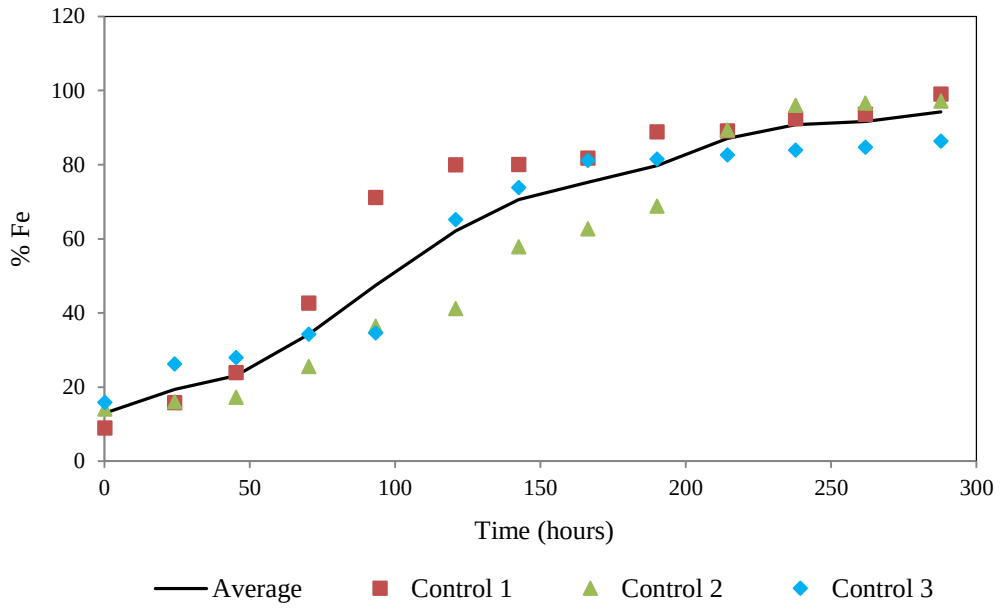


Figure 4-6: Trend in percentage iron released during the leaching experiment of the Control cultures of the one hour, three hours and 24 hours exposure time with triplicate Control experiments (shaded symbols) and the average values (straight line) to demonstrate inter-experiment reproducibility.

4.3 Results: Effect of acid stress on the bioleaching performance

4.3.1 *One hour exposure to acid stresses*

In these experiments, the inocula were subjected to acid stress using H_2SO_4 at concentrations of 0.34M, 0.51M and 0.68M for one hour before re-suspension into optimal conditions as described in Section 3.4. The Control culture, also left static with the stressed cultures, was inoculated with unstressed inoculum for comparison to assess the effect of the stress to which the microorganisms were subjected. The results of the bioleaching performance are presented in Figure 4.7 through Figure 4.10, while the detailed data are presented in Appendix F. The comparison of the microbial cell number in suspension, pH, redox potential and variations in Fe extraction are reported.

Upon inoculation, pH was adjusted to pH 1.40 to ensure that growth occurred under the same conditions such that only the acid stress effect on the leaching performance was observed. The pH profiles are presented in Figure 4.7. A rapid drop in the Control pH was observed when the leaching reaction started, reaching pH 1.00 within 120 h, then stabilised for the remaining 167 h of the experiment. An increase in time of the onset of the pH drop, shown as a shift of the profiles to the right, was observed as the acid stress concentration was intensified. The cultures stressed with 0.34M and 0.51M acid remained around pH 1.30 for 97 h before decreasing to pH 1.02 after 215 h. Following stress at 0.68M acid, the pH decrease was delayed till 145 h, reaching pH 1.04 after 241 h. The effect of the acid stress was clearly observed in the pH values depicting a delay in the decrease in pH with increasing stress, owing to delayed formation of H_2SO_4.

The profiles of redox potential as a function of time for the Control and acid stressed cultures are given in Figure 4.8. The redox potential in the Control reactor, with negligible lag, displayed an increase from an initial 540 mV to a maximum of 782 mV within 121 h, indicating that the microorganisms were active and efficient in converting the ferrous iron to ferric iron. For the stressed cultures, an initial decrease in redox potential was observed in the first 46 h due to the ferric iron leaching of the mineral with its conversion to ferrous iron before the microorganisms recovered from the acid stress to regenerate the ferric iron. The microbial ferrous conversion for the 0.34M and 0.51M acid stressed cultures only commenced after 46 h. The redox potential increased to 780 mV and 770 mV for the 0.34M and 0.51M acid stressed cultures respectively at 167 h. The redox potential following stress at 0.68M H_2SO_4 remained low for 97 h before ferrous iron oxidation commenced, indicating an extended recovery time. The redox potential reached 771 mV after 215 h. The results showed that the stress resulted in an extended lag period prior to the microorganisms efficiently oxidising the ferrous iron present following recovery.

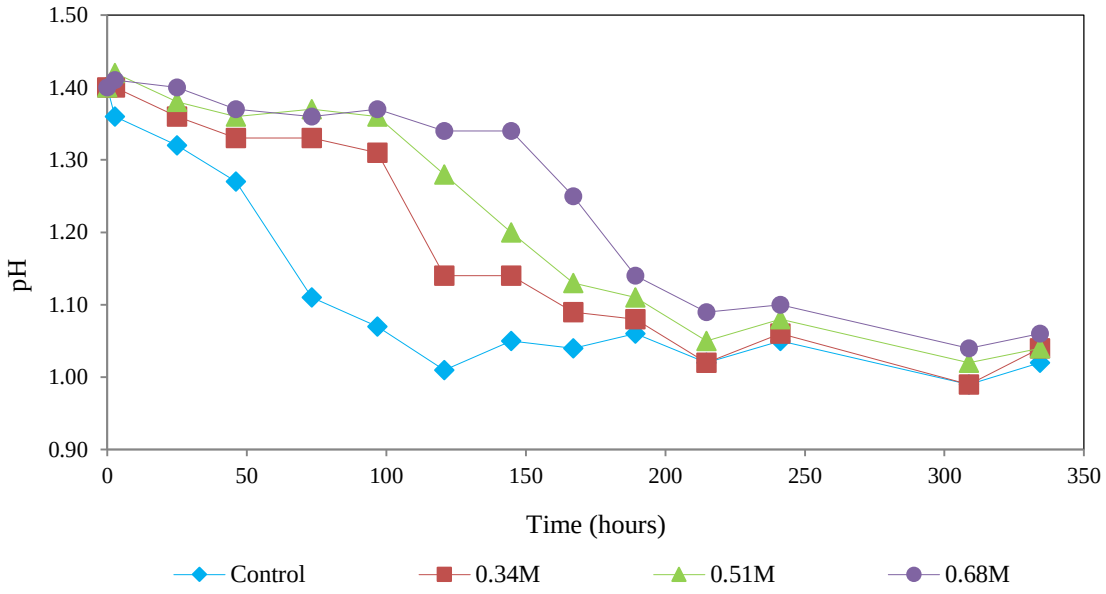


Figure 4-7: pH profiles of the stressed cultures following exposure to acid stress for one hour. Performance was observed in a 3% pyrite slurry as a function of time.

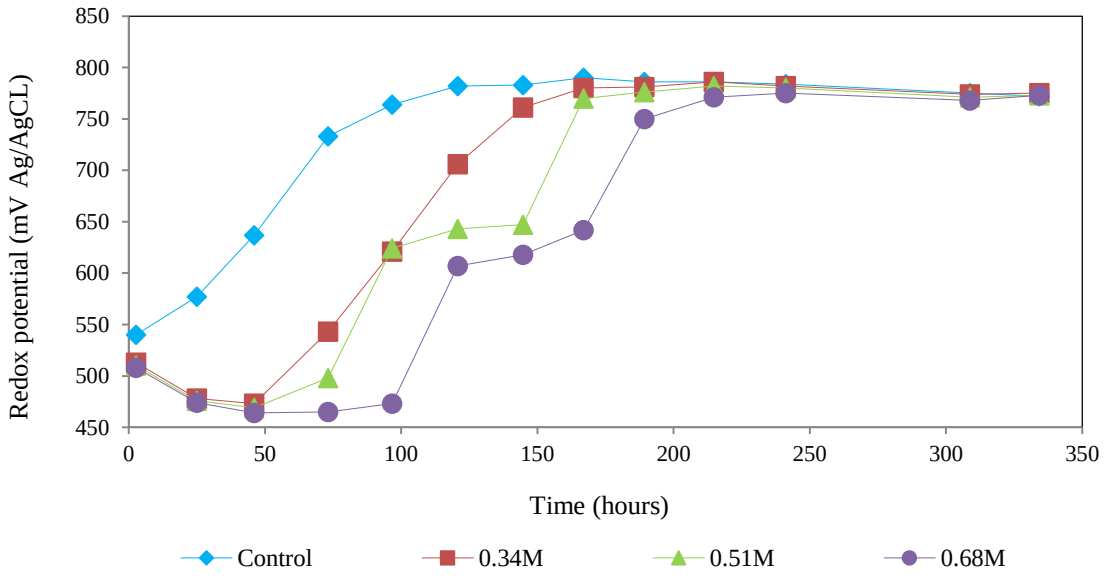


Figure 4-8: Redox potential profiles of the acid stressed cultures following one hour exposure. Performance was observed in a 3% pyrite slurry as a function of time.

The total iron leached as a function of time for the one hour acid stressed cultures are given in Figure 4.9. The maximum linear leach rates and corresponding correlation coefficients, R^2, are given in Table 4.1. The extent of pyrite solubilisation, calculated from the total iron released (iron in solution and precipitate) and the iron content in the mineral concentrate, is also presented in Table 4.1, with the time required to achieve these values.

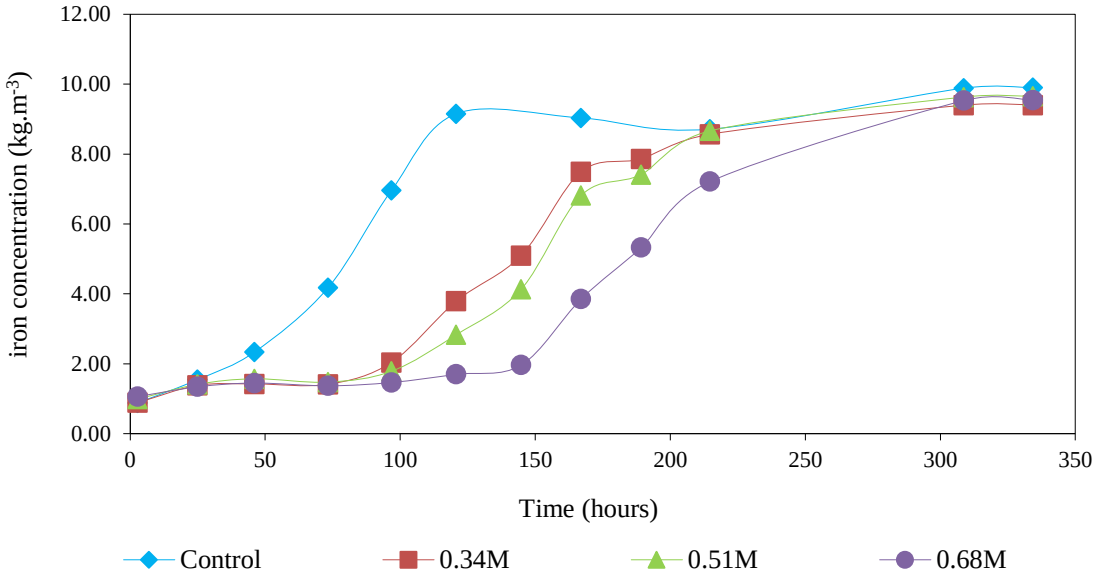


Figure 4-9: Trend of the total iron released (in solution and precipitate) as a function of time and acid stress following one hour exposure time.

The Control culture exhibited a bioleaching rate (Fig 4.9) more or less similar to that of the stressed cultures but displayed a higher extent of solubilisation. A calculated leaching rate of 0.093 kg Fe $m^{-3}h^{-1}$ (R^2= 0.99) and an extent of pyrite solubilisation of 94% was achieved within 120 h. The bioleaching performance of the 0.34M, 0.51M and 0.68M acid stressed cultures was reduced and the required time to achieve highest extent of solubilisation increased. The lag phase before the onset of iron solubilisation observed in the stressed cultures displayed an increase in lag time. An extended lag time was needed for the recovery of the stressed culture before a microbial oxidation and mineral leaching could be initiated. The 0.34M, 0.51M and 0.68M stressed cultures required 93 h, 105 h and 139 h respectively to initiate the microbial ferrous iron oxidation. The leaching rate of the 0.34M acid stressed culture was 0.075 kg Fe $m^{-3}h^{-1}$ (R^2= 0.98) with 88% pyrite solubilisation achieved after 214 h. The 0.51M acid stressed culture achieved a slightly lower leaching rate of 0.072 kg Fe $m^{-3}h^{-1}$ (R^2= 0.95) and the same extent of pyrite solubilisation of 88% after 214 h. The leaching performance of the 0.68M acid stressed culture was lowest. However, following a lag period of 139 h, its recovery allowed a leaching rate of 0.074 kg Fe $m^{-3}h^{-1}$ (R^2= 0.99), similar to that of the 0.34M and the 0.51M acid stressed cultures. A much smaller solubilisation of pyrite than the 0.34M and the 0.51M acid stressed cultures was achieved within the same period i.e. 82% after 214 h. In all cases, similar solubilisation (98 %) was eventually achieved by 310 h.

The results of this set of experiments indicated that acid stress had a negative effect on the overall performance of the culture. The stressing of the culture with 0.34M, 0.51M and 0.68M acid reduced the maximum leaching rate similarly, by 19.7 %, 22.9 % and 20.8 % respectively. However, a clear

indication of the extension of the lag period prior to iron oxidation for the acid stressed cultures was observed, increasing with the degree of stress. The Control began oxidising the iron within the first 40 h of the leaching experiment while iron oxidation was only established after 91 h, 103 h and 137 h for the 0.34M, 0.51M and 0.68M acid stressed cultures respectively.

Table 4-1: Maximum pyrite leach rates and extent of pyrite solubilisation following acid stress over an exposure time at one hour.

Acid concentration [M]	Maximum Leach Rate [kg Fe $m^{-3}h^{-1}$]	R^2	Lag phase before Fe oxidation [h]	Extent of Solubilisation [%]	Time to achieve solubilisation [h]
Control	0.093	0.99	40.1 ± 1.6	94	120
0.34	0.075	0.98	91.4 ± 2.0	88	214
0.51	0.072	0.95	103.3 ± 2.1	88	214
0.68	0.074	0.99	137.7 ± 2.0	82	214

The planktonic cell concentration is given as a function of time for the Control and acid stressed cultures following one hour exposure in Figure 4.10. Specific growth rates, yields, maximum microbial cell concentration and the lag phases are given in Table 4.2 as a function of acid stress concentration.

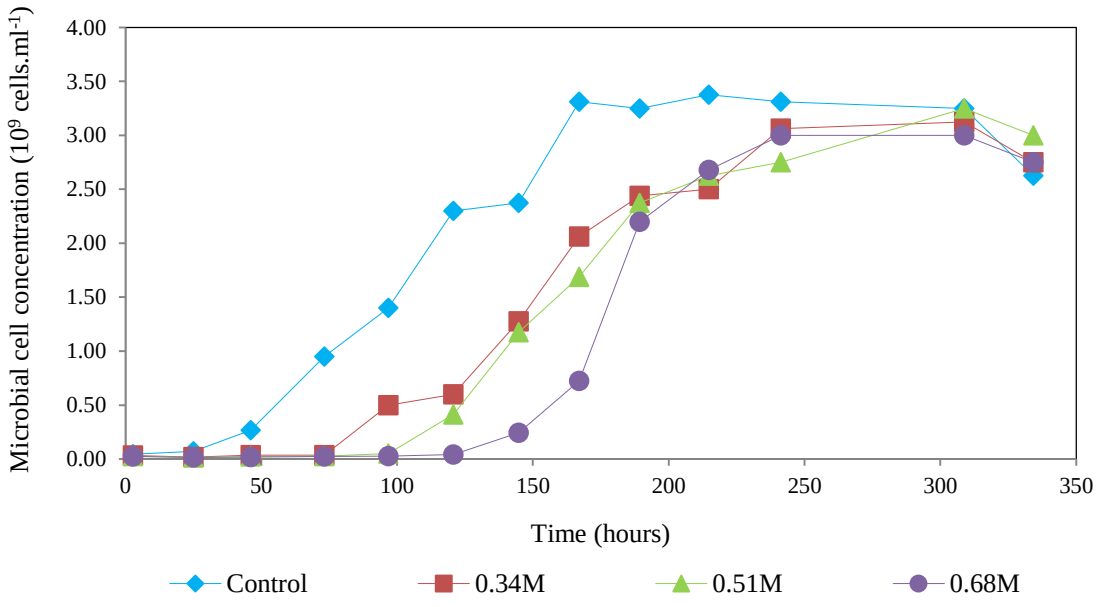


Figure 4-10: Planktonic microbial cell concentration profiles following exposure to acid stress for one hour. Performance was observed in a 3% pyrite slurry as a function of time.

The cell concentrations of the three stressed cultures were 5.0×10^7, 5.0×10^7 and 4.25×10^7 cells.mL^{-1} for 0.34M, 0.51M and 0.68M respectively and 7.13×10^7 cells.mL^{-1} for the Control at the commencement of the exponential phases. A short initial lag phase in microbial growth was observed for the Control and the total duration of the exponential phase was between 6 and 142 h when the planktonic cell number reached 3.31×10^9 cells.mL^{-1}. The Control remained in the stationary phase from 142 to 237 h before the cell concentration decreased. Lag phases of 73 h; 94 h and 143 h were observed for 0.34M, 0.51M and 0.68M acid stressed cultures respectively with exponential phases of between 73 and 214 h; 94 and 238 h; 121 and 261 h.

The 0.34M, 0.51M and 0.68M acid stressed cultures displayed exponential phase durations of 141, 144 and 140 h respectively in comparison to the 136 h for the Control culture. The decrease in cell concentrations after the stationary phase indicated the exhaustion of the energy source, in the form of Fe^{2+} generated from the pyrite or build-up of toxic or inhibitory products such as Fe^{3+}. The maximum planktonic cell numbers observed for this experiment were 3.38×10^9 cells.mL^{-1} at 190 h for the Control culture; 3.13×10^9 cells.mL^{-1} at 237 h for 0.34M acid stressed culture; 3.24×10^9 cells.mL^{-1} at 237 h for 0.51M acid stressed culture and 3.50×10^9 cells.mL^{-1} at 261 h for 0.68M acid stressed culture.

The lag phases indicated that a recovery time was required before the microbial growth and metabolism with associated generation of leach agents began. This lag time increased with severity of stress. No great difference was observed in the microbial growth following stress at 0.34M and 0.51M H_2SO_4. However the 0.68M acid stress required a longer time to recover in terms of cell growth, reaching similar cell concentrations to the lower stress conditions after 241 h. The specific growth rate of both the Control culture and the three acid stressed cultures had an average value of 0.058 h^{-1} (R^2=0.98). When the culture was stressed with 0.34M acid, the specific growth rate was 0.050 h^{-1} (R^2=0.99) compared to 0.056 h^{-1} for the Control culture, and 0.064 h^{-1} (R^2=0.98) and 0.062 h^{-1} (R^2=0.98) when stressed with 0.51M and 0.68M acid respectively. The calculated growth rates indicated similar rates following the recovery of stressed cultures during the extended lag phases.

Table 4-2: Microbial cell data for the various acid stressed cultures over one hour exposure time

Acid concentration [M]	Max specific growth rate [h^{-1}]	R^2	Lag phase [h]	Max cell concentration [10^9 cells mL^{-1}]	Time to achieved max cell conc [h]	Comparative Yield $_{X/Fe}$ calculated from 0 to C_x max Control culture [10^{14} cells kg^{-1}Fe]
Control	0.056	0.99	30.6 ± 5.9	3.38	190	3.67
0.34	0.050	0.99	73.2 ± 1.9	3.13	237	2.75
0.51	0.064	0.98	94.2 ± 2.9	3.24	237	2.48
0.68	0.062	0.98	143.3 ± 1.5	3.50	261	1.88

The yield calculated in terms of microbial cells produced per kg iron oxidised between inoculation and 167 h (the point at which the Control culture reached maximum cell number) was highest for the Control culture and decreased with exposure to more extreme acid stress (Table 4.2). The yield increased in the acid stressed cultures after the recovery stage. High yield of 3.95×10^{14} cells kg^{-1} Fe was recorded for 0.34M acid stressed culture after 241 h of leaching, 3.51×10^{14} cells kg^{-1} Fe for 0.51M stress after 241 h and 3.15×10^{14} cells kg^{-1} Fe for 0.68M stress after 308 h. This indicated that the acid stress had only delayed the leaching reaction to allow the recovery from the stress. The highest yield compared to the Control was in the same range.

4.3.2 *Three hour exposure to acid stress*

In this experiment the inocula were stressed using H_2SO_4 at concentrations of 0.34M, 0.51M and 0.68M for a three hour exposure period before re-suspension under optimal conditions. The Control culture, inoculated with an inoculum not exposed to stress was run concurrently. The pH was adjusted to 1.40 upon inoculation with the concentrations of Na_2CO_3 reported in Table 3.3. The results of the bioleaching performances, including the pH, redox potential, [Fe] and microbial cell number in suspension are presented in Figure 4.11 through Figure 4.14. These experiments were allowed to run for 390 h to observe the effect of the stress since lag phases became longer and the one hour experiment had established an expectation of resumption of normal leaching ability upon recovery.

The Control experiment displayed a rapid decrease in pH when the leaching reaction started, reaching pH of 1.00 within 143 h then stabilising for the remaining 239 h. An increase in time for onset of the pH drop as the acid concentration was intensified was shown as a shift to the right on the profiles. The cultures stressed with 0.34M, 0.51M and 0.68M lagged around pH between pH 1.25 and pH 1.30 for 120 - 168 h before decreasing to between pH 1.01 and pH 1.07 after 212 h. The culture stressed at 0.68M showed the slowest response as expected. The pH of these cultures never dropped much after

that and stabilised around pH 1.07 with the exception of the 0.51M acid stressed culture that dropped further to a low pH 0.99 after 336 h.

The redox potential profiles as a function of time are given in Figure 4.12. The Control redox potential rose rapidly from an initial 542 mV to a high of 782 mV within 121 h, indicating that the microorganisms were active and efficient in converting the ferrous to ferric iron. An initial drop in redox potential was observed in the first 47 h for the 0.34M acid stressed cultures and to a further 97 h for the 0.51M and the 0.68M acid stressed cultures before increase in redox potential. This was caused by the ferric iron leaching of the mineral with its conversion to ferrous iron before the microorganisms recovered from the acid stress to regenerate ferric iron. The difference in the observed decrease in redox potential was also a clear indication of the recovery time required before microbial oxidation.

The microbial ferrous conversion of the 0.34M acid stressed cultures was observed after 47 h, and that of the 0.51M and 0.68M acid stressed cultures only after 97 h. Following 0.34M acid stress, the redox potential reached 700 mV at 110 h, 757 mV after 240 h and a final high redox potential of 780 mV after 382 h, following 0.51M and 0.68M acid stress, the redox potential reached 700 mV at 170 h and 190 h respectively and 777 mV after 212 h of leaching. All stressed cultures reached a high redox potential of between 750 mV to 780 mV within 240 h and a high of between 770 mV and 780 mV at the end of experiment. The results confirmed that the microorganisms converted the ferrous iron present to ferric iron efficiently following recovery from the acid stress.

The total iron released into solution as a function of time following acid stress for three hours is given in Figure 4.13. The maximum linear leach rates and the corresponding correlation coefficients are given in Table 4.3. The extent of pyrite solubilisation, calculated from the total iron concentration in the suspension (iron in solution and precipitate) and the iron content in the mineral concentrate, is also presented in Table 4-3, with the time required to achieve these values.

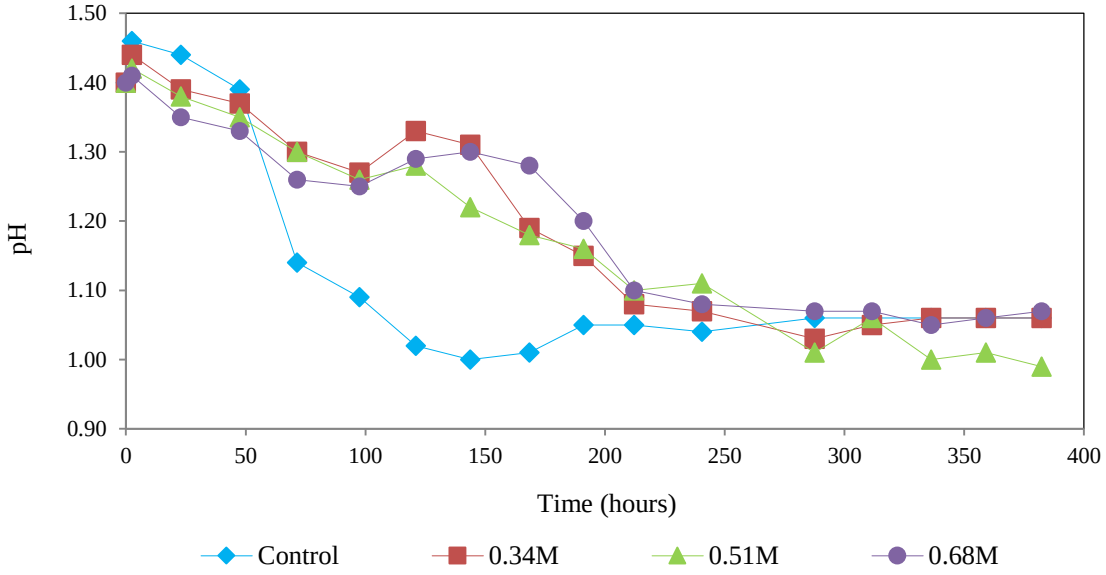


Figure 4-11: pH profiles of the stressed cultures following exposure to acid stress for three hours. Performance was observed in a 3% pyrite slurry as a function of time.

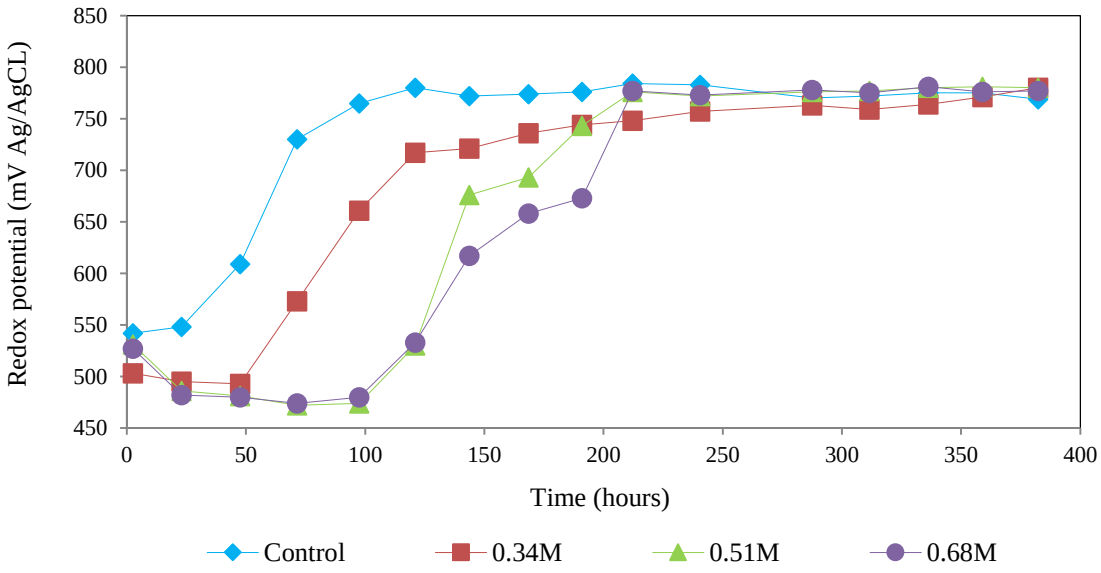


Figure 4-12: Redox potential profiles of the acid stressed cultures following three hour exposure. Performance was observed in a 3% pyrite slurry as a function of time.

Figure 4.13 shows that the Control exhibits the highest bioleaching rate and extent of solubilisation. The leaching rate of 0.090 kg Fe $m^{-3}h^{-1}$ (R^2=0.99) allowed 97 % of pyrite solubilisation within 142 hours. The bioleaching performance of the 0.34M, 0.51M and 0.68M stressed cultures was reduced and the required time to achieve highest extent of solubilisation increased from 142 to 382 h. The lag phase before the onset of iron solubilisation observed in the stressed cultures also increased. An

extended lag period was needed for the recovery of stressed culture before microbial oxidation and mineral leaching could be initiated. The 0.34M, 0.51M and 0.68M stressed cultures required 169 h, 175 h and 183 h respectively to initiate the microbial oxidation compared with 50 h for the Control. The leaching rate of the 0.34M and the 0.51M stressed cultures were 0.073 kg Fe $m^{-3}h^{-1}$ (R^2=0.96) and 0.071 kg Fe $m^{-3}h^{-1}$ (R^2=0.97) with 79% and 76% respective pyrite solubilisation achieved after 382 h. The leaching rate of the 0.68M stressed cultures was slightly higher at 0.084 kg Fe $m^{-3}h^{-1}$ (R^2=0.99) with an equal 77% pyrite solubilisation achieved after 382 h.

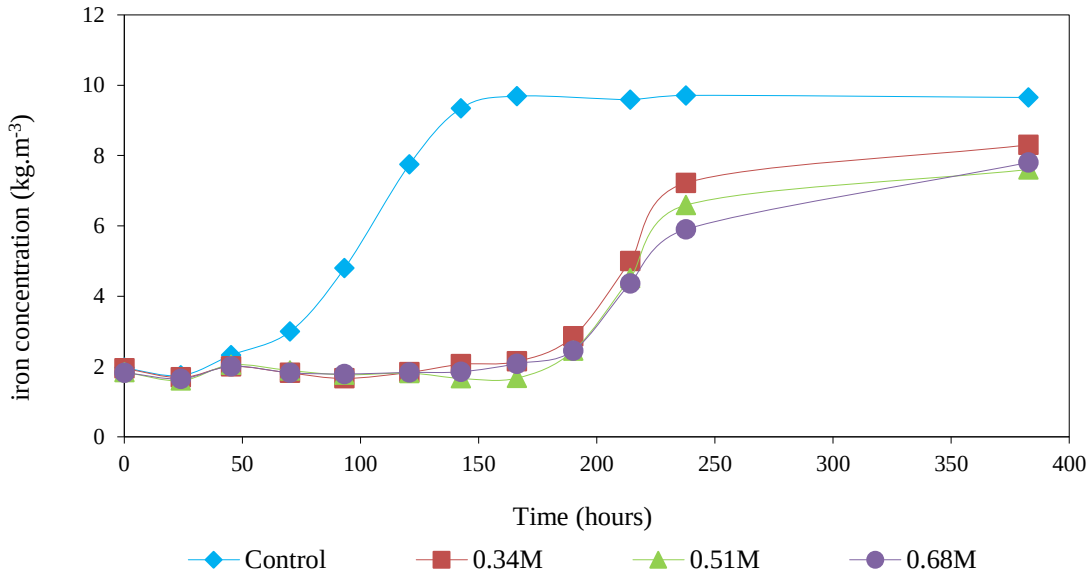


Figure 4-13: Trend of the total iron released (in solution and precipitate) as a function of time and acid stress following three hours exposure time.

Table 4-3: Maximum pyrite leach rates and extent of pyrite solubilisation following acid stress over an exposure time at three hours.

Acid concentration [M]	Maximum Leach Rate [kg Fe $m^{-3}h^{-1}$]	R^2	Lag phase before Fe oxidation [h]	Extent of Solubilisation [%]	Time to achieve solubilisation [h]
Control	0.090	0.99	57.8 ± 2.0	97	142
0.34	0.073	0.96	167.8 ± 2.4	79	382
0.51	0.071	0.97	173.6 ± 2.5	76	382
0.68	0.072	0.99	182.8 ± 2.1	77	382

The results of this set of experiments indicated that the negative effect of acid stress on the overall performance. The maximum leaching rate was reduced by 19.4 %, 21.9 % and 19.8 % for the 0.34M,

0.51M and 0.68M acid stress respectively. A clear indication of the extension of the lag period in the iron oxidation for the acid stressed cultures was also observed, increasing with the degree of stress.

The planktonic cell concentration as a function of time following the three hour exposure to stress is given in Figure 4.14. Specific growth rates, yields, maximum microbial cell concentration and the lag phases are given in Table 4.4 as a function of acid concentration.

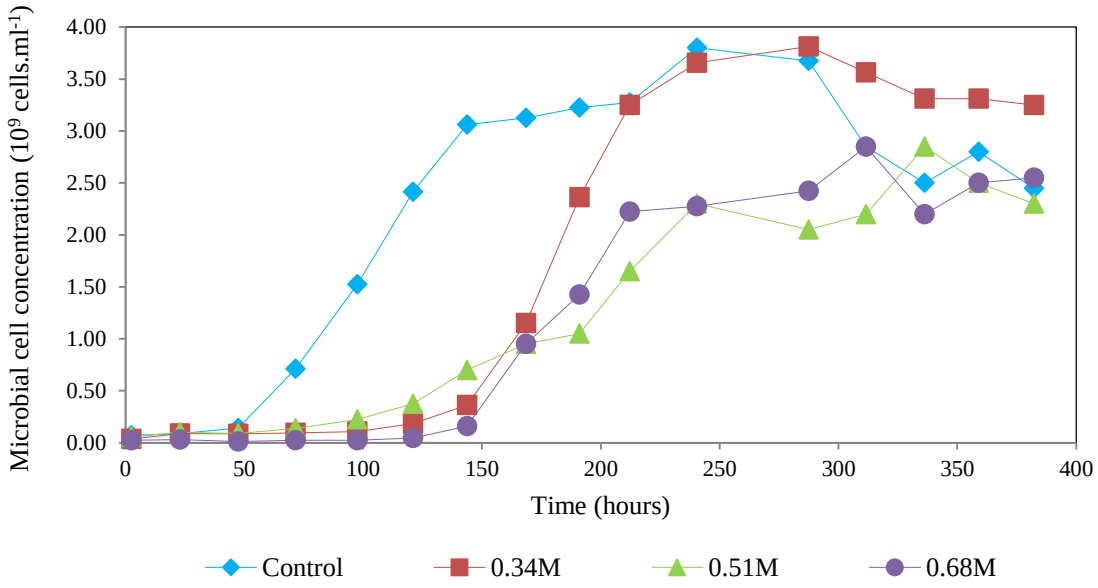


Figure 4-14: Microbial cell concentration profiles of the acid stressed cultures following three hour exposure. Performance was observed in a 3% pyrite slurry as a function of time.

The cell concentrations at the commencement of the exponential phase were 3.50×10^7, 5.63×10^7 and 2.38×10^7 cells.mL^{-1} for 0.34M, 0.51M and 0.68M cultures respectively compared with 7.13×10^7 cells.mL^{-1} for the Control. For the Control culture, a short initial lag phase was observed prior to the exponential phase from 47 to 143h when the planktonic cell number reached 3.75×10^9 cells mL^{-1}. Stationary phase lasted from 143 to 287 h before the cell concentration decreased. Lag phases of 119 h, 120 h and 144 h were observed for 0.34M, 0.51M and 0.68M acid stressed cultures respectively with exponential phases observed between 119 and 287 h, 120 and 336 h and 144 and 311 h for 0.34M, 0.51M and 0.68M acid stressed cultures respectively.

The stationary phases of more than 150 h could only be observed for the Control culture in contrast with the acid stressed cultures achieving a stationary phase of just 70 h for the 0.34M culture and less than 50 h for both 0.51M and 0.68M acid stressed cultures. The decrease in cell concentrations after the stationary phase indicated the exhaustion of the energy source, in the form of Fe^{2+} generated from the pyrite. The maximum planktonic cell numbers observed for this experiment were 3.75×10^9 cells.mL^{-1} at 287 h for the Control culture; 2.70×10^9 cells.mL^{-1} at 311 h for 0.34M acid stressed

culture; 3.00×10^9 cells.mL^{-1} at 382 h for 0.51M acid stressed culture and 1.50×10^9 cells.mL^{-1} at 382 h for 0.68M acid stressed culture.

The lag phases indicated that, following stress, a recovery time was required before the microbial growth and leaching process began. This lag time also increased with the severity of stress. No greater difference was observed in the microbial growth of the three stressed cultures following the lag period. The specific growth rates of the Control culture and that of the three stressed cultures had an average value of 0.035 h^{-1} (R^2=0.98). When the culture was stressed with 0.34M, 0.51M and 0.68M acid the calculated specific growth rates were 0.038 h^{-1} (R^2=0.99); 0.022 h^{-1} (R^2=0.99) and 0.040 h^{-1} (R^2=0.98) respectively and 0.040 h^{-1} (R^2=0.97) for the Control culture.

Table 4-4: Microbial cell data for the various acid stressed cultures over three hours exposure time

Acid concentration [M]	Max specific growth rate [h^{-1}]	R^2	Lag phase [h]	Max cell concentration [10^9 cells mL^{-1}]	Time to achieved max cell conc [h]	Comparative Yield $_{X/Fe}$ calculated from 0 to C_x max Control culture [10^{14} cells kg^{-1}Fe]
Control	0.040	0.97	43.6 ± 4.7	3.75	287	4.13
0.34	0.038	0.99	119.5 ± 3.2	2.70	311	2.01
0.51	0.022	0.99	120.8 ± 2.1	3.00	382	1.94
0.68	0.040	0.98	144.8 ± 2.1	1.50	382	0.25

The yields in terms of microbial cells produced per kg Fe oxidised between inoculation and 121 h was highest in the Control culture (4.13×10^{14} cells kg^{-1} Fe) and decreased with exposure to more extreme acid stress. The 0.34M and 0.51M acid stressed cultures displayed a similar yield of between 2.01×10^{14} – 1.94×10^{14} cells kg^{-1} Fe. The yield was greatly reduced to 0.25×10^{14} cells kg^{-1} Fe when the culture was stressed with 0.68M acid concentration. The yield increased in the acid stressed cultures after the recovery stages. A highest yield of 3.09×10^{14} cells kg^{-1} Fe was observed for 0.34M acid stressed culture after 212 h of leaching, 3.60×10^{14} cells kg^{-1} Fe was observed for 0.51M culture after 287 h and 3.62×10^{14} cells kg^{-1} Fe was observed for 0.68M culture after 311 h, indicating again that acid stress only delayed the commencement of the bioleaching reaction under optimal conditions. The highest yields following recovery compared favourably to the Control.

4.3.3 *24 hour exposure to acid stress*

In this experiment the inocula were subjected to a 24 hour exposure to acid stress using H_2SO_4 at concentration of 0.34M, 0.51M and 0.68M before re-suspension into optimal conditions. The Control, inoculated with unstressed inoculum, ran concurrently. The results of the bioleaching

performance, in terms of pH, redox potential, [Fe] and microbial cell number in suspension are presented in Figure 4.15 through Figure 4.18, while the detailed data are presented in Appendix F. These experiments were conducted for 410 h to observe the effect of exposure time and acid stress on performance since stress had had been shown to cause lag phase.

Upon inoculation, pH was adjusted to pH 1.40 to ensure that growth occurred under the same conditions such that the acid stress effect on the leaching performance was observed. The pH profiles are presented in Figure 4.15. A pH decreased when microbial activity started, and reached pH 0.90 within 237 h in the Control culture, then stabilised at pH 0.95 for the remaining 168 h of the experiment. An increase in time, shown as a shift of the profiles, to the right was observed in terms of the onset of the drop in pH as the acid stress intensified. The cultures stressed with 0.34M and 0.51M acid remained around pH 1.29 and pH 1.37 for 142 and 210 h before decreasing to pH 0.95 at 240 and 360 h respectively. The 0.68M acid stress displayed a delay before it decreased in pH, with the sharp decrease observed after 287 h to reach pH 1.02 after 405 h. The effect of the acid stress was clearly observed by the delay in the decrease in pH for the stressed cultures, owing to delayed formation of H_2SO_4.

The profiles of redox potential are given in Figure 4.16. The redox potential in the Control reactor increased from an initial 500 mV at 45 h through 700 mV at 100 h to a high of 782 mV at 142 h, indicating the good health of the microorganisms that were active and efficient in converting the ferrous iron to ferric iron. An initial decrease in redox potential was observed in the first 45 to 142 h for the stressed cultures due to ferric iron leaching of the mineral with its conversion to ferrous iron before the microorganisms recovered from the acid stress to regenerate the ferric iron. The microbial ferrous iron oxidation for the 0.34M, 0.51M and 0.68M acid stressed cultures was only observed after 70 h, 143 h and 214 h respectively. The redox potential of the 0.34M acid stressed culture increased from 480 mV at 70 h through 700 mV at 150 h to 787 mV at 190 h and reached a maximum high redox potential of 800 mV after 261 h. The redox potential following stress at 0.51M and 0.68M H_2SO_4 remained low for 142 h and 214 h before ferrous iron oxidation was observed, indicating that the recovery time following these stresses was much longer. The redox potential reached 700 mV at 225 h and 300 h respectively, further increasing to 795 mV after 287 h for the 0.51M acid stressed culture and 789 mV after 405 h for the 0.68M acid stressed culture. The microorganisms converted the ferrous iron present to ferric iron efficiently following recovery from the acid stress.

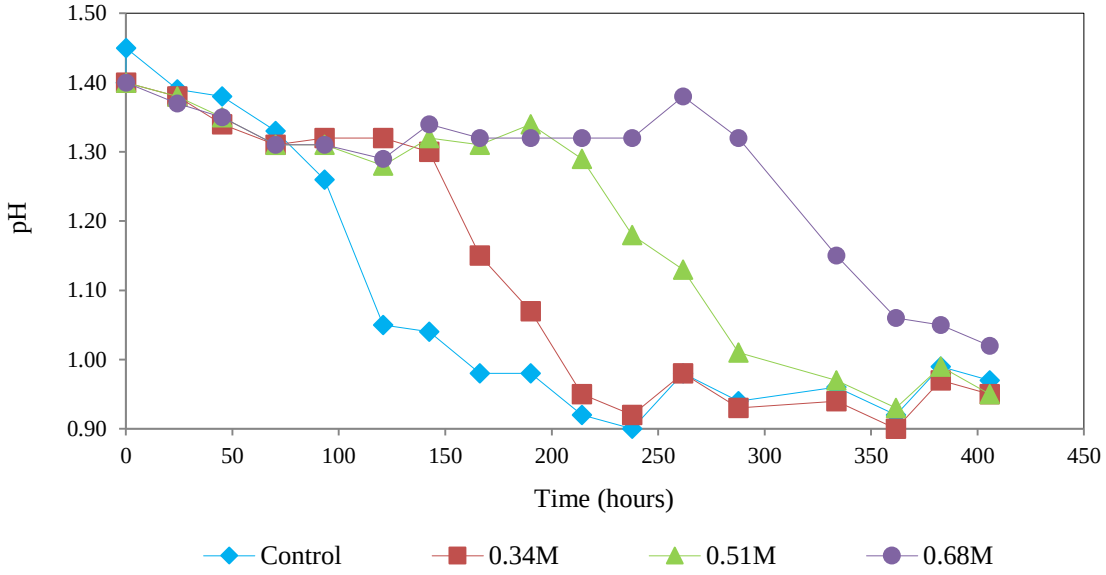


Figure 4-15: pH profiles of the stressed cultures as a function of time, following exposure to acid stress for 24 hour. Performance was observed in a 3% pyrite slurry as a function of time.

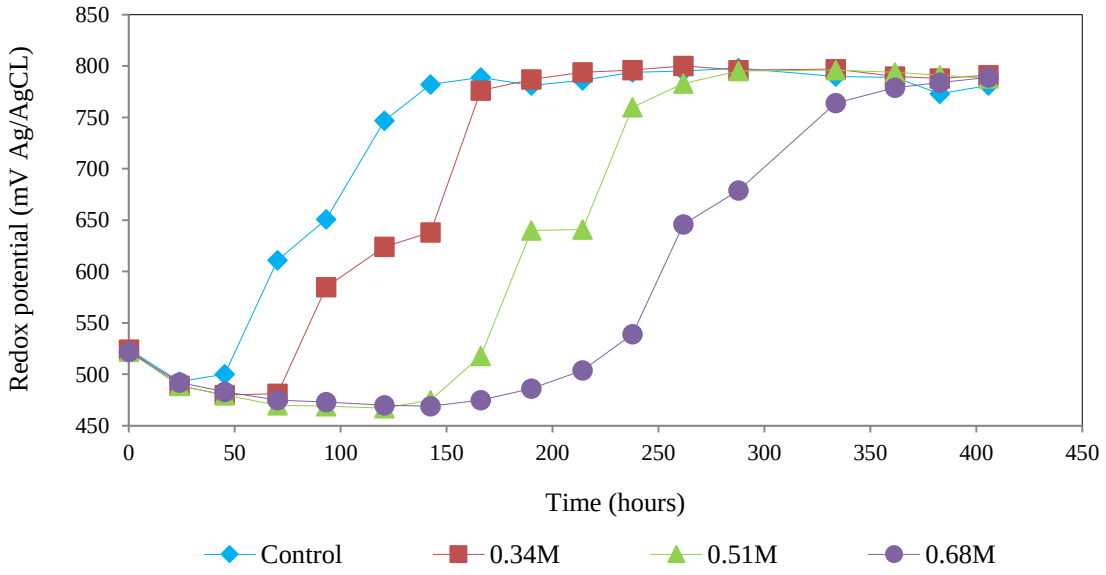


Figure 4-16: Redox potential profiles of the acid stressed cultures following 24 hour exposure. Performance was observed in a 3% pyrite slurry as a function of time.

The total iron released in solution as a function of time following 24 hour exposure to acid stress is given in Figure 4.17. The maximum linear leach rates and corresponding correlation coefficients are given in Table 4.5. The extent of pyrite solubilisation, calculated from the total iron concentration in the suspension (iron in solution and precipitate) and the iron content in the mineral concentrate, are presented with the time required to achieve these values.

Figure 4.17 shows that the Control exhibited a high bioleaching rate (0.90 kg Fe $m^{-3}h^{-1}$, R^2=0.99) and extent of solubilisation of 97 % achieved within 166 h. The bioleaching performance of 0.34M, 0.51M and 0.68M acid stressed cultures was reduced and required additional time to achieve highest extent of solubilisation. The lag phase before the onset of iron solubilisation observed in the stressed cultures increased with degree of stress. The cultures exposed to 0.34M, 0.51M and 0.68M stress required 70 h, 143 h and 214 h respectively to initiate the microbial oxidation, compared to 40 h for the Control. The leaching rate of 0.34M stressed culture was 0.071 kg Fe $m^{-3}h^{-1}$ (R^2=0.98) with 80 % pyrite solubilisation achieved after 405 h. The 0.51M stressed cultures achieved identical leaching rate of 0.074 kg Fe $m^{-3}h^{-1}$ (R^2=0.99) but a reduced pyrite solubilisation of 77 % after 405 h. The leaching performance following stress at 0.68M for 24 hours was greatly compromised, achieving a leaching rate of 0.061 kg Fe $m^{-3}h^{-1}$ (R^2=0.98) and a low pyrite solubilisation of 68 % after 405 h.

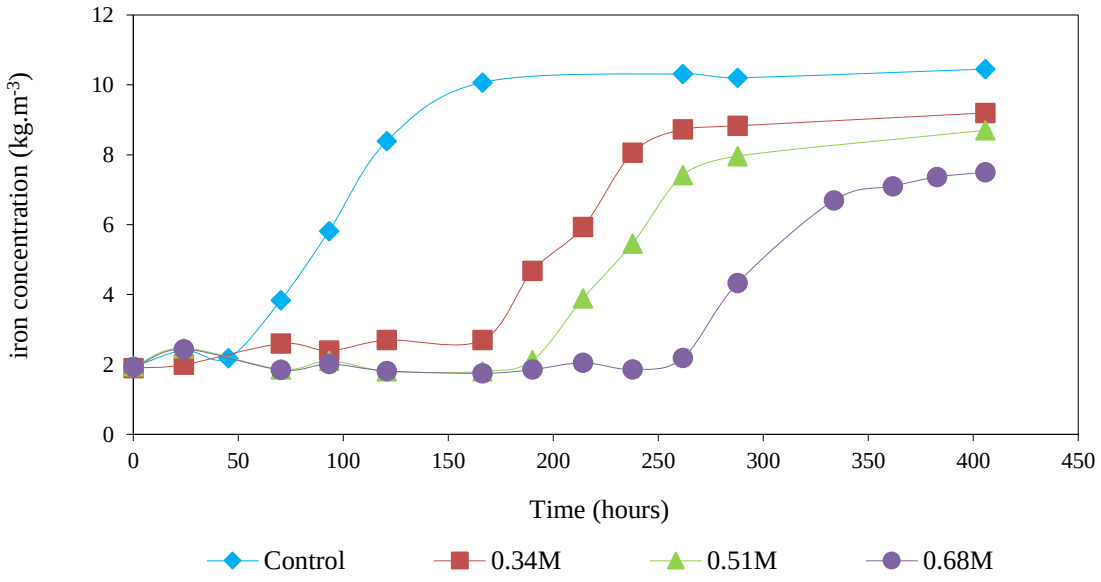


Figure 4-17: Trend of the total iron released (in solution and precipitate) as a function of time and acid stress following 24 hour exposure time.

The results of this set of experiments indicated that extended acid stress had an even more negative effect on the overall performance of the culture. The stressing of culture with 0.34M, 0.51M and 0.68M acid over 24 hours reduced the maximum leaching rate, by 21.6%, 17.7% and 31.9% respectively with respect to the Control. The extension of the lag period in the iron oxidation for the acid stressed cultures was observed in this experiment, increasing with the degree of stress.

Table 4-5: Maximum pyrite leach rates and extent of pyrite solubilisation following acid stress over an exposure time at 24 hour.

Acid concentration [M]	Maximum Leach Rate [kg Fe $m^{-3}h^{-1}$]	R^2	Lag phase prior to Fe oxidation [h]	Extent of Solubilisation [%]	Time to achieve solubilisation [h]
Control	0.090	0.99	53.7 ± 2.5	97	166
0.34	0.071	0.98	158.7 ± 3.2	80	405
0.51	0.074	0.99	193.8 ± 3.1	77	405
0.68	0.061	0.98	260.1 ± 3.7	68	405

Planktonic cell concentrations as a function of time for the Control and cultures exposed to a 24 hour acid stress are given in Figure 4.18. Specific growth rates, yields, maximum microbial cell concentration and the lag phases are given in Table 4.6 as a function of acid concentration.

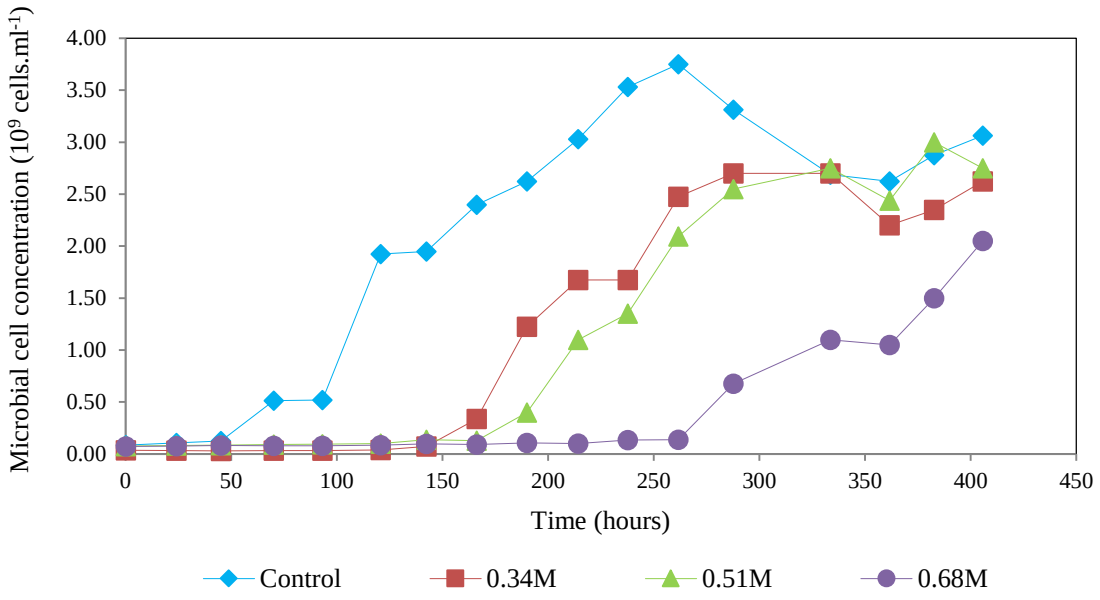


Figure 4-18: Microbial cell concentration profiles of the acid stressed cultures following 24 hour exposure. Performance was observed in a 3% pyrite slurry as a function of time.

The cell concentrations of the three stressed cultures were 1.84×10^8, 1.38×10^8 and 1.38×10^8 cells.mL^{-1} for 0.34M, 0.51M and 0.68M respectively and 1.24×10^8 cells.mL^{-1} for the Control at the commencement of the exponential phases. A short initial lag phase in microbial growth (13 h) was observed for the Control and the total duration of the exponential phase was from 13 to 120 h at which the planktonic cell number reached 3.53×10^9 cells.mL^{-1}. Further linear growth was observed from 140 to 250 h. After 260 h, cell concentration decreased. Lag phases of 135 h, 144 h and 205 h were

observed for 0.34M, 0.51M and 0.68M acid stressed cultures respectively with exponential phases extending from 135 to 190 h; 144 to 214 h; 205 h to the end of the experiments for 0.34M; 0.51M and 0.68M acid stressed cultures respectively.

The 0.34M and 0.51M acid stressed cultures displayed continuous stationary phases from 190 and 214 h to the termination of the experiment. The 0.68M acid stressed culture only displayed the exponential phase from 205 h to the time experiment was ended. The maximum planktonic cell numbers observed for this experiment were 3.75×10^9 cells.mL^{-1} at 261 h for the Control culture; 2.70×10^9 cells.mL^{-1} at 287 h for 0.34M acid stressed culture; 2.00×10^9 cells.mL^{-1} at 382 h for 0.51M acid stressed culture and 2.05×10^9 cells.mL^{-1} at 405 h for 0.68M acid stressed culture. The specific growth rate of the Control culture and that of the three acid stressed cultures had an average value of 0.039 h^{-1} (R^2=0.98). When the culture was stressed with 0.34M, 0.51M and 0.68M acid, the specific growth rate was 0.059 h^{-1} (R^2=0.99); 0.045 h^{-1} (R^2=0.99) and 0.019 h^{-1} (R^2=0.99) respectively and it was 0.035 h^{-1} (R^2=0.96) for the unstressed culture.

The yields calculated in terms of microbial cells produced per kg Fe oxidised between inoculation and 214 h was highest for the Control culture and decreased with exposure to more extreme acid stress, with the 0.34M stressed culture displaying a more consistent yield of 3.14×10^{14} cells kg^{-1} Fe after 287 h. It was considerably reduced in the 0.51M and 0.68M acid stressed culture to just 2.15×10^{14} cells kg^{-1} Fe and 0.39×10^{14} cells kg^{-1} Fe after 382 h and 405 h respectively. The Control culture produced a yield of 3.24×10^{14} cells kg^{-1} Fe after 261 h. The yield increased in the 0.34M acid stressed culture towards the end of the experiment reaching a value of 3.97×10^{14} cells kg^{-1} Fe after 382 h. However, it stayed very low in the 0.51 (2.61×10^{14} cells kg^{-1} Fe) and 0.68M (1.64×10^{14} cells kg^{-1} Fe) stressed culture at the end of the experiment as compared to 3.63×10^{14} cells kg^{-1} Fe for the Control culture.

Table 4-6: Microbial cell data for the various acid stressed cultures over 24 hour exposure time

Acid concentration [M]	Max specific growth rate [h^{-1}]	R^2	Lag phase [h]	Max cell concentration [10^9 cells mL^{-1}]	Time to achieved max cell conc [h]	Comparative Yield $_{X/Fe}$ calculated from 0 to C_x max Control culture [10^{14} cells kg^{-1}Fe]
Control	0.035	0.96	50.7 ± 4.2	3.75	261	3.24
0.34	0.059	0.99	167.9 ± 2.3	2.70	287	3.14
0.51	0.045	0.99	191.5 ± 2.4	2.00	382	2.15
0.68	0.019	0.99	259.4 ± 2.6	2.05	405	0.39

4.4 Microbial Speciation

4.4.1 *Introduction*

At completion of the leaching experiment, samples of the cultures were collected and processed as described in Section 3.5.3 such that the composition of the microbial community could be assayed using qPCR analysis. Data presented for the speciation, were collected for the highest exposure (24 hour) to acid stress.

A further study was conducted to determine the microbial composition through the recovery, exponential, stationary and death phases of the three hour acid stress experiment at 0.51M H_2SO_4. The three hour acid stress experiment was selected for this experiment as it was also selected to assess inter-experiment reproducibility.

4.4.2 *Microbial speciation at experiment completion*

All experiments were inoculated with a mixed mesophilic culture containing *At. ferrooxidans*, *A. cupricumulans, Archaea*, *F. acidiphilum,* and predominantly *L. ferriphilum*. The recovered cultures were analysed to determine the dominance of the surviving stressed microorganisms. Figure 4.19 shows the relative concentrations (calculated using determined copy numbers per genome) of the microorganisms observed.

The culture at the end of the Control experiment and the inoculum had a similar microbial species composition with *L. ferriphilum* remaining the dominant species and accounting for 90 % of the microorganisms identified. *L. ferriphilum* gradually increased in dominance as the acid stress increased from 0.34M to 0.68M H_2SO_4, increasing from 90% in the inoculum to 97% in the 0.34M acid stressed culture, to 98% in the 0.51M acid stressed culture, to 100% in the 0.68M acid stressed culture. This has indicated that the acid stress subjected to the mixed mesophilic cultures has favoured the dominance of *L. ferriphilum*.

Following 24 hour exposure of the mixed mesophilic cultures to acid stress *A. cupricumulans, Archaea* (*JTC Archaea 1)* and *At. ferrooxidans* were suppressed to below detection limits leaving only *Fe. acidiphilum* observable in addition to *L. ferriphilum*. The dominance of *Fe. acidiphilum* decreased from a 3% initially in the inoculum to 2% in the 0.34M and 0.51M acid stressed cultures to undetectable in the 0.68M acid stressed culture.

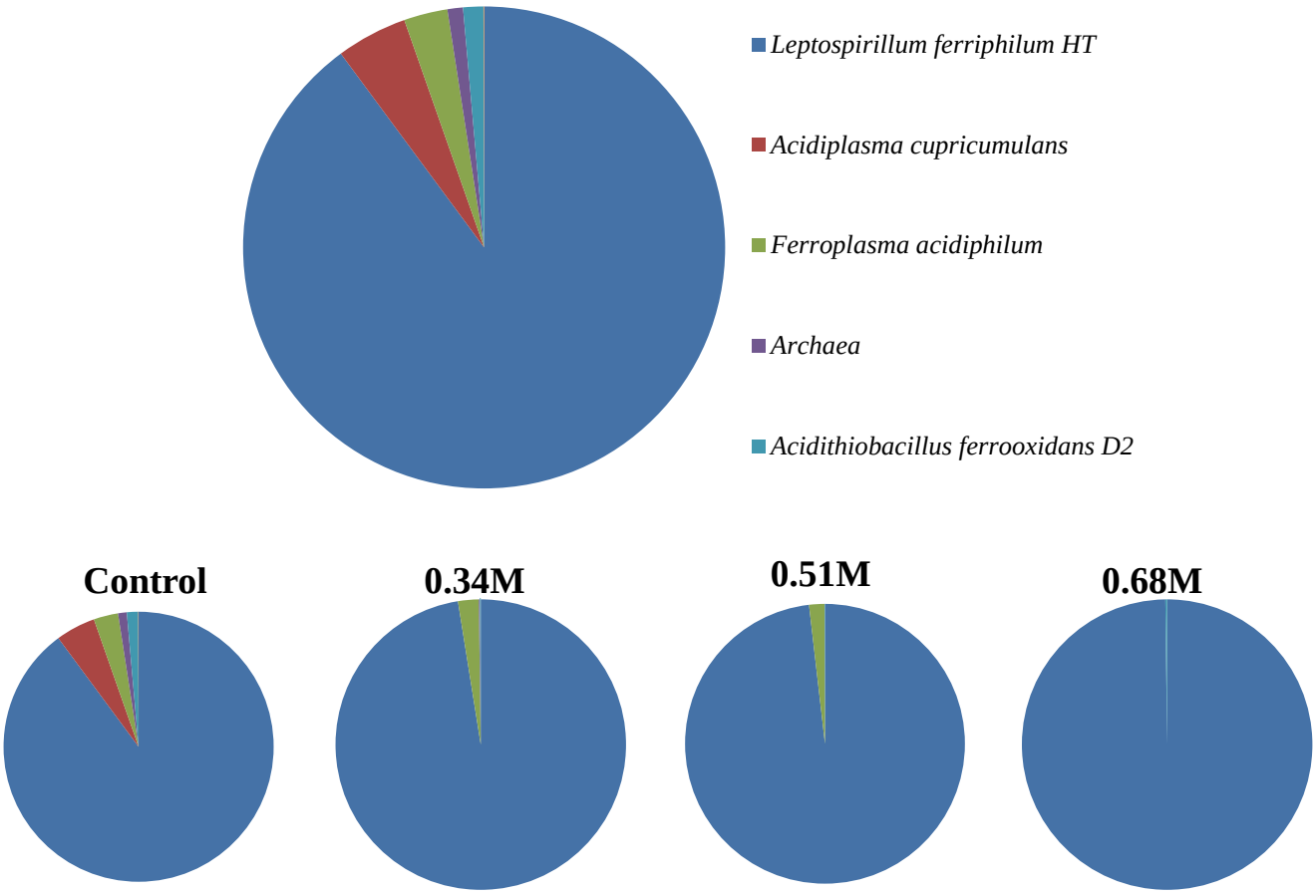


Figure 4-19: Microbial composition of the cultivated cultures following 24 hour exposure to acid stress.

4.4.3 *Changing microbial speciation across duration of experiment*

Following observation of the speciation collected at the end of the 24 hour experiment a question arose as to the time frame of the suppression of the species following being subjected to acid stresses. To answer this question, an experiment was set-up using the same mixed mesophilic cultures and acid stress protocol during which samples were collected at different stages of the growth of the culture for speciation analysis.

The mixed mesophilic culture containing *At. ferrooxidans*, *A. cupricumulans*, *Archaea*, *F. acidiphilum*, and predominantly *L. ferriphilum* was stressed with 0.51M H_2SO_4 for three hour and cultivated in triplicate, under optimal conditions, as described in Section 3.4. Samples were collected in triplicate and analysed for microbial composition using qPCR as described in Section 3.5.3. Figure 4.20 shows the growth curve and the microbial speciation analysis observed during the different growth phases of the experiment. Speciation analysis at 70 h indicated a reduction in microbial species from an initial five to three.

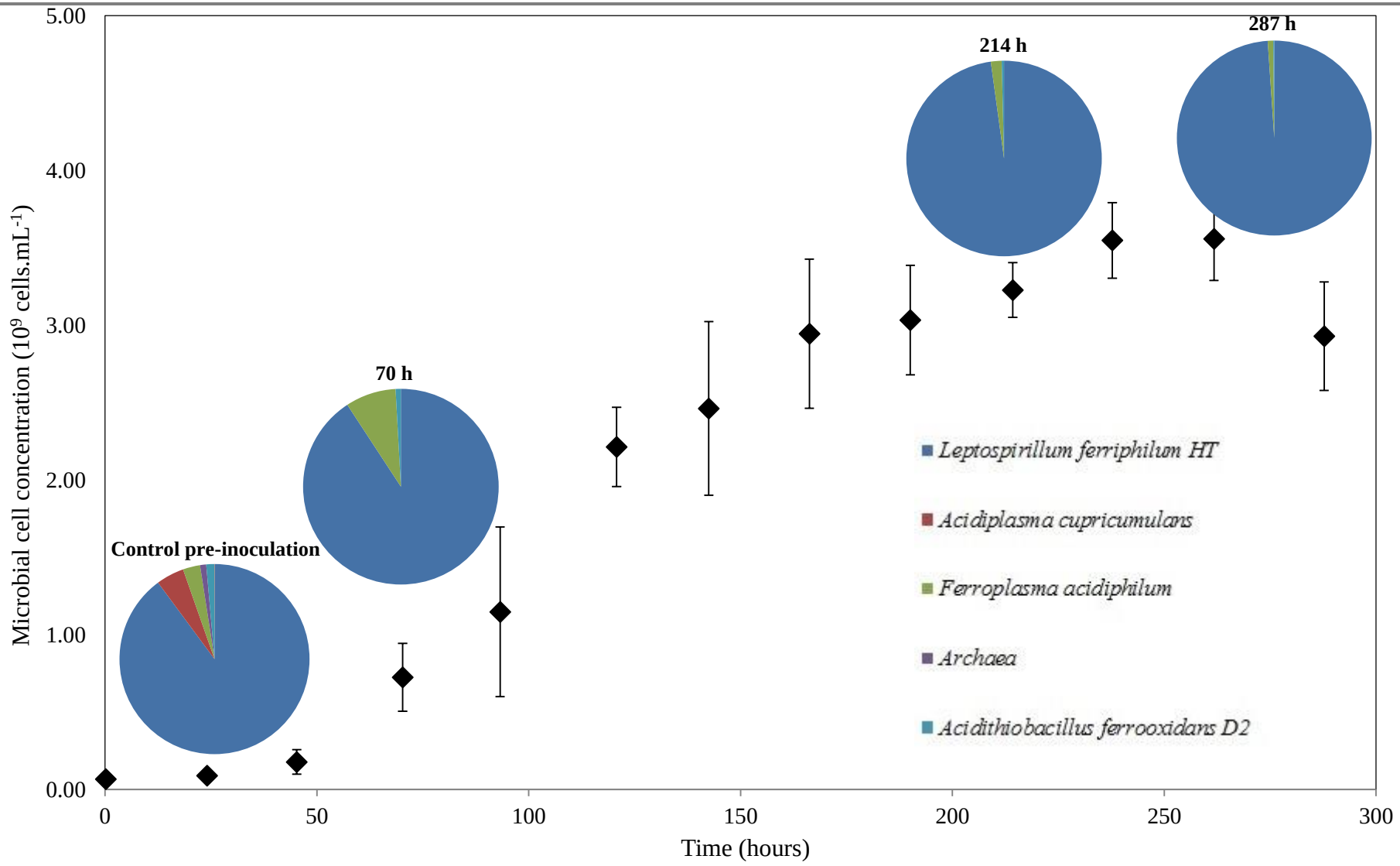


Figure 4-20: Microbial growth curve of an acid stressed and cultivated cultures depicting the microbial composition after 70, 214 and 287 h post acid stress

From the growth curve this phase is when the culture has entered the exponential phase with an average microbial concentration of 7.58×10^{8} cells.mL^{-1} (10 fold increase of the inoculum) and a specific growth rate of 0.043 h^{-1}. *A. cupricumulans* and *Archaea* were not detectable during the cultivation process. The detection limit is 1 %, so this may result from inability to multiply or death. *At. ferrooxidans*, *F. acidiphilum*, and predominantly *L. ferriphilum* remained present at 1 % or more. *L. ferriphilum* increased from an initial 90% in the inoculum to 91 % after 70 h of cultivation. The relative increase in dominance of *F. acidiphilum* was greatest from 3% in the initial inoculum to 8%. In both cases, the growth rates of these species outcompeted the other species. *At. ferrooxidans* was just detectable at 1% as in the inoculum. This suggested that the specific growth rate of *F. acidiphilum* was greatest in the early stage of the experiment. The survival of these three microorganisms is explained by the reduction in pH making the culture extremely acidic and reaching a low pH of 1.20. Hallberg and Johnson (2001) reported that reduction in culture pH can be deleterious to the growth of other, less acid-tolerant, microorganisms.

Speciation analysis at 214 h indicated an increase of the *L. ferriphilum* from 91% at 70 h to 98% at 214 h leaving just 2% of the *F. acidiphilum*, while *At. ferrooxidans* became undetectable. The remaining two species are also observed when a sample is analysed at 287 h with *L. ferriphilum* increasing to 99% and *F. acidiphilum* decreasing to just 1%. In competition for the remaining available nutrients, *L. ferriphilum* outcompeted *F. acidiphilum*, demonstrating a higher growth rate. *L. ferriphilum* is particularly able to scavenge low Fe^{2+} concentrations, enabling high growth rates to be maintained at high redox potential and corresponding low ferrous iron. At time 287 h, the microbial growth shows a decline in cell concentration indicating the death phase of the microorganisms involved. Microbial speciation is not changed significantly from late exponential phase.

Leptospirillum spp. is adapted at accelerating the oxidative dissolution of pyrite which is the most abundant of all sulphide minerals. Due to its high substrate (Fe^{2+}) affinity, tolerance of ferric iron and moderately thermal (>40^{o}C) environments, it is frequently the dominant iron-oxidising organism in mineral leaching environments (Hallberg and Johnson, 2001; Coram and Rawling, 2002). This is confirmed in our study with *L. ferriphilum* dominating the other species during the cultivation. The acid stress to which the mixed culture was subjected has also further advantaged *L. ferriphilum*, through the reduction of non-acid tolerant microorganism. The iron and sulphur oxidiser *At. ferrooxidans* is able to operate autotrophically using ferrous iron and sulphur and other reduced inorganic sulphur compounds as electron donors (Hallberg and Johnson, 2001). The observed dominance of *L. ferriphilum* can be attributed to its ability to scavenge lower concentrations of Fe^{2+} than *At. ferrooxidans* and its preference for the temperature, constantly maintained at 35^{o}C. In

addition, it has the ability to oxidise iron in a low pH range of between pH 0.5 and pH 3.5 (Plumb *et al*., 2008).

4.5 Discussion

4.5.1 *Introduction*

All organisms are subject to on-going changes in their environment. These changes may stress the organism and generate a stress response which is key to its survival. The agents causing stress may be physical, chemical, mechanical or combinations of the three (Schlegel, 1993). The stress response seen in nature is also found within the field of bioprocessing where practices such as the bioleaching of base metals, brewing, the recovery of valuable enzymes and antibiotics and biological waste treatment are highly dependent on the optimal performance of organisms under intensified conditions optimized to maximize productivity. It is postulated that many stress responses are similar. In addition, once an organism has survived the effect of one form of stress, tolerance to other forms of stress have been induced e.g. cells that have been exposed to substrate limitations have increased resistance to heat shock, osmotic stress or disinfecting agents (Schlegel, 1993). Conversely, exposure of the unadapted cells to multiple stresses can lead to cumulative stress effects (Harrison *et al*., 2007)

Organisms respond to the effects of stress in several ways. Most organisms possess defence mechanisms that are initiated once the stress has been "sensed". In general, the response to stress is transitory. A shift in an environmental parameter such as pH is first detected by the cell which subsequently adjusts gene expression to counteract the physical effects of the external change on its molecular components, finally recovering a steady state. The physical state of the membrane is known to be a very sensitive monitor of the most diverse environmental changes. This feature was suggested to render cell membranes an ideal location for the primary stress sensor (Horváth *et al*., 1998). Membranes have fluidity properties that permit the cell to sense changes in temperature, pH, osmotic and atmospheric pressure etc. (Vigh *et al*., 1998).

The initialisation of the stress response and the extent to which the microorganisms stay in the response state is usually the indication of the adaptation phase during which all the microorganisms' energy and metabolism is dedicated to self-preservation and recovery. This lag phase is a valuable indicator of the extent of stress endured by the microbial community.

4.5.2 *Observation of the microbial recovery time upon acid stress in terms of growth*

Three experiments were conducted to assess the effect of various acid stresses and exposure times on the subsequent microbial activities during the leaching of pyrite concentrate. The assessment was analysed on the level of iron oxidation and microbial growth and are reported in Section 4.4 in terms of recovery times, rates and extent of reaction.

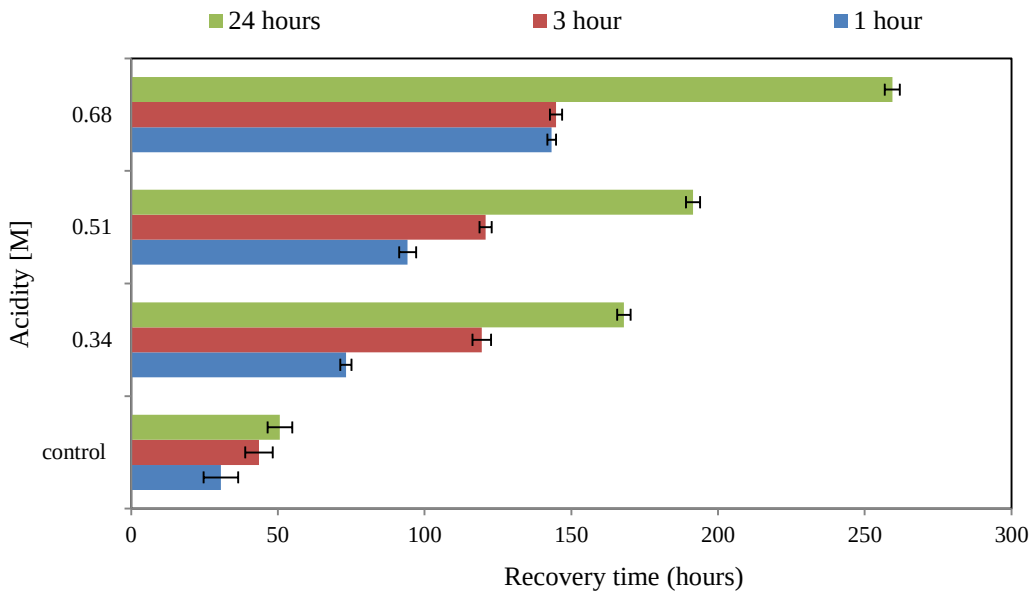


Figure 4-21: Calculated recovery time required before microbial growth was observed following acid stress for exposure times of one, three and 24 hour.

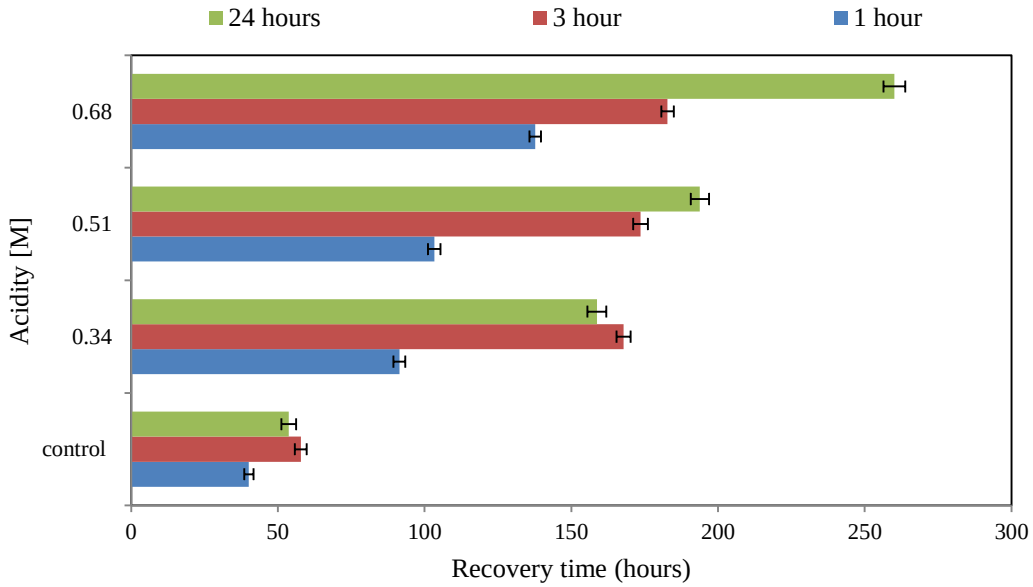


Figure 4-22: Calculated lag periods required before microbial leaching was initiated following acid stress for exposure times of one, three and 24 hour.

Figure 4.21 shows the recovery times required before microbial growth could be observed following acid stressed and Figure 4.22 shows the recovery times required by the stressed cultures before microbial oxidation could be initiated.

The microbial lag phase was an indicator of the beginning of the microbial growth during the experiment. The lag phase for iron oxidation was also an indicator of when the microbial ferrous iron oxidation was initiated. Here the ferric iron necessary for the metal solubilisation was produced by ferrous iron oxidation by the recovered microorganisms. The stress endured by the microorganisms required a recovery period once introduced in operational conditions. In the bioleaching environment, it is important for microorganisms to be active when introduced into the operation to ensure energy generation for growth and maintenance under challenging conditions. Microbial growth was observed rapidly in the Control cultures within a time period of 30-50 h after inoculation; however the oxidation of ferrous iron was only observed within an average time of 50 h (Figure 4.22), indicating that although microbial growth occurred rapidly after inoculation, an increase cell number was required before Fe^{2+} oxidation rate outweighed the ferric leach rate, resulting in microbial leaching of the mineral. The 0.34M stressed cultures displayed an increased lag period of between 73 and 167 h when exposed for one, three and 24 hour before microbial growth was observed. The microbial leaching was only observed after 91, 167 and 158 h for the one, three and 24 hour exposure time respectively. Similarly, the 0.51M stressed cultures displayed a much longer recovery period ranging between 94 and 191 h when exposed for various exposure times before microbial growth was observed. The microbial leaching was only observed after 103, 173 and 193 h for one, three and 24 hour exposure time respectively. The 0.68M stressed cultures showed an even much longer lag period before microbial growth was observed, ranging between 143 and 259 h when exposed for the various exposure times. The microbial leaching was observed after 137, 180 and 260 h for one, three and 24 hour exposure time respectively. It was expected that the acid stress would delay the commencement of the microbial growth and the microbial iron oxidation to allow sufficient microbial recovery from the stress to which the cultures were subjected.

A gradual increase in recovery prior to microbial oxidation can be observed. The recovery time increases with increased stress to the microbial culture in terms of concentration and exposure time. The 0.68M stressed culture exposed for 24 hours depicted a microbial leach lag period of over 260 h before any microbial leaching could be observed.

4.5.3 *Effect of acid stress on mineral leaching rate and extent*

Figure 4.23 shows the calculated iron leaching rates following acid stress for various exposure times, emphasising the acid concentration.

When considering the acid concentration effect on the rate of oxidation of the mesophilic cultures used for pyrite leaching, the rate of leaching of the Control cultures were in a similar range of 0.090 kg Fe $m^{-3}h^{-1}$.

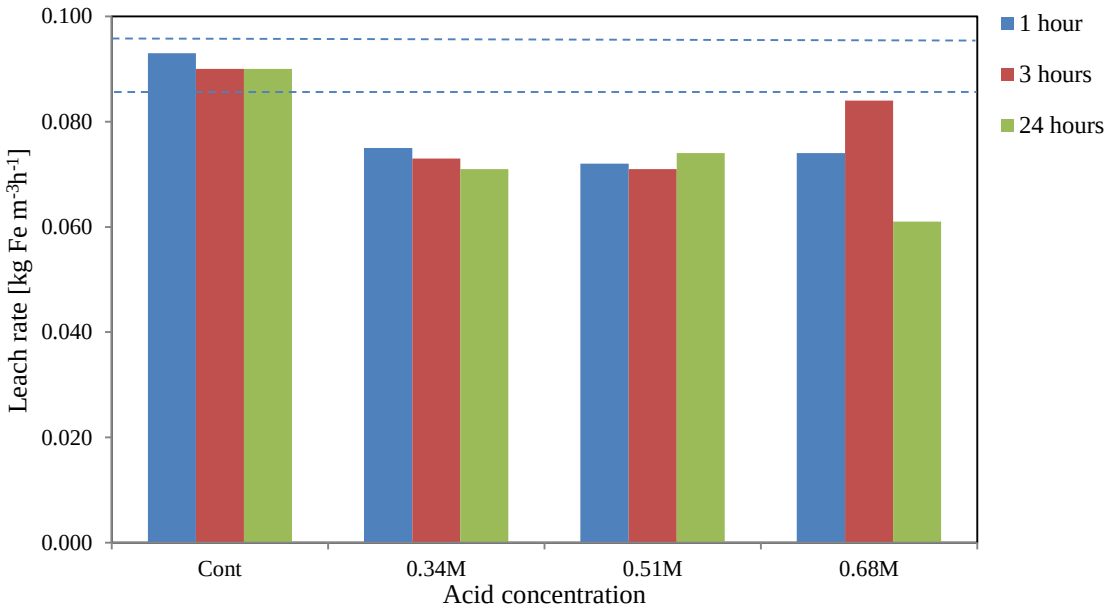


Figure 4-23: Calculated leaching rates following acid stress and exposure times with emphasis on the acid concentrations. Dotted lines represent 5% error on the average rates of the Control cultures.

A 5 % error margin was calculated from the leaching rate average of the three Control cultures and indicated that the leaching rate across all three experiments were in the same margin. The inocula for these Control cultures, like the stressed culture collected from stock reactors, were left outside optimal conditions for the chosen exposure time before being re-introduced into the reactor and subjected to optimal operation conditions. The acclimatisation becomes necessary regardless of the 0M stress on the Control and this can be observed in the time necessary to initiate the microbial iron oxidation. The lag period before microbial growth and microbial oxidation was observed in the Control cultures, following one, three and 24 hour "exposure time", increased from 30 h to 50 h. But the rates remained comparable with one another.

The rates of Fe oxidation for the 0.34M, 0.51M and 0.68M acid stressed cultures appeared to be similar across the exposure time at approximately 0.070 kg Fe $m^{-3}h^{-1}$ (77 % of Control) with just the 0.68M exposed for 24 hour showing a much reduced rate of 0.061 kg Fe $m^{-3}h^{-1}$ (67 % of Control). During the one hour exposure time the stress shock is assumed to be sudden and does not give sufficient time to the stressed cultures to adapt or go into self-preservation state. Microorganisms are then re-introduced in optimal conditions and this allowed a quick re-acclimatisation but much longer adaptation hence the lag phases extending from 91 h when the 0.34M acid stressed culture was subjected to one hour exposure time and 103 h when the 0.51M acid stressed culture was subjected to one hour exposure time. However when the exposure time was changed to three hours, the lag phases of both 0.34M and 0.51M acid stressed cultures extended to 167 and 173 h respectively and their rates

never changed. The rates of Fe oxidation for the 0.34M and 0.51M acid stressed cultures when exposed for three hours also remained in the same range as the one and three hours exposure time. However the extent of solubilisation of these two stresses (0.34M and 0.51M following three hours exposure time) is reduced by almost 15% compared to when they are subjected to the same concentration of acid but for just one hour. The same observation is reported when the cultures are subjected to 24 hours acid stress; the extent of solubilisation is reduced by 15% compare to the Control cultures. This indicates that acid shock is detrimental to the initialisation of the metal leaching with microorganisms requiring longer recovery time as the concentration of acid stress increases.

The rates of Fe oxidation for the 0.68M acid stressed cultures were comparable to the 0.34M and the 0.51M, however the stress endured following the 24 hours exposure time have considerably reduced the rate of Fe leaching to 32 % below the Control culture, 18 % below the average stressed cultures and required over 260 h of recovery time. Again here the shock brought by the extreme stress and long exposure time could not be contained and the microorganisms struggled to recover as seen in the long extended lag phase. The rate of Fe oxidation by these 0.68M acid stressed cultures dropped to 0.061 kg Fe $m^{-3}h^{-1}$ compare to the 0.074 kg Fe $m^{-3}h^{-1}$ and the 0.072 kg Fe $m^{-3}h^{-1}$ recorded for the same acid concentration stress following one hour and three hours exposure time.

Its microbial growth lag phase extended to over 259 h and before microbial oxidation could begin 260 h lag period were required. Its extent of metal solubilisation is greatly reduced from a 82 % when the 0.68M acid stressed culture was exposed to these extreme conditions for one hour to just 68% following the 24 hours exposure time; a drop of nearly 20%.

4.5.4 *Effect of exposure time of acid stress on mineral leaching*

When considering the exposure time to the various acid concentrations during the leaching of pyrite the Fe oxidation rates do not significantly vary from the Control to the different acid stressed cultures. The acid stressed cultures leaching rates are all below the Control leaching rate. A small decrease in the leaching rate was observed following acid stress and exposure time but was not significant to conclude a substantial compromise in the overall leaching ability of the stressed culture to leach the metal after the recovery time. The recovered cultures depict similar rates of microbial leaching with the exception of the 0.68M acid stressed culture following 24 hour exposure time that took a serious knock and dropped to a low 0.061 kg Fe $m^{-3}h^{-1}$. The major difference amongst the stressed culture was the lag period required for microbial growth and microbial leach reactions to initialise.

Figure 4.24 shows the calculated iron leaching rates following acid stresses and various exposure times emphasising one, three and 24 hours exposure time. The Control was conducted concurrently and was inoculated with unstressed cultures.

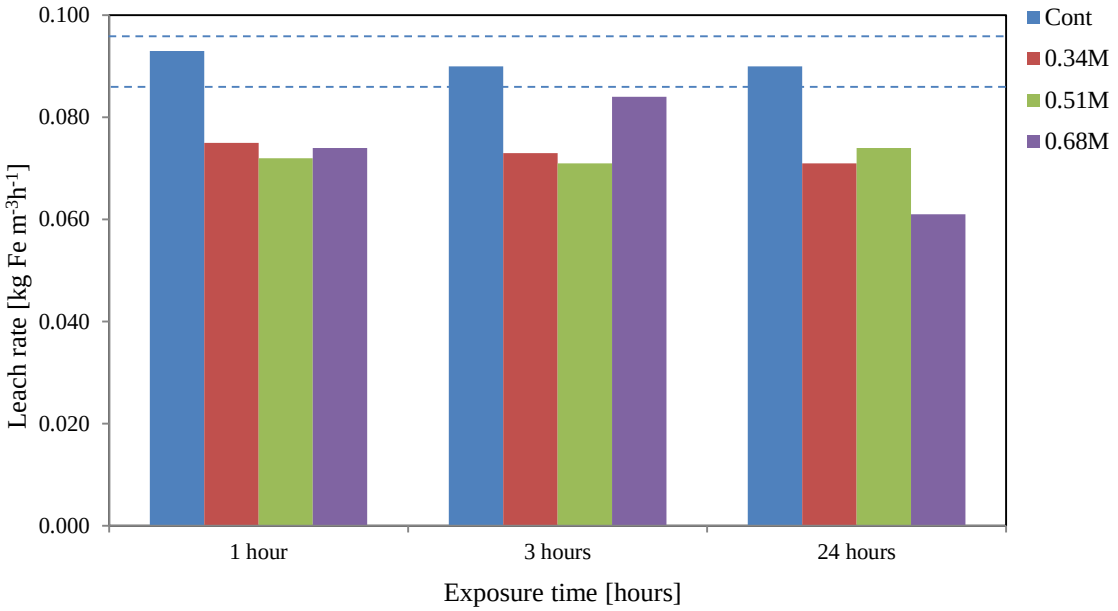


Figure 4-24: Calculated leaching rates following acid stress and exposure times with emphasising the exposure time. The two lines represent 5% error on the average rates of the Control cultures.

In the previous section it was observed that when placed under high acid concentration for one hour the culture lag for as long as 260 h but the rate stayed in the similar range to the other stressed cultures. This is also observed when the concentration of acid was 0.34M and 0.51M following three and 24 hours exposure time. The lag period before microbial leach was initiated for these acid stressed cultures (0.34M, 0.51M and 0.68M) extended from 91 to 137 h following one hour exposure time; from 167 to 182 h following three hours exposure time and from 158 to 260 h following 24 hours exposure time. The leaching rate stayed similar between 0.072 and 0.075 kg Fe $m^{-3}h^{-1}$ for the 0.34M, 0.51M and 0.68M following one hour exposure time. It also stayed similar between 0.071 and 0.073 kg Fe $m^{-3}h^{-1}$ for the 0.34M, 0.51M and 0.084 kg Fe $m^{-3}h^{-1}$ for the 0.68M following three hours exposure time. Only the 0.34M and the 0.51 M culture register similar leaching rate of 0.071 and 0.074 kg Fe $m^{-3}h^{-1}$ following 24 hours exposure time with the 0.68M only showing a leaching rate of 0.061 kg Fe $m^{-3}h^{-1}$. The extent of Fe solubilisation however is also gradually reduced across the exposure time. The recorded extends of solubilisation for the 0.34M, 0.51M and 0.68M acid stressed cultures were respectively 88, 88 and 82 % following one hour exposure time; 79, 76 and 77 % following three hours exposure time and 80, 77, 68 % following 24 hour exposure time.

Subjecting the cultures to the various acid concentrations for a period of 24 hours before being re-introduced to optimal operating conditions have affected negatively the culture subjected to the highest concentration of acid. The 0.68M acid stressed culture extended the microbial growth lag phase to 259 h and the microbial Fe oxidation to 260 h. The extent of Fe solubilisation when the experiment was stopped was just over half the total available Fe. The leaching rate was reduced to 0.061 kg Fe $m^{-3}h^{-1}$ compare to the 0.090 kg Fe $m^{-3}h^{-1}$ recorded by the Control culture and an average of 0.075 kg Fe $m^{-3}h^{-1}$ recorded for the rest of the stressed cultures. Both the 0.34M and the 0.68M acid stressed cultures displayed similar rate as they did in the one hour acid stress experiment. Their lag phases did not change significantly compare to the same stresses when exposed to three hours. The same was observed for their extent of Fe solubilisation.

An overall observation is that there is a considerable increase in recovery time necessary for the culture to initiate growth and microbial oxidation as the acid stress and exposure time increase with a resumption of normal leaching capability once recovered. Moreover, studies documented by Fagan et al. (2014) have shown that in drip irrigation, the lateral movement of the liquid increases with the bed depth through preferential flow. This in turn, impact on the leaching efficiency and the microbial colonisation of the ore.

4.5.5 *Microbial growth performance*

In the literature review, agglomeration was discussed extensively and the necessity of this application in the pre-treatment of mineral ore before it is stacked up into pile to further leach out the metal of interest was demonstrated. The bioleaching of copper from mineral such as chalcopyrite, chalcocite, covellite, enargite due to their low grade, is economically achieved using heap bioleaching. The agglomeration of these mineral ores is done with high concentration of sulphuric acid, to achieve strong binding of fine to coarse materials.

There are two ways of introducing the leaching microorganisms into these leaching operations. They could be added during the agglomeration process where the microorganisms community is added as the acid solution is added subjecting these microorganisms to spontaneous stresses lasting the entire time the heap is constructed until leaching solution is trickled through. However the amount of acid added varies due to the acid-neutralising capacity of the mineral. This stress can be assimilated to the 24 hours exposure time and longer stress hours than encountered in this study. The results show that the 24 hours exposure time and an acid concentration of 0.68M has extended the recovery time to 6 fold more that of a healthy unstressed culture (Control culture). However when the concentration was 0.34M or 0.51M and the exposure time was 24 hours, the recovery time was only increased 3 to 4 fold than that of the Control culture. This suggested that an early acid exposure, in this case, an inoculation of bioleaching microorganisms during the acid agglomeration will bring early stress to

these microorganisms and their recovery period will be prolonged since they will stay in the acid stress condition for a long period of time before metabolic conditions become favourable again. The leaching rate will not change regardless of the acid concentration used post recovery.

Figure 4.25 shows the summary of the calculated biomass yield in terms of microbial cells produced per kg iron oxidised as a function of the different acid concentrations. Yields data were calculated from inoculation to C_x max of the Control cultures.

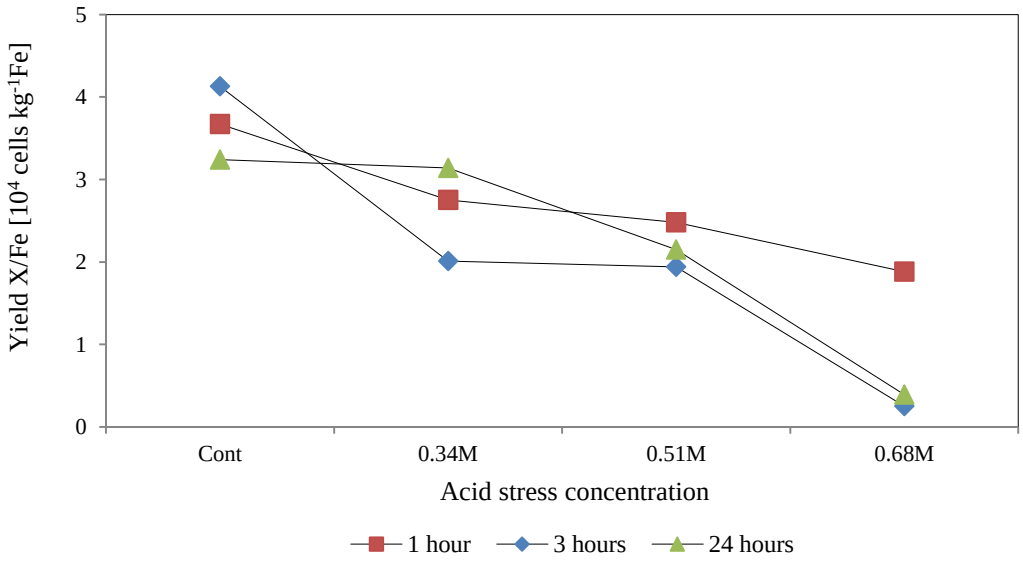


Figure 4-25: Biomass yield ($Y_{X/Fe}$) in terms of microbial cells produced per kg iron oxidised as a function of the different acid stress concentrations

Alternatively a heap operation is inoculated by dripping the microorganisms culture solution after the agglomerated ore has been stacked into a heap. Here the ore has been acid agglomerated and the medium pH is very low when microorganisms are introduced. It could be assimilated to the one and three hour acid stress encountered in this study since leaching solution is followed immediately after inoculation. The one and three hours acid stress experiment has demonstrated a slight increase in recovery time and initiation of microbial leach (2 fold that of the Control). Microorganisms will be shocked when inoculated but because the feed solution will be trickled soon after inoculation, conditions will quickly become favourable again for the microorganisms to recover and begin the leaching of the mineral.

The observation of the summarised biomass yield also indicates a substantial reduction in yield as the concentration of acid used to stress the culture increases until the recovery is complete and normal leaching rate resumes. In light of the above and with the observations of the experiment conducted the later inoculation method (drip inoculation) is recommended.

4.5.6 *Summary of the leaching performance*

The general observation of this work has shown little effect on the maximum specific growth rate following the time needed for recovery by the stressed microorganisms under all, except the most extreme, stress conditions. The iron leaching rate was decreased by a factor of 0.77 to 0.82 in all cases except the most extreme, following recovery. These recovery times were observed for both the growth of microorganisms and for the initiation of the ferrous oxidation. The lag time for the onset of microbial growth and ferrous oxidation were assessed in terms of time exposure and acid concentrations. The lag times were increased between 2.4 to 4.7 fold for microbial growth and 2.3 to 3.4 fold for ferrous oxidation following acid stress for one hour. Following the three hour stress, a 2.7 to 3.3 fold increase in lag time of microbial growth and 2.9 to 3.2 fold increase for ferrous oxidation were observed relative to the control. Similarly increases of 3.3 to 5.1 fold (microbial growth) and 3.0 to 4.8 fold (ferrous oxidation) were observed following the 24 hour stress. Equally the lag phases, when assessed in terms of acid concentrations and compared to the control cultures, are increased between 2.4 to 3.3 fold (microbial growth) and 2.3 to 3.0 fold (ferrous oxidation) when stressed with 0.34M acid; 3.1 to 3.8 fold (microbial growth) and 2.6 to 3.6 fold (ferrous oxidation) when stressed with 0.51M acid and 4.7 to 5.1 fold (microbial growth) and 3.4 to 4.8 fold (ferrous oxidation) when stressed with 0.68M acid over 1 to 24 hours.`

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The aim of this study was to investigate the effect of acid stress on a mixed mesophilic culture responsible for leaching sulphide mineral to gain a better understanding of the stresses endured by the leaching microorganisms. These effects were assessed as a function of the acid concentrations (and the resulting acidity) and of the exposure time to the various acid concentrations. In this study, a mixed mesophilic leaching culture was stressed using various acid stress levels and left exposed to them for different times before being re-suspended under optimal leaching conditions.

The effects of the stresses on the microbial growth and the leaching performance were investigated. Subsequently, the microbial speciation caused by the various acid stresses was elucidated. The overall findings of the study are consolidated in this chapter, summarised in Section 5.1 and 5.2 with ensuing key findings presented in Section 5.3 and the recommendations made in Section 5.4.

5.1 Effect of Acid Stress on Recovery Time, Microbial Activity and Leaching Rate

The effect of acid stress was investigated on the growth rate and leaching performance of the mixed mesophilic culture in the presence of 3% pyrite concentrate and by maintaining the physicochemical conditions constant while varying the acid concentrations (0.34M, 0.51M and 0.68M) to which the inoculum was exposed and its exposure time (one hour, three hour and 24 hour). The acid concentrations used to stress the microorganisms were selected to approximate the acid environment experienced during acid agglomeration.

A key observation of this study was the extension of the lag phase for microbial growth and in the observed initiation of the leaching rate when the acid stressed cultures were re-suspended in optimal conditions. The Control cultures exhibited a lag period of between 40 to 50 h before the onset microbial iron oxidation. These lag period were extended to between 90 and 140 h, 160 to 180 h and 160 and 260 h when the cultures were exposed to 0.38 M, 0.51M and 0.68M H_2SO_4 for one, three and 24 hour respectively. The metabolic activities were reduced during the recovery stage. The average yield calculated for the control cultures was 3.68×10^4 cells kg^{-1} Fe at the end of the exponential phase (C_x max). During the same interval from the onset of the microbial oxidation the yields were reduced to between 0.4 and 3×10^4 cells kg^{-1} Fe following stress. Upon adaptation of the cultures to the stress, no significant change was observed in terms of leaching rate and microbial growth rate during the exponential phase in the stressed cultures.

The extent of metal leaching over a defined period was reduced in the stressed cultures. The Control cultures were capable of leaching 96 % of the metal within 142 h. The stressed cultures were required

an extended time varying between 214 and 405 h to leach out between 68 and 88 % of the metal with the most stressed cultures needing a longer leaching time and releasing less metal into solution.

5.2 Effect of Acid Stress on Microbial Speciation

The effect of acid stress on microbial speciation was investigated at the end of the 24 hours stress experiment and during the course of the reproducibility experiment to assess the change in microbial species at different growth phases of the experiment.

L. ferriphilum was observed to be the dominant surviving species when the cultures were stressed. Accounting for 90% in the inocula, its concentration increased to 97, 98 then 99 % following stress of 0.34, 0.51 and 0.68M acid concentration respectively. *At. ferrooxidans, A. cupricumulans, Archaea* and *F. acidiphilum* accounted in the inoculum for just 10 % and were subsequently reduced to minimal or undetectable levels following stress, with 2 % recorded for *F. acidiphilum* following stress of 0.34 and 0.51M acid concentration, but subsequently becoming undetectable following stress of 0.68M acid concentration.

L. ferriphilum remained the dominant species throughout the cultivation of the stressed culture during the different growth stages. A key observation was that *F. acidiphilum*, initially detected at 3 % in the inoculum, increased to 8% at 70 h (initiation of exponential phase) following inoculation, suggesting the highest growth rate during this period. Subsequently it was reduced to 2% at 214 h. The result obtained at completion of all stressed cultures indicated *L. ferriphilum* (99%) to be the dominant species with just 1% of *F. acidiphilum*.

5.3 Summary of Key Findings

The key findings of this study in terms of the resulting effects of acid stress on the bioleaching performance, microbial activity and speciation are summarised as follows:

- Under all stress conditions used, the mixed mesophilic cultures showed a recovery. The recovery period was a function of the acid concentration and the exposure time to which the microorganisms were subjected. Initiation of microbial growth and bioleaching occurred after this recovery period.
- The growth rates of the Control cultures (unstressed) were all in the 5 % error margin necessary to validate the results of the experiment. Following adaptation from the stress, little change was observed in the instantaneous specific growth rate during the exponential phase.
- The stressed cultures showed a similar rate of mineral leaching following adaptation to the stress. The yield in terms of microbial cells produced per kg iron oxidised was reduced as the

acid concentration was increased but not necessarily with increased exposure time.

- The extent of iron solubilisation over a defined time period was reduced, thus extending the leach period.
- A decrease in the microbial diversity of the mixed mesophilic culture implicated in leaching was observed, demonstrating the robustness with respect to acid stress. *L. ferriphilum* remained the dominant species at all growth stages of the cultivation.
- The different speciation performed at different stages of the cultivation of a stressed culture had indicated that at exponential phase, only *At. ferrooxidans*, *Fe. acidiphilum* and *L. ferriphilum* survived the acid stress.

5.4 Recommendations For Further Study

Improved experimental conditions in the laboratory

- Acid stress effects should also be tested on simulated heap leaching experiments using agglomerated ore and assessed with both inoculations during agglomeration and after agglomeration.
- Acid concentration and exposure time should be increased to assess the extent of microbial recovery and acid tolerance levels of the bioleaching microorganisms.
- A similar experiment should be conducted using moderate thermophile and thermophile cultures since the microbial consortium in a heap operation varies from the mesophile microorganisms to the thermophiles (Tupikina *et al.*, 2013a).
- A conglomerate of a more defined mixed culture should be used to assess the acid resistant species as a preliminary experiment to determine the acid-tolerant microorganisms. These acid resistant species should be used and recommended.
- Microbial speciation as a mean of identifying surviving microbial species to different stresses should be used more rigorously throughout the experiments to determine clearly the effect of acid stress on the microbial community and provide a clear identification of the critical stages where microbial species are suppressed or where microbial species are encourage
- Physico-chemical conditions resulting from the acid agglomeration, such as shear stress, increased temperature, and increased concentrations of liberated metals, ions and conductivity should be assessed further.
- Further analysis using factorial design is recommended to be applied to add value to the data and substantiate the study. This will be done with additional work currently underway.

REFERENCES

1. Acevedo, F. (2000). The use of reactors in biomining processes: A review. *Biotechnology*, **3**(3), 184-194.
2. Afewu, K.I., Dixon, D.G. (2008). Calibrating a 3D axisymmetric water and solute transport model for heap leaching. In Hydrometallurgy, Proceedings of the Sixth International Symposium, C.A. Young, P.R. Taylor, C.G. Anderson and Y. Choi, (ed)., SME, Littleton, CO, pp. 1098–1109.
3. Africa, C.J., van Hille, R.P., Harrison, S.T.L. (2013). Attachment of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferriphilum* cultured under varying conditions to pyrite, chalcopyrite, low-grade ore and quartz in a packed column reactor. *Applied Microbiology and Biotechnology*, **97**, 1317-1324.
4. Bailey, A.D., Hansford, G.S. (1993). Factors affecting bio-oxidation of sulphide minerals at high concentrations of solids: A review. *Biotechnology and Bioengineering*, **42**(10), 1164-1174.
5. Bailey, J.E., Ollis, D.F. (1986). Biochemical Engineering Fundamentals. McGraw Hill, New York.
6. Baker, B.J., Banfield, J.F. (2003). Microbial communities in acid mine drainage. *FEMS Microbial Ecology*, **44**, 139-152.
7. Bartlett, R.W. (1992). Leaching and fluid recovery of materials. Solution Mining. Gordon & Breach Science Publishers.
8. Blancarte-Zurita, M.A., Branion, R.M.R., Lawrence, R.W. (1986). Particle size effects in the microbiological leaching of sulphide concentrates by *Thiobacillus ferrooxidans*. *Biotechnology and Bioengineering*, **28**, 751-755.
9. Bouffard, S.C. (2005). Review of agglomeration practice and fundamentals in heap leaching. *Mineral Processing and Extractive Metallurgy Review*, **26**, 233-294.
10. Breed, A.W., Dempers, C.J.N., Searby, G.E., Gardner, M.N., Rawlings, D.E., Hansford, G.S. (1999). The effect of temperature on the continuous ferrous iron oxidation kinetics of a predominantly *Leptospirillum ferrooxidans* culture. *Biotechnology and Bioengineering*, **65**, 44-53.
11. Breed, A.W., Hansford, G.S. (1999). Effect of pH on ferrous iron oxidation kinetics of *Leptospirillum ferrooxidans* in continuous culture. *Biochemical Engineering Journal*, **3**, 193-201.
12. Brierley, C.L. (2001). Bacterial succession in bioheap leaching. *Hydrometallurgy*, **59**, 249-255.

13. Bromfield, L., Africa, C.-J., Harrison, S.T.L., van Hille, R.P. (2011). The effect of temperature and culture history on the attachment of *Metallosphaera hakonensis* to mineral sulphides with application to heap bioleaching. *Minerals Engineering*, **24**, 1157-1165.

14. Bryan, C. G., Davis-Belmar, C. S., van Wyk, N., Fraser, M. K., Dew, D. W., Rautenbach, G. F. and Harrison, S. T. L., 2012. The effect of CO_2 availability on the growth, iron oxidation and CO_2-fixation rates of pure cultures of *Leptospirillum ferriphilum* and *Acidithiobacillus ferrooxidans*. *Biotechnology and Bioengineering*, **109**, 1693-1703.

15. Bryan, C.G., Jones, G.C., van Wyk, N., Tupikina, O.V., van Hille, P., Harrison, S.T.L. Development and critical evaluation of techniques for the recovery and analysis of sessile and planktonic microbial communities involved in heap bioleaching. Unpublished data.

16. Chamberlin, P.D. (1980). Heap leaching and pilot testing of gold and silver ore. In: Precious Metals Symposiums, Sparks, Nevada, November 17-19, 77-83.

17. Chiume, R., Minnaar, S.H., Ngoma, I.E., Bryan, C.G., Harrison, S.T.L. (2012). Microbial colonisation in heaps for mineral bioleaching and the influence of irrigation rate. *Minerals Engineering*, **39**, 156-164.

18. Clark, D.A., Norris, P.R. (1996). *Acidimicrobium ferrooxidans* gen. nov. sp. nov.: mixed culture ferrous iron oxidation with *Sulfobacillus* species. *Microbiology*, **141**, 785-790.

19. Coram, N.J., Rawlings, D.E. (2002). Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. no. dominates in South African commercial biooxidation tanks that operate at 40^{o}C. *Applied Environmental Microbiology*, **68**, 838-845.

20. Córdoba, E.M., Muñoz, J.A., Blázquez, M.L., González, F., Ballester, A. (2008). Leaching of chalcopyrite with ferric iron. Part II: Effect of redox potential. *Hydrometallurgy*, **93**, 88-96.

21. Dempers, C.J.N., Breed, A.W., Hansford, G.S. (2003). The kinetics of ferrous iron oxidation by *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*; effect of cell maintenance. *Biochemical Engineering Journal*, **16**, 337-346.

22. Devasia, P., Natarajan K.A., Sathyanarayana D.N., Rao, G.R. (1993). Surface chemistry of *Thiobacillus ferrooxidans* relevant to adhesion to mineral surfaces. *Applied & Environmental Microbiology*, **59** (12), 4051-4055.

23. Dew, D.W., Rautenbach, G.F., van Hille, R.P., Davis-Belmar, C.S. (2011). High temperature heap leaching of chalcopyrite: Method of evaluation and process model validation. The Southern African Institute of Mining and Metallurgy. Percolation Leaching: The Status globally and in Southern Africa 2011, 201-220.

24. Dhawan, N., Safarzadeh, M.S., Miller, J.D., Rajamani, R.K., Moats, M. (2012). Insights into

heap leaching. SME Annual Meeting – Feb 19 – 22, 2012, Seattle, Washington. Preprint 12 - 112.

25. Du Plessis C.A., Batty, J.D., Dew, D.W. (2007). Commercial applications of thermophile bioleaching. In: Biomining, Rawlings D.E. and Johnson D.B. (ed), Springer-Verlag, pp 57-78.
26. Escobar, B., Lazo, D. (2003). Activation of bacteria in agglomerated ores by changing the composition of the leaching solution. *Hydrometallurgy*, **71**, 173-178.
27. Fagan, M.A., Ngoma, I.E., Chiume, R.A., Minnaar, S.H., Sederman, A.J., Johns, M.L., Harrison, S.T.L. (2014). MRI and gravimetric studies of hydrology in drip irrigated heaps and its effect on the propagation of bioleaching microorganisms. *Hydrometallurgy*, in press.
28. Franzmann, P.D., Haddad, C.M., Hawkes, R.B., Robertson, W.J., Plumb, J.J. (2005). Effects of temperature on the rates of iron and sulphur oxidation by selected bioleaching bacteria and archaea: Application of the Ratkowsky equation. *Mineral Engineering*, **18**, 1304-1314.
29. Gericke, M., Pinches, A. (1999). Bioleaching of copper sulphide concentrate using extremely thermophilic bacteria. *Mineral Engineering*, **12**, 893-904.
30. Goebel, B.M., Norris, P.R., Burton, N.P. (2000). Acidophiles in Biomining. In: Applied Microbial Systematics, Priest, F.G., Goodfellow, M. (ed). 293-314. Kluwer, Dordrecht.
31. Golovacheva, R.S., Golyshina, O.V., Karavaiko, G.I., Dorofeev, A.G., Pivovarova, T.A., Chernykh, N.A. (1993). A new iron-oxidizing bacterium, *Leptospirillum thermoferrooxidans* sp. nov. *Microbiology*, **61**, 744-750.
32. Golovacheva, R.S., Karavaiko, G.I. (1979). A new genus of thermophilic spore-forming bacteria, *Sulfobacillus*. *Microbiology*, **48**, 658-665.
33. Govender, E., Bryan, C.G., Harrison, S.T.L. (2013). Quantification of growth and colonisation of low grade sulphidic ores by acidophilic chemoautotrophs using a novel experimental system. *Minerals Engineering*, **48**, 108-115.
34. Halinen, A.K., Rahunen, N., Kaksonen, A.H., Puhakka, J.A. (2009). Heap bioleaching of complex sulphide ore: Part II. Effect of temperature on base metal extraction and bacterial compositions. *Hydrometallurgy*, **98**, 101-107.
35. Hallberg, K.B., Johnson, D.B. (2001). Biodiversity of acidophilic prokaryotes. *Advances in Applied Microbiology*, **49**, 37-84.
36. Han, C.J., Kelly, R.M. (1998). Biooxidation capacity of the extremely thermophilic archaeon *Metallosphaera sedula* under bioenergetics challenge. *Biotechnology and Bioengineering*, **58**, 617-624.
37. Harrison, A.P.Jr, Norris, P.R. (1985). *Leptospirillum ferrooxidans* and similar bacteria: some characteristics and genomic diversity. *FEMS Microbiology Letters*, **30**, 99-102.

38. Harrison, S.T.L. (2012). Microbial attachment, colonisation and activity as key steps in establishing the desired microbial community for attaining a well-functioning heap. Keynote lecture at the 6th International Symposium on Biohydrometallurgy (Biohydromet'12), June 18-20, 2012. Falmouth, Cornwall, United Kingdom.

39. Harrison, S.T.L., Coram-Uliana, N.J., Ntshabele, K., van Hille, R.P. (2007). The effect of multiple stresses on *Sulfolobus*-like cultures in bioleaching systems: super-imposing stress responses to metal concentration and solids loading. 16th International Biohydrometallurgy Symposium 2007, Frankfurt, Germany, Sept 2007.

40. Harrison, S.T.L., Sissing, A., Raja, S., Pearce, S.J.A., Lamaignere, V., Nemati, M. (2003a). Solids loading in the bioleach slurry reactor: mechanisms through which particulate parameters influence slurry bioreactor performance. 15th International Biohydrometallurgy Symposium 2003, Athens, Hellas, Sept 2003.

41. Harrison, S.T.L., Sissing, A.A., Raja, S., Nemati, M. (2003b). Identifying and quantifying biological responses of *Sulfolobus* to high pulp densities in the slurry bioleach reactor. Proceedings of International Mineral Processing Conference (ed. Lorenzen, Bradshaw and Thom).

42. Hawkes, R.B., Franzmann, P.D., Plumb, J.J. (2004). Microbiology of an industrial-scale chalcocite heap bioleaching operation. Proceedings of the Bac-Min Conference 8th-10th November 2004 Bendigo, Australia, Victoria. Australasian Institute of Mining and Metallurgy, 11-17.

43. High Pure PCR Template Preparation Kit Version 16.0 Booklet, 12-14. Available at: https://www.roche-applied-science.com/shop/home. [Accessed 12 March 2013].

44. Historical Copper Prices and Price Chart – InvestmentMine. 2014. *Historical Copper Prices and Price Chart – InvestmentMine*. [ONLINE] Available at: http://www.infomine.com/investment/metal-prices/copper/all/. [Accessed 09 October 2014].

45. Horváth, I., Glatz, A., Varvasovszki, V., Török, Z., Páli, T., Balogh, G., Kovács, E., Nádasdi, L., Benkö, S., Joó, F., Vigh, L. (1998). Membrane physical state controls the signalling mechanism of the heat shock response in *Synechocystis* PCC 6803: Identification of *hsp17* as a "fluidity gene". Proceedings of the National Academy of Sciences, **95**, 3513-3518.

46. Ingledew, W.J. (1982). *Thiobacillus ferrooxidans*: the bioenergetics of an acidophilic chemolithotroph. *Biochimica et Biophysica Acta*, **683**, 89-117.

47. Johnson, D.B. (1998). Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbial Review*, **27**, 307-317.

48. Jones, G., van Hille, R.P., Harrison, S.T.L. (2009). Sulphide mineral induced oxidative stress as a limiting factor in tank bioleaching performance. *Advanced Materials Research*, **71-73**,

365-368.

49. Kappes, D.W. (2005). Heap leaching of gold and silver ores. In: Development in Mineral Processing, Vol 15, Adams, M.P. (ed). Chapter 19, 456-478. Elsevier.
50. Karavaiko, G.I., Dubinina, G.A., Kondratieva, T F. (2006). Lithotrophic microorganisms of the oxidative cycles of sulphur and iron. *Microbiology*, **75**(5), 512-545.
51. Karavaiko, G.I., Tourova, T.P., Kondrat'eva, T.F., Lysenko, A.M., Kolganova, T.V., Ageeva, S.N., Muntyan, L.N., Pivovarova, T.A. (2003). Phylogenetic heterogeneity of the species *Acidithiobacillus ferrooxidans*. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 113-119.
52. Kodali, P., Depci, T., Dhawan, N., Wang, X., Lin, C.L., Miller, J.D. (2011). Evaluation of stucco binder for agglomeration in the heap leaching of copper ore. *Minerals Engineering*, **24**(11), 886-893.
53. Komadel, P., Stucki, J.W. (1988). Quantitative assay of minerals for Fe^{2+} and Fe^{3+} using 1,10 phenantroline: III. A rapid photochemical method. *Clays and Clay Minerals*, **36**(4), 379-381.
54. Konishi, Y., Yoshida, S., Asai, S. (1995). Bioleaching of pyrite by acidophilic thermophile *Acidianus brierleyi*. *Biotechnology and Bioengineering*, **48**, 592-600.
55. Lempe, J., Lachowiec, J., Sullivan, A.M., Queitsch, C. (2013). Molecular mechanisms of robustness in plants. *Current Opinion in Plant Biology*, **16**, 62-69.
56. Lewandowski, K.A., Kawatra, S.K. (2009). Binders for heap leaching agglomeration. *Minerals and Metallurgical Processing*, **26**(1), 1-24.
57. Liu, C.Q., Plumb, J., Hendry, P. (2006). Rapid specific detection and quantification of bacteria and archaea involved in mineral sulphide bioleaching using real-time PCR. *Biotechnology and Bioengineering*, **94**(2), 330-336.
58. Lizama, H.M. (2001). Copper bioleaching behaviour in an aerated heap. *International Journal of Mineral Processing*, **62**, 257-269.
59. Lizama, H.M., Harlamovs, J.R., McKay, D.J., Dai, Z. (2005). Heap leaching kinetics are proportional to the irrigation rate divided by heap height. *Minerals Engineering*, **18**, 623-630.
60. Macario, A.J.L., Lange, M., Ahring, B.K., Conway de Macario, E. 1999. Stress genes and proteins in archaea. *Microbiology and Molecular Biology Reviews* **63**, 923-967.
61. Markosyan, G.E. (1972). A new acidophilic iron bacterium, *Leptospirillum ferrooxidans*. *Biologicheskii Zhurnal Armenii* **25**, 26-29.
62. Masel, J., Siegal, M.L. (2009). Robustness: mechanisms and consequences. *Trends in Genetics*, **25**, 395-403.
63. McClelland, G.E. (1988). Testing of ore. In: Introduction to Evaluation, Design and

Operation of Precious Metal Heap Leaching Projects, van Zyl, D., Hutchison, I.A., Kiel, J.E. (eds), SME, Littleton, CO, 61-67.

64. Meruane, G., Vargas, T. (2003). Bacterial oxidation of ferrous iron by *Acidithiobacillus ferrooxidans* in the pH range 2.5-7.0. *Hydrometallurgy*, **71**, 149-158.
65. Minnaar S., Tupikana O.V., van Hille R.P., van Wyk N., Janse van Rensberg S. Harrison S.T.L. (2010). Microbial growth rates on mesophilic acidophiles on low grade whole ore containing chalcopyrite. In Proceedings of Bio & Hydrometallurgy Conference, Cape Town, South Africa.
66. Moats, M.S., Janwong, A. (2008). The art and science of crushed ore agglomeration for heap leaching. In: Proceedings of Hydrometallurgy 2008, Young, C., Taylor, P., Anderson, C., Choi, Y. (ed) – 6th International Symposium, August 17-21, Phoenix, Arizona, 912-917.
67. Monod, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology*, **3**, 371-394.
68. Muira, Y., Hiraiwa, M.N., Ito, T., Watanabe, Y., Okabe, S. (2007). Bacterial community structures in MBRs treating municipal wastewater: relationship between community stability and reactor performance. *Water Research*, **41**, 627-637.
69. Ndlovu, S. (2008). Biohydrometallurgy for sustainable development in the African minerals industry. *Hydrometallurgy*, **97**, 20-27.
70. Nemati, M., Harrison, S.T.L. (2000). Effect of solid loading on thermophilic bioleaching of sulphide minerals. *Chemical Technology and Biotechnology*, **75**, 526-532.
71. Nemati, M., Harrison, S.T.L., Hansford, G.S., Webb, C. (1998). Biological oxidation of ferrous sulphate by *Thiobacillus ferrooxidans*: a review on kinetic aspects. *Biochemical Engineering*, **1**, 171-190.
72. Nemati, M., Lowenadler, J., Harrison, S.T.L. (2000). Particle size effects in bioleaching of pyrite by acidophilic thermophile *Sulfolobus metallicus* (BC). *Applied Microbiology and Biotechnolgy*, **53**, 173-179.
73. Ngoma, I.E., Ojumu, T.V., Harrison, S.T.L. (2014). Investigating the effect of acid stress on selected mesophilic bioleaching microorganisms. *Minerals Engineering* (Submitted).
74. Norris, P.R. (1983). Iron and mineral oxidation studies with *Leptospirillum*-like bacteria. In: Recent Progress in Biohydrometallurgy, Rossi, G., Torma, A.E. (ed). 83-96. Associasione Mineraria Sarda, Iglesias.
75. Norris, P.R. (1990). Acidophilic bacteria and their activity in mineral sulphide oxidation. In: Microbial Mineral Recovery, Ehrlich, H.L., Brierley, C.L. (ed). 3.27. McGraw-Hill,, New York.

76. Norris, P.R. (1997). Thermophiles in bioleaching. In: Biomining, Rawling, D.E. (ed). 247-258. Springer, Berlin Heidelberg New York.

77. Norris, P.R. (2007). Acidophile diversity in mineral sulphide oxidation. In: Biomining, Rawlings, D.E., Johnson, D.B. (ed). 199-216. Springer-Verlag Berlin Heidelberg.

78. Norris, P.R., Johnson, D.B. (1998). Acidophillic microorganisms. In: Extremophiles: Microbial Life in Extreme Environments, Horikoshi, K., Grant, W.D. (ed). 133-154. Wiley, New York.

79. O'Kane Consultants Inc. (2000). Demonstration of the application of unsaturated zone hydrology for heap leach optimization. Industrial Research Assistance Program Contract # 332407, Report No. 628-1.

80. Ojumu, T.V., Petersen, J. (2011). The kinetics of ferrous iron oxidation by *Leptospirillum ferriphilum* in continuous culture: The effect of pH. *Hydrometallurgy*, **106**, 5-11.

81. Ojumu, T.V., Petersen, J., Searby, G.E., Hansford, G.S. (2006). A review of rate equations proposed for microbial ferrous iron oxidation with a view to application to heap bioleaching. *Hydrometallurgy*, **83**, 21-28.

82. Ongendangenda, H.N., Ojumu, T.V. (2011). The effect of initial pH on the kinetics of ferrous iron biooxidation at low temperature. *African Journal of Biotechnology*, **10**, 1679-1683.

83. Oyekola, O.O., van Hille, R.P., Harrison, S.T.L. (2007). Effect of sulphate concentration on the community structure and activity of sulphate reducing bacteria. *Advanced Materials Research*, **20-21**, 513-515.

84. Özkaya, B., Sahinkaya, E., Nurmi, P., Kaksonen, A.H., Puhakka, J.A. (2007). Kinetics of iron oxidation by *Leptospirillum ferriphilum* dominated culture at pH below one. *Biotechnology and Bioengineering*, **97** (5), 1121-1127.

85. Penev, K., Karamanev, D. (2010). Batch kinetics of ferrous iron oxidation by *Leptospirillum ferriphilum* at moderate to high total iron concentration. *Biochemical Engineering*, **50** (1-2), 54-62.

86. Petersen, J., Dixon, D.G. (2003). The dynamics of chalcocite heap bioleaching. In: Hydrometallurgy 2003, Proceedings of the 5th International Symposium, Young, C.A., Alfantazi, A.M., Anderson, C.G., Dreisinger, D.B., Harris, B., and James, A. (Ed), 351-364.

87. Petersen, J., Dixon, D.G. (2007a). Principles, mechanisms and dynamics of chalcocite heap bioleaching, In: Microbial Processing of Metal Sulphides, Donati, D.E., Sand, W. (ed), 193-218. Springer.

88. Petersen, J., Dixon, D.G. (2007b). Modelling and optimization of heap bioleach processes. In: Biomining, Rawlings D.E., Johnson D.B. (Ed). 153-175. Springer-Verlag.

89. Petersen, J., Minnaar, S.H., du Plessis, C.A. (2010). Carbon dioxide and oxygen consumption during the bioleaching of copper ore in a large isothermal column. *Hydrometallurgy*, 104, 356-362.

90. Pirt, S.J. (1975). Principles of Microorganism and Cell Cultivation. Blackwell Scientific Publications, Oxford.

91. Plumb, J.J., McSweeney, N.J., Franzmann, P.D. (2008a). Growth and activity of pure and mixed bioleaching strains on low grade chalcopyrite ore. *Minerals Engineering*, **21**, 93-99.

92. Plumb, J.J., Muddle, R., Franzmann, P.D. (2008b). Effect of pH on rates of iron and sulphur oxidation by bioleaching organisms. *Minerals Engineering*, **21**, 76-82.

93. Pradhan N., Nathsarma, K.C., Srinivasa Rao, K., Sukla, L.B., Mishra, B.K. (2008). Bacterial heap bioleaching of chalcopyrite: A review. *Minerals Engineering*, **21**, 355-365.

94. Purkiss, S., Anthony, R. (2004). Heap leaching base metals from oxide ores. International Patent. No. WO/2004/031422 A1. International Publication Date. 15 April 2004.

95. Raja, S.B. (2005). The effect of particulate-induced hydrodynamic stress on the bioleaching of chalcopyrite by a *Sulfolobus* sp. PhD thesis, University of Cape Town.

96. Rawlings, D.E. (2002). Heavy metal mining using microorganisms. *Microbiology*, **56**, 65-91.

97. Rawlings, D.E., Dew, D., du Plessis, C. (2003). Biomineralization of metal-containing ores and concentrates. *TRENDS in Biotechnology*, **21**, 38-44.

98. Rawlings, D.E., Johnson, D.B. (2007). Review: The microbiology of biomining: development and optimization of mineral-oxidizing microbial consortia. *Microbiology*, **153**, 315-324.

99. Rockhold, M.L., Yarwood, R.R., Niemet, M. R., Bottomley, P.J., Selker, J.S. (2002). Review: Considerations for modelling bacterial-induced changes in hydraulic properties of variably saturated porous media. *Advances in Water Resources*, **25**, 477-495.

100. Rodriguez, Y., Ballester, A., Blazquez, M.L., Gonzalez, F., Munoz, J.A. (2003). Study of bacterial attachment during the bioleaching of pyrite, chalcopyrite and sphalerite. *Geomicrobiology Journal*, **20**, 131-141.

101. Rodríguez, Y., Ballester, A., Blázquez, M.L., González, F., Muñoz, J.A. (2003). New information on the chalcopyrite bioleaching mechanism at low and high temperature. *Hydrometallurgy*, **71**, 47-56.

102. Rohwerder, T., Sand, W. (2007). Mechanisms and biochemical fundamentals of bacterial metal sulphide oxidation, In: Microbial Processing of Metal sulphides, Donati, D.E., Sand, W. (ed), 193-218. Springer.

103. Rossi G. (1990). Biohydrometallurgy, McGraw-Hill, Hamburg

104. Roychoudhury, A.N. (2004). Sulphate respiration in extreme environments: A kinetic study.

Geomicrobiology, **21**, 33-43.

105. Sand, S., Gehrke, T. (2006). Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. *Research in Microbiology*, **157**, 49-56.

106. Sand, W., Gehrke T., Jozsa P.G., Schippers A. (2001). (Bio)chemistry of bacterial leaching – direct vs. indirect bioleaching. *Hydrometallurgy*, **59**, 159-175.

107. Schippers, A. (2007) Microorganisms involved in bioleaching, In: Microbial Processing of Metal Sulphides, Donati, D.E., Sand, W. (ed), 193-218. Springer.

108. Schippers, A., Jozsa, P-G., Sand, W. (1996). Sulphur chemistry in bacterial leaching of pyrite. *Applied and Environmental Microbiology*, **62**, 3424-3431.

109. Schlegel, H.G. (1993). General Microbiology, 7th Edition. University Press, Cambridge, UK.

110. Schlitt, W.J. (1992). Solution mining: surface techniques. In: SME Mining Engineering Handbook, Hartman, H.I. (ed). 1478-1492. Society for Mining, Metallurgy and Exploration, Inc.

111. Shuler, L., Kargi, F. (1992). Bioprocess Engineering – Basic Concepts. Prentice-Hall, pp. 232-248.

112. Simmons, S., Norris, P.R. (2002). Acidophiles of saline water at thermal vents of Vulcano, Italy. *Extremophiles*, **6**, 201-207.

113. Sissing, A. (2002). An investigation into the effect of solid particulate phase on the bioleaching performance of *Sulfolobus metallicus*. MSc (Eng) dissertation, University of Cape Town.

114. Sissing, A., Harrison, S.T.L. (2003). Thermophilic mineral bioleaching performance: A compromise between maximizing mineral loading and maximizing microbial growth and activity. *South African Institute of Mining and Metallurgy*, **103**, 139-142.

115. Stott, M.B., Sutton, D.C., Watling, H.R., Franzmann, P.D. (2003). Comparative leaching of chalcopyrite by selected acidophilic bacteria and archaea. *Geomicrobiology*, **20**, 215-230.

116. Tibbals, R.L. (1987). Agglomeration practice in the treatment of precious metal ores. In: Proceedings of the International Symposium on Gold Metallurgy, vol 1. Winnipeg, Canada, August 23-26. 77-86. Pergamon Press, New York.

117. Tributsch, H. (2001). Direct versus indirect bioleaching. *Hydrometallurgy*, **59**, 177 – 185.

118. Tupikina, O.V., Minnaar, S.H., Rautenbach, F.G., Dew, D.W., Harrison, S.T.L. (2014). Effect of inoculum size on the rates of whole ore colonisation of mesophilic, moderate thermophilic and thermophilic acidophiles. *Hydrometallurgy* **149**, 244 - 251.

119. Tupikina, O.V., Minnaar, S.H., van Hille, R.P., van Wyk, N., Rautenbach, G.F., Dew, D.,

Harrison, S.T.L. (2013). Determining the effect of acid stress on the persistence and growth of thermophilic microbial species after mesophilic colonisation of low grade ore in a heap leach environment. *Minerals Engineering* 53, 152-159.

120. Tupikina, O.V., Ngoma, I.E., Minnaar, S., Harrison, S.T.L. (2011). Some aspects of the effect of pH and acid stress in heap bioleaching. *Minerals Engineering*, **24**, 1209-1214.
121. Tyson, G.W., Lo, I., Baker, B.J., Allen, E.E., Hugenholtz, P., Banfield, J.F. (2005). Genome-directed isolation of key nitrogen fixer *Leptospirillum ferrodiazotrophum* sp. Nov. from an acidophilic microbial community. *Applied Environmental Microbiology*, **71**, 6319-6324.
122. van Aswegen, P.C., van Niekerk, J., Olivier, W. (2007). The BIOX™ process for the treatment of refractory gold concentrates. In: Biomining, Rawlings, D.E., Johnson, D.B. (ed), Springer, Berlin, 425-433.
123. van Hille, R.P., van Wyk, N., Froneman, T., Harrison, S.T.L. (2013). Dynamic evolution of the microbial community in BIOX leaching tanks. *Advanced Materials Research*, **825**, 331-334.
124. van Hille, R.P., van Zyl, A.W., Spurr, N.R.L., Harrison, S.T.L. (2010). Investigating heap bioleaching: Effect of feed iron concentration on bioleaching performance. *Minerals Engineering*, **23**, 518-525.
125. Van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Zehnder, A.J.B. (1990). Influence of interfaces on microbial activity. *Microbiological Reviews*, **54**(1), 75-87.
126. Viera, M., Pogliani, C., Donati, E. (2007). Recovery of zinc, nickel, cobalt and other metals by bioleaching. In: Microbial processing of metal sulphides, Donati, D.E., Sand, W. (ed), 193-218. Springer.
127. Vigh, L., Maresca, B., Harwood, J.L. (1998). Does the membrane's physical state control the expression of heat shock and other genes? *Trends in Biochemical Sciences*, **23**(10), 369-374.
128. Watling, H.R. (2006). The bioleaching of sulphide minerals with emphasis on copper sulphides: A review. *Hydrometallurgy*, **84**, 81-108.
129. Watling, H.R., Collinson, D.M., Shiers, D.W., Bryan, C.G., Watkin, E.L.J. (2013). Effects of pH, temperature and solids loading on microbial community structure during batch culture on a polymetallic ore. *Minerals Engineering*, **48**, 68-76.
130. Whitacre, J.M. (2012). Biological robustness: paradigms, mechanisms, and systems principles. *Frontiers in Genetics*, **3**(67), 1-15.
131. Zhang, R., Wei, M., Ji, H., Chen, X., Qui, G., Zhou, H. (2009). Application of real-time PCR to monitor population dynamics of defined mixed cultures of moderate thermophiles involved in bioleaching of chalcopyrite. *Applied Microbiology and Biotechnology*, **81**, 1161-1168.

APPENDICES

Appendix A – Culture Stock and Media Preparation

Norris Media

The media solution used for microbial stock sub-culturing and throughout all the experiment was the "Norris medium" (Clark and Norris, 1996) and was prepared with the following composition:

Table A.1: Media composition

Chemical Compound	Composition
Deionised H_2O	5 L
Conc. H_2SO_4	Adjust to pH 2.50
$MgSO_4.7H_2O$	0.50 $g.L^{-1}$
$(NH_4)_2SO_4$	0.40 $g.L^{-1}$
KH_2PO_4	0.20 $g.L^{-1}$
KCl	0.10 $g.L^{-1}$
Yeast extract	0.15 $g.L^{-1}$

All the salts were dissolved in deionised water. The solution was then supplemented with yeast extract and the pH was adjusted to pH 2.50 using concentrated H_2SO_4. The prepared media was then autoclaved at 120°C, under pressure of 1 bar (gauge) for 30 min.

Microbial Cultures

A mixed mesophilic culture was used (UCT stock) containing *At. ferrooxidans, A. cupricumulans, Archaea, Fe. acidiphilum,* and predominantly *L. ferriphilum,* confirmed by qPCR and grown on pyrite concentrate in a 1 litre batch stirred tank reactor at 35°C and agitation rate of 550 rpm at solid loading of 0.75 %. The reactor was sub-cultured once weekly to ensure activity, by removing approximately 150 mL slurry and re-filling the volume up to the 1 L mark with fresh Norris media and 3.5g of pyrite concentrate.

Appendix B – Size Distribution of Pyrite

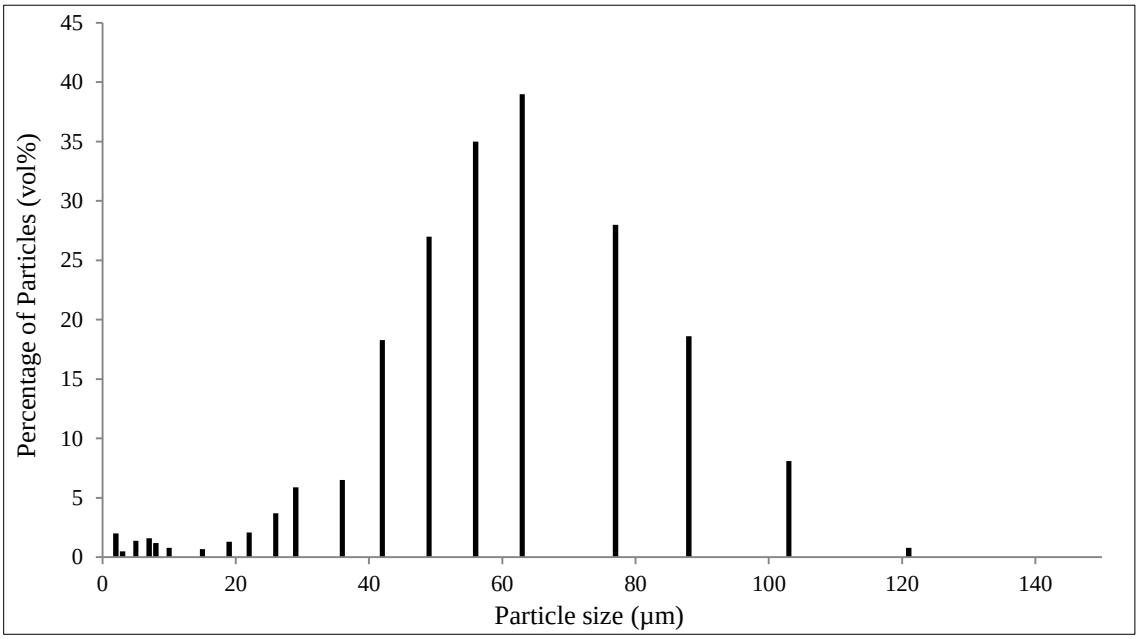


Figure B.1: Particle size distribution of pyrite

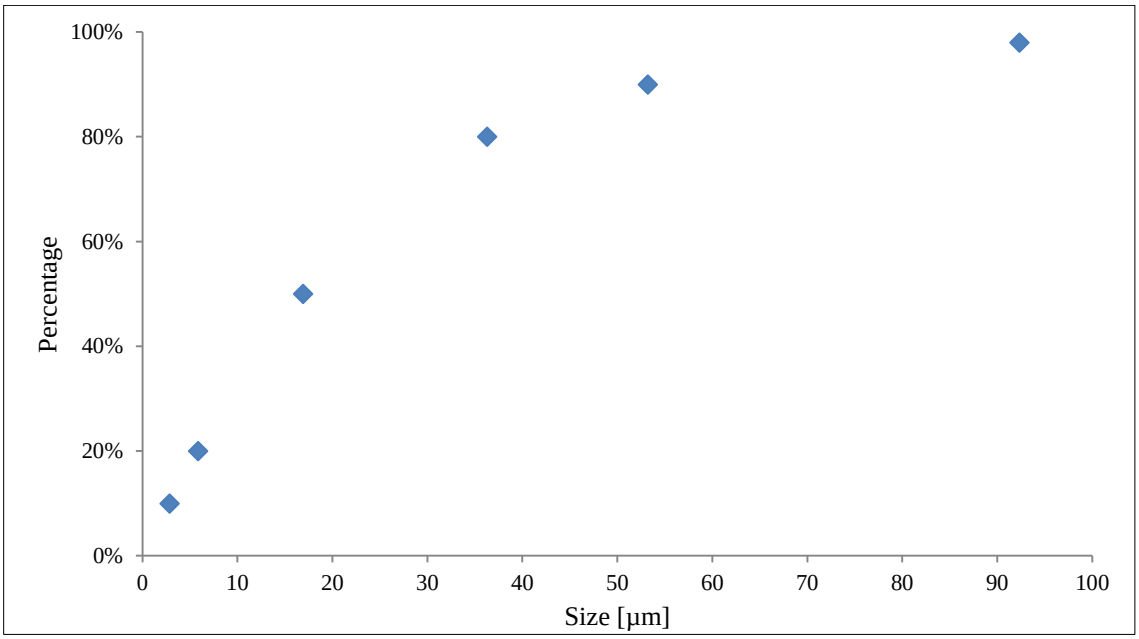


Figure B.2: Cumulative particle size distribution of pyrite, analysed using the Malvern Particle Size Analyser

Appendix C – Analytical methods

Microscopic Cell Counting Method

Cell counts were done using a Thoma Counting Chamber under an Olympus BX40 Microscope. The microscopic analysis of the daily sample was done after it was allowed to settle for two hours to remove the fine particles capable of interfering with microbial suspension.

Formulae for calculation cell concentration:

$$Volume\_of\_1\_small\_square\_[mm^3] = depth \times area$$

$$Cell\_concentration\_[cells/ml] = \frac{cells\_counted \times \frac{total\_no\_of\_big\_squares\_(16)}{no\_of\_squares\_counted\_(4)}}{volume\_of\_1\_small\_square \times total\_no.\_of\_small\_squares} * \frac{1}{d}$$

d = dilution ratio

Ferrous and Total Iron Concentration via Spectrophotometry

The protocol presented here-in was obtained from the CeBER database of analytical laboratory methods. The ferrous and total iron concentrations of the collected samples were determined colormetrically using the 1-10 phenanthroline method developed by Komadel and Stucki (1988). The reagents were prepared as follows:

1. A fresh stock of ferrous iron solution at 1000 ppm was used as the standard. It was obtained from Merck (cat no: SAAR3255000KF)
2. An ammonium acetate buffer solution is made by dissolving 250 g of ammonium acetate ($NH_4C_2H_3O_2$) in 150 mL of distilled water, followed by the addition of 700 mL of concentrated glacial acetic acid.
3. To make the 1-10 phenanthroline indicator solution, 2127.7 mg of 1-10 phenanthroline ($C_{12}H_8N_2.H_2O$) was dissolved in 100 mL of distilled water in a 1000 mL. volumetric flask. The solution was diluted with distilled water to 1000 mL, providing a concentration in excess of the stoichiometric requirements.
4. To obtain the total iron concentration, a spatula tip of hydroxylamine was added to each sample to convert all iron present to the ferrous form, after the ferrous assay was performed.

Standard curves of the relationship between ferrous concentration and absorbance were constructed by adding 2 mL aliquot of acetate buffer, followed by 2 mL of 1-10 phenanthroline solution into test-

tubes. The standard ferrous iron stock solution was diluted to provide samples of 0, 6.25, 12.5, 25 and 50 ppm. From these, 1 mL was pipetted into the respective test-tubes. On addition of samples containing ferrous iron, solution reacted to form an orange-red colour. The concentration of 0 ppm was made up of pure Millipore water, and was required to zero the spectrophotometer. The absorbance maximum was determined through a scan of a ferrous sample and confirmed to be 510 nm. The standard curves generated are presented in Figures B1 and B2. Note that the concentrated daily collected samples should be diluted to determine the concentration from the standard curves, i.e $A_{510} < 2$ results.

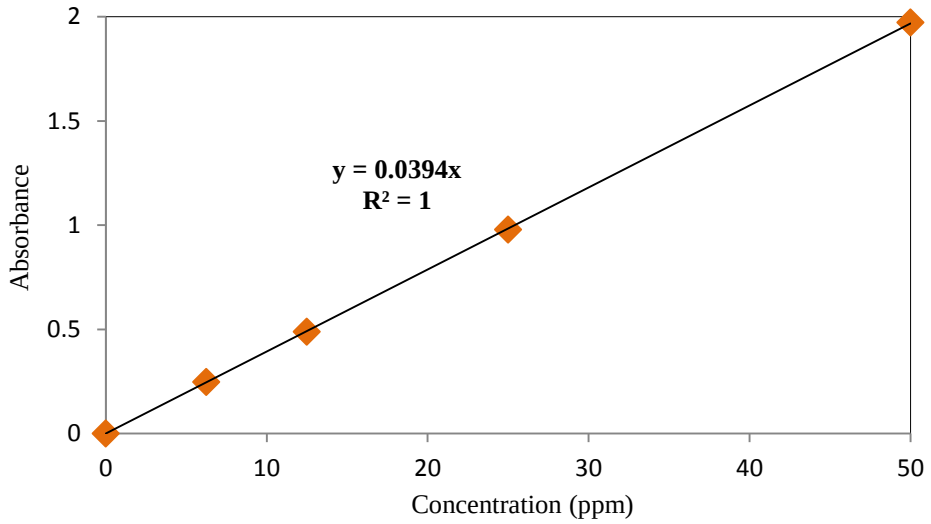


Figure C.1: Standard curve used to obtain the ferrous and total iron concentrations during the one hour exposure time experiment

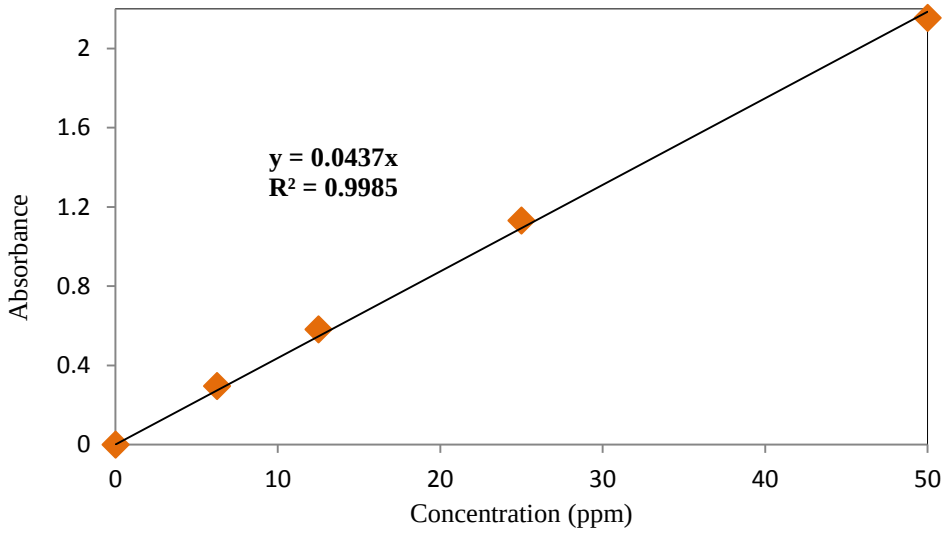


Figure C.2: Standard curve used to obtain the ferrous and total iron concentrations during the three hour exposure time experiment

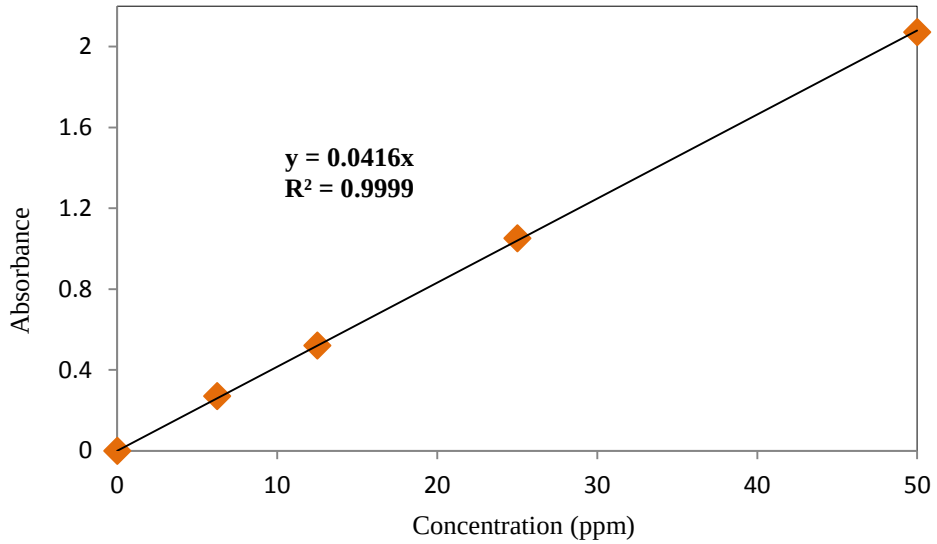


Figure C.3: Standard curve used to obtain the ferrous and total iron concentrations during the 24 hour exposure time experiment

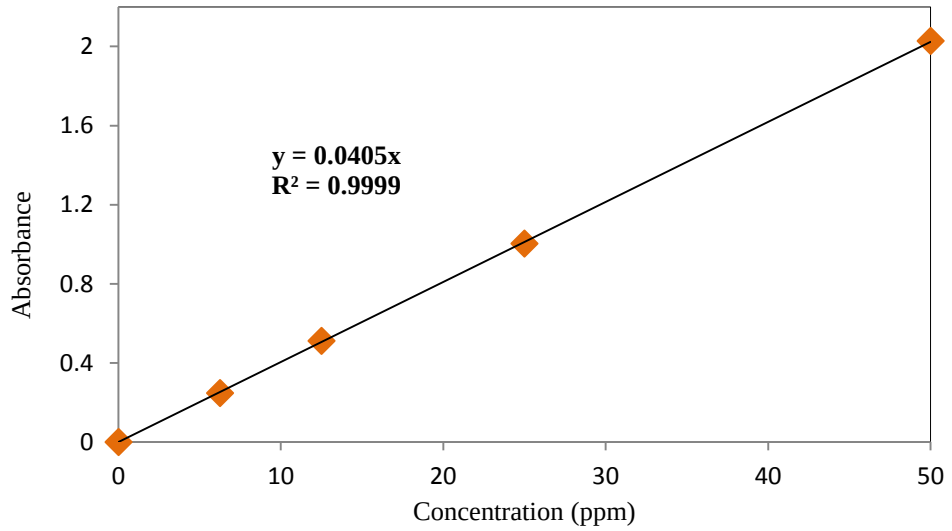


Figure C.4: Standard curve used to obtain the ferrous and total iron concentrations during the reproducibility experiment

Table C.1: Data generated to produce the standard curve for the determination of ferrous and total iron concentrations during the one hour exposure time experiment

Concentration [mg/l]	Absorbance			
	Sample 1	Sample 2	Sample 3	Average
0	0	0	0	0
6.25	0.246	0.249	0.249	0.248
12.5	0.488	0.487	0.489	0.488
25	0.976	0.980	0.978	0.978
50	1.974	1.973	1.969	1.972

Table C.2: Data generated to produce the standard curve for the determination of ferrous and total iron concentrations during the three hour exposure time experiment

Concentration [mg/l]	Absorbance			
	Sample 1	Sample 2	Sample 3	Average
0	0	0	0	0
6.25	0.299	0.294	0.295	0.296
12.5	0.581	0.585	0.580	0.582
25	1.134	1.134	1.128	1.132
50	2.153	2.156	2.156	2.155

Table C.3: Data generated to produce the standard curve for the determination of ferrous and total iron concentrations during the 24 hour exposure time experiment

Concentration [mg/l]	Absorbance			
	Sample 1	Sample 2	Sample 3	Average
0	0	0	0	0
6.25	0.269	0.272	0.269	0.270
12.5	0.521	0.520	0.519	0.520
25	1.053	1.050	1.050	1.051
50	2.063	2.081	2.072	2.072

Table C.4: Data generated to produce the standard curve for the determination of ferrous and total iron concentrations during the reproducibility experiment

Concentration	Absorbance			
[mg/l]	Sample 1	Sample 2	Sample 3	Average
0	0	0	0	0
6.25	0.246	0.249	0.246	0.247
12.5	0.510	0.511	0.512	0.511
25	1.004	1.002	1.003	1.003
50	2.026	2.029	2.029	2.028

Appendix D – Raw Data Calculations

The pH, redox potential, ferrous iron concentration and total iron concentration were all provided as measured values.

Extent of solubilisation calculation:

$$Extent\_of\_solubilisation\_[\%] = \frac{Conc\_Fe^{tot}\_in\_solution\_[kg.m^{-3}]}{Conc\_Fe\_in\_0.021kg\_pyrite[kg.m^{-3}]} \times 100\%$$

The yield in terms of microbial cells produced per kg iron oxidised (X/Fe) calculations:

$$Yield_{(X/Fe)}\_[cells.kg^{-1}Fe] = \frac{Microbial\_cell\_concentration\_[cells.L^{-1}])}{Concentration\_Fe^{tot}\_[kg.L^{-1}]}$$

Plots of ln [cell number] as a function of the leaching time were used to obtain the growth rates, assuming exponential growth illustrated by the Malthus equation.

$$\frac{dX}{dt} = \mu X$$

The specific growth rates were estimated assuming a linear relationship between natural logarithm cell concentration and time over the steepest gradient, given correlation coefficient is greater than 0.95. Initial growth rates were determined over the lag phase of microbial growth and estimated using similar linear trends for the change in cell concentration with time (R values). The intersection of this baseline curve and the specific growth rate curve was used to estimate the lag period for the microbial growth curve and the baseline was calculated as an average of the intersection of the Y axis with the highest and the lowest linear trend for the change in cell concentration with time.

Figure C1, C2 and C3 show the growth rates attained from the gradient of the straight line graph. Units were converted from day^{-1} to h^{-1}.

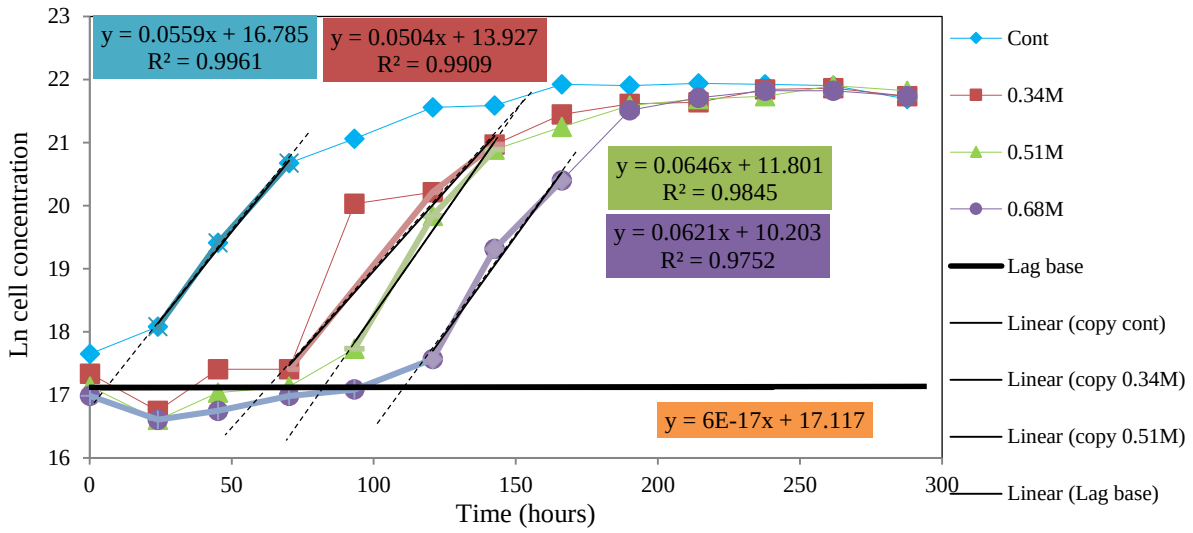


Figure D.1: Growth rates calculation for the different acid stressed cultures following one hour exposure time

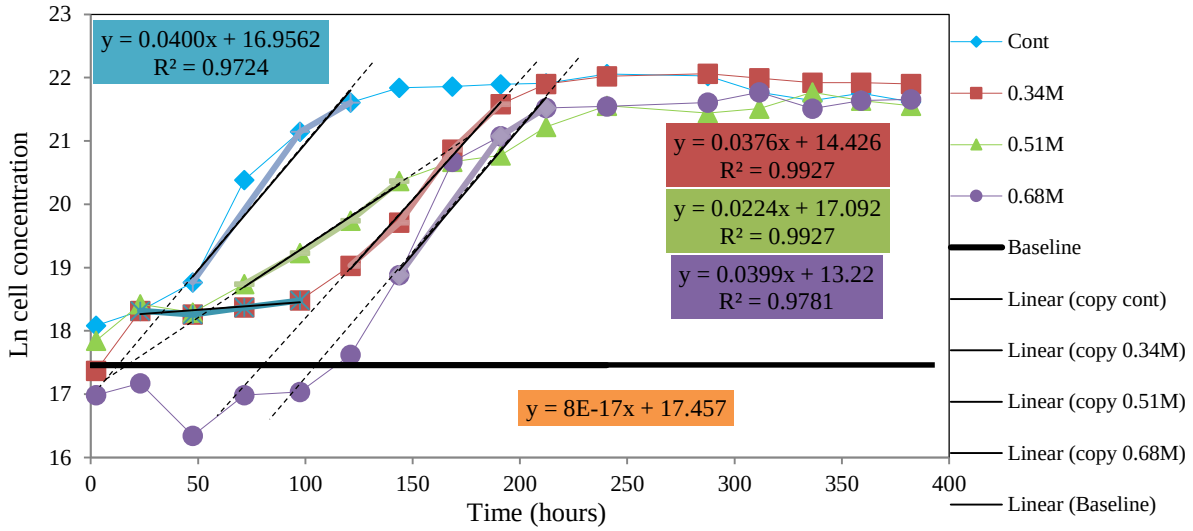


Figure D.2: Growth rates calculation for the different acid stressed cultures following three hour exposure time

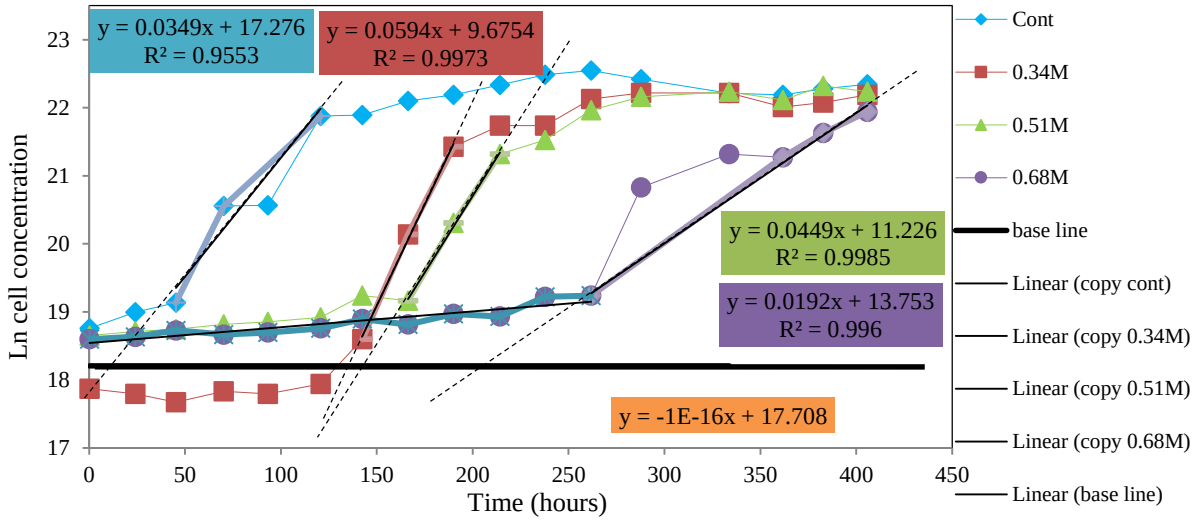


Figure D.3: Growth rates calculation for the different acid stressed cultures following 24 hour exposure time

The maximum leach rates were estimated assuming a linear relationship between iron concentration and time over the steepest gradient, given correlation coefficient is greater than 0.95. Initial iron oxidation rates were determined over the lag phase of microbial growth and estimated using similar linear trends for the change in iron concentration with time (R values). The intersection of this baseline curve and the maximum leach rate curve was used to estimate the lag period for the microbial ferrous iron kinetics and the baseline was calculated as an average of the intersection of the Y axis with the highest and the lowest linear lag trend for the change in iron concentration with time. Figure C4, C5 and C6 show the maximum leaching rates attained from the gradient of the straight line graph. Units were converted from day^{-1} to h^{-1}.

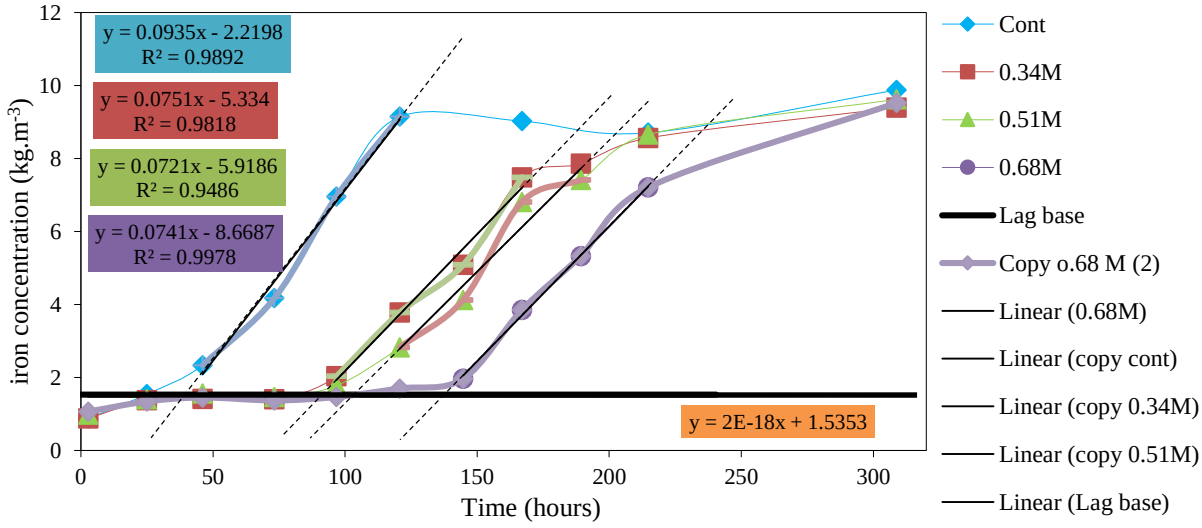


Figure D.4: Maximum leaching rates calculation for the different acid stressed cultures following one hour exposure time

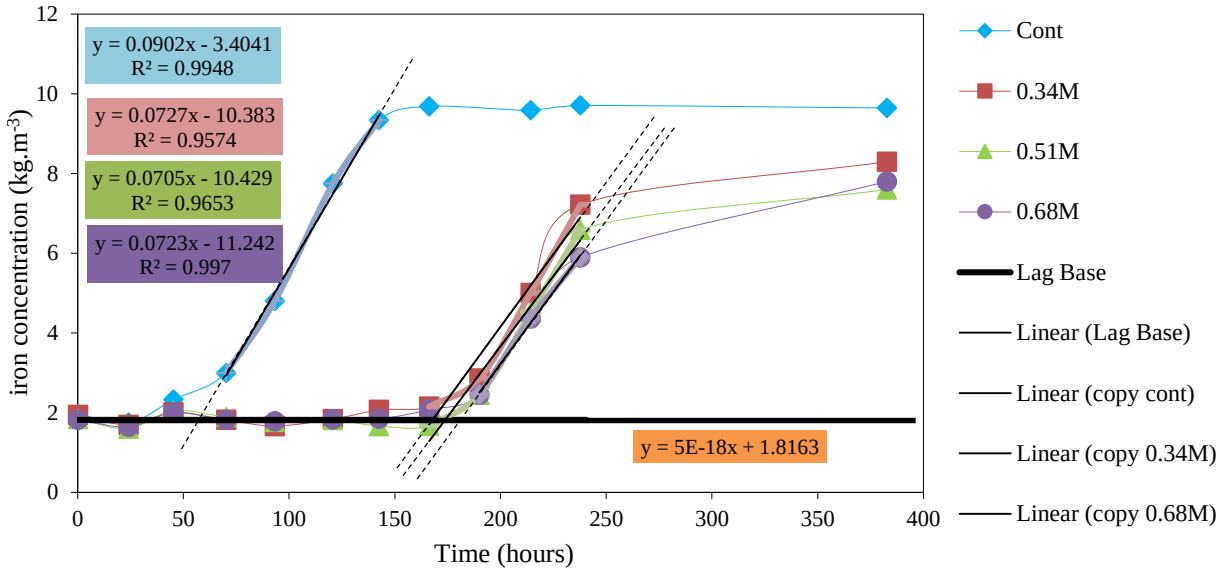


Figure D.5: Maximum leaching rates calculation for the different acid stressed cultures following three hour exposure time

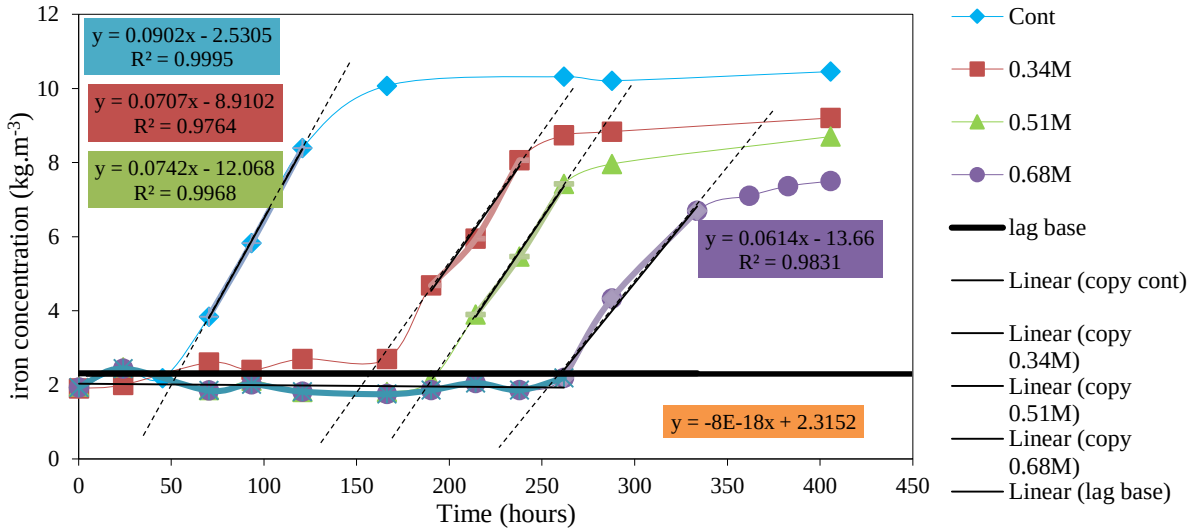


Figure D.6: Maximum leaching rates calculation for the different acid stressed cultures following 24 hour exposure time

Appendix E – Quantitative real-time polymerase chain reaction (qPCR)

qPCR Calculations

If for an example;

Concentration of extracted gDNA = β ng/µL

Amount of the diluted gDNA sample analysed = 50 µL (i.e. the final elution volume)

Amount of sample filtered for DNA extraction = 2 mL

Calculation of gDNA Concentration

$$\text{Concentration of gDNA in the sample} = \frac{\beta \frac{\text{ng}}{\mu\text{L}} \text{ x } 50\mu\text{L}}{2 \text{ mL}}$$

$$= 25\beta \text{ ng/mL sample}$$

Calculation of Genomic Copy Number

$$\text{Genomic copy number of Atc in the sample} = \frac{5.00\text{E+06 genes x } 0.5\beta \frac{\text{ng}}{\text{mL sample}}}{\omega \text{ ng x } \frac{1 \text{ gene}}{\text{genomic copy}}}$$

$$= \frac{2.50\text{E+06 } \beta}{\omega} \text{ genomic copies/mL sample}$$

In the calculating of genomic copy numbers, the gene dosage of microbial species was considered. *L. ferriphilum* is reported to have one copy of the 16S rRNA gene per genome (Liu *et al*., 2006; Zhang *et al*., 2009). *At. ferrooxidans* has two copies of the 16S rRNA gene per genome. Many known leaching acidophiles contain only one to two copies of 16S rRNA gene per genome, therefore there is an approximately 1:1 relationship between the number of microbial cells and the number of 16S rDNA copies present in samples (Liu *et al*., 2006).

Appendix F – Raw data

One Hour Acid Stress Run Data

Time (h)	pH				Eh				Biomass Conc [cell.mL^{-1}]			
	Cont	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**
0.00	1.4	1.4	1.4	1.4								
2.75	1.36	1.4	1.42	1.41	540	513	510	508	4.63E+07	3.38E+07	2.75E+07	2.38E+07
25.00	1.32	1.36	1.38	1.4	577	478	476	474	7.13E+07	1.88E+07	1.63E+07	1.63E+07
46.08	1.27	1.33	1.36	1.37	637	473	469	464	2.69E+08	3.63E+07	2.50E+07	1.88E+07
73.25	1.11	1.33	1.37	1.36	733	543	498	465	9.50E+08	3.63E+07	2.75E+07	2.38E+07
96.75	1.07	1.31	1.36	1.37	764	621	624	473	1.40E+09	5.00E+08	5.00E+07	2.63E+07
120.75	1.01	1.14	1.28	1.34	782	706	643	607	2.30E+09	6.00E+08	4.13E+08	4.25E+07
144.75	1.05	1.14	1.2	1.34	783	761	647	618	2.38E+09	1.28E+09	1.18E+09	2.44E+08
167.00	1.04	1.09	1.13	1.25	790	780	770	642	3.31E+09	2.06E+09	1.69E+09	7.25E+08
189.25	1.06	1.08	1.11	1.14	786	781	776	750	3.25E+09	2.44E+09	2.38E+09	2.20E+09
214.75	1.02	1.02	1.05	1.09	786	786	782	771	3.38E+09	2.50E+09	2.63E+09	2.68E+09
241.25	1.05	1.06	1.08	1.1	784	782	780	775	3.31E+09	3.06E+09	2.75E+09	3.00E+09
308.75	0.99	0.99	1.02	1.04	775	774	771	768	3.25E+09	3.13E+09	3.25E+09	3.00E+09
334.25	1.02	1.04	1.04	1.06	772	775	773	773	2.63E+09	2.75E+09	3.00E+09	2.75E+09

Fe^{tot} [$g.L^{-1}$]				Fe^{2+} [$g.L^{-1}$]				Fe^{3+} [$g.L^{-1}$]				Percentage Fe solub [%]			
Cont	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.88	0.88	0.99	1.07	0.08	0.13	0.13	0.13	0.80	0.75	0.85	0.94	9.00	9.05	10.09	10.91
1.55	1.38	1.41	1.34	0.02	0.39	0.38	0.36	1.53	0.98	1.03	0.98	15.83	14.10	14.41	13.74
2.34	1.42	1.57	1.45	0.02	0.46	0.46	0.47	2.32	0.96	1.10	0.98	23.94	14.41	15.05	14.03
4.18	1.41	1.47	1.37	0.03	0.06	0.23	0.52	4.15	1.35	1.25	0.85	42.71	14.52	16.03	14.85
6.96	2.04	1.78	1.47	0.04	0.02	0.02	0.44	6.92	2.02	1.77	1.02	71.21	20.83	18.25	15.01
9.15	3.79	2.83	1.70	0.05	0.03	0.03	0.06	9.10	3.76	2.80	1.64	79.97	38.75	28.94	17.39
7.82	5.10	4.13	1.96	0.04	0.03	0.03	0.02	7.77	5.07	4.10	1.95	80.05	52.13	42.24	20.10
9.03	7.49	6.82	3.86	0.05	0.04	0.04	0.03	8.98	7.45	6.78	3.83	81.79	76.63	69.72	39.44
7.82	7.86	7.41	5.33	0.05	0.04	0.04	0.04	7.78	7.82	7.37	5.29	88.92	79.39	75.83	59.69
8.71	8.56	8.66	7.21	0.05	0.05	0.05	0.05	8.66	8.51	8.61	7.16	89.13	80.41	80.05	80.77
7.99	7.76	7.82	7.90	0.05	0.04	0.05	0.04	7.95	7.72	7.78	7.85	92.41	87.61	87.90	82.88
9.89	9.39	9.63	9.53	0.06	0.06	0.06	0.06	9.83	9.34	9.57	9.47	93.64	88.77	88.63	86.15
8.69	8.68	8.59	8.42	0.05	0.05	0.05	0.05	8.64	8.63	8.54	8.37	101.13	96.11	98.51	97.50

Three Hour Acid Stress Run Data

Time (h)	pH				Eh				Biomass Conc [cell.mL^{-1}]			
	Cont	0.34M	0.51M	0.68M	Cont	0.34M	0.51M	0.68M	Cont	0.34M	0.51M	0.68M
0.00	1.40	1.40	1.40	1.40								
2.50	1.46	1.44	1.42	1.41	542	503	531	527	7.13E+07	3.50E+07	5.63E+07	2.38E+07
23.00	1.44	1.39	1.38	1.35	548	495	486	482	9.00E+07	9.00E+07	1.00E+08	2.88E+07
47.50	1.39	1.37	1.35	1.33	609	493	481	480	1.41E+08	8.50E+07	8.75E+07	1.25E+07
71.50	1.14	1.30	1.30	1.26	730	573	472	474	7.13E+08	9.50E+07	1.38E+08	2.38E+07
97.50	1.09	1.27	1.26	1.25	765	661	474	480	1.53E+09	1.06E+08	2.25E+08	2.50E+07
121.00	1.02	1.33	1.28	1.29	780	717	530	533	2.42E+09	1.84E+08	3.75E+08	4.50E+07
143.75	1.00	1.31	1.22	1.30	772	721	676	617	3.06E+09	3.63E+08	7.00E+08	1.59E+08
168.50	1.01	1.19	1.18	1.28	774	736	693	658	3.13E+09	1.15E+09	9.50E+08	9.50E+08
191.00	1.05	1.15	1.16	1.20	776	744	743	673	3.23E+09	2.36E+09	1.05E+09	1.43E+09
212.17	1.05	1.08	1.10	1.10	784	748	776	777	3.28E+09	3.25E+09	1.65E+09	2.23E+09
240.50	1.04	1.07	1.11	1.08	783	757	772	773	3.80E+09	3.66E+09	2.30E+09	2.28E+09
287.50	1.06	1.03	1.01	1.07	770	763	776	778	3.68E+09	3.81E+09	2.05E+09	2.43E+09
311.50	1.06	1.05	1.06	1.07	772	759	777	775	2.85E+09	3.56E+09	2.20E+09	2.85E+09
336.25	1.06	1.06	1.00	1.05	775	764	780	781	2.50E+09	3.31E+09	2.85E+09	2.20E+09
359.00	1.06	1.06	1.01	1.06	775	771	781	776	2.80E+09	3.31E+09	2.50E+09	2.50E+09
382.25	1.06	1.06	0.99	1.07	769	780	780	777	2.45E+09	3.25E+09	2.30E+09	2.55E+09

Fe^{tot} [$g.L^{-1}$]				Fe^{2+} [$g.L^{-1}$]				Fe^{3+} [$g.L^{-1}$]				Percentage Fe solub [%]			
Cont	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.96	1.95	1.84	1.83	0.10	0.13	0.12	0.12	1.87	1.82	1.72	1.71	14.10	17.05	13.31	16.86
1.75	1.69	1.60	1.65	0.08	0.48	0.46	0.45	1.67	1.21	1.15	1.20	16.06	17.31	14.36	18.27
2.33	2.01	2.05	2.01	0.02	0.64	0.61	0.54	2.30	1.37	1.44	1.46	17.32	18.63	14.75	18.63
3.90	1.82	1.89	1.82	0.04	0.84	0.82	0.70	3.86	0.98	1.07	1.12	25.58	18.81	14.89	18.69
4.89	1.67	1.77	1.79	0.05	0.71	0.83	0.54	4.84	0.96	0.94	1.25	36.44	19.92	17.40	18.70
6.84	1.84	1.82	1.83	0.04	0.10	0.89	0.08	6.80	1.74	0.92	1.75	41.25	20.54	20.12	18.91
8.12	2.07	1.66	1.85	0.05	0.01	0.82	0.00	8.07	2.06	0.84	1.84	57.88	21.16	30.25	20.52
9.69	2.15	1.67	2.08	0.06	0.02	0.92	0.02	9.63	2.13	0.76	2.06	62.73	26.85	41.38	25.10
8.84	2.86	2.45	2.45	0.05	0.03	0.76	0.03	8.79	2.83	1.69	2.42	68.86	33.23	47.99	26.90
9.59	5.00	4.52	4.37	0.06	0.04	0.04	0.04	9.53	4.96	4.48	4.32	89.45	51.19	51.78	44.66
9.71	7.22	6.59	5.90	0.05	0.05	0.02	0.05	9.66	7.17	6.57	5.95	96.06	62.23	52.26	57.48
8.85	6.66	6.13	6.31	0.05	0.05	0.04	0.05	8.80	6.60	6.09	6.27	96.71	68.09	59.63	64.60
8.59	6.08	6.27	5.62	0.05	0.04	0.03	0.04	8.54	6.04	6.24	5.58	97.18	70.09	73.27	65.12
9.05	6.85	6.92	6.37	0.06	0.05	0.04	0.05	8.99	6.80	6.87	6.31	97.18	73.88	94.89	65.86
9.45	7.46	6.05	7.80	0.05	0.05	0.05	0.05	9.40	7.41	6.00	7.75	97.22	76.33	95.30	79.81
9.65	9.46	8.58	8.80	0.05	0.06	0.05	0.05	9.60	9.40	8.53	8.75	97.32	96.73	95.41	90.05

24 Hour Acid Stress Run Data

Time (h)	pH				Eh				Biomass Conc [cell.mL^{-1}]			
	Cont	0.34M	0.51M	0.68M	Cont	0.34M	0.51M	0.68M	Cont	0.34M	0.51M	0.68M
0.00	1.4	1.4	1.4	1.4								
0.17	1.45	1.4	1.4	1.4	524	524	522	522	8.50E+07	3.50E+07	7.63E+07	7.25E+07
24.08	1.39	1.38	1.38	1.37	493	489	489	492	1.08E+08	3.25E+07	8.13E+07	7.50E+07
45.25	1.38	1.34	1.35	1.35	500	480	480	483	1.24E+08	2.88E+07	8.38E+07	8.25E+07
70.25	1.33	1.31	1.31	1.31	611	481	470	475	5.13E+08	3.38E+07	9.00E+07	7.75E+07
93.25	1.26	1.32	1.31	1.31	651	585	469	473	5.19E+08	3.25E+07	9.38E+07	8.00E+07
120.75	1.05	1.32	1.28	1.29	747	624	467	470	1.93E+09	3.75E+07	1.00E+08	8.50E+07
142.50	1.04	1.3	1.32	1.34	782	638	475	469	1.95E+09	7.25E+07	1.38E+08	9.75E+07
166.25	0.98	1.15	1.31	1.32	789	776	518	475	2.40E+09	3.38E+08	1.28E+08	9.00E+07
190.08	0.98	1.07	1.34	1.32	781	787	640	486	2.63E+09	1.23E+09	4.00E+08	1.05E+08
214.25	0.92	0.95	1.29	1.32	786	794	641	504	3.03E+09	1.68E+09	1.10E+09	1.01E+08
237.75	0.9	0.92	1.18	1.32	794	796	760	539	3.53E+09	1.68E+09	1.35E+09	1.35E+08
261.75	0.98	0.98	1.13	1.38	795	800	783	646	3.75E+09	2.48E+09	2.09E+09	1.38E+08
287.75	0.94	0.93	1.01	1.32	798	796	795	679	3.31E+09	2.70E+09	2.55E+09	6.75E+08
333.75	0.96	0.94	0.97	1.15	790	797	796	764	2.69E+09	2.70E+09	2.75E+09	1.10E+09
361.75	0.92	0.9	0.93	1.06	789	790	794	779	2.63E+09	2.20E+09	2.44E+09	1.05E+09
382.75	0.99	0.97	0.99	1.05	773	788	791	784	2.88E+09	2.35E+09	3.00E+09	1.50E+09
405.75	0.97	0.95	0.95	1.02	781	791	788	789	3.06E+09	2.63E+09	2.75E+09	2.05E+09

Fe^{tot} [g.L^{-1}]				Fe^{2+} [g.L^{-1}]				Fe^{3+} [g.L^{-1}]				Percentage Fe solub [%]			
Cont	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**	**Cont**	**0.34M**	**0.51M**	**0.68M**
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.93	1.90	1.96	1.93	0.57	0.00	0.00	0.00	1.36	1.90	1.96	1.93	25.94	0.00	0.00	0.00
2.40	2.00	2.47	2.44	0.76	0.18	0.18	0.16	1.64	1.82	2.28	2.28	26.28	19.85	18.45	17.92
2.18	2.10	2.78	2.58	0.59	0.61	0.55	0.50	1.59	1.49	2.23	2.08	28.04	20.03	18.50	18.61
3.84	2.60	1.86	1.85	0.02	0.88	0.96	0.70	3.86	1.72	0.90	1.14	34.33	21.68	19.05	18.90
5.82	2.40	2.11	2.02	0.03	0.70	0.82	0.72	5.79	1.70	1.29	1.29	34.69	24.90	19.85	19.01
8.39	2.70	1.81	1.82	0.04	0.03	1.03	0.88	8.35	2.67	0.78	0.94	65.27	26.32	20.02	19.05
7.74	2.40	1.94	1.93	0.04	0.02	1.03	0.85	7.69	2.38	0.91	1.08	73.88	27.14	21.54	19.75
10.07	2.70	1.80	1.75	0.05	0.04	0.93	0.90	10.02	2.66	0.87	0.85	81.20	47.91	21.74	19.76
9.66	4.68	2.12	1.86	0.05	0.04	0.23	0.72	9.62	4.65	1.89	1.14	81.54	60.78	25.25	20.63
9.35	5.94	3.89	2.05	0.05	0.05	0.02	0.59	9.29	5.89	3.87	1.46	82.66	78.81	28.45	20.99
11.50	8.06	5.46	1.86	0.06	0.06	0.03	0.38	11.44	8.00	5.43	1.48	83.98	82.08	39.84	22.39
10.31	8.73	7.42	2.19	0.05	0.05	0.03	0.11	10.26	8.68	7.39	2.08	84.75	82.45	55.91	24.96
10.21	8.83	7.96	4.33	0.06	0.06	0.05	0.02	10.15	8.77	7.91	4.35	86.42	88.84	75.90	26.36
9.86	10.06	9.22	6.70	0.05	0.06	0.05	0.03	9.81	10.00	9.17	6.67	87.47	89.35	81.43	50.46
10.98	8.02	8.14	7.10	0.06	0.07	0.06	0.04	10.92	7.96	8.08	7.06	90.61	90.37	83.32	67.76
8.46	8.68	8.65	7.36	0.05	0.06	0.05	0.04	8.41	8.63	8.60	7.32	97.99	94.59	88.48	70.38
10.45	9.20	8.70	7.50	0.06	0.06	0.06	0.05	10.40	9.14	8.64	7.45	102.17	102.88	94.30	75.32

Data Nucleic Acid and DNA Concentration for Microbial Speciation at Completion of Experiment

	Nucleic acid concentration [ng.µL^{-1}]						260/280	260/230
	conc 1	conc 2	conc 3	conc 4	conc 5	Average		
Control	148.6	148.5	148.3	147.3	148.4	148.22	1.964	2.05
0.34M	60.9	61.4	61.2	61.5	60.7	61.14	1.884	2.056
0.51M	65.1	65.6	65.4	65.6	65.5	65.44	1.824	1.476
0.68M	56.2	55.8	55.7	55.7	56.3	55.94	1.856	1.798

	DNA concentration [ng.mL^{-1}]				
	Leptospirurilum ferriphilum HT	***Acidiplasma cupricumulans***	***Ferroplasma acidiphilum***	**Archaea**	***Acidithiobacillus ferrooxidans D2***
Control	1.60E+07	8.46E+05	5.21E+05	1.85E+05	2.41E+05
0.34M	2.12E+07	0.00E+00	5.01E+05	2.70E+04	2.01E+04
0.51M	3.09E+07	0.00E+00	5.57E+05	0.00E+00	1.79E+04
0.68M	1.78E+07	0.00E+00	0.00E+00	0.00E+00	3.54E+04

Data of Nucleic Acid and DNA Concentration for Microbial Speciation During Experiment

	Nucleic acid concentration [ng.µL^{-1}]						260/280	260/230
	conc 1	**conc 2**	**conc 3**	**conc 4**	**conc 5**	**Average**		
Inoculum	148.6	148.5	148.3	147.3	148.4	148.22	1.964	2.05
70 Hours	55.3	55.5	55.5	54.6	54.2	55.02	2.026	2.036
214 Hours	53.5	53.3	53.2	53.3	52.6	53.18	1.932	1.924
287 Hours	105.8	105.6	105.5	106	105.9	105.76	1.908	1.778

	DNA concentration [ng.mL^{-1}]				
	Leptospirurilum ferriphilum HT	***Acidiplasma cupricumulans***	***Ferroplasma acidiphilum***	**Archaea**	***Acidithiobacillus ferrooxidans D2***
Inoculum	1.60E+07	8.46E+05	5.21E+05	1.85E+05	2.41E+05
70 Hours	8.50E+06	0.00E+00	7.83E+05	0.00E+00	8.15E+04
214 Hours	5.36E+06	0.00E+00	9.75E+04	0.00E+00	2.12E+04
287 Hours	8.55E+06	0.00E+00	7.83E+04	0.00E+00	1.46E+04