Growth of mixed cultures of moderate thermophiles for commercial heap bioleaching of low-grade copper-sulphide ores

By

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DECLARATION

I know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own. This has not been submitted for any degree or examination in any other university.

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04 October 2013

SYNOPSIS

Heap bioleaching has been demonstrated as an economic technology for processing low-grade and recalcitrant copper-sulphide ores which would otherwise be uneconomic to process using conventional methods of mineral extraction such as concentration followed by smelting or autoclaving. Heap bioleaching has lower operating costs compared to other metallurgical methods. Naturally, a bioleach heap can be colonised by the indigenous micro-organisms but the need to shorten process times and to increase efficiency has resulted in a need to inoculate the heaps. The aim of inoculation is to deliver micro-organisms best suited for the conditions expected in a heap as leaching progresses and as the heap ages. Successful leaching of primary copper-sulphide ores such as chalcopyrite has been shown to require thermophilic temperatures. Therefore, an inoculum with micro-organisms that facilitate the increase of temperature in the bioleach heap is required. Successful inoculation of a thermophilic bioleach heap requires a fundamental understanding of microbial growth on the ore at different temperature ranges. Furthermore, successful preparation and maintenance of suitable inocula also requires an understanding of culture conditions that could affect the micro-organisms in the stock cultures.

This study focused on investigating the growth of bioleaching micro-organisms at 50°C, a moderate thermophilic temperature. This temperature is of particular interest as a ramping temperature from ambient to thermophilic temperatures. It is well known that efficient progression through the moderately thermophilic region can be a challenge. In the first part of the study, the aim was to characterise microbial growth in a bioleach heap environment. This was carried out in a controlled environment to allow for an independent study to determine growth rates of the component species in the community on low-grade ore as well as studying the effect of different microbial compositions of the inocula and the copper concentrations on the growth rates of a consortium of selected bioleaching micro-organisms. A simulated heap environment was created by packing a series of identical laboratory glass columns with low grade chalcopyrite ore and inoculating the columns with consortia of moderate thermophiles implicated in bioleaching. Physicochemical parameters such as Eh, pH, soluble copper and iron were monitored every second day in the pregnant liquid solution from the columns for a maximum of 50 days in each experiment. Assuming uniformity across all the columns in a series, columns were sacrificed at regular intervals to determine the microbial composition on the ore. Microbial assays included cell counts and quantification of microbial species using quantitative real-time polymerase chain reaction (qPCR) analysis with universal and species-specific primers. The microbial community from a single column was taken to be representative of all the columns in the series at a particular instance.

Microbial community analyses using qPCR showed that all the four major microbial species detected in the inocula i.e. *Acidithiobacillus caldus, Acidiplasma cupricumulans, Acidithiomicrobium* species and *Metallosphaera* species colonised the ore, albeit at different rates. The growth rates of individual microbial species were observed to vary in response to the changing physicochemical and biological environment in the ore. The maximum specific growth rates in the whole ore bioleach heap environment were reported. Regardless of the starting composition of the inoculum, *At. caldus,* a sulphur-oxidiser was shown to be the first species to dominate the columns and was succeeded by *Acidithiomicrobium* spp. or *Metallosphaera* spp., the iron- and sulphur-oxidisers over time.

Copper concentration was observed to affect microbial growth. Substantial growth and attachment of micro-organisms to the ore took place up to 5.0 g/L Cu in the irrigation solution but beyond this concentration the growth was minimal. At 10.0 and 15.0 g/L Cu, no detectable microbial growth occurred on the ore and the micro-organisms were only detected in the PLS, indicating that the micro-organisms could not attach strongly to the ore at higher Cu concentration.

The second part of the study assayed microbial growth in a liquid culture environment used to prepare inocula for the heaps, paying particular attention to *Acidithiomicrobium* species, a key iron- and sulphur-oxidising moderate thermophile. The effect of carbon dioxide concentration and agitation speed on the microbial composition of a mixed moderate thermophilic culture was investigated. Three stirred tank reactors (STRs) and a shake flask were inoculated with the culture and maintained at 50 °C. Two STRs were sparged with air enriched to 1 % CO_2 content while the third STR was sparged with normal air. One CO_2 enriched STR was agitated at 550 rpm whilst the other two STRs were agitated at 250 rpm. The shake flask was not sparged and was agitated at 180 rpm. The cultures were monitored for 98 days and their microbial composition assayed weekly using cell counts and qPCR analysis. Eh, pH, iron and copper were also monitored. Similar physicochemical conditions occurred across all the reactors. Microbial community analysis using qPCR showed that the CO_2 concentration in normal air did not limit the growth of *Acidithiomicrobium* spp. at an agitation speed of 250 rpm i.e. sparging with air enriched to 1 % CO_2 was not beneficial at low agitation speed. A higher agitation speed of 550 rpm was detrimental to *Acidithiomicrobium* spp. Furthermore, the effect of Cu concentration on the microbial composition of the mixed culture was investigated. Growth of *Acidithiomicrobium* spp. was inhibited by an additional 2.5 g/L Cu in solution and the inhibition increased with increasing Cu concentration. On day 20 of the experiment, the presence of *Acidithiomicrobium* spp. in the reactors with the initial Cu concentration of 2.5, 5.0 and 7.5 g/L was reduced by 17 %, 50

% and 88 %, respectively with respect to the Control. The amount of *Acidithiomicrobium* spp. present was observed to recover over the duration of the experiment, up to 5 g/L Cu suggesting the potential for acclimatisation to Cu. At higher Cu concentration, this species did not recover for the entire 127 days of the experiment.

This work describes both microbial growth and colonisation of a bioleach heap, and inocula preparation. The work contributes a fundamental understanding of microbial growth on the ore and in liquid culture at species level. This is important for a successful design of suitable inocula and for optimisation of microbial succession during thermophilic heap bioleaching particularly to get to high temperatures through the moderate thermophile zone. The process management of a heap is particularly important at 50 to 55 °C owing to less biodiversity and activity of acidophiles in this temperature range. This work shows microbial succession at 50 °C. Sulphur-oxidisers dominate first as sulphur oxidation produces more energy for biomass growth than iron oxidation. Sulphur oxidation produces the heat required to raise the temperature to levels conducive for the growth of moderate thermophiles; subsequently the sulphur-oxidisers are succeeded by the combined iron- and sulphur-oxidisers over time. Finally, the importance of culture conditions was also shown. It was indicated that microbial species respond differently to copper and agitation speed, affecting the ecology of the inoculum, whilst the CO_2 was shown to have no effect across the conditions studied. Through understanding microbial growth and colonisation of low-grade ore, and inocula preparation, minimisation of the time required to raise the temperature in thermophilic bioleach heap, and therefore allow value to be derived from the heap, can be achieved.

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GLOSSARY

Acidophile	A micro-organism that thrives in environments with a low pH
Autotroph	An organism that acquires its carbon by fixing carbon dioxide
Chemolithoautotroph	A microorganism that fixes CO_2 and obtains its energy by the oxidation of inorganic compounds
Chemolithotroph	An organism that uses inorganic compounds as a source of chemical energy
Consortium	A group of micro-organisms living together in which each individual benefits from the others.
Eh	Redox potential relative to the standard hydrogen electrode (SHE)
Genomic copy number	The number of 16S rRNA genes per genome of a single cell of a microbial species
Hydrometallurgy	Extraction of metals using water-based processes
Planktonic	Floating in solution
pH	Measure of acidity or alkalinity
Pyrometallurgy	Extraction of metals using heat-based processes
Redox potential	The tendency of a chemical species to acquire electrons and therefore be reduced
Sessile	Attached to a surface
Supernatant	The liquid lying above the solid residue after centrifugation
Vortexing	Turbulent shaking or spinning

CHEMICAL AND TECHNICAL ABBREVIATIONS

$(NH4)_2SO_4$	Ammonium sulphate
AAS	Atomic absorption spectroscopy
$Ca(NO_3)_2$	Calcium nitrate
CO_2	Carbon dioxide
$CuFeS_2$	Chalcopyrite
Cu	Copper
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
DNA	Deoxy-ribose nucleic acid
$(NH_4)_2HPO_4$	Di-ammonium hydrogen phosphate
K_2HPO_4	Di-potassium hydrogen phosphate
Fe^{2+}	Ferrous iron
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
Fe^{3+}	Ferric iron
gDNA	Genomic DNA
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MeS	Metal sulphide
O_2	Oxygen
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
K_2SO_4	Potassium sulphate
PLS	Pregnant leach solution
ppm	Parts per million (= mg/L)
qPCR	Quantitative real-time polymerase chain reaction
FeS_2	Pyrite
rcf	Relative centrifugal force
rpm	Revolutions per minute
STR	Stirred tank reactor
H_2SO_4	Sulphuric acid
Fe^{tot}	Total iron

1. INTRODUCTION

1.1 Background

Heap bioleaching is a method of extracting metals from ore using micro-organisms. Leaching is essentially a chemical process in which the leach agents (ferric ions and/or an acid) leach the metal from the mineral. Micro-organisms are used to regenerate the leach agents. If a mineral is recalcitrant to mesophilic (low-temperature) leaching, the answer is in the realm of chemistry rather than in biology i.e. raising the temperature would increase chemical leach rates. The biology needs to follow the chemistry and micro-organisms capable of regenerating leach agents at high temperatures are required (Rawlings, 2007). Bioleaching has been applied successfully commercially for secondary copper-sulphide minerals at mesophilic temperatures but this has not been the case with primary copper-sulphide minerals such as chalcopyrite. Effective leaching of primary sulphides has been demonstrated to be possible at elevated temperatures, preferably above 55°C. Good copper extraction has been achieved with thermophilic micro-organisms in stirred tank reactors (van Staden *et al.*, 2008) and in thermophilic bioleach heaps at laboratory and demonstration scale. Thermophilic bioleaching requires high temperature to be reached in the heap. To achieve this, micro-organisms must regenerate the leach agents and generate heat through sulphur oxidation. The presence of complementary and temperature-sequential microbial communities is required to facilitate heating from mesophilic through moderately thermophilic to thermophilic temperatures (Franzmann *et al.*, 2005; Brierley, 2008; Dew *et al.*, 2011). A fundamental understanding of microbial growth on the ore as a function of temperature is required at species level for a successful design of suitable inocula and for optimisation of microbial succession during thermophilic heap bioleaching.

Despite the commercial success of heap bioleaching of gold ore and some copper ores, there is limited understanding of the microbiology in full-scale bioheap operations. Understanding the microbiology is one of the keys to advancing commercial bioheap applications as it would enable better control of conditions to improve leach rates, metal recoveries and costs (Brierley, 2001). An analysis of the relationship between microbial activity and the rate of metal extraction is more difficult in heaps than in liquid bioreactors. This is due to the practical challenges of representative sampling of large areas and various depths of heaps and obtaining unbiased extraction of micro-organisms (or their nucleic acids) from the minerals (Norris, 2007). Most studies on heaps have relied on samples from leach solutions from columns operated at optimal conditions or leach solutions recycled through the heaps. Other studies have been done on mineral concentrate suspensions and on enrichment media. The limitation of enrichment is that it might select for a narrow range

of micro-organisms that grow better under the imposed culture conditions and this might give a false impression of the relative importance of some organisms (Pradhan *et al.*, 2008).

Previously, studies were conducted at the Centre for Bioprocess Engineering Research (CeBER) in the University of Cape Town by Minnaar *et al.* (2013) and Tupikina *et al.* (2011, 2013) to develop a method to investigate microbial growth on ore in a simulated heap environment. The results obtained were reproducible confirming the reliability of the method developed, and gave an insight of microbial growth rates in the mesophilic and thermophilic temperature ranges. The process management of a heap is particularly important at 50 to 55 °C owing to less biodiversity and activity of acidophiles in this temperature range, particularly sulphur-oxidisers (Franzmann *et al.*, 2005; du Plessis *et al.*, 2007). This study therefore expanded the research work done at CeBER by focusing specifically on the growth of moderate thermophiles at 50°C. The objectives of this study were:

(i) To investigate the growth rates and dominance of a consortium of bioleaching micro-organisms in a bioleach heap environment at 50 °C using inocula with different compositions and irrigation solutions with different copper concentrations, on a low-grade copper-sulphide ore.

(ii) To investigate the growth and dominance of a consortium of bioleaching micro-organisms in a liquid stock culture at 50 °C under different carbon dioxide concentrations, copper concentrations, and agitation speeds.

1.2 Dissertation structure

Chapter 2 presents a review of the literature relevant to this study, in which the gaps in the current knowledge were identified. A comparison of different copper ore processing methods, the biochemistry of bioleaching and the set-up of a heap bioleach are included. The bioleaching micro-organisms are reviewed, including the description of individual species, the factors affecting bioleaching micro-organisms, study cases of prevalence and dominances of moderate thermophiles both on whole ore and in liquid cultures, and the methods of identifying and quantifying bioleaching micro-organisms. The hypotheses and objectives of the thesis are presented. Chapter 3 describes the materials, the reactor set-ups, the experimental protocols and analytical procedures used in the first part of this study in which column leaching is used, including the rationale behind the choice of the particular materials and methods. In Chapter 4, the results obtained from the investigation carried out on whole ore are reported. The growth rates, the effect of using different compositions of the

inoculum and of varying copper concentrations in the irrigation solution are discussed. Chapter 5 describes the materials, the reactor set-ups, the experimental protocols and analytical procedures used to study submerged culture. Chapter 6 is a presentation of the results obtained on investigating development of the liquid stock culture. The effects of CO_2 concentration, agitation speed and copper concentration on microbial growth are discussed. Chapter 7 presents the conclusions drawn from the study in light of the key questions raised during literature review, and in light of the objectives and hypotheses of the study. Recommendations for future research related to this study are also presented in Chapter 7.

2. LITERATURE REVIEW

2.1 Methods of copper mining

Various methods are used in mining copper including pyrometallurgical methods such as smelting, and hydrometallurgy e.g. in-situ, concentrate, dump and heap leaching.

Smelting is the most common method of processing copper because the use of high processing temperatures makes this method insensitive to the mineralogy of the ore, thereby applicable to process a wide variety of ores. The ore is pre-treated by crushing, milling and flotation to produce a concentrate which is processed at high temperature. Smelting has high operating costs because of the high temperatures, the pre-treatment of the ore and possible loss of some metal into tailings. Smelting is also hazardous to the environment because it produce may toxic gases and residues as well as active residues containing metals that can still be leached in mine dumps leading to acid mine drainage (du Plessis *et al.*, 2007).

Concentrate leaching is normally used for high-value and high-grade ores. It is an expensive method because of the preparation of a concentrate similar to the ore pre-treatment in smelting. Also, there are costs incurred in controlling process parameters such as aeration, agitation, pH and temperature. After pre-treatment, the ore is bioleached or abiotically leached in a tank or in a heap (du Plessis *et al.*, 2007). Well controlled continuous stirred tank reactors (CSTRs) are normally used. The throughput of the CSTRs is limited in that the pulp density of the concentrate must not exceed 20 % to enable oxygen (O_2) requirements to be met and to prevent damage to microbial cells due to shear stress. Also, operating CSTRs at high temperature is negatively affected by evaporation and a need for special construction material to withstand corrosion at high temperatures. High operating temperatures also decrease the solubility of O_2 and carbon dioxide (CO_2), both needed for bioleaching. Therefore there is a need to sparge CSTRs with enriched air at high temperatures which is expensive (Rawlings *et al.*, 2003).

In heap bioleaching, micro-organisms colonise the ore stacked into a heap, enabling continued regeneration of leach agents in proximity to the site of leaching. This is a low cost technology because it requires simple equipment, and produces acceptable yields. The method can be used on refractory ("difficult to process") ores which can be readily leached at high temperatures in the heap, hence this method is viable for marginal ores which would be expensive to process through other mining methods (du Plessis *et al.*, 2007; Watling, 2006). This is a growing technology e.g. more than 10 % of copper produced in Chile is currently

extracted by heap bioleaching, compared to 5 % in 2002 (Demergasso *et al.*, 2010). This technology is also environmentally-friendly because no harmful off-gases and residues such sulphur dioxide and arsenic, are produced (Rawlings *et al.*, 2003). In Table 2.1 are typical operation costs for the afore-mentioned methods of mining copper.

Table 2. 1: Comparison of operating costs for different methods of copper production in 2003 (Rawlings *et al.*, 2007)

Mineral processing method	Operating cost (US cents per pound)
Concentrate leaching	49 - 77
Smelting	45 - 65
Heap bioleaching	34 - 47

2.2 Bioleaching

2.2.1 The biochemistry and mechanisms of bioleaching

In bioleaching, metals are extracted from the ore using micro-organisms to generate the leach agents. This can be carried out either in a highly aerated, continuous flow, stirred tank reactor containing finely ground ore or in a heap of whole ore (Pradhan *et al.*, 2008; Suzuki, 2001; Watling, 2006; du Plessis *et al.*, 2007). Bioleaching micro-organisms act as catalysts to regenerate the leach agents by utilising ferrous ions and/ or reduced forms of sulphur as energy sources. Iron-oxidising micro-organisms oxidise ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) as shown in a simplified way by Equation (1) and the sulphur-oxidising micro-organisms oxidise sulphur to an acid (H^+) as shown by Equation (2).

$$4Fe^{2+} + 4H^+ + O_2 \rightarrow 4Fe^{3+} + 2H_2O \quad (1)$$

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+ \quad (2)$$

The ferric ions and the acid go on to chemically leach the mineral as exemplified by the leaching of chalcopyrite ($CuFeS_2$) indicated in the Equations (3) and (4).

$$CuFeS_2 + 4Fe^{3+} \rightarrow Cu^{2+} + 2S^0 + 5Fe^{2+} \quad (3)$$

$$CuFeS_2 + 4H^+ + O_2 \rightarrow Cu^{2+} + Fe^{2+} + 2S^0 + 2H_2O \quad (4)$$

During leaching, Fe^{3+} ions are reduced to Fe^{2+} ions and the acid is used up, hence these leach agents need to be regenerated for leaching to continue. The exothermic oxidation of sulphur contributes to generating acid and the heat essential in increasing the temperature in

bioleach heaps. Figure 2.1 shows the activity of micro-organisms in the bioleaching of a metal sulphide (MS).

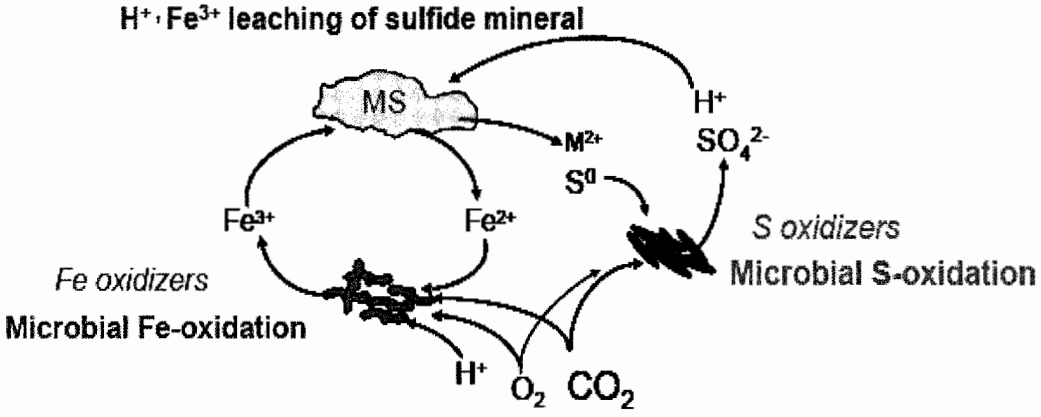


Figure 2. 1: Schematic presentation of microbial activity in the bioleaching of a metal sulphide, MS (Schippers *et al.*, 1996; Rohwerder and Sand, 2007).

The action of micro-organisms on the ore during leaching may be described by two mechanisms; the direct mechanism whereby micro-organisms leach the mineral through an enzymatic action and the indirect mechanism in which the micro-organisms facilitate leaching by generating leach agents. The latter is the largely accepted mechanism. The indirect mechanism is further divided into the non-contact mechanism in which planktonic micro-organisms regenerate the leach agents and the contact mechanism in which sessile micro-organisms regenerate the leach agents (Crundwell, 2003).

Two pathways of leaching are applicable to both the planktonic and sessile micro-organisms depending on the ore type. Acid-insoluble sulphide minerals e.g. pyrite (FeS_2) and tungstenite (WeS_2) are leached through the thiosulphate pathway in which the mineral is oxidised only by the ferric ion, whilst acid-soluble sulphide minerals e.g. sphalerite (ZnS) and chalcopyrite ($CuFeS_2$) are leached through the polysulfide pathway by a combination of the action of ferric ions and the acid as indicated in Figure 2.2 (Schippers and Sand, 1999; Rohwerder *et al.*, 2003).

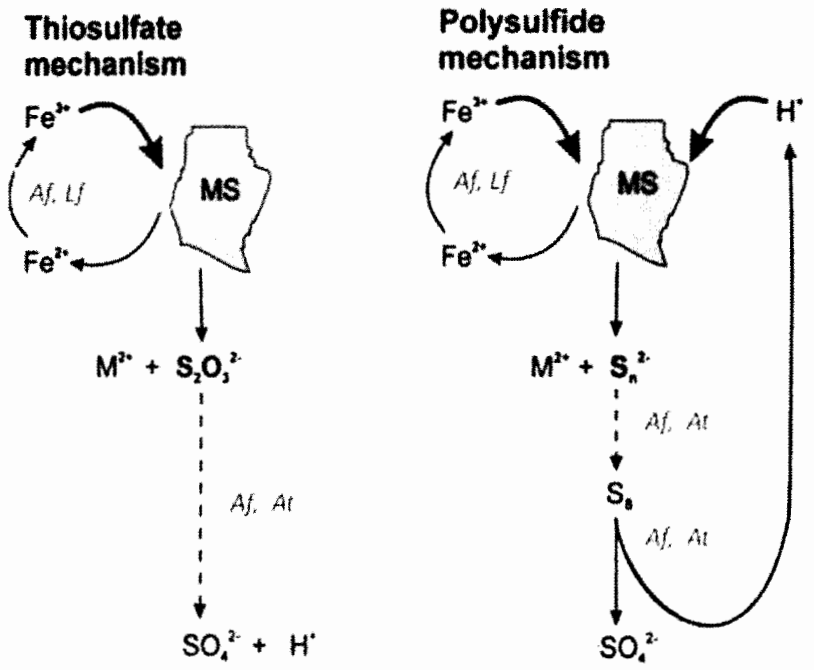


Figure 2. 2: Schematic comparison of the thiosulfate and polysulfide mechanisms in bioleaching of a metal sulfide, MS (Schippers and Sand, 1999; Rohwerder *et al.*, 2003).

2.2.2 Heap bioleaching

2.2.2.1 Construction of a bioleach heap

In the construction of a bioleach heap, the ore is crushed to small fractions (usually 80 % smaller than 10 mm). The ore is moistened with water and cured using concentrated sulphuric acid such that the ore is agglomerated i.e. fine particles are attached to the bigger ones and a highly permeable substrate is formed (Domic, 2007). The ore is stacked into a heap on top of an impervious bed and air supplied as the source of O_2 and CO_2 using a network of pipes at the base of the heap. Air can also be passively drawn into the heap. (Rohwerder *et al.*, 2003; Pradhan *et al.*, 2008). An acidic leaching solution containing the required nutrients is a medium for transport of the leached metal and is supplied by spray or drip irrigation. This percolates and is collected from the bottom as a pregnant leach solution (PLS) that contains the metal of interest. The PLS is sent for further processing by solvent extraction and electrowinning to recover the metal. A schematic diagram of heap leaching system is illustrated in Figure 2.3.

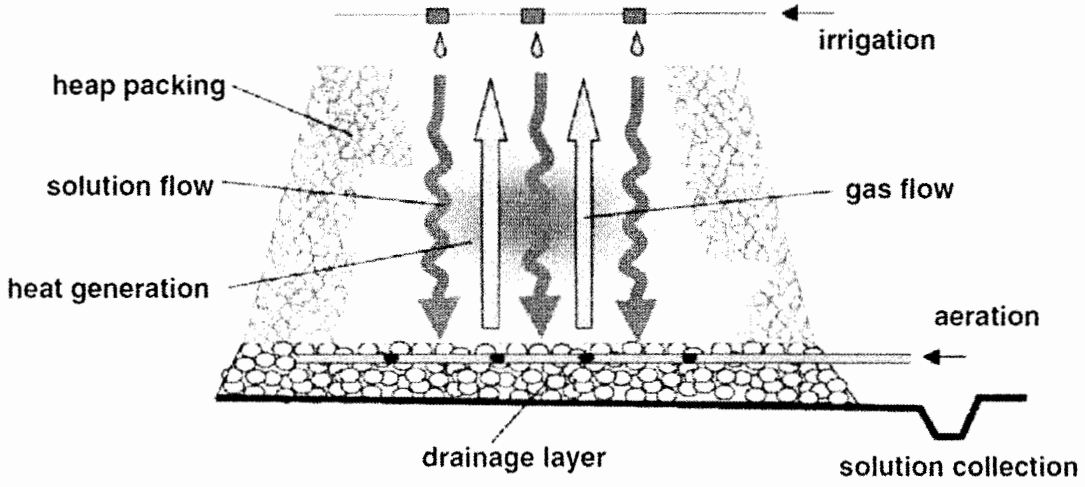


Figure 2. 3: Schematic presentation of a heap leaching system (Petersen and Dixon, 2007a)

2.2.2.2 Inoculation of a bioleach heap

The aim of inoculation is to promote colonisation of the ore by providing sufficient microbial diversity suitable for the range of conditions expected in the heap as it ages. This would ensure that adequate numbers of active bioleaching micro-organisms are present throughout the life of a heap so that optimum heap performance is achieved (Rawlings and Johnson, 2007). The process management of a heap is particularly important at 50°C to 55°C because the biodiversity and activity of acidophiles and particularly sulphur-oxidisers is less in this temperature range than both at lower and higher temperatures (Franzmann *et al.*, 2005; du Plessis *et al.*, 2007).

In the last decade it has been noted that metal solubilisation is promoted by the concerted effort of a microbial consortium rather than by single strains. Microbial consortia have been observed to be more robust and efficient than pure cultures (Rawlings and Johnson, 2007). Pure and mixed cultures of bacteria and archaea have been grown on chalcopyrite ore. It has been observed that the growth rates in pure cultures are slower than the growth rates in mixed cultures although the growth rates of individual strains in the mixed cultures vary (Plumb *et al.*, 2008a).

Frequently, consortia of micro-organisms capable of growth at a required temperature and pH are adapted to a suitable mineral before inoculating a bioleaching process. The ease of adaptation to the new process and the dominance of micro-organisms from the inoculum in the consortium that persists in the process after years of operation is still under investigation (Rawlings, 2007). However, some recent studies noted that bacteria pre-adapted to pyrite or

chalcopyrite attached in greater numbers on the surface of pyrite or chalcopyrite with more rapid metal dissolution, compared to the unadapted bacteria (Xia *et al.*, 2008).

Various aspects of the inoculation process e.g. how and when best to inoculate are not yet fully understood and further investigation is needed (Rawlings and Johnson, 2007). Inoculation methods have been researched and some applied to achieve uniform distribution of microbial activity across the heap which is important for the generation, distribution and maintenance of heat (du Plessis *et al.*, 2007). Inoculation during agglomeration or stacking has been applied. This method allows uniform distribution of micro-organisms but the moderate thermophiles and thermophiles are introduced into the heap before temperatures suitable for their optimal activity are achieved. This could result in these organisms not surviving in adverse conditions before they are optimally active or being washed out of the system. Inoculation via the leaching solution has also been applied and has the advantage of introducing specific microbial communities at the time required. The drawback of this type of inoculation is that some acidophiles especially sulphur-oxidisers have a tendency of strongly attaching to the minerals and therefore their migration downwards might be restricted, this is referred to as the tall heap phenomenon (Bouffard and Dixon, 2001). A solution to the tall heap could be the StickiBugs™ method of inoculating with partially deactivated micro-organisms allowing them to migrate deeper into the ore before attaching (Gomez and Blazquez, 1999).b

2.2.2.3 Monitoring of a bioleach heap

The extent of bioleach heap monitoring varies widely from operation to operation and also changes with the maturity and performance of the bioheap operation. A greater degree of monitoring takes place when the bioheap operation is in start-up or if problems arise during operation (Brierley, 2001).

The following techniques are employed in the industry, although rarely are all these applied at any singular heap operation. The PLS from bioheaps is analysed for pH, redox potential, acidity, ferrous and total iron concentrations, copper and arsenic (for arsenic-bearing ores) in solution. The significance of some of these measurements is discussed in Section 2.3.2. Analyses at different depths and locations of the heap are also carried out and these include assay for residual copper and other constituents such as sulfide-sulphur, arsenic and iron, measurements of temperature and oxygen, and microbial counts. The mineral assays provide inventory measurements as well as performance information i.e. the extent and rates of sulfide and mineral oxidation. Temperature measurements provide information on pyrite

oxidation e.g. high temperatures indicate active pyrite oxidation. Oxygen measurements indicate levels of aeration and when coupled with solids analyses may provide information on sulfide mineral oxidation. Oxygen uptake measurements of solid samples, PLS and raffinate using a respirometer are also done and provide information on the activity of the micro-organisms (Brierley, 2001).

The mining industry has become aware of the availability and potential value of molecular methods for determining the microbial communities in bioheaps (Brierley, 2001; Schippers, 2007). The validity of these monitoring techniques depends on obtaining representative samples. Valuable information may be obtained by correlating specific samples, the chemical and physical conditions of the bioheap with data from microbial and molecular biology assays. However, conclusions drawn from analyses of these samples may not necessarily represent the bulk of the bioheap due to the inherent lack of uniformity of the ore in bioheaps (Brierley, 2001).

2.2.3 Bioleaching of copper sulphide ores

High-grade and commercially accessible copper deposits are diminishing and an increasing proportion of copper is extracted from marginal ores (less than 1 % Cu composition) of which an increasing proportion occurs as refractory primary sulphide minerals, mainly chalcopyrite. The most comprehensively demonstrated technology for extracting copper from the marginal refractive ores has been thermophilic heap bioleaching (du Plessis *et al.*, 2007).

Secondary sulphide ores such as covellite (CuS), chalcocite (Cu_2S) and bornite (Cu_5FeS_4) are readily leached in the presence of acid and ferric ions whilst the primary copper sulphide chalcopyrite is not chemically leached at mesophilic (< 40°C) temperatures (Antonijevic and Bogdanovic, 2004). Effective leaching of the primary copper sulphides is possible at elevated temperatures, preferably at greater than 55 °C (Clark and Norris, 1996). Therefore to achieve effective copper recovery from chalcopyrite, micro-organisms are not only required to regenerate the leach agents but also to generate heat by sulphur oxidation (van Staden *et al.*, 2005). Increased leach rates have been observed at high temperatures using small particle size in stirred tank reactors as indicated in Figure 2.4 and extraction rates up to 96 % of copper from chalcopyrite have been achieved with thermophilic micro-organisms in stirred tank reactors. However, these extents of leaching are seldom achieved in heap leaching due to challenges in raising and maintaining the temperature at the required levels (Watling, 2006).

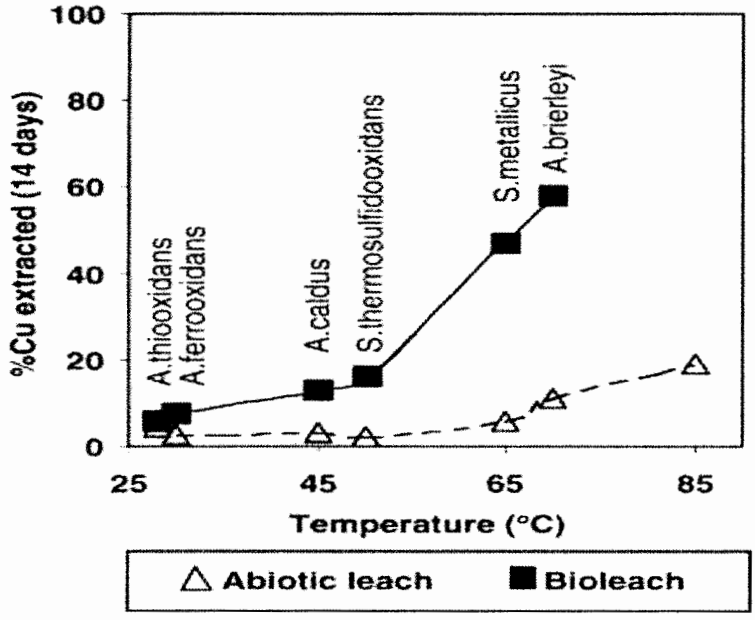


Figure 2. 4: Comparison of abiotic leaching, and bioleaching using iron- and sulphur-oxidising microorganisms, on a chalcopyrite concentrate (Watling, 2006).

At the centre of the heap, temperatures of 45 – 50 °C can be attained through sulphur oxidation by the mesophilic micro-organisms. Moderately thermophilic micro-organisms can grow at these temperatures and take part in sulphur oxidation to extend heat production further to around 60 °C. Thermophiles then need to be available to carry on with sulphur oxidation to even further raise the temperature. Petersen and Dixon (2007b) carried out a study on thermophilic heap leaching of a chalcopyrite concentrate coated onto inert support rocks (the GEOCOAT™ process) in small heated columns. The temperature was gradually increased to 70 °C, while successively introducing various mesophile and thermophile cultures. The residual concentrates were analysed and it was found that copper extractions in excess of 90 % were achieved within 100 days. MINTEK has indicated a possibility to recover up to 60 % copper in heap bioleaching of the Sarcheshmeh (predominantly chalcopyrite) ore at temperatures above 65 °C (Pradhan *et al.*, 2008).

2.3 Bioleaching micro-organisms

2.3.1 Characteristics of bioleaching micro-organisms

Bioleaching micro-organisms include bacteria and archaea, and a variety of these are found and have been isolated from natural leaching environments such as acid mine drainage (Pradhan *et al.*, 2008). The micro-organisms found in the heaps are similar to those found in stirred tank processes at similar temperatures although their proportions vary depending on the heap conditions (Rawlings, 2007). Bioleaching micro-organisms are often acidophilic, autotrophic chemolithotrophs that obtain energy for reproduction and maintenance by

oxidising ferrous iron or reduced inorganic sulphur compounds (RISCs) or both, as electron donors, and fix atmospheric carbon dioxide (CO_2) as their sole carbon source for biomass. The ability of bioleaching microbes to fix atmospheric CO_2 is advantageous because there is no need to use organic carbon sources which complicate bioleach management. These organisms also require oxygen (O_2) as their electron acceptor and some nutrients such as nitrogen (N), phosphorus (P), potassium (K) and micronutrients for their growth. In commercial applications, fertiliser-grade mineral salts are added to ensure that N, P, K and trace element limitation does not occur. The mineral (ore) is the source of iron, sulphur and other trace elements (Rawlings, 2007). Mixotrophic or heterotrophic acidophiles have also been found in bioheaps. To date, these have been reported to be present in smaller quantities than autotrophs and appear to have an important role of metabolising organic materials that might inhibit some of the more sensitive autotrophs from which these organics derive. Since many of these mixotrophs or heterotrophs also catalyse iron or sulphur oxidation or both, they may also contribute to mineral dissolution (Rawlings and Johnson, 2007).

The optimal pH for bioleaching acidophiles is in the range 0.5 - 2.5. Low pH is important in leaching for the solubility of ferric ions which are insoluble at neutral or higher pH (Brierley, 2001; Rawlings, 2007). Although the acidophiles grow at low pH, their internal (cytoplasmic) pH is close to neutral, hence a steep pH gradient exists across the cell membrane which allows transport of nutrients (Rawlings, 2007).

Micro-organisms inhabiting a bioleaching operation are selected based on temperature owing to the specific optimal temperatures (T_{OPT}) where they function. Below T_{OPT}, the micro-organisms may show reduced activity. At temperatures above T_{OPT}, they may be destroyed (Pradhan *et al.*, 2008; Franzmann *et al.*, 2005). The relationship between chemical rates and temperature is often described by the Arrhenius Equation. Similarly, microbial growth rates obey the Arrhenius rule until T_{OPT} is exceeded whereafter cell death becomes dominant. These interacting reactions may be described by the Ratkowsky Equation (Franzmann *et al.*, 2005), discussed in Section 2.3.3.

Bioleaching micro-organisms tend to attach to the ore (Schippers, 2007). During attachment, these micro-organisms secrete an extracellular polymeric substance (EPS) forming a biofilm which serves as a reaction space where mineral dissolution takes place more efficiently than in the bulk solution (Sand and Gehrke, 2006; Rawlings, 2007). This attachment and biofilm formation may provide a mechanism through which the micro-organisms can locate themselves near the energy sources (Watling, 2006).

2.3.2 Physical and chemical parameters that affect bioleaching micro-organisms

A heap is a complex bioreactor with spatial variations characterised by gradients of physicochemical parameters such as temperature, pH, redox potential, nutrients, O_2 and CO_2. This is due to the non-homogeneity of the heap caused by variations in liquid transfer, gas transfer, heat distribution, ore mineralogy and physical and chemical changes that take place as leaching progresses and as the heap ages (Olson *et al.*, 2003). These variations affect microbial growth resulting in diverse microbial communities in a heap. Some of these parameters are discussed in the following sub-sections.

2.3.2.1 Nutrient availability and metal ion concentration

The significance of some nutrients has already been introduced in Section 2.3.1, and CO_2 is discussed in detail in Section 2.3.2.2. Bioleaching micro-organisms generally tolerate a wide range of metal ions, vital for their survival in ores which are a source of various ions especially at low pH and in the presence of ferric ions, where leaching of the ore easily takes place (Dopson *et al.*, 2003; Rawlings, 2007). Generally, microbial cultures are pre-grown or adapted to a particular ore in a laboratory or pilot plant. This way, selection for organisms able to oxidise iron and sulphur in the presence of potentially inhibitory agents is achieved. Acidophiles have a unique physiology that makes them sensitive to inhibition by organic acids and certain ions when they exceed certain levels (Olson et al., 2003). For example, fluoride ions must be below 0.5 g/L, and chloride and nitrate must be below 1.5 g/L. Silver and anions of tellurium, arsenic and selenium are toxic at 50 -100 mg/L and molybdenum (as molybdate) at 5 mg/L. Potentially inhibitory compounds also come from process waters, the target ore and solvent extraction organics in the recycled irrigation solution (Tuovinen *et al.*, 1971; Gomez and Blazquez, 1999; Suzuki *et al.*, 1999). Copper toxicity is discussed in Section 2.3.2.2.

2.3.2.2 Carbon-dioxide availability: solubility and mass transfer

Oxygen and CO_2 have low solubility i.e. only 0.26 mM (8.32 mg/L) O_2 and 0.0172 mM (0.756 mg/L) CO_2 can dissolve in water at 25°C in an air/water mixture. However due to the influence of culture or media ingredients and mineral pulp, the maximum O_2 and CO_2 contents are usually lower than would be in pure water (Witne and Phillips, 2000). The situation is further worsened when elevated operating temperatures are used as the solubility of these gases decreases with increasing temperature (Witne and Phillips, 2000; du Plessis *et al.*, 2001). Furthermore, CO_2 solubility is reduced by an increase in acidity (Naik, 2010). Low solubility of gases reduces the gas-liquid transfer rate thereby limiting

bioleaching efficiency; hence CO_2 enrichment is often used in tank leaching operations (d'Hugues *et al.*, 2002).

To date, there has been no general consensus on the upper limit where CO_2 becomes inhibitory or the lower limit where CO_2 becomes a limiting substrate thus slowing down microbial growth rate and ultimately the metal extraction rate. Differences in the results reported in literature indicate that CO_2 demand in bioleaching varies with different cultures, bacterial adaptation and the feed material e.g. the presence of another carbon source (Witne and Phillips, 2000). However, many studies have reported increases in bioleaching rates by the supplementation of sparged air with CO_2. Petersen *et al.* (2010) observed that CO_2 was consumed bottom up resulting in CO_2-depleted zones in large-scale columns during bioleaching of a low-grade copper ore using air enriched to between 1000 and 2000 ppm CO_2. Metal oxidation rates declined in the CO_2-depleted zones and this decline was postulated be linked to a decrease in microbial community due to CO_2 deficiency. The results implied that CO_2 supplementation stimulates microbial growth and CO_2 consumption, but not necessarily O_2 uptake and leaching. Witne and Phillips (2000) reported that Cu and Fe dissolution from a copper ore concentrate improved with increasing aeration CO_2 content up to an optimum of 10 vol % beyond which any increase in the CO_2 content resulted in the decrease of Cu dissolution. Their study was done on a mesophile (*Acidithiobacillus ferrooxidans*), a moderate thermophile (*Sulfobacillus acidophilus*) and an extreme thermophile (*Sulfolobus* species) culture and all the cultures showed an increase in the dissolution rate compared with normal air (0.03 vol % CO_2). Nagpal *et al.* (1993) observed during bioleaching of a pyrite-arsenopyrite concentrate in continuous-flow reactors that the optimal aqueous phase CO_2 concentration for bacterial growth was 3 - 7 mg/L and that CO_2 in excess of 10 mg/L (ca. 0.7 vol %) inhibited bacterial growth. Nagpal *et al.* (1993) also stated that Holuige *et al.* (1987) reported that an increase of CO_2 concentration from 0.03 to 0.07 and 0.10 vol % increased the growth of *At. ferrooxidans* two-fold and no further increase was observed above 0.35 % whilst growth was reduced when 5.4 % CO_2 was used. Nagpal *et al.* (1993) also stated that their previous study done in 1991 showed that bacteria grown on a sulfide ore concentrate utilise O_2 and CO_2 in a molar ratio of approximately 20 moles of O_2 per mole of CO_2 suggesting that air supplemented with 1.0 vol % CO_2 would provide the appropriate ratio of these two gases. They observed that supplementation with 1.0 vol % CO_2 enabled up to a ten-fold increase in cell growth rates over that achieved without CO_2 supplementation at similar gas sparge rates.

Mass and heat transfer profiles exist in leaching but tank leaching provides better mixed systems that overcome some of the mass transfer limitations encountered in heap leaching

(Rawlings, 1997). Naik (2010) stated that "mass transport encompasses the transport of ferrous iron to microbes at the mineral surface, the gas-liquid mass transfer of oxygen and carbon dioxide, and the dissolution and transport of the leach solution out of the heap". Petersen and Dixon (2007) stated that "Gas (i.e. O_2 and CO_2) uptake from the gas phase into the liquid phase is a simple mass-transfer step governed primarily by temperature". Boon and Heijnen (1997) reviewed literature on kinetic experiments, to assess whether gas–liquid transfer of O_2 or CO_2 determined the bacterial oxidation rate of sulphide minerals in STRs and shake flasks. The conclusion was that in bacterial oxidation of sulphide minerals gas–liquid transfer of O_2 and CO_2 easily becomes reaction rate-determining. It was shown that CO_2 exhaustion in the aeration air and CO_2 gas–liquid transfer limitation occurs rather than O_2 limitation. It was also noticed that possibly other factors may cause a decrease of the bacterial oxidation rate, such as nutrient limitation, low pH values, a relatively low concentration of bacteria due to the size of the inoculum, bacterial shear or inhibition by substances that leach from the mineral. However, these factors can only be examined if sufficient mass transfer is provided.

2.3.2.3 Copper toxicity

Copper is a heavy metal. Many heavy metals are essential for microbial growth, but they are also reported to have toxic effects on cells, mainly as a result of their ability to denature protein molecules (Gadd and Griffiths, 1978). Cu concentrations in heap or dump leachates are typically in the range of 2 - 6 g/L (30 - 90 mM) and in agitated tanks reach at least 19 g/L (ca. 300 mM). Metal tolerance varies significantly between microbial species, and between strains of the same species. This is accentuated through adaptation to different levels of metal exposure (Jerez, 2011; Rawlings, 2005).

In a study of metal toxicity on *At. caldus,* half-maximal inhibitory concentrations (IC50s) for lead (Pb), zinc (Zn) and Cu were observed to be 0.9, 39 and 120 mM, respectively and minimum inhibitory concentrations were 7.5, 75 and 250 mM (i.e. ca. 1.5, 5.0 and 16 g/L) respectively (Aston *et al.*, 2010). When metals were presented in binary mixtures, the toxicities were less than additive. The importance of inoculum history was evaluated by pre-adapting cultures in Pb, Zn and Cu at their respective IC50s. After adaptation, cultures had growth rates 39±11, 32±7 and 28±12 % higher in the presence of Pb, Zn, and Cu IC50s respectively, compared with un-adapted cultures. Metal chlorides showed more toxicity than metal sulfates suggesting an additive combination of metal and chloride toxicities. Tuovinen *et al.* (1971) reported that during iron oxidation, *At. ferrooxidans* tolerated Zn, Ni, Cu, Co, Mn and Al concentrations greater than 10 g/L. Adaptation to Zn, Ni or Cu was found to result in

increased tolerance to other metals. Published data on metal tolerance suggests that all heterotrophs except some fungi are affected by heavy metals above 0.1 mM concentrations while some autotrophic *Acidithiobacillus* species are unique in their high tolerance.

2.3.2.4 Solution pH

The solution pH in the heap is determined by the balance between acid-producing and acid-consuming components of the ore and also by the input of the mine-site operator e.g. the acid used during agglomeration, the acid concentration of the feed leach solution, the irrigation rate and the microbial activity established. Dissolution of the mineral sulphides can be acid-producing leading to a decrease in pH e.g. pyrite dissolution, or acid-consuming leading to pH increase e.g. chalcocite dissolution. Also, some gangue minerals such as carbonates are acid-consuming (Plumb *et al.*, 2008b). The pH and acidity measurements indicate the extent of acid conditioning of the bioheap and provide insight into the oxidation of pyrite (Brierley, 2001). Low pH values optimal for acidophiles are important because the activity of some micro-organisms especially archaea is adversely affected at pH values greater than 2. Too high a pH contributes to formation of jarosite (a ferric precipitate) and too low a pH might cause dissolution of gangue. The accumulation of the gangue minerals and the jarosite precipitation might cause clogging and negatively impact on gas-liquid transfer in the heap (du Plessis *et al.*, 2007). It has also been demonstrated that bioleaching of mineral sulphides occurs favourably at pH less than 3 (high concentration of the acid). At such low pH values, abiotic oxidation of Fe^{2+} is negligible and elemental sulphur is inert to abiotic oxidation hence the activity of acidophilic ferrous iron-oxidisers and sulphur-oxidisers is important at these pH values (Plumb *et al.*, 2008b).

2.3.2.5 Redox potential

A high redox potential shows a high Fe^{3+}/ Fe^{2+} ratio and this could indicate that iron-oxidising micro-organisms are very active. However a high concentration of Fe^{3+} at high temperatures could lead to jarosite formation and passivation (coating) of the mineral leading to decreased diffusion rates, decreased microbial growth rates and hence low leach rates (Rawlings and Johnson, 2007). Redox potential, iron concentrations and ferrous to ferric ratios also provide information on dissolution of pyrite and other iron-bearing minerals (Brierley, 2001).

2.3.2.6 Irrigation and aeration

Management of the rates of irrigation and aeration is important as these affect heap temperature profiles (Petersen and Dixon, 2007b). The balance of the downward liquid flow and upward gas flow in a heap is important for heat distribution and some ways that have

been employed to reduce heat loss from the heat surfaces are covering the heaps with insulating material and use of solar mats (du Plessis *et al.*, 2007).

2.3.2.7 Temperature

Physical and chemical changes within a heap or liquid culture select for the fittest micro-organisms, effecting a succession of organisms. The microbial response to these changes is poorly understood with the exception of temperature (Brierley, 2001; Franzmann *et al.*, 2005). As temperature increases, a succession from mesophiles to moderate thermophiles to thermophiles can occur. This occurrence in a mineral sulphide ore depends on the availability of these micro-organisms which can either be indigenous to the ore or are introduced to the ore as an inoculum (Franzmann *et al.*, 2005; Watling, 2006). Microbial response to temperature is largely understood from tests done using pure cultures in ideal laboratory conditions and more studies are required to define this in the heap environment.

2.3.3 Classification of bioleaching micro-organisms with respect to temperature

Most of the familiar bioleaching acidophiles can be placed in one of the three broad groups based on their temperature range of optimal growth. Generally, two or three species specific to a temperature range predominate in various mixed cultures (Norris, 2007). The three groups of bioleaching organisms are; mesophilic bacteria that live and reproduce at about 10 to 45°C; moderately thermophilic bacteria that function at about 45 to 60°C; and the thermophilic archaea, which grow in the 60 to 90°C range (Brierley, 2000). A number of Fe- and S-oxidising micro-organisms have been isolated from sulphide mineral ores, analysed physiologically and phylogenetically, and deposited in data banks (Schippers, 2007). A selection of these micro-organisms is listed in Table 2.2.

The response of micro-organisms to temperature is often described by Equation (5), the empirical Ratkowsky Equation;

$$\sqrt{\frac{1}{t}} = b(T - T_{min})(1 - e^{c(T - T_{max})}) \quad (5)$$

where T is the temperature
t is the doubling time or the time taken to reach a specific condition e.g. time taken by a culture to reach a certain optical density
T_{min} is the theoretical extrapolated minimum temperature for growth
T_{max} is the theoretical extrapolated maximum temperature for growth
b and *c* are fitting parameters.

Table 2.3 shows results obtained using the Ratkowsky Equation on some moderate thermophile bioleaching bacteria and archaea (Franzmann *et al.*, 2005).

2.3.3.1 Mesophilic bacteria

In the mesophile range, the most active acidophilic microbial species are the *Acidithiobacillus* and *Leptospirillum*. The most commonly reported in natural and commercial bioleaching systems below 40 °C are *At. ferrooxidans, L. ferriphilum* and *L. ferrooxidans* (Coram and Rawlings, 2002; Watling, 2006). *Leptospirillum* species are widespread in ore deposits, ore dumps and solutions from Cu leaching processes (Brierley, 2001). *L. ferrooxidans* tolerates lower pH and higher metal concentrations, Fe^{3+} (higher redox potentials), uranium, molybdenum, silver and sulphates than *At. ferrooxidans* but is more sensitive to Cu. *At. ferrooxidans* is more tolerant of low temperatures and less tolerant of high temperatures than *L. ferrooxidans* (Rawlings *et al.*, 1999). The activity of both *At. ferrooxidans* and *Leptospirillum* species is strongly inhibited above 45 °C (Brierley, 2001). *L. ferriphilum* was reported to dominate in arsenopyrite biooxidation stirred tanks together with *At. caldus* and also to grow faster when attached to the ore than when free in the PLS in mesophilic conditions (Zeng *et al.,* 2010). *L. ferriphilum* has a slightly higher temperature tolerance and a faster Fe-oxidation rate than *L. ferrooxidans* which could explain its dominance over *L. ferrooxidans* in commercial bioleach tanks (Plumb *et al.*, 2008a).

Acidithiobacillus ferrooxidans is the most studied bioleaching micro-organism because it was the first micro-organism to be isolated from an acidic leaching environment and is widespread in bioleaching systems especially batch systems. *Acidithiobacillus* spp. were viewed as the most important species in bioleaching systems until other species were discovered (Coram and Rawlings, 2002; Brierley, 2007). *At. ferrooxidans* are Gram-negative bacteria found naturally living in acid mine drainage waters of sulphide ore deposits, bituminous coal mines and commercial bioleaching systems (Rawlings *et al.*, 1999). This species grows on Fe^{2+} and RISCs, can also oxidise hydrogen, formic acid and other metal ions, and can fix atmospheric nitrogen explaining its activity in the absence of any obvious N source (Brierley, 2008). *At. ferrooxidans* grows at 4 - 40°C, optimally around 33°C, and at pH 1.5 - 6.0 with an optimum pH of 2.0 - 2.5. This species has metal tolerance with metabolic activity reported in 84 mM As^{3+}, 800 mM Cu^{2+}, 1071 mM Zn^{2+}, 500 mM Cd^{2+} and 1000 mM Ni^{2+} (Dopson *et al.*, 2003).

Table 2. 2: Optima and ranges of pH and temperature for growth, and physiological properties of metal sulfide oxidising, acidophilic micro-organisms (adapted from Schippers, 2007; Dew *et al.*, 2011).

Micro-organism	pH optimum	pH range	Temperature optimum	Temperature range	Substrates oxidised*	Type of growth*
Acidithiobacillus ferrooxidans	1.7 - 2.5	1.3 - 4.5	30 - 35	10 - 37	Fe, S, Pyr, MS	A
Acidithiobacillus thiooxidans	2.0 - 3.0	0.5 - 5.5	28 - 30	10 - 37	S, MS	A
Leptospirillum ferrooxidans	1.5 - 3.0	1.3 - 4.0	28 - 30	20 - 35	Fe, Pyr, MS	A
Acidimicrobium ferrooxidans	1.7 - 2.5	1.0 - 3.5	50	40 - 55	Fe, Pyr, MS (na)	F
Acidithiomicrobium species	1.7 - 2.5	1.0 - 3.5	50	40 - 55	Fe, Pyr, S	F
Acidithiobacillus caldus	2.0 - 2.5	1.0 - 3.5	45	32 - 52	S, MS	F
Leptospirillum ferriphilum	1.4 - 2.1	1.4 - 4.0	37	30 - 45	Fe, Pyr, MS	A
S. thermosulfidooxidans	1.7 - 2.4	1.0 - 3.0	45 - 58	28 - 60	Fe, S, Pyr, MS	F
Acidiplasma cupricumulans	1.0 - 1.2	0.4 - 1.8	54	22 - 63	Fe, S, Pyr (na), MS	F
Metallosphaera hakonensis	3.0	1.0 - 4.0	70	50 - 80	Fe/Pyr (na), S, MS	F
Metallosphaera sedula	2.0 - 3.0	1.0 - 4.5	75	50 - 80	Fe, S, Pyr, MS	F

* Fe = ferrous iron; S = sulphur; Pyr = pyrite; MS = other metal sulfides besides pyrite, A = autotroph; F = facultative autotroph and/or mixotroph; na = data not available

Table 2. 3: Extrapolated values of cardinal temperatures for the activity of some moderate thermophile bioleaching micro-organisms derived from the application of the Ratkowsky Equation (adapted from Franzmann *et al.*, 2005).

Micro-organism	Substrate	Cardinal temperatures (°C)		
		T_{min}	T_{OPT}	T_{max}
Acidimicrobium ferrooxidans	Fe^{2+}	7.4 ± 3.0	48.8	59.5 ± 0.3
Acidithiobacillus caldus	S^0	-32.4 ± 11.0	48.8	53.6 ± 0.1
Leptospirillum ferriphilum	Fe^{2+}	10.7 ± 1.1	38.6	48.5 ± 1.0
Sulfobacillus thermosulfidooxidans	Fe^{2+}	11.7 ± 5.3	51.2	63.5 ± 1.4
Ferroplasma acidiphilum	Fe^{2+}	12.7 ± 6.1	39.6	47.2 ± 0.7
Ferroplasma cyprexacervatum *	Fe^{2+}	15.0 ± 1.4	55.2	63.0 ± 0.1

*also once referred to as *Ferroplasma cupricumulans* (Hawkes *et al.*, 2006) but now referred to as *Acidiplasma cupricumulans* (Golyshina *et al.*, 2009)

Leptospirillum ferriphilum was isolated from commercial biooxidation tanks in South Africa. *L. ferriphilum* are aerobic, obligately chemolithotrophic bacteria which use Fe^{2+} or pyrite as their energy source. These bacteria are optimally active at pH 1.4 - 1.8 and at 30 - 37 °C but some isolates are able to grow at 45 °C and therefore *L. ferriphilum* can also be classified under moderate thermophiles. *Leptospirillum* spp. have been found as the dominant Fe-oxidising bacteria in industrial continuous-flow biooxidation tanks such as those used in treatment of gold-bearing arsenopyrite concentrates (Coram and Rawlings, 2002).

2.3.3.2 Moderately thermophilic bacteria and archaea

Moderate thermophiles are found in the same types of natural environments as the mesophilic bacteria, becoming abundant as the temperature increases (Brierley, 2001). *Sulfobacillus* species dominate in this temperature range although some bacteria that are active from ± 25 to 55°C e.g. *At. caldus* and *Acidimicrobium ferrooxidans* are also found in this range. In recent years, many reports have shown that moderate thermophiles such as *L. ferriphilum*, *At. caldus* and *Ferroplasma thermophilum* can greatly improve reaction kinetics, avoid excessive passivation and thus improve Cu extraction during bioleaching of chalcopyrite (Gomez and Blazquez, 1999; Hawkes *et al.*, 2006). Moderately thermophilic micro-organisms are routinely used along with mesophilic bacteria and thermophilic archaea for bioleaching of pyrite in refractory sulfidic gold ores (Brierley, 2008).

Acidithiobacillus caldus are Gram-negative proteobacteria that grow autotrophically and oxidise various sulphur compounds (elemental sulphur, sulfide, sulphite, thiosulfate and tetrathionate). This species grows optimally at 45°C and at pH 2.0 - 2.5. *At. caldus* were the first species of the moderately thermophilic *Acidithiobacillus* genus to be isolated and characterized. The growth rate of *At. caldus* measured in tetrathionate media at 45°C at various pH values was highest at pH 2.0 - 2.5 where the doubling time was 3 hours. Growth was slow at pH 4.0 and 1.0 where the doubling time was 46 hours and 6.7 hours respectively whilst no growth was observed at pH 0.5. The growth of this species was found to be enhanced when sparged with air supplemented with 2 % v/v CO_2 (Hallberg and Lindström, 1994). *At. caldus* is generally considered to be the most active acidophilic, thermotolerant S-oxidising bacterium in mineral leaching environments and has been found in tank bioleaching operations, geothermal sites and acid mine drainage (Norris *et al.*, 2011).

Acidimicrobium ferrooxidans are Gram-positive Fe-oxidising bacteria that were first found in a commercial Cu leach dumps (Norris, 2007). This species is optimally active at 48 °C but

can also be found growing at mesophilic temperatures and its maximum doubling time on yeast extract at its optimum temperature is about 6 hours (Clark and Norris, 1996).

Acidithiomicrobium is a species of autotrophic Fe- and S-oxidising actinobacteria that has not been formally named but is referred to as "*Acidithiomicrobium*". This species which is related to *Acidimicrobium ferrooxidans* was the first acidophilic actinobacteria observed to grow autotrophically on sulphur and their extent of S-oxidation is similar to that of *At. caldus.* This species also grows autotrophically with Fe^{2+} and degrades pyrite efficiently. The extensive dissolution of pyrite during growth contrasted with a requirement for yeast extract for significant growth of the related *Am. ferrooxidans*. *Acidithiomicrobium* species was first isolated from geothermal springs but has also been found dominating in mixed cultures of moderate thermophiles growing on copper sulfide and polymetallic sulfide ores in leaching columns. *Acidithiomicrobium* species grows at an optimum temperature around 50°C but can operate at a range of 40 - 55 °C and grow optimally at a pH of 1.7 - 2.5 but can operate at a pH of 1.0 - 3.5 (Davis-Belmar and Norris, 2009; Norris *et al.*, 2011; Dew *et al.*, 2011).

Acidiplasma cupricumulans was formerly classified in the *Ferroplasma* genus which is composed of single-celled archaea with no nucleus or cell-bound organelles (Hawkes *et al.*, 2006a; Golyshina *et al.*, 2009). *A. cupricumulans*, isolated from a chalcocite bioleaching heap in Myanmar, was then the first moderately thermophilic strain of the *Ferroplasma* genus. This species grows optimally at 53.6 °C and at pH 1.0 - 1.2, and has a maximum growth temperature of 63 °C. *A. cupricumulans* exhibits chemomixotrophic growth oxidising ferrous iron, pyrite and other metal sulfides as a source of energy and also oxidises ferrous sulphate, in the presence of yeast extract. This species is a facultative aerobe which reduces ferric iron under anaerobic conditions (Hawkes *et al.*, 2006a).

Sulfobacillus genus is composed of aerobic Gram-positive, endospore forming, facultatively autotrophic and extremely acidophilic bacteria that prefer to utilise organic carbon, although they are able to fix CO_2. This genus uses both Fe^{2+} and reduced sulphur compounds even in the presence of yeast extract as energy sources. Currently there are three recognized species, *S. thermosulfidooxidans, S. acidophilus* and *S. sibiricus* (Brierley, 2001; Bogdanova *et al.*, 2006). *S. thermosulfidooxidans* discovered in Cu mine leach dumps in Russia in the 1970s was the first species of the *Sulfobacillus* genus to be isolated and characterized. This species grows optimally at 45 - 58 °C and at pH 1.7 - 2.4, and is widely distributed in exposed sulfidic mineral deposits and in bioleaching tanks of Cu-containing mineral concentrates. *S. thermosulfidooxidans* prefers mixotrophic conditions and may not grow if these conditions are not met (Schippers, 2007; Plumb *et al.*, 2008a; Brierley, 2008;

Ñancucheo and Johnson, 2010). The mean doubling time of this species was observed to be 8 - 12 hours when heterotrophic growth was assayed on yeast extract (Norris *et al.*, 1996). This species is also metal tolerant, with metabolic activity reported in 6 mM Cu^{2+}, 43 mM Zn^{2+} and 5 mM Ni^{2+}. *Sulfobacillus* spp. probably have only a minor role in mineral oxidation, and their importance is in degrading organic compounds which when accumulated would potentially be toxicity to other chemolithoautotrophs (Dopson *et al.*, 2003; Schippers, 2007).

2.3.3.3 Thermophilic archaea

The dominant thermophilic archaea recognised to occur in the bioleaching environment are *Sulfolobus* spp. and *Metallosphaera* spp. These archaea grow under extreme high temperatures close to the boiling point of water and are acidophilic, mixotrophic chemolithoautotrophs which can use both Fe^{2+} and RISCs as energy sources and can survive under low oxygen levels and high ionic strengths (Brierley, 2001).

Metallosphaera sedula was isolated from a solfataric field in Italy and is an aerobic, facultative chemolithoautotroph which uses metal sulfides, elemental sulphur and organic compounds as substrates. *M. sedula* extracts metal ions from minerals with very high efficiency and grows between 50 °C and 80 °C with an optimum around 75 °C, and at a pH between 1.0 and 4.5. The doubling time for *M. sedula* at its optimum temperature and pH in a suspension of an ore mixture of pyrite, sphalerite and pitch blend is 5.25 hours. *M. sedula* is also metal tolerant with metabolic activity reported in 1.3 mM As, 0.9 mM Cd, 0.85 mM Co, 16 mM Cu, 0.0005 mM Hg, 0.1 mM Mo, 0.8 mM Sb, 0.4 mM U and 150 mM Zn (Huber *et al.*, 1989; Dopson *et al.*, 2003; Schippers, 2007).

Metallosphaera hakonensis was originally described as *Sulfolobus hakonensis* by Takayanagi *et al.* (1996) and reclassified as *M. hakonensis* by Kurosawa *et al.* (2003). This species, isolated from an acidic hot spring in Hakone, Japan, is also an aerobic, facultative chemolithoautotroph, and uses metal sulfides, elemental sulphur, tetrathionate, hydrogen sulfide and organic compounds as substrates. *M. hakonensis* grows between 50 °C and 80 °C with an optimum around 70 °C, and at pH 1.0 - 4.0 with an optimum pH of 3.0 (Kurosawa *et al.*, 2003; Takayanagi *et al.*, 1996).

2.3.4 Growth rates and microbial dynamics of bioleaching moderate thermophiles

Most of the published data on microbial dynamics of bioleaching micro-organisms is on the prevalence and dominance of microbial species. There is scarcity of published data on growth rates. Some published data on growth rates determined in liquid cultures is available

but information on growth rates in whole ore environments typical of the heap leach processes is scarce. Growth rates found in literature have been stated in the descriptions of some of the microbial species in Section 2.3.3. Case studies of prevalence and dominance of moderate thermophiles on whole ore and in liquid cultures are discussed below.

At. caldus, S. thermosulfidooxidans and *S. montserratensis*-like bacteria were found to dominate consortia on a chalcopyrite, pyrite and arsenic pyrite concentrates at 45 °C (Dopson *et al.*, 2003). *At. caldus* was also found dominating the consortia colonising the ore and also prevalent in the PLS on chalcopyrite at 28, 35 and 45 °C (Plumb *et al.*, 2008a) and also being the dominant S-oxidiser in bioleach tanks at 40 – 55 °C (Rawlings *et al.*, 1999).

An enumeration and characterization was done on micro-organisms isolated from a 45 °C pilot-scale stirred-tank bioleach of a polymetallic concentrate whose major minerals were chalcopyrite, sphalerite and pyrite. Four distinct species were isolated; three bacteria (an *At. caldus*-like organism, *Leptospirillum* spp. and *Sulfobacillus* spp.), and one archaeon (a *Ferroplasma*-like organism). The microbial composition changed as leaching progressed and *Ferroplasma* spp. became increasingly dominant eventually accounting for at least 99 % of the isolated community in the third of the 3 in-line reactors (Okibe *et al.*, 2003).

In a mixed culture of moderate thermophiles used to bioleach finely ground chalcopyrite ore in a stirred reactor at 50 °C, *L. ferriphilum* and *At. caldus* were found to be the dominant bacteria and *F. thermophilum* the only archaea. On investigating the microbial dynamics over a 40-day period, *At. caldus* was observed to dominate both the mineral surface and the solution in the early stages and *L. ferriphilum* dominated the later stages and was observed to increase faster on the ore than in the PLS. *F. thermophilum* was found throughout the whole period but was observed to increase faster in PLS than on the ore towards the end of the experiment (Zeng *et al.*, 2010).

The effect of temperature on community dynamics on non-sterile low grade chalcopyrite ore in columns at 30, 40, 50 and 60 °C was studied using a consortium of ten microbial strains as an inoculum. Only *At. caldus*, *L. ferriphilum* and *F. acidiphilum* were identified both in the PLS and ore whilst the rest of the organisms identified were adventitious growths. In the PLS, *L. ferriphilum* was dominant in all the columns except for the 50 °C column where *At. caldus* dominated. The archeon *F. acidiphilum* was also dominant at 40 and 50°C and was also found in low quantity at 60°C where an unidentified archeon was dominant. On the ore, *At. caldus* and *F. acidiphilum* dominated all the columns (Mutch *et al.*, 2010). The conclusion was that the three surviving microbial species grew because their growth requirements were

met and the adventitious growths were either from the columns or were indigenous to the ore. The reason for the failure of the other micro-organisms were in some instances not known but *Acidimicrobium ferrooxidans* which was expected in all columns could have been hindered by the acid-consuming nature of the ore because it requires low pH to grow. *S. thermosulfidooxidans* could have been hindered because the columns did not offer the mixotrophic conditions it requires and could have also been hindered by low levels of CO_2 owing to reduced solubility at 50 °C. The thermophilic archaea *Acidianus brierleyi* and *Metallosphaera hakonensis* did not survive, probably because their optimal temperatures were not reached (Clark and Norris, 1996; Franzmann *et al.*, 2005; Mutch *et al.*, 2010).

Growth and activity of pure and mixed cultures were investigated on finely ground low grade chalcopyrite ore in Erlenmeyer flasks at 28, 35, 45, 55 and 65 °C. Eleven strains tested showed slow growth in pure cultures and fast growth in mixed cultures with the fastest growth observed at 65 °C. Dominant strains in the mixed cultures were found to be *S. thermosulfidooxidans, Am. ferrooxidans, At. caldus* and *L. ferriphilum* at 45 – 55 °C, and *M. hakonensis and Sulfolobus metallicus* at 65 °C (Plumb *et al.*, 2008b).

Dew *et al.* (2011) reported on observations made when special tall pilot scale columns were used to simulate heap leach operating conditions in an investigation of the process of microbial succession during heap bioleaching of chalcopyrite ore. The microbial community in the ore was estimated from the microbial analysis of the PLS. The results showed that initially (at the starting temperature of 15 - 20 °C), the microbial community was dominated by the mesophile *At. ferrooxidans*. As the temperature increased to 35 °C and to 50 °C, the community transitioned such that *L. ferriphilum* and *Acidithiomicrobium* species became the dominant species. At higher temperatures (above 55 °C), thermophiles became dominant, most notably *M. hakonensis,* and *S. metallicus* at much lower numbers. *Acidiplasma cupricumulans* a moderate thermophile was also prominent throughout the operation.

2.4 Techniques of identifying and quantifying bioleaching micro-organisms

In order to achieve an understanding of the community structure and dynamics as discussed above, quick and reliable methods to identify and quantify single microbial species in bioleaching communities are needed. Microbial communities can be analysed using physical, biological and nucleic acid-based molecular methods described below.

2.4.1 Physical and biological techniques

2.4.1.1 Microscopy

Cells can be counted using either light microscopy with morphology as a discerning feature or fluorescence microscopy using nucleic acid-staining fluorochromes. However, fluorochromes bind unspecifically to DNA and therefore cell counts could also include dormant or dead cells (Schippers, 2007). Two more draw-backs of microscopy are the difficulty to differentiate cells from particulate matter and the difficulty encountered in detaching the micro-organisms from mineral surfaces, particularly those that synthesize copious amounts of EPS strengthening their attachment (Johnson and Hallberg, 2007).

2.4.1.2 Biomass measurements

There are standard methods to measure microbial biomass and these can be modified for use in bioleaching. In these techniques, cellular components e.g. proteins are measured to assay the amount of biomass. The major draw-back is the inability to differentiate between different types of micro-organisms (Johnson and Hallberg, 2007).

2.4.1.3 Activity measurements

Microbial activity, particularly the redox transformations of Fe and sulphur are important in mineral-leaching environments. Fe oxidation can be assayed by monitoring decrease of Fe^{2+} or an increase of Fe^{3+} concentrations, while sulphur oxidation can be assayed by measuring changes in sulphate concentrations. Also, a dissolved-oxygen probe can be used to measure O_2 consumption associated with Fe or sulphur oxidation. By coupling measurements of redox transformation of Fe or sulphur with those of biomass, specific rates of transformations can be determined. The heat generated through the biologically catalyzed oxidation of pyrite and other sulfidic minerals may also be measured to quantify the activity of these acidophiles (Johnson and Hallberg, 2007).

2.4.1.4 Cultivation techniques

The pre-requisite of these techniques is that the micro-organisms to be assayed are able to grow under the defined laboratory conditions. Enrichment liquid media are formulated to encourage growth of target micro-organisms. Solid media can also be used e.g. the dual-layer agarose plate technique for enriching micro-organisms from bioleaching communities. Most probable number (MPN) counts can also be used. This is a statistical method whereby samples are diluted and inoculated into a series of tubes containing growth media. After incubation the tubes are scored for positive or negative growth and the results compared

with standard tables to determine the MPN of micro-organisms present (Johnson and Hallberg, 2007). The draw-back of cultivation techniques is that only a subset of the whole microbial community can be detected because the media are designed to select for certain groups. Furthermore, cultivation techniques are labour-intensive and the analysis results become available after long incubation times (several days or weeks) which does not allow timely monitoring of bioleaching operations (Johnson and Hallberg, 2007; Schippers, 2007).

2.4.1.5 Immunological assays

Specific antibodies are used to target and enumerate micro-organisms. However, these assays are time-consuming and require thorough prior characterisation of the micro-organisms occurring in the bioleaching operation as only those micro-organisms targeted will be enumerated (Schippers, 2007).

2.4.2 Nucleic acid-based molecular techniques

Over recent years, nucleic acid-based molecular techniques have been used increasingly to identify and quantify micro-organisms in the environment and in other technical applications. These techniques allow for rapid analysis of microbial communities without prior cultivation. Most of these techniques are based on gDNA extraction from cells, followed by DNA amplification using polymerase chain reaction (PCR), and finally an analysis of the DNA amplification products. In most cases the SSU rRNA (small subunit of ribosomal RNA) gene is targeted for use in identification, but also functional genes coding for key enzymes can be targeted. The SSU rRNA gene is common to all organisms i.e. the 16S rRNA gene of prokaryotes (bacteria and archaea), and the 18S rRNA gene of eukaryotes. This gene is a highly conserved molecule that is made up of regions of near identity interspersed with regions of high sequence variability. The nearly identical regions are targets for universal PCR primers that facilitate the amplification of this gene. Universal primer pairs to amplify nearly the entire 16S rRNA gene from bacteria and archaea, as well as internal portions of the gene are commercially available. In contrast, the regions of high variability can serve as targets for species-specific PCR primers. The resulting PCR products are subjected to various analyses to quantify the micro-organisms e.g. real-time PCR and fluorescence in-situ hybridization (FISH). The drawback of these methods is that they cannot provide information about unknown microorganisms. Therefore, a combined use of molecular and cultivation techniques is recommended (Johnson and Hallberg, 2007; Schippers, 2007). Alternatively, complementing nucleic acid based techniques able to quantify the range of micro-organisms present, but not their abundance, should be used in a complementary manner.

2.4.2.1 PCR-based molecular methods for identification of microorganisms

Biodiversity in bioleaching microbial communities and the identification of new species can be addressed by cloning PCR products. The 16S rRNA gene of the various clones can be sequenced and the similarities of the sequences can then be shown in a phylogenetic tree, to address the phylogenetic affiliation of the microorganisms in a sample. Alternatively, the PCR products can be separated in DGGE (denaturing gradient gel electrophoresis) which allows a separation of DNA fragments of the same length but different base-pair sequences. Bands in the DGGE can be excised and the 16S rRNA gene sequenced to address the phylogenetic affiliation. DNA fingerprinting techniques which allow only the identification of known organisms can also be used e.g. RFLP (restriction fragment length polymorphism) and ARDREA (amplified ribosomal DNA restriction enzyme analysis) both which have been applied to identify bioleaching organisms. These techniques include the digestion of PCR products with one or more restriction enzymes to produce fragments of varying sizes that can be resolved on appropriate gels (Johnson and Hallberg, 2007; Schippers, 2007).

2.4.2.2 FISH (Fluorescence in-situ hybridization) for quantification of microorganisms

FISH targets rRNA, an indicator of active metabolising micro-organisms, hence provides quantitative information on viable micro-organisms in a sample. Cells are fixed with ethanol or formalin to preserve the community structure. A gene probe coupled with a fluorophore is applied to the sample to bind to complementary sequences in the 16S rRNA molecules in the target micro-organisms. The sample is viewed under a fluorescence microscope to count fluorescing cells. A draw-back is that a sufficient content of cellular ribosomes is a prerequisite for this technique (Johnson and Hallberg, 2007; Schippers, 2007).

2.4.2.3 Real-time PCR for quantification of microorganisms

Real-time PCR (RT-PCR) is highly sensitivity and can be used to quantify different, previously identified, phylogenetic groups and genera. The technique is based on the online fluorescence detection of PCR products and allows rapid detection and quantification of gene sequences. DNA is extracted from samples, purified, and specifically amplified with a thermocycler using sequence-specific fluorescently labelled probes. There are different chemical assays for RT-PCR but the most common are the sequence-specific TaqMan® probes and the intercalating non-specific SYBR® Green. The detection limit depends on the target of interest, sample purity and PCR conditions, but theoretically allows detection of a single DNA molecule. RT-PCR has been applied to quantify micro-organisms and archaea in mine heaps. Furthermore, SYBR® Green-based protocols have been developed to quantify single species in bioleaching communities (Johnson and Hallberg, 2007; Schippers, 2007).

2.5 Scope of study

The literature review revealed the importance of having adequate microbial communities that operate optimally at different temperature ranges in order to facilitate the heating of bioleach heaps from mesophilic to thermophilic temperatures. It was noted that some test work has been done concerning growth of bioleaching micro-organisms in liquid media (bioleach tanks and pure liquid cultures), but there is paucity of published data on the growth rates of key microbes in each temperature range in a bioleach heap environment. Also, there is limited publication on test work done concerning maintenance of stable microbial communities in mixed bioleaching stock cultures, used for inoculating bioleach heaps. The limited nature of these data is most marked at 50 - 60°C. This study aimed to investigate the growth of bioleaching moderate thermophiles with the view to add to an understanding of microbial growth and activity during the transition from mesophilic through moderate thermophilic to thermophile temperatures in a bioleach heap environment. The study also investigated microbial growth in a mixed bioleaching stock culture with the view to add data which could be useful in the optimisation of inoculum preparation. Review of the literature revealed a prevalence of *Acidithiomicrobium* species in mixed cultures of moderate thermophiles growing on sulfide ores; however there is paucity of data on growth of this organism. Therefore the study in liquid culture paid particular attention to *Acidithiomicrobium* species.

2.5.1 Objectives

To address the gaps identified through the literature review the objectives of the study were:

1. To investigate the growth and dominance of a consortium of bioleaching micro-organisms in a bioleach heap environment at 50 °C using inocula with different compositions and irrigation solutions with different copper concentrations, on a low-grade copper-sulphide ore;

2. To investigate the growth and dominance of a consortium of bioleaching micro-organisms in a liquid stock culture at 50 °C under different carbon dioxide concentrations, copper concentrations, and agitation speeds.

2.5.2 Key questions

In light of the objectives, the following key questions were raised:

1. Which microbial species from the selected consortium colonise the ore at 50 °C?

2. What will be the growth rates and dominance of the attached microbial species?

3. Will the growth rates and dominance of the attached micro-organisms change when inocula with different compositions are used?

4. Will the growth rates and dominance of the attached micro-organisms change when different concentrations of copper are used in the irrigation solution?

5. Which microbial species from a consortium dominate in a mixed culture under submerged culture conditions at 50 °C?

6. How will the dominance and quantity of the *Acidithiomicrobium* species, a key Fe- and S-oxidising moderate thermophile, change when the environment in the culture is changed by introducing different concentrations of CO_2, and using different agitation speeds?

7. How will the dominance and quantity of the *Acidithiomicrobium* spp. change when the environment in the culture is changed by introducing different concentrations of copper?

8. Do similar copper concentrations have a similar effect on micro-organisms in a whole ore environment and in liquid culture?

2.5.3 Hypotheses

In order to guide and evaluate the study, the following set of hypotheses was formulated:

1. The growth rates and dominance of the microbial species on whole ore are not affected by using different compositions of inocula, owing to these being influenced most strongly by the physicochemical environment.

2. The changes in the physicochemical environment of the culture affect the microbial species to different extents such that the dominance changes, owing to the differing optimal physicochemical conditions required for different species.

3. Different copper concentrations affect the microbial species to different extents such that the dominance changes in both the whole ore and the liquid culture, owing to the differing critical inhibition concentrations for different species.

4. Similar copper concentrations affect the dominance of microbial species similarly in both the whole ore and the liquid culture, owing to the same critical concentrations for the same species regardless of the bioleach system.

3. MATERIALS AND METHODS I: COLUMN TEST WORK ON WHOLE ORE

This chapter presents a description of the materials, the reactor set-ups, the experimental protocols and analytical procedures used in the first part this study i.e. the investigation carried out on whole ore in columns. The rationale behind the choice of the particular materials and methods is also discussed in this chapter.

3.1 Microbial cultures

The seven mixed stock cultures of moderate thermophile bioleaching micro-organisms used in this study were obtained from BHP Billiton and maintained at CeBER for different periods of time. These stock cultures were all maintained at 50°C in 1 L stirred tank reactors (STRs) sparged with air and stirred with pitched 4-blade impellers at 550 rpm. The cultures were fed with a basal salts medium adapted from Norris *et al.* (1996); i.e. 0.4 g/L $(NH4)_2SO_4$, 0.2 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$ and 0.1 g/L KCl in water, with pH adjusted to pH 1.7 using concentrated H_2SO_4. This medium was supplemented with 10 g/L chalcopyrite and 3 g/L pyrite concentrates as energy sources for the micro-organisms, and to adapt the micro-organisms to these two minerals. The cultures were sub-cultured once every week by taking out 15 % of the volume from each reactor and replacing with the same volume (15 %) of a fresh medium containing the chalcopyrite and the pyrite concentrate.

The microbial composition of the cultures was determined using qPCR analysis. On analysis, the same types of microbial species were detected in these cultures but there were differences in the composition of the microbial species. The compositions of these cultures are discussed under qPCR analysis in Sections 4.2, 4.3, 4.4, 6.2 and 6.3. Culture 1 was used to inoculate the ore in the investigation of the growth rates on whole ore. Cultures 2, 3 and 4 were used in the investigation of the effect of inoculum composition on microbial growth on whole ore. Culture 5 was used in the investigation of the effect of copper concentration on microbial growth on whole ore. Cultures 6 and 7 were used as the inocula in the investigations carried out in liquid culture.

3.2 Ores

Low-grade chalcopyrite ore samples, ore type A and type B, from two different copper mines in Chile were used in this study. This ore was supplied by BHP Billiton already crushed to a particle size of 80 % passing 12.5 mm. The composition of the ore, shown in Tables 3.1 and 3.2, was supplied by BHP Billiton. The preparation of the ore for the experiments is described in Section 3.2.2.

3.2.1 Ore composition

Table 3. 1: Elemental composition of the ores used in the study of growth on whole ore

	Composition (%)	
Element	**Ore A**	**Ore B**
Cu	0.68	0.46
Fe	3.74	2.48
S	3.48	2.45

Table 3. 2: Mineral composition of the ores used in the study of growth on whole ore

	Composition (%)	
Mineral	**Ore A**	**Ore B**
Chalcocite	0.09	0.20
Chalcopyrite	1.66	0.50
Covellite	0.05	0.30
Pyrite	4.69	4.00
Iron oxides	0.59	n/a
Muscovite	n/a	28.60
Kaolinite	n/a	7.40
Quartz	n/a	44.80
Gangue	91.41	n/a

n/a = not analysed

3.2.2 Ore preparation

Blending and splitting: Each 60 kg ore sample was mixed thoroughly using a spade and split into two 30 kg portions using a 2-way riffle splitter. Each 30 kg was further split into two 15 kg portions and so on until obtaining 1.875 kg portions which were then used to make up 5 kg representative portions.

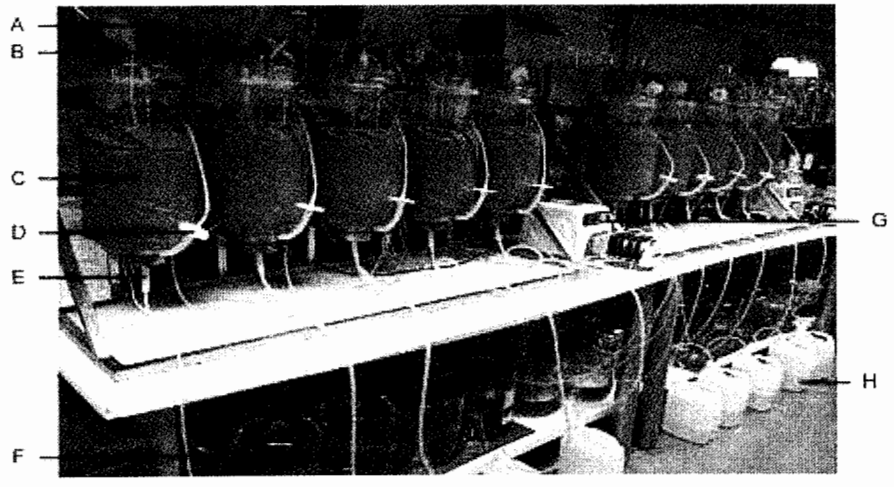
Agglomeration and sterilisation: Agglomeration was done to attach fines to the bigger particles so as to create a permeable substrate as well as to treat the acid-consuming gangue (Domic, 2007). The agglomeration was done to achieve 3 kg acid/ ton ore and 4.5 % moisture for ore A, and 2 kg acid/ ton ore and 5.5 % moisture for ore B. For 5 kg of ore A, 15 g of concentrated H_2SO_4 was mixed with 210 mL of H_2O and for 5 kg of ore B, 10 g of H_2SO_4 was mixed with 265 mL of H_2O. The acid to moisture ratio was as per the supplier's specifications for these particular ores, based on the acid neutralising capacity of the ore. The agglomerate was packed in plastic bags and sent for sterilisation by exposure to 50 kGy of gamma radiation (HEPRO, Cape Town, South Africa).

3.3 Column set-up

A series of identical laboratory-scale glass columns (0.4 m height and 0.16m internal diameter) were used to simulate a bioleach heap. These columns were designed so that the experiments could be conducted in a sterile environment. Table 3.2 indicates the ores, the inocula and the irrigation solutions used in the experiments. Figures 3.1 and 3.2 show a series of columns and a schematic representation of one column, respectively.

Table 3. 2: The ore, inocula and irrigation solutions used in the study of growth on whole ore

Experiment	Column description
1	All the 9 columns contained ore type A. The Control was not inoculated whilst the rest of the columns were inoculated with Inoculum 1.
2	Ten columns including the Control contained ore type A and one column contained ore type B (to determine the effect of mineralogy on microbial growth). The Control was not inoculated; six columns containing ore type A and the column containing ore type B were inoculated with Inoculum 2. Of the remaining three columns containing ore type A, two were inoculated with Inoculum 3 and one was inoculated with Inoculum 4.
3	All the ten columns contained ore type A and were all inoculated with Inoculum 5 including the Control column. The Control column was irrigated with a solution containing no copper, three columns were irrigated with a solution containing 5 g/ L Cu, three were irrigated with 10 g/L Cu and the other three were irrigated with 15 g/L Cu.



A- thermostat, B- irrigation solution inlet, C- heating jacket, D- air inlet filter, E- PLS outlet, F- irrigation solution reservoir, G- peristaltic pump, H- PLS reservoir

Figure 3. 1: The column set-up for the experiments at 50 °C

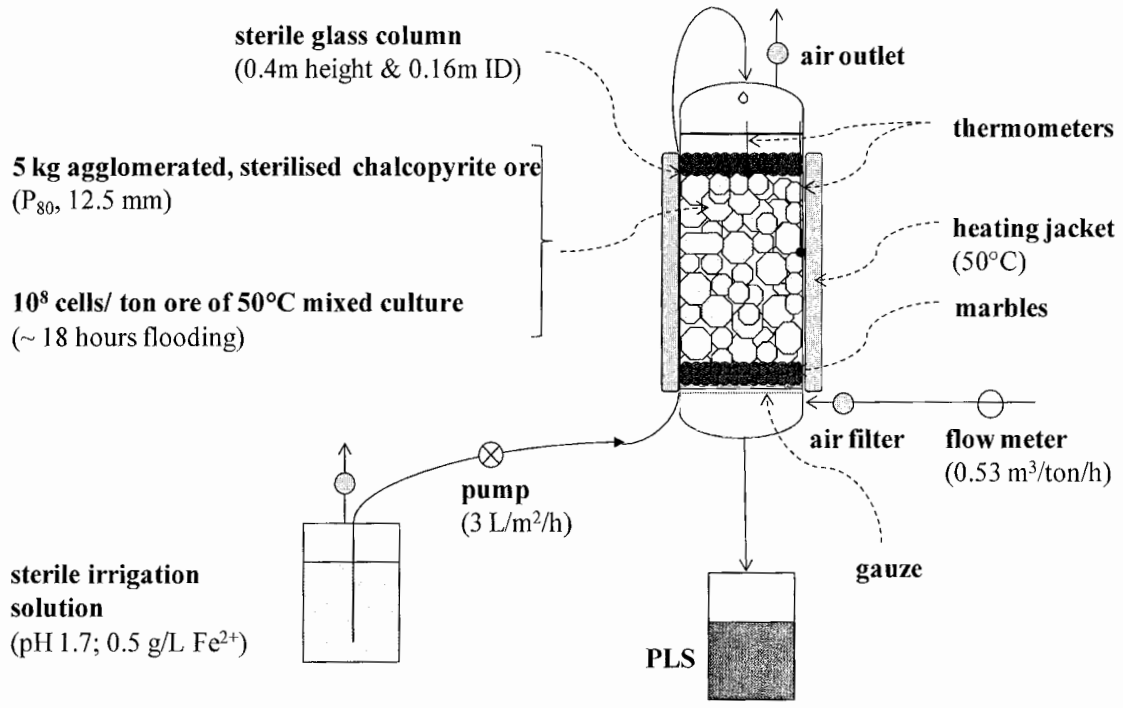


Figure 3. 2: The schematic diagram of a single column set-up for experiments at 50 °C

3.4 Media

3.4.1 The irrigation solution, inoculation solution and detachment solution

The irrigation solution contained 0.18 g/L $(NH_4)_2SO_4$, 0.06 g/L $(NH_4)_2HPO_4$, 0.15 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L K_2SO_4 and 0.5 g/L (500 mg/L) Fe^{2+} as $FeSO_4.7H_2O$ in water. The amount of Fe^{2+} was consistent with a suggestion that "availability of a reactive mineral surface may be more important than iron concentration for the bioleaching of low grade ores and heap leach operations may be effective at iron concentrations as low as 0.2 g/L" (van Hille *et al.*, 2010). van Hille *et al.* (2010) observed that after approximately 30 days operation, the rate of Cu recovery from a predominantly chalcopyrite ore was similar across columns operated at feed iron concentrations ranging from 0.2 to 5.0 g/L. The pH of this solution was adjusted to 1.7 using concentrated H_2SO_4, to be consistent with the pH used in other studies done at CeBER that are part of the bigger study of the colonisation of low-grade ores. The solution was sterilised by autoclaving before adding the Fe^{2+} which was prepared as separate solution and sterilised by filtering through a 0.22 µm membrane and added to the autoclaved irrigation solution. The two solutions were mixed in a laminar flow cabinet to avoid contamination from the surrounding air. The irrigation solution for the third experiment was prepared to contain varying concentrations of copper i.e. 5.0, 10.0 and 15.0 g/L added as $CuSO_4.5H_2O$. The Cu solution was also prepared separately and sterilised by filtering through a 0.22 µm membrane. The inoculation and detachment solutions were the same as the irrigation solution except that they did not contain iron.

3.4.2 0K medium

The 0K medium is the "9 K medium (Silverman and Lundgren, 1959) without ferrous sulfate" (Giaveno *et al.*, 2003). This medium contained 3 g/L $(NH_4)_2SO_4$, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L KCl and 0.01 g/L $Ca(NO_3)_2$ in water. The pH of this solution was adjusted to pH 1.7 using concentrated H_2SO_4 and the solution was sterilised by autoclaving.

3.4.3 Activity test medium

This medium contained 0.4g/L $(NH_4)_2SO_4$, 0.1 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 5 g/L Fe^{2+} (as $FeSO_4.7H_2O$) in water, and the pH was adjusted to 1.7 using H_2SO_4 (Norris, 1986 unpublished). This medium was sterilised by autoclaving before adding the iron which was prepared as separate solution and sterilised by filtering through a 0.22 µm membrane.

3.5 Experimental protocol

3.5.1 Sterilisation

The columns and all their accessories except for thermometers were sterilised by autoclaving. Autoclaving was done at 121°C at a pressure of 1 atmosphere for 20 minutes. The thermometers were sterilised by immersing in 70 % alcohol solution. The ore was irradiated. Media was autoclaved with the exception of the ferrous iron and copper solutions which were filter-sterilised.

3.5.2 Packing

Each column was packed with 5 kg of the agglomerated sterilised ore. Sterile marbles were placed on top of and beneath the ore to assist with liquid and gas distribution (Bouffard and Dixon, 2001). A long thermometer was inserted on the inside wall of each column to a level half the height of the ore bed. A pocket thermometer was placed at the top centre of the ore. Each column was closed with a sterile lid. Parafilm and vacuum grease applied where the lid and column come into contact were used to seal the reactor preventing contamination from the surrounding air.

3.5.3 Heating

A heating jacket connected to a thermostat was wrapped around each column with the aim to maintain all columns at 50 ± 2 °C. Thermometers inserted into the ore bed as described in Section 3.5.2 and shown in Figure 3.2 allowed measurement of the temperature profile in the ore bed. The measurements showed that there were temperature gradients in the columns, in the range 40 – 53 °C, the coolest regions being the top part of the ore bed.

3.5.4 Inoculation

A cell count on the stock culture was used to determine the volume of the culture required to attain an inoculum size of 3 x 10^8 cells/ ton ore (Appendix B1). The inoculum was aseptically mixed with 3 L sterile inoculation solution in a laminar flow cabinet. The mixture was poured over the packed ore, the flooded columns left to stand for 18 hours to allow microbial attachment to the ore and then the solution slowly drained before commencing the irrigation.

3.5.5 Irrigation

An open circuit irrigation was used in this study i.e. the irrigation solution was not recycled. A fresh sterile feed solution was dripped onto the ore from the top of the columns at a rate of 3 L/m^2/h using a calibrated peristaltic pump. The feed line from the pump passed under the heating jacket so that the feed solution could be heated up before reaching the ore. The irrigation rate of 3 L/m^2/h is lower than that applied in many industrial operations i.e. 5 - 20 L/m^2/h (Petersen and Dixon, 2007b). This was used because it is within an ideal range for studies involving microbial attachment on a laboratory scale as indicated by a study done at CeBER by Chiume *et al.* (2012). Chiume *et al.* (2012) investigated the effects of different flow rates on microbial attachment to whole ore.

3.5.6 Aeration

Normal air was pumped into the columns from the bottom at a standard normalised flow rate of 0.53 m^3/ton/h using flow meters. This aeration rate was chosen to be comparable to the aeration rates used in pilot scale studies. The air was not pre-humidified and was sterilised by passing it through a 0.22 µm filter membrane.

3.5.7 Column maintenance

The columns were checked daily for temperature using thermometers inserted into the ore, leakages, blockages, air flow by measuring the outlet flow using a bubble meter, irrigation rate by checking the volumes of the one-hour PLS samples and the over-night PLS.

3.5.8 Sampling and testing

The experiments were monitored regularly using the sampling procedure described below. The assays carried out on the samples are described in detail in Section 3.6.

Inoculum: At the beginning of each experiment, the inoculum was sampled and assayed for Eh, pH, cell count, activity (Section 3.6.4) and microbial composition using qPCR analysis.

Drained solution: The solution drained after inoculation was weighed, and assayed for Eh, pH, the concentration of ferrous iron (Fe^{2+}) and total iron (Fe^{tot}), and cell count.

Pregnant leach solution (PLS): The PLS was collected at the bottom of the columns into 5 L containers. Fresh samples were collected from the draining PLS over one hour every second day and assayed for Eh, pH, Fe^{2+}, Fe^{tot} and cell count for selected columns. One-hour samples were used so as to avoid erroneous measurements due to additional oxidation that would have taken place overnight in the PLS reservoir. The volume of the overnight PLS was measured and a sample taken for the soluble metal (Cu and Fe) assay and the PLS was then discarded. The PLS drained from a stopped column was assayed instead of the one-hour sample and then combined with the overnight PLS for qPCR analysis.

Irrigation solution: When a feed bottle ran out it was replaced with a new bottle and a sample of the out-going feed was taken and assayed for Eh, pH, Fe^{2+}, Fe^{tot}, Fe and Cu to check for contamination.

Ore: The ore from each column was analysed when the column was sacrificed. At an interval, a selected column was stopped by switching off the heating jacket, irrigation and the air flow. The column was left standing for 2 hours to allow the PLS to drain from the column and also to allow the column to cool down. The ore was weighed and mixed, and representative samples taken for moisture analysis and for detachment. Duplicate 500 g samples were dried at 80 °C overnight to determine the dry weight of the ore for use in qPCR calculations. Microbial cells were detached from another pair of ore samples, using the detachment protocol described in Section 3.5.9.

3.5.9 Detachment protocol

Cells were detached from the ore using a detachment protocol developed at CeBER (Chiume *et al.*, 2012; Govender *et al.*, 2013; Tupikina *et al.*, 2013b). This protocol was developed to distinguish between the interstitial, weakly-attached and strongly-attached micro-organisms present within the ore bed. A 500 g (or 300g) ore sample was placed in a sterile 2 L conical flask and treated with 6 washing steps. Each washing step entailed adding 250 mL (or 150 mL) of the sterile detachment solution into the flask, vortexing the flask for 2 minutes (except the first step where the flask was swirled smoothly), allowing the ore to settle and decanting the solution into a clean centrifuge tube. The supernatant was centrifuged at a relative centrifugal force (rcf) of 800 *g* for 1 minute, to remove the remaining suspended ore without pelleting the suspended cells. The settled residue was discarded. In the last two washing steps, 250 mL (or 150 mL) detachment solution containing 0.4 v/v %

Tween 20 was used. The centrifugation was done using a JA-10 rotor in a Beckman Avanti™ J-25 centrifuge. The supernatant from washing step 1 was regarded as the interstitial phase; the solution from washing steps 2, 3 and 4 was regarded as the weakly attached phase, and the supernatant from washing steps 5 and 6 was regarded as the strongly attached phase. A cell count was done on each of the 6 washing steps of detachment. The microbe-containing supernatant from all the washing steps was combined and cleared of suspended solid particles by centrifuging four times at 900 *g* for 2 minutes each time. The clear supernatant was prepared as detailed in Section 3.6.3.1, for the extraction of genomic deoxyribonucleic acid (gDNA) and qPCR analysis.

3.6 Analytical procedures

To monitor the performance of the bioleaching systems, samples were analysed using techniques that can be categorised into; physicochemical tests, cell counts, quantitative real-time polymerase reaction (qPCR) analysis and activity tests determined by ferrous iron oxidation rates. These tests are discussed in Section 3.6.1 to 3.6.4.

3.6.1 Physicochemical tests

3.6.1.1 Eh (redox potential, SHE)

The redox potential was measured with reference to a saturated Ag/AgCl electrode using a Pt electrode attached to a Metrohm 827 pH meter. The Eh i.e. redox potential relative to the standard hydrogen electrode (SHE), was calculated from the redox potential by adding 207 mV, a correction factor for the Ag/AgCl system at 25°C (Metrohm, South Africa). The electrode was checked daily for precision using a Crison standard redox buffer (468 mV at 25°C) and the error was found to be < 1 %.

3.6.1.2 pH

The pH was measured using a Ag/AgCl electrode attached to a Metrohm 704 pH meter. The electrode was daily calibrated to 95 - 105 % accuracy using Metrohm buffer solutions (pH 4.00 and pH 7.00 at 25°C).

3.6.1.3 Ferrous iron (Fe^{2+}) and total soluble iron (Fe^{tot}) concentration

The concentrations of Fe^{2+} and Fe^{tot} were assayed using the 1,10-phenanthroline colorimetric method (Komadel and Stucki, 1988) and the ferric iron (Fe^{3+}) concentration was calculated as the difference between Fe^{2+} and Fe^{tot}. The 1,10-phenanthroline method is based on the principle that 1-10 phenanthroline chelates Fe^{2+} forming an orange-red

complex that absorbs maximally between 400 nm and 600nm, and hydroxylamine reduces Fe^{3+} to Fe^{2+}. The test procedure was as follows: 2 mL each of the ammonium acetate buffer solution and the 1,10-phenanthroline indicator were added into a test tube with 1mL of an appropriately diluted sample and vortexed. The absorbance was measured at 510 nm against a blank in which the sample solution was replaced by 1 mL of de-ionised water. The Fe^{2+} concentration was determined from a calibration curve prepared from known concentrations of Fe^{2+} (Appendix A1). To measure Fe^{tot}, a scoop of hydroxylamine chloride was added to the same sample and blank used for the Fe^{2+} assay. These mixtures were vortexed and left to stand for 5 minutes to allow the hydroxylamine to reduce all the Fe^{3+} to Fe^{2+}. The absorbance was measured at 510 nm to determine the Fe^{tot} concentration. The absorbance was measured using the HeλIOS α UV-visible spectrophotomer with version 7.09 software (Thermo Scientific, South Africa).

3.6.1.4 Soluble Fe and Cu metal concentration

Soluble metal (Cu and Fe) concentrations in the PLS were measured using a Varian Spectra AA-30 atomic absorption spectrophotometer with version 01.30.203 software. The analytical error for both metals was < 1 %. This allowed calculation of the metal extracted from the ore according to calculations shown in Appendix A1.

3.6.2 Cell counts

Total viable cells were counted directly using a THOMA counting chamber under an Olympus CX-41 phase contrast microscope at 1500 X magnification. The cell concentration was as specified in Appendix A2. Samples with a high cell concentration were diluted to count between 30 and 300 cells, to minimise the counting error. The detection limit of this method is 3.125×10^5 cells/mL with a standard error < 25 %.

3.6.3 Molecular analysis: Quantitative real-time polymerase chain reaction (qPCR)

The composition of the microbial community was assayed using qPCR analysis. This method is highly sensitive, with a detection limit that theoretically allows the detection of a single DNA molecule (Johnson and Hallberg, 2007; 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. The samples in this study were subjected to procedures

described below in preparation for qPCR analysis. Similar studies have been reported from CeBER (Tupikina *et al.*, 2010; van Hille *et al.*, 2011; Tupikina *et al.*, 2013b).

3.6.3.1 Sample preparation for gDNA extraction

Cells harvested from the inocula, the PLS or detached cells, were prepared for gDNA extraction using a procedure modified from Liu *et al.* (2006). A sample was filtered through a 0.22 µm GV Durapore® (cellulose acetate) filter membrane and washed with 5 mL 0K medium. The membrane was transferred into a 15 mL sterile tube, the sample washed off with 4 mL 0K medium and the membrane discarded. The sample was transferred into a 2 mL Eppendorf® tube and centrifuged in an Eppendorf® Mini-Spin Plus centrifuge at 5000 *g* for 60 seconds to settle the fines. The supernatant was transferred into a 2 mL Eppendorf® tube and centrifuged at 14100 *g* for 5 minutes to pellet the microbial cells and the supernatant discarded. Tissue-lysis buffer (250 µL) was added to the pellet and the microbial mass re-suspended by vortexing. The cell suspension was subjected to the gDNA ex traction procedure (Section 3.6.3.2) immediately or stored in a freezer at -60 °C to be extracted later.

3.6.3.2 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 14100 *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 a Collection Tube. Inhibitor-removal buffer (500 µL) was added into the upper reservoir of the Filter Tube, and centrifuged at 8000 *g* for 60 seconds. The Filter Tube was transferred into another Collection Tube and 500 µL Wash buffer added into the upper reservoir, 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 14100 *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, and incubated at 70 °C for 30 minutes, and

centrifuged at 14100 *g* for 60 seconds to elute DNA into the micro-centrifuge tube. This step was repeated once. 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 (Thermo Scientific, South Africa). The gDNA was analysed using qPCR immediately or stored at -20 °C to be analysed later.

3.6.3.3 qPCR analysis

The microbial composition of the samples was determined by analysing the extracted gDNA using qPCR according to a CeBER protocol (Tupikina *et al.*, 2013b). The samples, where applicable were diluted to 10 ng/ µL 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) per quantification. Ten nanograms (1 µL of a 10 ng/ µL dilution) of gDNA used as a template per 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 x (95 °C, 6 min), 40 x (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 3.3.

Table 3. 3: PCR primers used to assay microbial composition in the inocula, PLS and the ore

Primer title		Microbial	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
	M.sed	*M. sedula/ hakonensis*	CTC GCA AGA GGG CTT TTC TCC A
	JTC1/ 2	*Thermoplasmatales* spp.	AGA AAA ATT CTC CCG CTC AAC GG
Bacterial primers	JTC4/SAR	*Acidithiomicrobium* spp.	CAA GGA CGA AAC TGA CGG TACT T
	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	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

Species-specific primers allowed for detection of the species sought whilst the universal primers for bacteria and archaea allowed for detection of the total bacterial and archaeal species present. The specific species detected made up a high proportion (> 95 %) of the total showing that there was a low presence of unexpected species. In the calculation of genomic copy numbers (Appendix A.3.1.2), the gene dosage of microbial species was considered e.g. *At. caldus, L. ferriphilum* and S. *thermosulfidooxidans* each have one copy of the 16S rRNA gene per genome (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).

3.6.4 Microbial activity characterised by ferrous iron oxidation rates

An activity test, to track the metabolic activity of the microbial community over time, was done by measuring the rate of Fe^{2+} oxidation by cells drawn from the inocula and the PLS, and cells detached from the ore. The test set up was as follows: Erlenmeyer flasks were filled with 250 mL sterile activity test medium (Section 3.4.3) i.e. 5 g/L Fe^{2+} in solution. The flasks were inoculated with 10^9 cells and incubated at 50 °C and 180 rpm in an orbital shaking oven. An un-inoculated flask (Control) was run concurrently. The flasks were gravimetrically topped up with sterile de-ionised water daily before sampling to make up for evaporation loss. Eh, pH, Fe^{2+} and Fe^{tot} were measured daily over a maximum of 14 days. The Fe^{2+} oxidation rates were calculated using Equation (5) (Doran, 1995). The biological Fe^{2+} oxidation rate ($\mathbf{r_{Fe^{2+}bio}}$) was calculated by subtracting the Fe^{2+} oxidation rate in the blank (abiotic) flask from the oxidation rates in the inoculated flasks. Specific oxidation rates could not be calculated. This is because after the inoculation, the cell concentration in the shake flasks was not determined in the course of the experiment.

$$r_{Fe^{2+}} = \frac{dFe^{2+}}{dt} \qquad (5)$$

where $r_{Fe}{}^{2+}$ is the rate of ferrous iron oxidation (g Fe^{2+}/ L/ day)
dFe^{2+} is the change in the concentration of Fe^{2+} over a time interval (g Fe^{2+}/ L).
dt is the time interval (day).

3.6.5 Growth rates

Growth rates of individual microbial species were calculated using genomic copy numbers detached from the ore. Total community growth rates were calculated using the cell counts. The assumptions were that (i) the microbial growth on the ore was typical of batch microbial cultures, (ii) the microbial growth in the PLS was typical of continuous culture systems and (iii) the microbial growth followed first-order kinetics with respect to cell numbers. Specific growth rates (μ) and corresponding doubling times (t_d) on the ore and the apparent (combined) growth rates in the ore and the PLS were calculated using the Equations (6) and (7) (Doran, 1995). The growth rates in the PLS were calculated using Equation (8);

$$\mu = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)} \quad (6)$$

$$t_d = \frac{\ln 2}{\mu} \quad (7)$$

$$X_{out} - X_{in} = \mu X V \quad (8)$$

where μ is the specific growth rate constant (h^{-1}).

t_d is the doubling time (h).

X_1 and X_2 are cell concentrations at time t_1 and t_2, respectively.

X_{in} and X_{out} are cell concentrations going into and coming out of a column respectively.

X is the cell concentration in the PLS held up inside the column.

V is the volume of the PLS held up inside the column.

4. RESULTS AND DISCUSSION I: Microbial growth kinetics on whole ore

4.1 Introduction

This chapter presents and discusses the results obtained from the investigation carried out on whole ore. Three experiments were conducted to investigate the growth of moderate thermophiles on low-grade copper-sulphide ore using columns packed with 5 kg ore, to represent a bioleach heap system. The first experiment investigated the growth rates of the microbial species identified in the 50 °C inoculum used to inoculate the ore. The second experiment investigated the effect of using inocula with different compositions of the same microbial species, on the growth and dominance on the ore. The third experiment investigated the effect of solution copper concentration on the growth and dominance of the microbial species introduced on low-grade ore. Throughout this study, particular attention was given to the growth and dominance of *Acidithiomicrobium* species, a key iron- and sulphur-oxidising moderate thermophile. The results of this study are presented and discussed in the categories: physicochemical parameters, cell counts, quantitative real-time polymerase reaction (qPCR) analysis, growth rates and microbial activity measured through ferrous iron oxidation rates.

4.2 The growth rates of moderate thermophiles on whole ore

Experiment 1, set-up as described in Section 3.3, was conducted to investigate the growth rates of moderate thermophiles on whole ore. Eight columns were packed with ore type A of which seven were inoculated with Inoculum 1. The Control column was not inoculated. The experiment was run for 34 days. The microbial composition of the inoculum, analysed using qPCR analysis, shown in Figure 4.1, was dominated by *Acidithiobacillus caldus* (55 %) followed by *Acidiplasma cupricumulans* (42 %), *Acidithiomicrobium* spp. (2 %) and *Metallosphaera* spp. (< 1 %). An inoculum size of 3 x 10^8 cells/ ton ore was used. This was previously found to be an ideal inoculum size for investigating growth rates at laboratory scale from a scoping study (Tupikina *et al.*, 2013a) investigating the effects of inoculum size on microbial colonisation of whole ore, as part of the overall study into which this study fits. The columns were numbered in the order of their take-down. Column 1.1 to 1.7 were sacrificed on day 7, 10, 13, 15, 20, 30 and 34 respectively whilst the Control column was sacrificed on day 34.

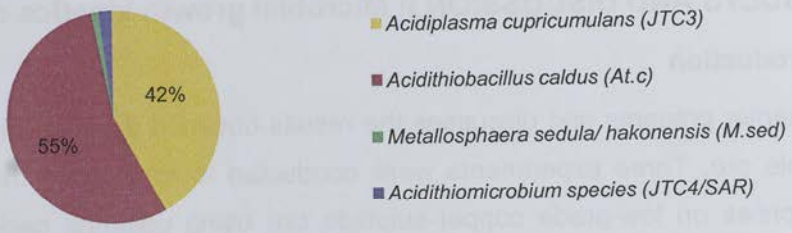


Figure 4. 1: Composition of Inoculum 1 used in Experiment 1

4.2.1 Physicochemical parameters

The Eh, pH, Fe^{2+}, Fe^{tot}, soluble Cu and Fe metal in the pregnant liquid solution (PLS) draining from the columns were measured every second day. The Eh is reported with reference to the standard hydrogen electrode (SHE). The results of these physicochemical measurements are shown the Figures 4.2 to 4.8.

Eh, iron concentrations and Fe^{3+}/Fe^{2+} ratios provide information on dissolution of pyrite and other iron-bearing minerals (Brierley, 2001) as well as activity of ferrous-iron oxidising micro-organisms. Therefore the Eh and iron concentration of the PLS was used as an indicator of microbial activity and the extent of mineral oxidation, respectively. The major redox couple in this study was Fe^{3+}/Fe^{2+}, hence an increase in Eh indicated an increasing oxidation of Fe^{2+} to Fe^{3+} i.e. Fe^{3+} production exceeded Fe^{3+} consumption. Fe^{3+} is a leach agent and is consumed as leaching takes place.

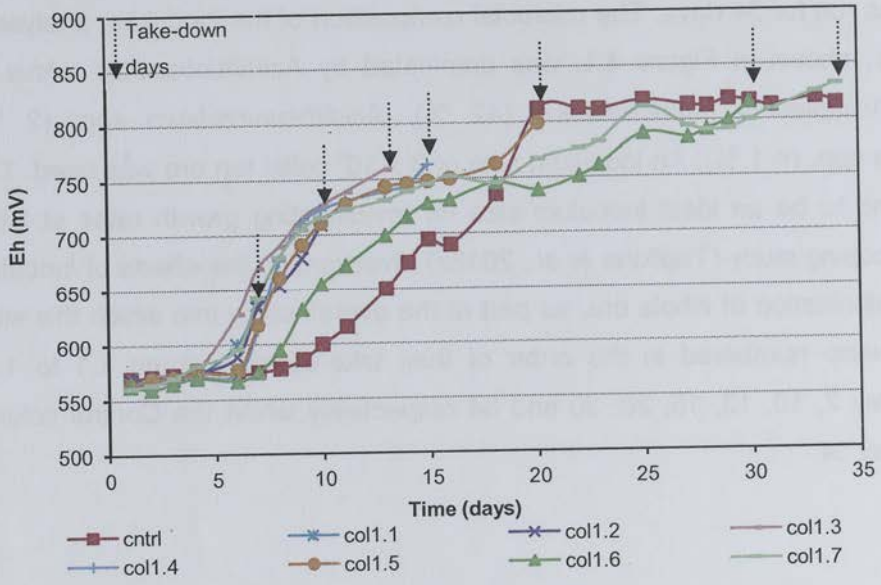


Figure 4. 2: Eh measured in the PLS of Experiment 1

The Eh in all the columns, shown in Figure 4.2, started around 560 mV, initially lagged and reached an average of 580 mV by day 6 except for Col 1.6 and the Control (Cntrl) both which lagged longer and reached 580 mV by day 7 and 8 respectively. Thereafter, the Eh of Col 1.1 to 1.5 and 1.7 increased similarly, rising quickly to reach 730 mV by day 11 after which the increase was slower, reaching 830 mV by day 34 the last day of the experiment. The Eh increase in Col 1.6 was similar to that of the other five inoculated columns but delayed by a two day increase in lag. The Eh in the Control increased consistently after the 8-day lag and reached 812 mV by day 20, where it remained constant until day 34.

An inference can be made from these Eh profiles that adequate microbial attachment to the ore took place in the inoculated columns after 6 days except in Col 1.6. The longer lag in Eh in Col 1.6 could have been caused by a faulty thermostat for this column resulting in heating being introduced a day after other columns had been started. This could have affected microbial colonisation of the ore and the iron oxidation rates. The Eh of the Control was not expected to rise significantly since this column was not inoculated. The increase in Eh of the Control after day 8 suggests microbial activity of the contaminant micro-organisms detected when the ore from this column was subjected to the detachment protocol.

The pH in all the columns, shown in Figure 4.3, lay between 1.7 and 2.1 throughout the experiment. The stability of the pH indicated a balance between acid-consuming and acid-generating reactions, partly due to prior conditioning of the ore by acid-agglomeration.

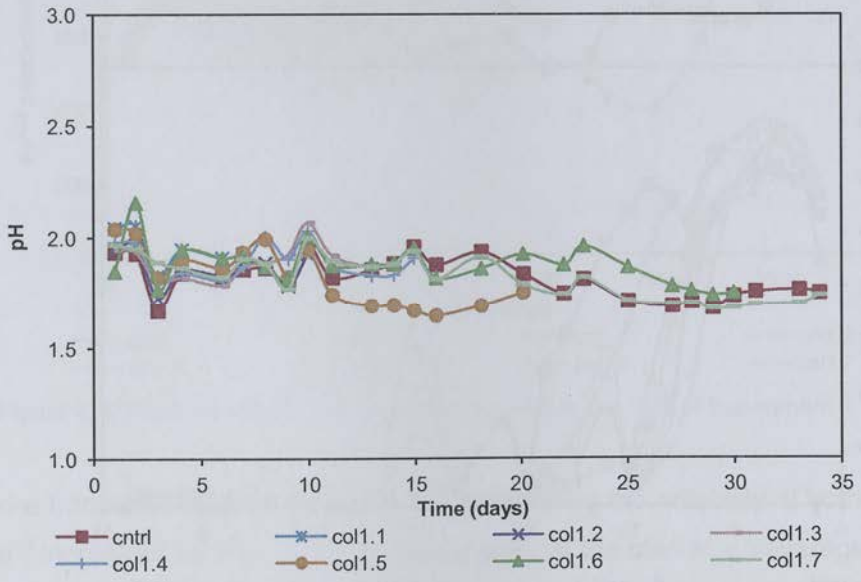


Figure 4. 3: pH measured in the PLS of Experiment 1

Dissolution of mineral sulphides can be acid-producing causing pH decrease or acid-consuming causing pH increase (Plumb *et al.*, 2008b). Therefore, pH and acidity measurements indicate the extent of acid conditioning of the bioheap (Brierley, 2001).

The Fe^{2+} concentration in the PLS, shown in Figure 4.4, increased from an average of 500 mg/L at the start to between 600 and 700 mg/L on day 3, in all the columns. After day 3, the Fe^{2+} decreased; in Col 1.1 to 1.5 the Fe^{2+} decreased rapidly approaching 0 mg/L by day 10. In Col 1.6, the Fe^{2+} consumption delayed by the delayed heating, approached 0 mg/L by day 15. In Col 1.7 and Control, the Fe^{2+} decreased more gradually approaching 0 mg/L on day 20. Bioleaching of mineral sulphides occurs favourably at pH < 3 (Plumb *et al.*, 2008) and such pH values were realised in this experiment as shown in Figure 4.3. At such low pH values, abiotic oxidation of Fe^{2+} is negligible and elemental sulphur is inert to abiotic oxidation hence the activity of acidophilic Fe-oxidisers and S-oxidisers is important (Plumb *et al.*, 2008b). The initial increase of Fe^{2+} (in the first 3 days) could be attributed to the easily-leachable component of the ore releasing additional Fe^{2+} into solution. In agreement with the low Eh (Figure 4.2), there was little Fe^{2+} oxidation, due to negligible abiotic Fe^{2+} oxidation and limited microbial activity since cell numbers were still low in the system. The Eh in the inoculated columns was similar to the Eh in the abiotic control in the first 6 days. The further depletion of Fe^{2+} to levels lower than the irrigation Fe concentration after day 6, could be attributed to biotic Fe^{2+} oxidation. This is supported as the Fe^{2+} depletion was now more pronounced in the inoculated columns compared to the Control as shown in Figure 4.4. Also, the depletion of Fe^{2+} after day 6 was accompanied by an increase in Eh (Figure 4.2).

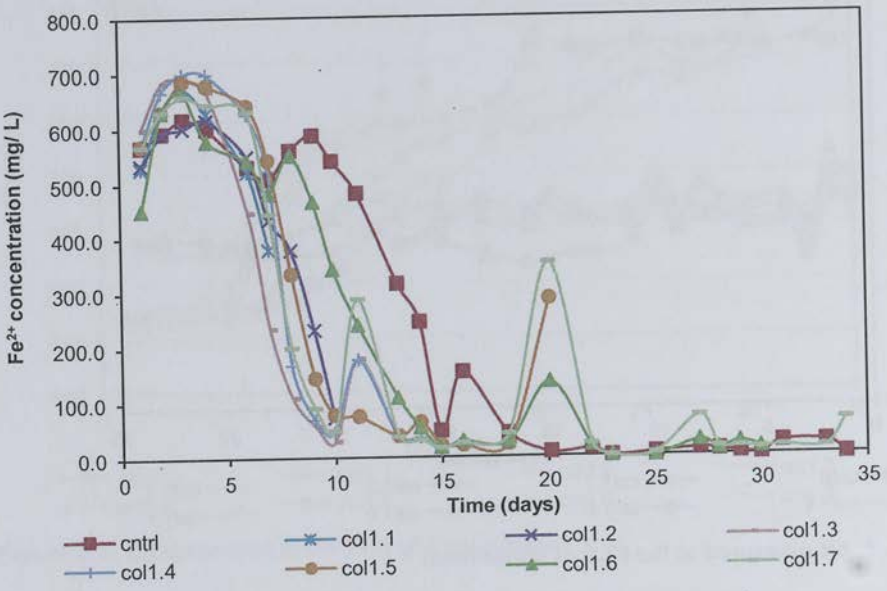


Figure 4. 4: Ferrous iron (Fe^{2+}) measured in the PLS of Experiment 1

The total iron concentration, Fe^{tot}, provides information on dissolution of pyrite and other iron-bearing minerals (Brierley, 2001). Fe^{tot} in the PLS was measured colorimetrically (Figure 4.5) and by atomic absorption spectroscopy (AAS), Fe^{AAS} (Figure 4.6).

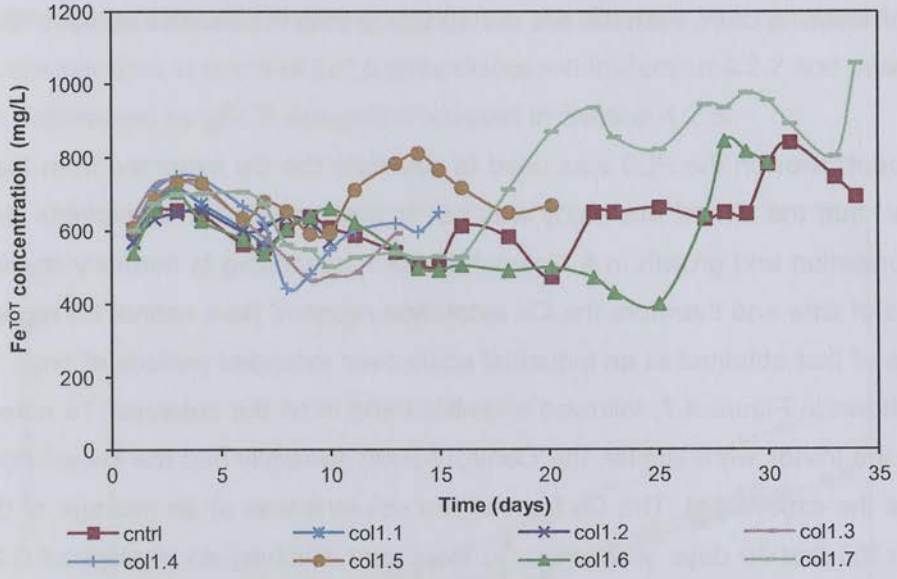


Figure 4. 5: Total iron (Fe^{tot}) measured colorimetrically in the PLS of Experiment 1

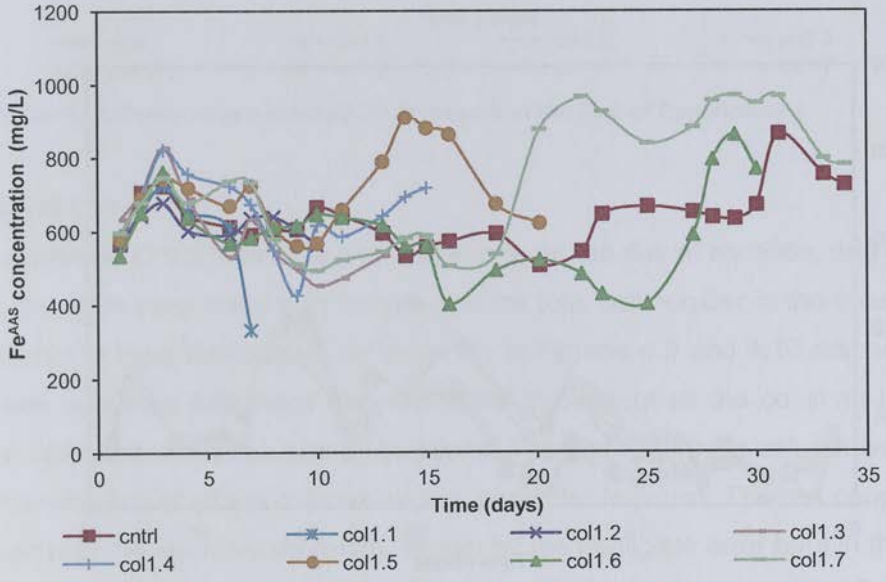


Figure 4. 6: Total iron (Fe^{AAS}) measured using AAS in the PLS of Experiment 1

Fe^{tot} had a trend and values similar to Fe^{AAS} confirming the reliability of both colorimetry and AAS. Fe^{tot} increased from an average of 600 mg/L at the start to an average of 700 mg/L by day 3 then decreased to an average of 600 mg/L by day 6 in all the columns and remained constant until day 15. After day 15, Fe^{tot} increased to an average of 800 mg/L by day 34. The trend and amount of Fe^{tot} in the first 6 days (shown in the raw data in Table B.1 in the

Appendix) was similar to that of Fe^{2+} shown in Figure 4.4 indicating that there was low Fe^{2+} oxidation. After day 6, the Eh and Fe^{tot} (Figures 4.2 and 4.3) indicated biotic Fe^{2+} oxidation. However, between day 6 and day 15, the Fe^{tot} was constant indicating a constant, though small, rate of leaching of Fe from the ore. The rise in the Fe^{tot} after day 15 is consistent with an increase of leaching of Fe from the ore during which time the Eh was at least 750 mV in all the columns.

The Cu concentration in the PLS was used to calculate the Cu extracted from the ore. It must be noted that the aim of this study was not to leach Cu but to investigate the initial microbial colonisation and growth in a bioleach heap. Cu leaching is normally studied over longer periods of time and therefore the Cu extraction reported here cannot be regarded as representative of that obtained at an industrial scale over extended periods of time. The Cu in the PLS, shown in Figure 4.7, followed a similar trend in all the columns. To note also is that although the trends were similar, the Control (Cntrl) generally had the lowest amount of Cu throughout the experiment. The Cu from all the columns was at an average of 0.10 g/5 kg ore/day for the first 10 days. After this, Cu increased reaching an average of 0.35 g by day 25 thereafter dropped to an average of 0.15 g/5 kg ore/day and remained constant until the end.

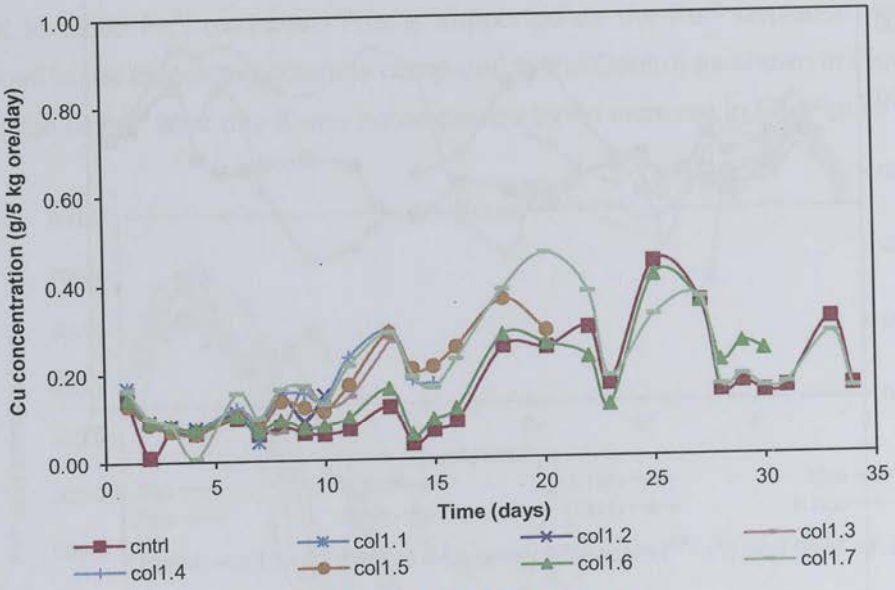


Figure 4. 7: Total Cu measured in the PLS of Experiment 1

The cumulative Cu extracted, shown in Figure 4.8, indicated that leaching progressed slowly from the start until day 10 to 15. Thereafter the linear extraction rate increased and remained constant until the end of the experiment. The total Cu extracted from the Control by the end of the experiment was 4.00 g/5 kg ore and from Col 1.7 was 5.30 g/5 kg ore. The

increase in the leach rate coincided with complete oxidation of Fe^{2+} as shown in Figure 4.4, indicating the link between the leach rate and Fe^{3+} availability. The higher copper extraction in the inoculated columns, compared to the Control column, was expected because microbial activity in the inoculated columns ensures rapid cycling of Fe^{2+} to Fe^{3+} and adequate sulphur oxidation (Watling, 2006). As the Control was not sterile, this requires further consideration in terms of cell counts discussed in Section 4.2.2 and microbial species present, determined by qPCR analysis discussed in Section 4.2.3.

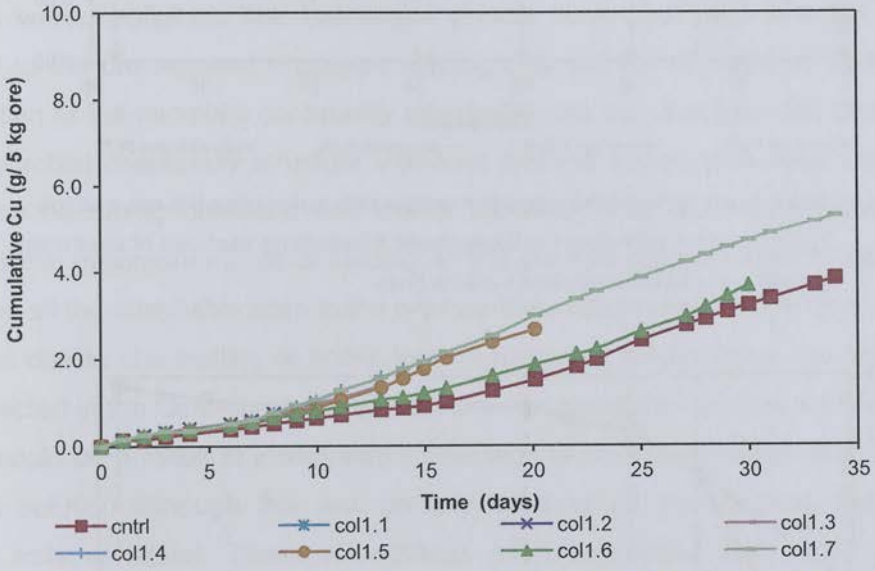


Figure 4. 8: Cumulative extracted Cu measured in the PLS of Experiment 1

4.2.2 Cell counts

The cell numbers, in both the PLS and on the ore, on the day of sacrifice, and a comparison between the cells associated with the ore and the total cell number in the column (5 kg ore) are presented in logarithmic scale (to base 10) in Figures 4.9 and 4.10 respectively. In this study it was assumed that there was uniformity throughout all the columns in a series as they were operated under the same conditions. Therefore, a single column was considered to be representative of all the columns at any particular instance. The cell counts were done in duplicate and the standard deviation, shown by the negligible error bars in the graphs and also in the raw data in Appendix B, was less than 1 %.

The columns were inoculated with an inoculum size of 3.00×10^5 cells/ kg ore. This inoculum size was arbitrarily regarded as the starting microbial concentration attached to the ore. The number of detached cells, shown in Figure 4.9, exhibited a general growth curve typical of batch microbial cultures (Doran, 1995).

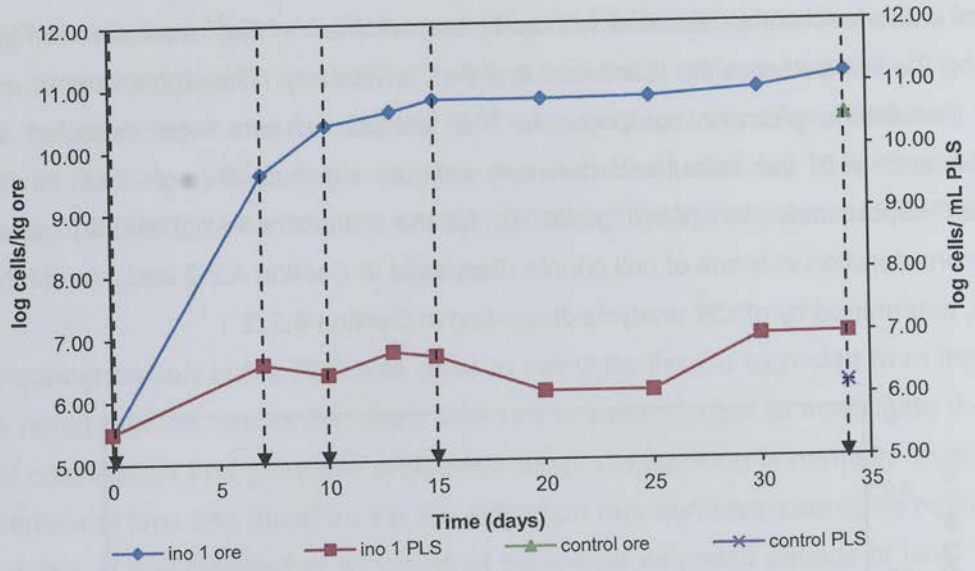


Figure 4. 9: The growth curve of the microbial community colonising the ore and the PLS at column take-down in Experiment 1 indicating sections of consistent growth rates between dotted vertical lines.

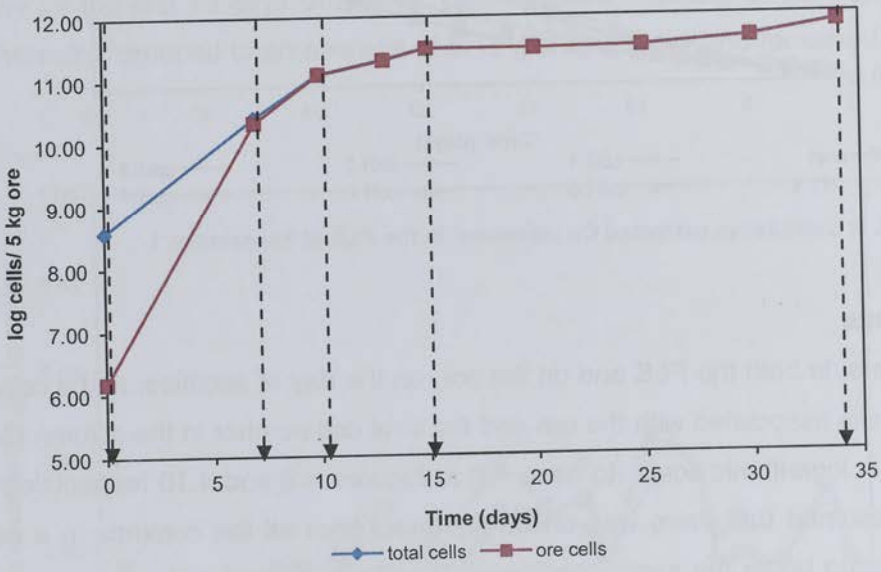


Figure 4. 10: The apparent growth curve of the total microbial community colonising the ore and the PLS, and the community colonising the ore alone, at column take-down in Experiment 1.

The highest microbial growth was exhibited in the beginning i.e. an increase from 3.00×10^5 at the start to 4.26×10^9 cells/ kg ore by day 7. After day 7, the growth rate slowed and the cell numbers reached 2.46×10^{10} cells/ kg ore by day 10 after which the growth rate further decreased with cell numbers finally reaching 4.05×10^{10} by day 13 and 6.19×10^{10} cells/ kg ore by day 15. After day 15 the growth was minimal and the cell numbers reached 1.47×10^{11} cells/ kg ore by day 34. The Control column (not inoculated) was analysed on day 34

and found to contain 3.12 x 10^{10} cells/ kg ore. The cell numbers in the PLS increased from 3.13 x 10^{5} cells/mL at the start to 3.75 x 10^{6} cells/mL on day 7. After day 7, the cell numbers fluctuated between 2.50 x 10^{6} and 4.70 x 10^{6} cells/mL finally reaching 9.69 x 10^{6} cells/mL by day 34. The PLS from the Control column contained 1.56 x 10^{6} cells/mL on day 34.

Figure 4.10 shows that in the beginning the highest cell numbers reported to the PLS; however in this period there was rapid attachment of cells to the ore such that by day 7 until the end, the attached cells accounted for the total cells in the columns whilst cell numbers in the PLS were negligible. The batch-type growth curve indicated that the cell numbers attached to the ore reached a plateau although, as will be indicated in Section 4.2.3, the composition of the microbial community attached to the ore changed with time. The change in the microbial community structure indicated that the microbial species were competing, with some becoming dominant and others receding. This batch-type growth curve also indicated that maximum microbial loading on the ore was reached after 15 days. This could mean that all the attachable sites in the ore had been taken and/or there were no avenues to new sites due to channelling or some form of restricted solution flow. No microbial growth was expected in the Control column since it was not inoculated and the microbial load in this column could be a result of incomplete sterilisation of the ore from the columns or from the irrigation solution although this was unlikely because all the columns and the irrigation solution were sterilised. These adventitious micro-organisms were likely to have been indigenous to the ore because a similar incomplete sterilisation of the ore was observed in other work done at CeBER by Govender *et al.* (2012 unpublished data).

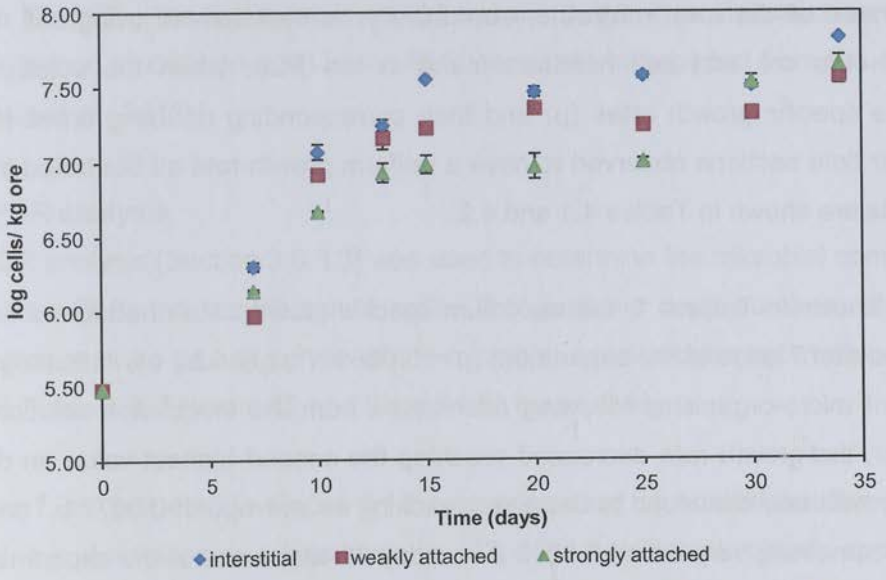


Figure 4. 11: The growth curves of the detachment phases in Experiment 1.

The detachment protocol (Section 3.5.9) was used to distinguish between the detachment phases of the micro-organisms attached the ore. Highest cell numbers were observed to report to the interstitial phase, followed by weakly attached and finally strongly attached phases, from the start up to day 25. After day 25, numbers increased in the strongly attached phase to reach values similar to the interstitial phase by day 30. After day 30, the strongly attached numbers decreased to lower than interstitial but were slightly higher than weakly attached numbers. This indicated that the micro-organisms were strengthening attachment to the ore with time or that in the EPS there was multiplication of micro-organisms already strongly attached to the ore, or both (Sand *et al.*, 1995; Rawlings, 2007). Similar trends whereby the highest cell numbers were in the interstitial followed by the weakly attached and finally the strongly attached phase were also observed in other heap bioleaching experiments conducted at CeBER, using the same detachment protocol used in this study (Chiume *et al.*, 2012; Govender *et al.*, 2013; Tupikina *et al.*, 2013b). This indicates the tendency of micro-organisms to attach to the ore rather than staying planktonic and this could be a means of the micro-organisms to draw closer to their energy source (Sand and Gehrke, 2006; Rawlings, 2007). A limitation of cell counts using a microscope is the limited ability to distinguish between different microbial species present in a sample. The cell counts could therefore not be used in calculating growth rates of individual microbial species and the growth rates were calculated using genomic copy numbers from qPCR analysis, as detailed in Section 4.2.4. However, the cell counts could be used for calculating the growth rates of the total microbial community discussed in Section 4.2.3.

4.2.3 The growth rates of the total microbial community

The growth rates of the total microbial community were calculated using cell numbers detached from the ore and cell numbers found in the PLS, when the columns were sacrificed. The specific growth rates (μ) and their corresponding doubling times (t_d) were calculated over time sections observed to have a uniform growth rate as illustrated in Figure 4.9. The results are shown in Tables 4.1 and 4.2.

In the ore, as shown in Table 4.1, the maximum specific growth rate of attached cells was observed in the first 7 days of the experiment ($\mu = 0.0569\ h^{-1}$; $t_d = 12.2$ h), indicating a rapid initial growth of micro-organisms following attachment from the inoculation solution to the ore. Thereafter, the growth rate decreased reaching the second highest value on day 7 to day 10. The growth rate continued to decrease reaching an average of $0.0077\ h^{-1}$ on day 10 to day 15 and remaining very low at $0.0019\ h^{-1}$ on day 15 to the end of the experiment. The apparent growth rate was estimated from the total cells in the columns (cells on the ore plus cells in the PLS). The apparent growth rate, as shown in Table 4.2, was also maximal in the

first 7 days (μ = 0.02517 h^{-1}; t_d = 27.5 h). This value was substantially lower than the growth rate on only the ore, owing to the fact that the highest number of cells initially reported to the PLS. After 7 days there was little increase in the PLS owing to more attachment to the ore.

Table 4. 1: Specific growth rates (μ) and doubling times (t_d) of the total cells colonising the ore in Experiment 1

Time (d)	μ (per h)	t_d (h)
0 - 7	0.0569 ± 0.0003	12.2 ± 0.1
7 - 10	0.0243 ± 0.0003	28.5 ± 0.4
10 - 15	0.0077 ± 0.0002	90.2 ± 2.4
15 - 34	0.0019 ± 0.0001	364.1 ± 19.4

Table 4. 2: Specific growth rates (μ) and doubling times (t_d) estimated from the apparent total (combined) cells colonising the ore and the PLS in Experiment 1

Time (d)	μ (per h)	t_d (h)
0 - 7	0.02517 ± 0.0002	27.5 ± 0.2
7 - 10	0.02202 ± 0.0004	31.5 ± 0.6
10 - 15	0.00764 ± 0.0002	90.8 ± 2.3
15 - 34	0.00197 ± 0.0001	365.1 ± 19.7

The maximum specific growth rate in PLS, calculated in the first 7 days, was 0.500 h^{-1}. This is a high value which is an over-estimation of the growth rate in the PLS. The columns were operated as an open flow through system with the PLS having a residence time about 10 minutes. Therefore, an assumption could be made that cell multiplication was negligible in the PLS, hence all cells counted in the PLS were cells transported from the ore to the PLS (Govender *et al.*, 2013).

4.2.4 qPCR analysis

The qPCR analysis (Section 3.6.3.3) was used to determine the microbial composition of the inocula, the PLS and the detached cells. The genomic copy numbers, the percentage composition and the growth curves of the microbial species detected in these samples are shown in Table 4.3, Figure 4.12 and Figure 4.13 respectively.

Inoculum 1, as indicated in Figure 4.12, was composed of *Acidithiobacillus caldus* (55 %) followed by *Acidiplasma cupricumulans* (42 %), *Acidithiomicrobium* spp. (2 %) and *Metallosphaera* spp. (< 1 %). All the four major microbial species detected in Inoculum 1 were found to colonise the ore, albeit at different rates. The same species were also found

planktonic in the PLS throughout the experiment. *At. caldus* dominated in both the ore and the PLS close to 100 % in the early stages i.e. on day 7, 10, 13, 15 and 20. The compositions of *A. cupricumulans*, *Metallosphaera* and *Acidithiomicrobium* spp. increased beyond 0.1 % of the total in the ore and PLS from day 20 onwards. *Acidithiomicrobium* spp. took over from *At. caldus* and dominated in both the ore and the PLS later i.e. on day 30 and 34. On day 34, major species present on the ore were *Acidithiomicrobium* spp. (82 %), *At. caldus* (13 %) and *A. cupricumulans* (5 %) whilst the PLS showed the same species at 82, 17 and 1 % respectively. The ore and the PLS in the Control were both dominated by *S. thermosulfidooxidans* close to 100 % whilst *At. caldus*, *A. cupricumulans, Acidithiomicrobium* spp. and *Thermoplasmatales* spp. were also present at < 1 %.

Table 4. 5. Genomic copy numbers (average) of microbial species in the inocula, in the PLS and in the ore in Experiment 1

	Acidiplasma cupricumulans (JTC4)		*Acidithiobacillus caldus* (At.c)		*Metallosphaera sedula/ hakonensis* (M.sed)*		*Acidithiomicrobium* species (JTC3/SAR)		*Sulfobacillus thermosulfidooxidans* (G2/STO)	
	PLS	ore	PLS	ore	PLS	ore	PLS	ore	PLS	ore
Day	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg
0[a]		9.90E+06		1.29E+07		2.60E+05		4.89E+05		-
7	5.71E+02	1.05E+05	5.71E+02	2.15E+09	5.71E+02	1.10E+04	-	3.79E+06	-	-
10	-	6.95E+05	1.71E+07	3.69E+10	5.06E+01	3.32E+05	2.93E+03	3.80E+06	-	-
13	-	3.00E+05	1.60E+07	5.76E+10	2.66E+01	4.23E+06	1.33E+03	7.81E+06	-	-
15	-	2.92E+06	2.74E+06	7.71E+10	2.82E+01	6.55E+05	8.11E+02	2.45E+07	-	-
20	5.23E+02	4.93E+07	6.14E+06	4.22E+10	1.79E+03	2.42E+07	3.76E+03	6.64E+08	-	-
30	1.02E+03	3.86E+08	2.50E+06	1.03E+10	8.59E+04	3.25E+09	8.70E+06	5.85E+10	-	-
34	9.39E+04	1.06E+10	1.31E+06	2.42E+10	3.10E+03	1.82E+07	6.57E+06	1.56E+11	-	-
34[b]	-	3.29E+04	4.11E+01	4.88E+05	-	6.70E+03*	2.55E+01	3.09E+05	1.70E+04	2.59E+08

a- Inoculum 1; *b*- Control column

* Archaea (JTC 1/2) instead *Metallosphaera* species in the Control column

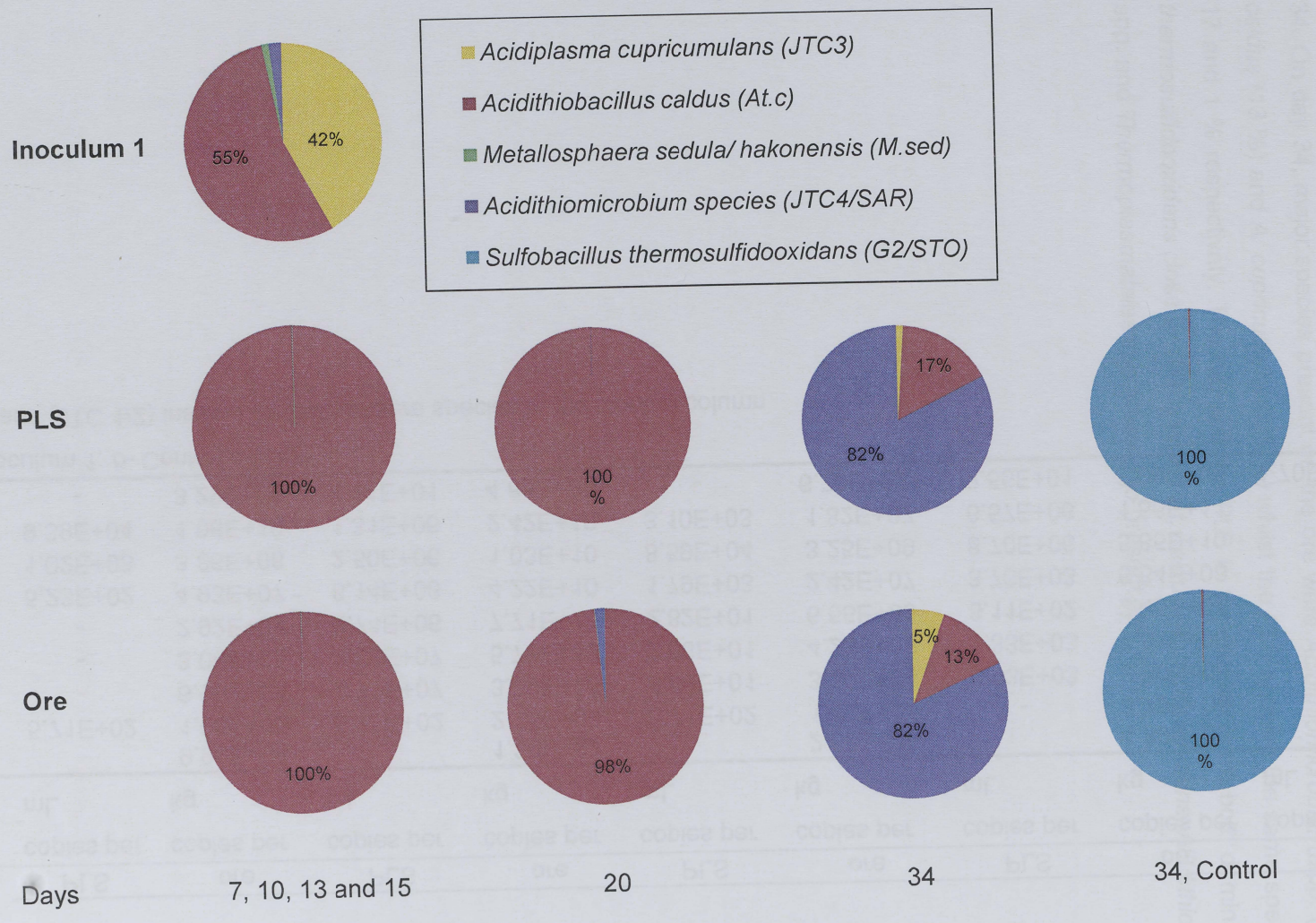


Figure 4. 12: Microbial composition of the inocula, the PLS and the ore in Experiment 1

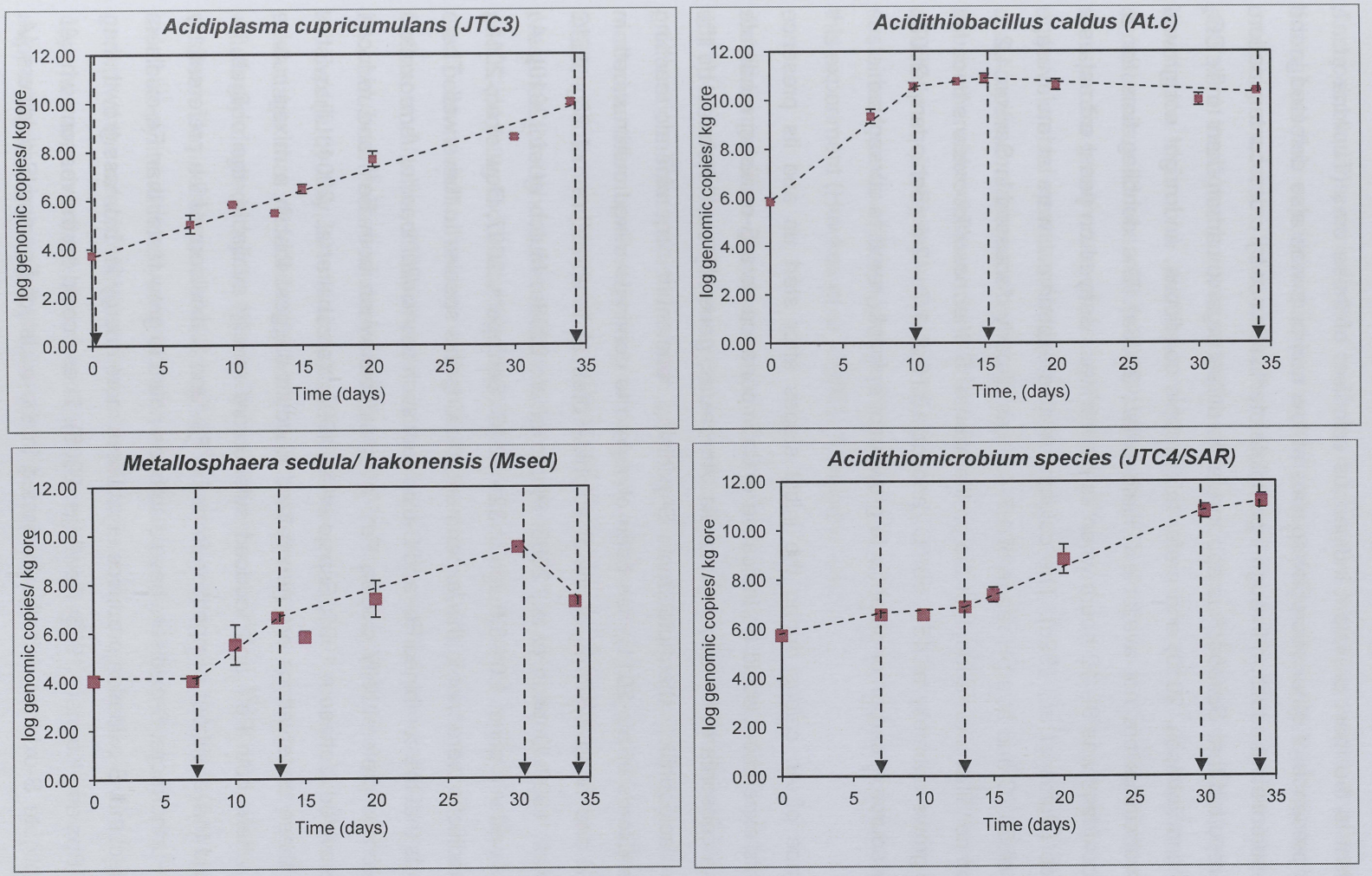


Figure 4. 13: The growth curves of the major microbial species colonising the ore in Experiment 1 indicating sections of consistent growth rates between dotted vertical lines and the corresponding lines of best fit for the particular sections.

S. thermosulfidooxidans was not detected in the inoculum and, as already discussed, the likely source of this spore-former was the ore. The *Sulfobacillus* spores (Bogdanova *et al.*, 2006) could be resistant to sterilisation by gamma radiation. It has been shown that this micro-organism is the most prevalent indigenous coloniser of these ores (Tupikina *et al.*, 2013b). The presence of other microbial species at low numbers was also detected in the Control with potential introduction through incomplete sterilisation or by cross contamination. It has been reported that *Sulfobacillus* spp. prefer to utilise organic carbon than to fix CO_2 (Ñancucheo and Johnson, 2010) and prefer mixotrophic conditions, and might not grow if mixotrophic conditions are not available (Plumb *et al.*, 2008a). The doubling time for *S. thermosulfidooxidans* was 8 - 12 hours when its growth was assayed on yeast extract as a carbon source (Norris *et al.*, 1996). The columns in this experiment were at an average temperature of 50 °C and at a pH range of 1.7 - 2.1 as already discussed in Section 4.2.1. These column conditions were ideal for the proliferation of *S. thermosulfidooxidans* because this species grows optimally at 45 - 58 °C, and at a pH of 1.7 - 2.4 (Schippers, 2007). However, the source of carbon or potential to grow autotrophically remains unresolved.

The dominance of *At. caldus* during the initial stages after start up and its presence throughout this experiment could be indicative of its importance as a S-oxidising moderate thermophile in consortia of bioleaching inocula. *At. caldus,* generally considered to be the most active acidophilic, thermotolerant S-oxidising bacterium in mineral leaching environments (Norris *et al.*, 2011), has been observed to dominate mixed cultures both in bioleach tank and heap systems of metal sulphide ores at temperatures of 28 - 55°C (Rawlings *et al.*, 1999; Dopson *et al.*, 2003; Plumb *et al.*, 2008a; Mutch *et al.*, 2010). *At. caldus* thrives well in a pH of 1.0 - 3.5 and at 32 - 52 °C (Schippers, 2007; Dew *et al.*, 2011), and these conditions were met in this experiment allowing this species to thrive well. There are also reports that the combined Fe- and S-oxidisers such as *Acidithiobacillus ferrooxidans* and *At. ferrivorans* preferentially oxidise Fe^{2+} over sulphur when both Fe^{2+} and reduced sulphur are provided (Johnson, 1995; Espejo *et al.*, 1998; Yarzábal *et al.*, 2004; Liljeqvist *et al.*, 2013). If these observations are generalized it would suggest that in a mixed culture environment where both Fe^{2+} and reduced sulphur are readily available, the obligate S-oxidisers would thrive without competition from the Fe- and S-oxidisers which preferentially oxidise Fe^{2+}. Furthermore, S-oxidisers have also been noted to grow faster than Fe-oxidisers owing to the fact that S-oxidation produces eight times more energy for biomass growth than Fe-oxidation (Bos and Kuenen, 1983; Rawlings, 2005). These could be the reasons why *At. caldus,* an obligate S-oxidiser, initially dominated in the midst of Fe- and S-oxidisers (*A. cupricumulans, Acidithiomicrobium, Metallosphaera* and *Sulfobacillus* spp.). The easily accessible sulphur was possibly depleted with time and also the Fe- and S-oxidisers

reached a biomass level adequate to compete with *At. caldus* hence the growth rate of *At. caldus* was decreased and combined Fe- and S-oxidisers increased in dominance.

A. cupricumulans, as indicated in Figure 4.13, had a steady growth rate and was prevalent throughout this experiment indicating that this species could be an important Fe- and S-oxidising moderate thermophile in bioleaching consortia. This species was also found prevalent throughout an experiment run for at least 180 days by Dew *et al.* (2011) to investigate microbial succession during heap bioleaching of chalcopyrite ore. *A. cupricumulans* thrives well in copper environments (Hawkes *et al.*, 2006b). Although steady, the specific growth rate of *A. cupricumulans* was the lowest of the growth rates of all the major microbial species of interest in the experiment as shown in Table 4.4. The growth rate of *A. cupricumulans* was low probably because the temperature of 50 °C and a pH of 1.7 - 2.1 in the columns were less than ideal for its optimal proliferation. This species grows optimally at a temperature of 54 °C and at a narrow pH range of 1.0 - 1.2. Furthermore, sustained growth of this species requires a reduced organic carbon source not supplied in this experiment (Hawkes *et al.*, 2006b; Schippers, 2007).

Acidithiomicrobium grows optimally around 50 °C and at a pH of 1.7 - 2.5 (Norris *et al.*, 2011); therefore the columns in this experiment provided the ideal temperature and pH for its proliferation. This species has also been found prevalent and dominant in mixed cultures of moderate thermophiles growing on copper sulfide and polymetallic sulfide ores in ore leaching columns (Norris *et al.*, 2011). The dominance of *Acidithiomicrobium* spp. and its presence throughout this experiment indicated that this species could be an important Fe- and S-oxidising moderate thermophile in bioleaching consortia. *Acidithiomicrobium* spp. has also been noted to have an extent of sulphur oxidation similar to that of *At. caldus* (Davis-Belmar and Norris, 2009) and this could explain why this species showed up in a previously *At. caldus*-dominated environment by day 20 and consequently took over and out-dominated *At. caldus* by day 30 of the experiment. This could have also been a result of an added advantage that *Acidithiomicrobium* spp. has due to its ability to also utilise Fe^{2+} and efficiently degrade pyrite (Davis-Belmar and Norris, 2009) whilst *At. caldus* is only a S-oxidiser. Davis-Belmar and Norris (2009) also noted that *Acidithiomicrobium* spp. extensively oxidised pyrite without yeast extract in contrast to a yeast extract requirement for significant growth of the related *Acidimicrobium ferrooxidans.* This could mean that *Acidithiomicrobium* spp. has an advantage over microbial species that require yeast extract such as *M. sedula* supporting its dominance by the end of the experiment.

M. sedula and *M. hakonensis* are closely related and their 16S rRNA genes have a high similarity (Dew *et al.*, 2011). It has not yet been possible to design target PCR primers capable of distinguishing between these two species; therefore throughout this write-up these two species are grouped under the umbrella name "*Metallosphaera* spp.". Nevertheless, *M. sedula* has been shown to never grow without yeast extract as a carbon source whilst *M. hakonensis* can grow on Fe^{2+} only (Dew *et al.*, 2011). In this experiment no yeast extract was added into the columns, therefore the genomic copies of *Metallosphaera* spp. were likely to be *M. hakonensis*. However still, similarly to *S. thermosulfidooxidans* as already discussed, *M. sedula* could have obtained organic carbon from dead microbial cells in the ore. *Metallosphaera* spp. was present throughout, although in the early stage it was out-dominated by *At. caldus*, and subsequently by *Acidithiomicrobium* spp. at the later stage of the experiment. However, *Metallosphaera* spp., as shown by the growth curves in Figure 4.13, steadily grew to a significant composition in the PLS and ore by day 30 of the experiment as shown in Figure 4.12. *Metallosphaera* spp. can grow at a temperature of 50 - 80 °C and at pH 1.0 - 4.5, but grows optimally at 70 - 75 °C and at pH 2.0 - 3.0 (Schippers, 2007; Dew *et al.*, 2011). The average temperature of 50 °C and a pH of 1.7 - 2.1 in the columns of this experiment were therefore not optimal for the proliferation of *Metallosphaera* spp. Although the temperature and pH were not optimal, *Metallosphaera* spp. had the second highest specific growth rate compared to the highest specific growth rates of the other three major microbial species of interest as shown in Table 4.4. The high growth rate of *Metallosphaera* spp. could result from the very high efficiency of this species in extracting metal ions from minerals (Huber *et al.*, 1989; Takayanagi *et al.*, 1996).

4.2.5 The growth rates of individual microbial species

The growth rates of individual microbial species were calculated using only the genomic copy numbers extracted from cells detached from the ore (Table 4.3). The genomic copy numbers extracted from the PLS were not used because they were erratic owing to low concentrations. The batch nature of the microbial growth on ore was supported by the growth curves generated from cell counts shown in Figure 4.9. The specific growth rates (μ) and the corresponding doubling times (t_d) were calculated over time sections observed, from Figure 4.13, to have a uniform growth rate. The qPCR analysis of detached cells was done in duplicate. The standard deviation of the copy numbers of individual microbial species is shown as error bars in Figure 4.13 and in the raw data in Table B.6 (Appendix). The growth rates and the corresponding doubling times of the microbial species are shown in Table 4.4.

Table 4. 4: Specific growth rates (μ) and doubling times (t_d) of the major microbial species colonising the ore in Experiment 1 (the maximum specific growth rates of individual species are in bold)

Microbial species	Time (d)	μ (per h)	t_d (h)
Acidiplasma cupricumulans (JTC3)	0 - 34	**0.0177 ± 0.0003**	**39.1 ± 0.7**
Acidithiobacillus caldus (Atc)	0 - 10	**0.0453 ± 0.0014**	**15.3 ± 0.5**
	10 - 15	0.0061 ± 0.0009	113.0 ± 16.8
	15 - 34	negative	-
Metallosphaera spp. (Msed)	0 - 7	lag	-
	7 - 13	**0.0413 ± 0.0001**	**16.8 ± 0.05**
	13 - 30	0.0163 ± 0.0004	42.6 ± 1.00
	30 - 34	negative	-
Acidithiomicrobium spp. (JTC4/SAR)	0 - 7	0.0115 ± 0.0000	60.1 ± 0.0
	7 - 13	0.0050 ± 0.0008	138.0 ± 23.4
	13 - 30	**0.0248 ± 0.0014**	**28.0 ± 1.7**
	30 - 34	0.0102 ± 0.0000	67.7 ± 0.0

A. cupricumulans had a steady specific growth rate (μ = 0.0177 h^{-1}; t_d = 39.1 h) throughout the experiment as shown in Figure 4.13. *At. caldus* had the maximum growth rate (μ = 0.0453 h^{-1}; t_d = 15.3 h) from the start of the experiment up to day 10. This was the highest growth rate observed in the heap system. This resulted in *At. caldus* dominating the ore from the start up to day 25. *Metallosphaera* spp. also had a high growth rate (μ = 0.0413 h^{-1}; t_d =16.8 h), on day 7 - 13. After day 15, the growth rate of *At. caldus* decreased, and at the same time the growth rate of *Metallosphaera* spp. also decreased whilst that of *Acidithiomicrobium* spp. started to increase. The data suggests that *Metallosphaera* spp. and *Acidithiomicrobium* spp. exhibited competitive behaviour because when one thrived well the other had a lower growth rate. *Metallosphaera* spp. lagged from the start until day 7 whilst *Acidithiomicrobium* spp. had its second highest growth rate (μ = 0.0115 h^{-1}; t_d = 60.1 h) during this period. After day 7 until day 13 *Metallosphaera* spp. had its maximum growth rate (μ = 0.0413 h^{-1}; t_d =16.8 h) whilst *Acidithiomicrobium* spp. had its lowest growth rate (μ = 0.0050 h^{-1}; t_d =138.0 h). Between day 13 and 30, *Acidithiomicrobium* spp. had its maximum growth rate (μ = 0.0248 h^{-1}; t_d =28.0 h) whilst *Metallosphaera* decreased in quantity during this period, hence *Acidithiomicrobium* spp. dominated both the ore and PLS until the end of the experiment.

The growth rate of *At. caldus* in the first 10 days (μ = 0.0453 h^{-1}; t_d = 15.3 h) was similar to the total microbial growth rate (μ = 0.04714 h^{-1}; t_d = 14.7 h) in the first 10 days, shown in Table 4.4. This indicated that *At. caldus* being the dominant microbial species influenced the

growth rate of the total microbial community. Although *Metallosphaera* spp. had the highest growth rate (μ = 0.0413 h^{-1}; t_d =16.8 h) on day 7 - 13, *At. caldus* still dominated the total microbial growth rate; the growth rate of *At. caldus* on day 10 - 15 (μ = 0.0061 h^{-1}; t_d =113.0 h) was similar to the total microbial growth rate (μ = 0.0077 h^{-1}; t_d = 90.2 h) in the same period, as the *Metallosphaera* spp. cell numbers were still too low similarly. Again, between day 13 and 30, the cell numbers of *Metallosphaera* spp. and *Acidithiomicrobium* spp., in spite of high growth rates, were still too low to influence the total microbial growth rate which remained dominated by the growth rate of *At. caldus*. In the last section of the experiment i.e. day 30 – 34, the growth rate of *Acidithiomicrobium* spp. (μ = 0.0102 h^{-1}; t_d = 67.7 h), was almost double that of the total microbial community (μ = 0.0056 h^{-1}; t_d = 124.7 h).

There is little published data on growth rates of bioleaching micro-organisms in whole ore environments typical of heap leach processes, making comparison of these findings with literature difficult. The growth rates found on low grade ore are compared to the maximum specific growth rates found in literature for liquid cultures. The growth rates of only *At. caldus* and *Metallosphaera* spp. in liquid culture were found in literature and are shown in Table 4.5.

Table 4. 5: Comparison of doubling times (t_d) of the *At. caldus* and *Metallosphaera* spp. in Experiment 1, with doubling times found in literature for liquid cultures

Microbial species and media	Temperature	pH	t_d (h)
Acidithiobacillus caldus			
Chalcopyrite whole ore at day 0 - 10	50 °C	1.7 - 2.1	15.3
Tetrathionate media*	45 °C	2.0 - 2.5	3.0
		1.0	6.7
		4.0	46.0
***Metallosphaera* spp.**			
Chalcopyrite whole ore at day 7 - 13	50 °C	1.7 - 2.1	16.8
1 g ore mixture of pyrite, sphalerite and pitch in 30 mL Allen medium	75 °C	1.0 - 4.5	5.25

* Hallberg and Lindström, 1994; ** Huber *et al.*, 1989

In Table 4.5 it is shown that the doubling time of *At. caldus* (t_d =15.3 h) in Experiment 1 was about 5 times the shortest doubling time in the liquid medium (t_d = 3.0 h) indicating that the growth of *At. caldus* was 5 times slower on the ore than in the liquid medium. Likewise, the doubling time of *Metallosphaera* spp. indicated that the growth of this species was 3 times slower on the ore than in the liquid medium. Growth in the whole ore was slower probably because physicochemical conditions were not controlled and kept optimal as was done for

conditions in liquid media. Also, the energy source in the whole ore was not as readily available to the micro-organisms as it was in liquid media.

4.2.6 The activity of the microbial community

The activity of the microbial community in the heap system was monitored by measuring the rate of Fe^{2+} oxidation. The test was set up as described in Section 3.6.4. The activity of the following samples was tested: Inoculum 1 at the start, the cells detached from the ore in the columns sacrificed on day 7, 20 and 34. These samples (10^9 cells) were used to inoculate shake flasks containing Fe^{2+}. The Fe^{2+} depletion was monitored over 14 days (Figure 4.14).

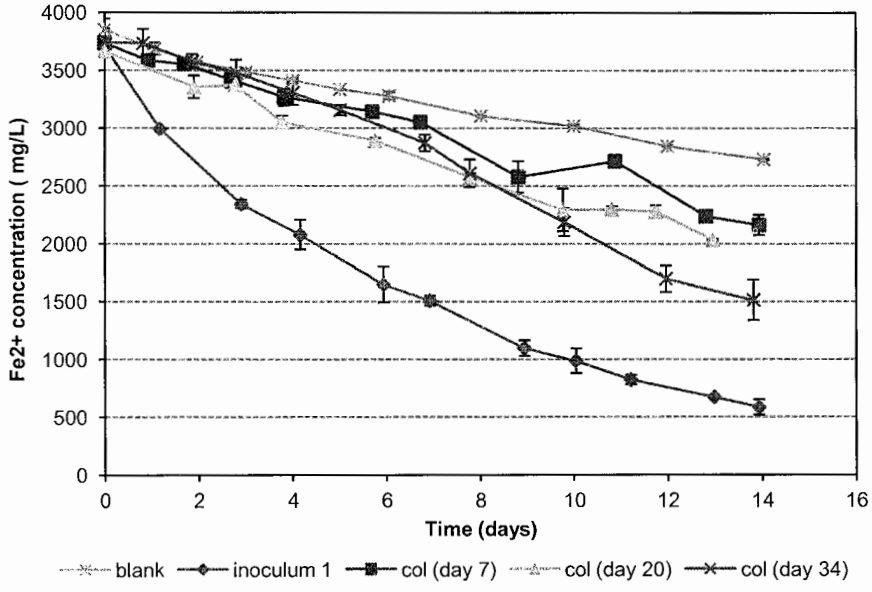


Figure 4. 14: Fe^{2+} depletion by different samples from Experiment 1

Typically, there was a linear rate of Fe^{2+} depletion throughout the experiment with the exception of the Control (Inoculum 1) where exponential depletion was observed. The rate of Fe^{2+} depletion was calculated over the first 3 days for Inoculum 1 to obtain the maximum oxidation rate whilst for the detached cells the oxidation rate was calculated over the 14 days. The biological Fe^{2+} oxidation rates of the samples are shown in Figure 4.15.

The activity test showed that Inoculum 1 had the highest activity with an iron oxidation rate of 0.144 $gFe^{2+}/L/day$, shown in Figure 4.15. Although the activity of the detached cells was lower, it was shown to increase with time i.e. 0.033, 0.046 and 0.081 g/L/day by day 7, 25 and 34 respectively. The increase in the Fe^{2+} oxidation rate by the detached samples could be indicative of either an increase in the composition of Fe-oxidisers or an increase in the specific activity of these Fe-oxidisers in the microbial community colonising the ore, with

time. The increasing composition of Fe-oxidisers was confirmed by the qPCR results (Figures 4.12 and 4.13) which indicated an increase of *A. cupricumulans*, *Acidithiomicrobium* spp. and *Metallosphaera* spp., all which are both Fe- and S-oxidisers.

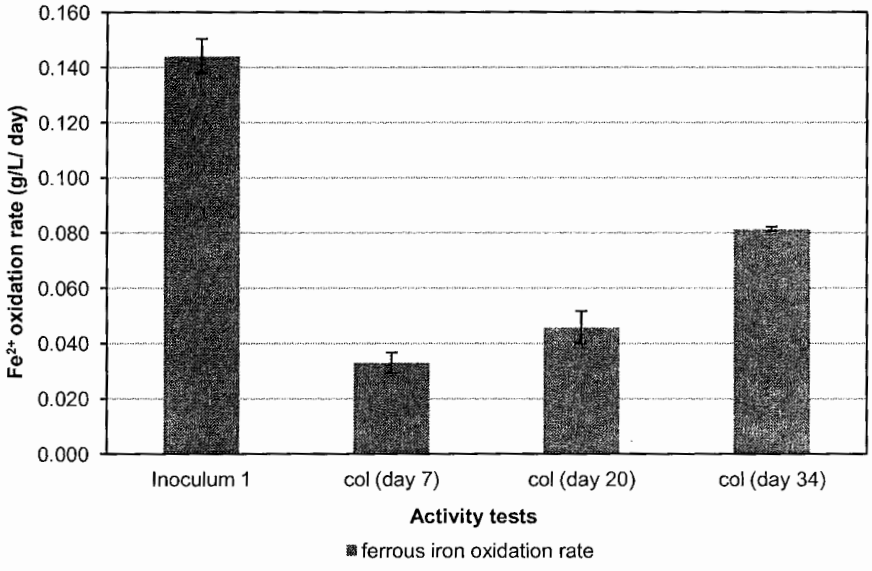


Figure 4. 15: Ferrous iron oxidation rates by Inoculum 1 and detached cells in Experiment 1

4.2.7 Discussion of the results from Experiment 1

The trends from physicochemical test results (Eh, pH, Fe^{2+}, Fe^{tot} and Cu) generally suggested that adequate microbial attachment to the ore in the inoculated columns had taken place after 6 days. A stable pH throughout the experiment indicated that there was a balance between acid-consuming and acid-generating reactions. This could partly be due to the fact that the ore used in this study had been conditioned by acid-agglomeration.

Counts of cells detached from the ore supported batch-type microbial growth on the ore. Cell numbers on the ore reached a plateau after about 15 days. This could suggest that all available sites for attachment on the ore had been taken up or that channelling restricted solution flow and limited access to available sites. Although there was a little change in cell numbers, the composition of the microbial community attached to the ore changed over time indicating that microbial species were competing, with some becoming dominant and others receding. No microbial growth was expected in the Control column since it was not inoculated and the microbial load that was detected in this column was likely to be from microbial species indigenous to the ore raising the questioning of the efficiency of gamma radiation in sterilising the ore.

Highest cell numbers were generally observed to report to the interstitial phase initially, followed by the weakly attached and finally the strongly attached phase. Later i.e. after day 25, the numbers were observed to increase in the strongly attached phase suggesting that the micro-organisms were attaching more to the ore over time and/or that in the EPS there was multiplication of microbial cells already attached to the ore.

All the four major microbial species detected in the inoculum i.e. *At. caldus, A. cupricumulans, Acidithiomicrobium* spp. and *Metallosphaera* spp., attached to the ore. The ore used in this experiment was largely chalcopyrite and had some component of pyrite, and attachment of the micro-organisms to this ore was quick probably because the microbial species had been adapted to chalcopyrite and pyrite both which were used as their energy sources in the stock culture. This is in agreement with the observation made by Olson *et al.* (1982) that when microbial cultures are pre-grown in a particular ore feed the micro-organisms able to oxidise iron and sulphur in the presence of inhibitory present in this feed are selected for. Also, the microbial species in this experiment grew probably because their growth requirements had been met although their different growth rates were probably because the conditions in the columns were not optimal for all the species. *At. caldus* dominated the columns in the earlier stages of the experiment and this could suggest that in mixed cultures S-oxidisers have a competitive advantage over Fe-oxidisers. However, the dominance of *Acidithiomicrobium* and *Metallosphaera* species in the later stages of the experiment indicated that Fe- and S-oxidisers finally take over from S-oxidisers becoming the dominant species the bioleach heap system over time. Activity tests showed that the ability of the attached cells to oxidise Fe^{2+} was proportional to the composition of Fe-oxidisers in the microbial community colonising the ore.

4.3 The effect of inoculum composition on the growth of moderate thermophiles on whole ore

The effect of inoculum composition on the growth and dominance of the constituent microbial species on whole ore was investigated. This experiment was necessitated by an observation that the composition of the bioleaching stock culture maintained at 50 °C changed over time. This resulted in inocula with varying concentrations of the same microbial species, under the same temperature, as shown in Figure 4.16. Its impact on the start up of an inoculated bioleach heap operation and the resultant composition of the ore-associated microbial community in a bioleach heap is of interest.

The experiment set-up was similar to the set-up of Experiment 1 described in Section 3.3. Ten columns were packed with ore type A, and one column (Col 2.7) was packed with ore type B. Three different inocula (Inoculum 2, 3 and 4) were used to inoculate the columns and the experiment was run for 50 days. The composition of the inocula, shown in Figure 4.16, was determined by qPCR analysis. Inoculum 2 was dominated by *Acidithiomicrobium* spp. (74 %), *Metallosphaera* spp. (24 %), and *A. cupricumulans* and *At. caldus* both at 1 % composition; and negligible amounts of *Thermoplasmatales* spp. and *Leptospirillum ferriphilum* both at < 1 %. Inoculum 3 was dominated by *Metallosphaera* spp. (92 %), *Acidithiomicrobium* spp. (6 %), and *A. cupricumulans* and *At. caldus* both at 1 %, and *Thermoplasmatales* spp. and *L. ferriphilum* both at < 1 %. Inoculum 4 was dominated by *At. caldus* (55 %), *Acidithiomicrobium* spp. (44 %), *Metallosphaera* spp. (1 %) and *A. cupricumulans* (< 1 %). The Control column was not inoculated. An inoculum size of 3×10^8 cells/ ton ore was used to inoculate Column 2.1 to 2.7 using Inoculum 2, and Column 3.1 and 3.2 using Inoculum 3. Column 4.1 was inoculated with an inoculum size of 3×10^9 cells/ ton ore using Inoculum 4. A bigger inoculum size was used because Inoculum 4 was still at sub-optimal performance i.e. still at an Eh of 714 mV, whilst Inoculum 2 and 3 were above 800 mV. The columns were numbered in the order of their take-down. Column 2.1 to 2.7 were sacrificed on day 6, 12, 18, 26, 38, 49 and 50 respectively whilst Column 3.1 and 3.2 were sacrificed on day 11 and 49 respectively. Column 4.1 was sacrificed on day 34 and the Control column was sacrificed on day 50.

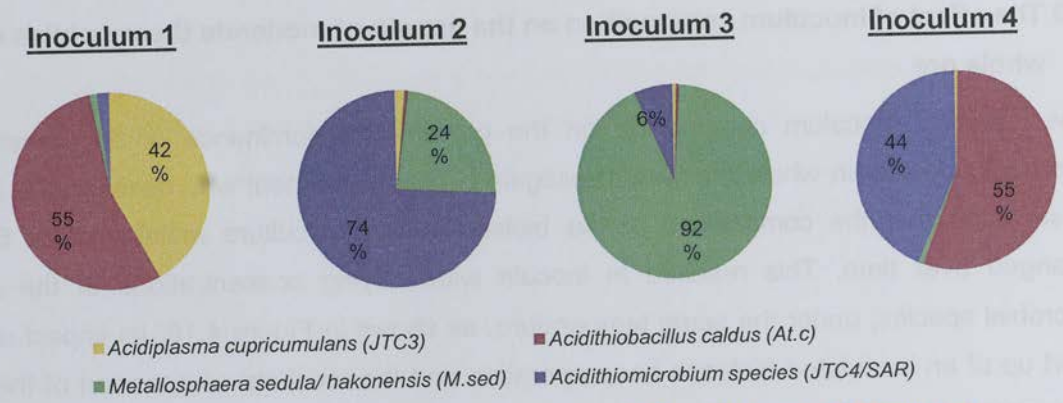


Figure 4. 16: Composition of Inoculum 1, and inocula (2, 3 and 4) used in Experiment 2

4.3.1 Physicochemical parameters

The Eh, pH, Fe^{2+}, Fe^{tot}, soluble Cu and Fe metal in the PLS from the columns were measured every second day. The results of these measurements including the cumulative amount of copper extracted from the ore are shown the Figures 4.17 to 4.22.

The Eh in all the columns including the Control, shown in Figure 4.17, increased in a similar trend except in Col 4.1 (inoculated with Inoculum 4). The Eh in all the columns started at an average of 570 mV and lagged for at about 4 days, then increased from day 6 to reach an average of 780 mV by day 12 except for the Control, Col 2.4 and Col 2.7 (containing ore type B). The Control and Col 2.4 reached 782 and 775 mV on day 23 and 18 respectively, and Col 2.7 reached 695 mV on day 12. After this rapid increase, the Eh increased at a slower rate until the end of the experiment. Column 2.6 and Col 3.2 both reached 817 mV by day 49. The Control and Col 2.7 reached 822 and 805 mV by day 50 respectively. Col 2.7 had the lowest Eh of all columns, except Col 4.1, throughout the experiment. After the 6-day lag, the Eh in Col 4.1 slowly increased and reached 650 mV by day 12 with little increase to 690 mV by day 25 after which the Eh increased quickly to reach 820 mV by day 34 where it plateaued.

The similar trends and values of Eh between columns inoculated with Inoculum 2 and Inoculum 3 indicated that the composition of the inocula had no effect on the microbial Fe^{2+} and/or pyrite oxidation. The Control column had a lower Eh than all the inoculated columns for the first 25 days, except for Col 2.7 containing ore type B and Col 4.1 inoculated with Inoculum 4. This suggested a better performance by columns inoculated with an active inoculum with demonstrated microbial activity. The performance of the Control suggests microbial activity by contaminant micro-organisms that were detected in this column . The contamination of the Control has already been discussed in Section 4.2 and will also be further discussed under cell counts and qPCR analysis (Section 4.3.2 and 4.3.3 respectively). Col 2.7 had a low Eh probably due to deterred microbial activity caused by the

initial high Cu concentration in the system (Gadd and Griffiths, 1978). The Cu was high in Col 2.7 because, as indicated in Table 3.2, ore type B had a higher content of easily leachable secondary copper-sulphide minerals; chalcocite at 0.20 % and covellite at 0.30 % composition compared to ore type A which contained 0.09 % chalcocite and 0.05 % covellite. Col 4.1 initially had a low Eh probably because the inoculum used had lower microbial activity resulting in sub-optimal performance initially until adequate colonisation after 25 days whereafter the Eh began to increase rapidly.

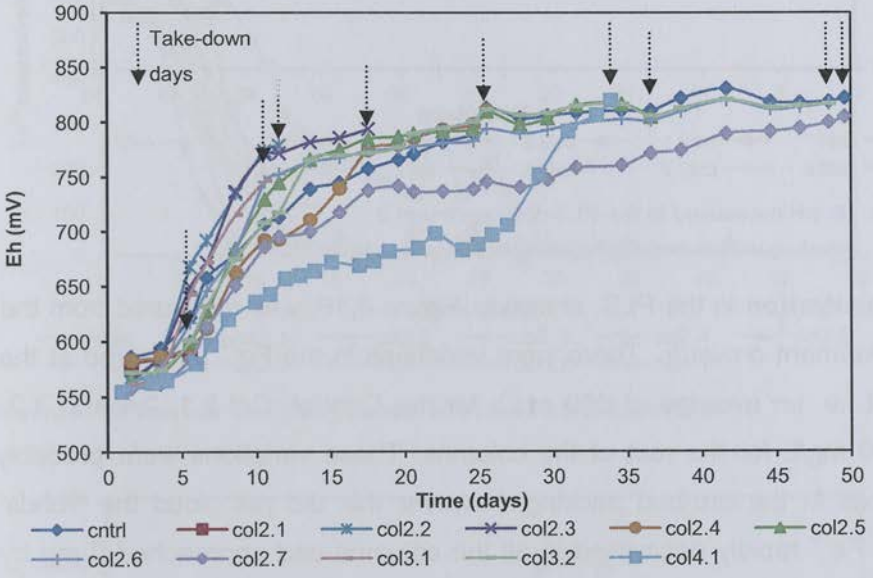


Figure 4. 17: Eh measured in the PLS of Experiment 2

The pH in all the columns except for Col 2.7 and Col 4.1, as shown in Figure 4.18, started at an average of pH 2.00, gradually decreased to around pH 1.70 by day 25 and remained constant until day 50. Although following a similar trend as the other columns, the pH of Col 2.7 (containing ore type B) was slightly higher starting off at pH 2.20, gradually decreasing to pH 1.75 on day 25 and thereafter remaining constant. The higher pH in Col 2.7 could have been attributed to gangue mineralogy. The pH of Col 4.1 was initially maintained at an average of pH 2.10 until day 21 and thereafter the pH decreased rapidly reaching values similar to other columns by day 26. The initially high pH in Col 4.1 may be due to the low initial metabolic activity owing to the inoculum used, but after 21 days adequate microbial growth had taken place in the ore and the pH decreased. The general pH profile of all the columns except Col 4.1 indicates an initial increase of acidity in the PLS probably due to the depletion of the acid-consuming gangue that was left untreated when the ore was pre-conditioned by acid-agglomeration. The stability of the pH after day 25 was an indicator that there was a balance between acid-consuming and acid-generating reactions.

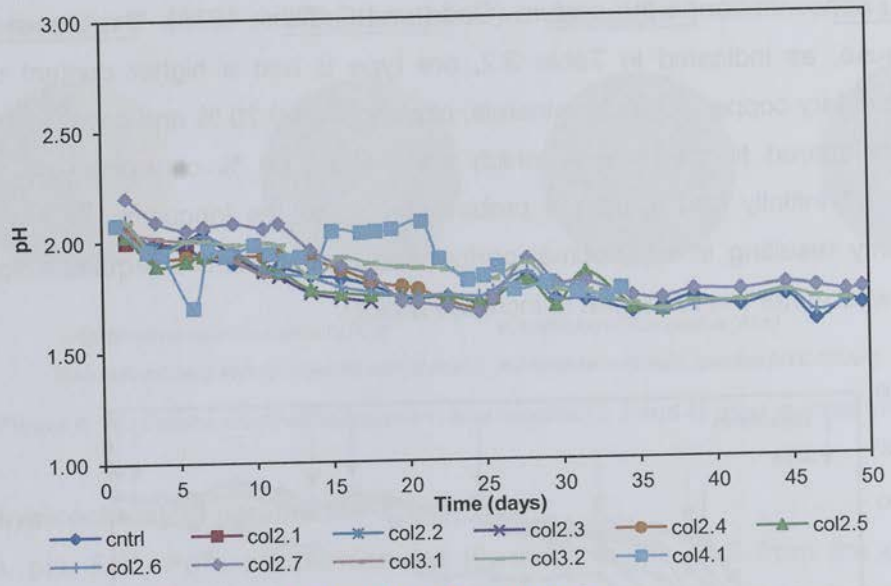


Figure 4. 18: pH measured in the PLS of Experiment 2

The Fe^{2+} concentration in the PLS, shown in Figure 4.19, was measured from the second day of the experiment onwards. There were variations in the Fe^{2+} measured at the start of the experiment i.e. an average of 850 mg/L for the Control, Col 2.1, 2.4 and 3.2, and an average of 600 mg/L for the rest of the columns. These variations were probably due to slight differences in the ore-bed packing; however this did not cloud the trends of Fe^{2+} depletion. The Fe^{2+} rapidly decreased in all the columns and approached 0 mg by day 14 except for Col 2.7 and 4.1 which approached 0 mg/L on day 20 and 31 respectively. The delay in the decrease of Fe^{2+} in Col 2.7 indicated a slower rate of Fe^{2+} oxidation which could be due to reduced microbial activity caused the negative effect of the high Cu concentration in this column whilst the delay in Col 4.1 was due to sub-optimal microbial activity of the inoculum. The initial high Fe^{2+} in all the columns could be attributed to the easily-leachable component of the ore releasing the Fe^{2+} into the PLS.

The Fe^{tot} concentration in the PLS, shown in Figure 4.20, in the first 12 days was similar to Fe^{2+} in all columns. After day 12, the Fe^{tot} was at an average of 500 mg/L and remained at this value until day 20, except for Col 2.7 which was at an average 400 mg/L for the rest of the experiment. After day 20, Fe^{tot} gradually increased in all other columns reaching an average of 600 mg/L by day 50, with the exception of Col 4.1. The increase of Fe^{tot} was more pronounced in Col 4.1 where the Fe^{tot} increased from 516 mg/L on day 21 to reach 1200 mg/L by day 34. The lower Fe^{tot} in Col 2.7 could be attributed to precipitation and a hold-up of Fe due to the influence of the high Cu concentration in this column. The sudden increase of Fe^{tot} in Col 4.1 after 21 days could be attributed to an increase in microbial growth and colonisation of the ore. The decrease of Fe^{tot} after day 12, coincided with the

decrease of Fe^{2+} to about 0 mg/L, as shown in Figure 4.19. This indicated that all the Fe^{2+} in the system was oxidised to Fe^{3+}. The gradual increase of Fe^{tot} after day 20 indicated that some additional Fe was now being leached from the ore into the PLS.

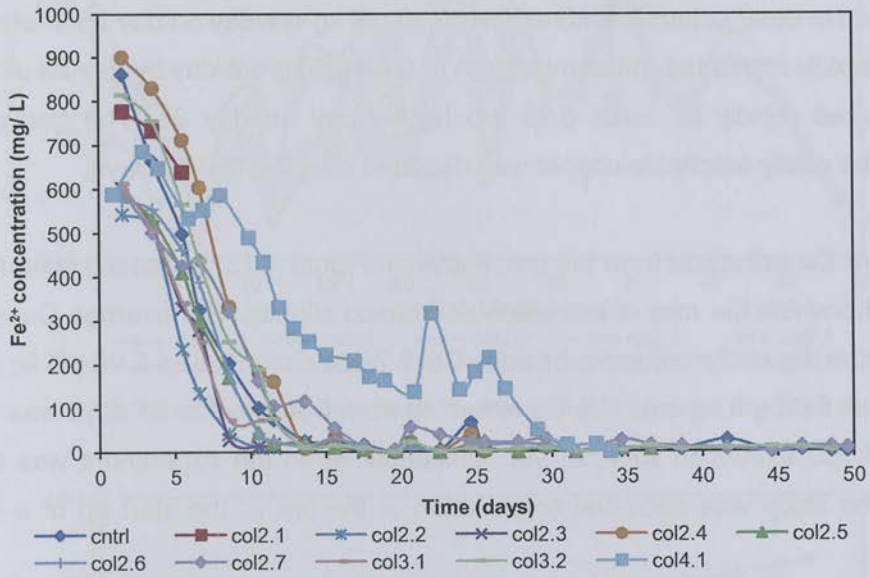


Figure 4. 19: Ferrous iron (Fe^{2+}) measured in the PLS of Experiment 2

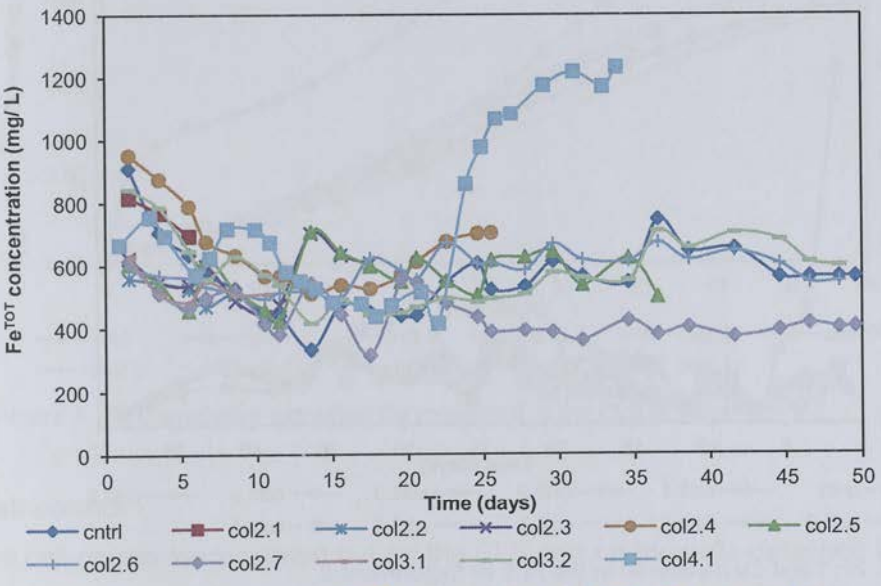


Figure 4. 20: Total soluble iron (Fe^{tot}) measured in the PLS of Experiment 2

The Cu concentration in the PLS was used to calculate the Cu extraction from the ore. The Cu concentration, shown in Figures 4.21 and 4.22, started at an average of 0.30 g/5 kg ore/day by day 2 and decreased to an average of 0.10 g/5 kg ore/day by day 12 in all columns except for Col 2.7 (containing ore type B). After this, the Cu gradually increased and reached an average of 0.40 g/5 kg ore/day on day 35 except in Col 4.1 after which the

Cu decreased again finally reaching an average of 0.10 g/5 kg ore/day by day 50. The Cu in Col 2.7 started at 2.00 g/5 kg ore/day on day 2 and quickly decreased and reached 0.10 g/5 kg ore/day, an amount similar to the other columns, by day 12. After this, the amount of Cu in Col 2.7 was similar to the rest of the columns. After 12 days the Cu in Col 4.1 increased more slowly than in other columns and reached 0.30 g/5 kg ore/day on day 25 afterwhich the Cu in Col 4.1 rapidly increased to the maximum of 0.66 g/5 kg ore/day by day 29 after which the Cu decreased rapidly to reach 0.25 g/5 kg ore/day on day 34. The general trend indicates that the easily-leachable copper was depleted over the first 12 days.

The cumulative Cu extraction from the ore, shown in Figure 4.23, increased linearly for the first 35 days afterwhich the rate of extraction decreased slightly. The average Cu extracted after 50 days from the all the columns, besides Col 2.7 and Col 4.1, was 5.90 g/5 kg ore, and from Col 2.7 was 8.60 g/5 kg ore. The Cu extracted from Col 4.1 after 34 days was 6.00 g/5 kg ore. Overall, Cu extraction was not yet substantial when the experiment was stopped. The focus of the study was microbial colonisation of the ore at the start up of a bioleach heap.

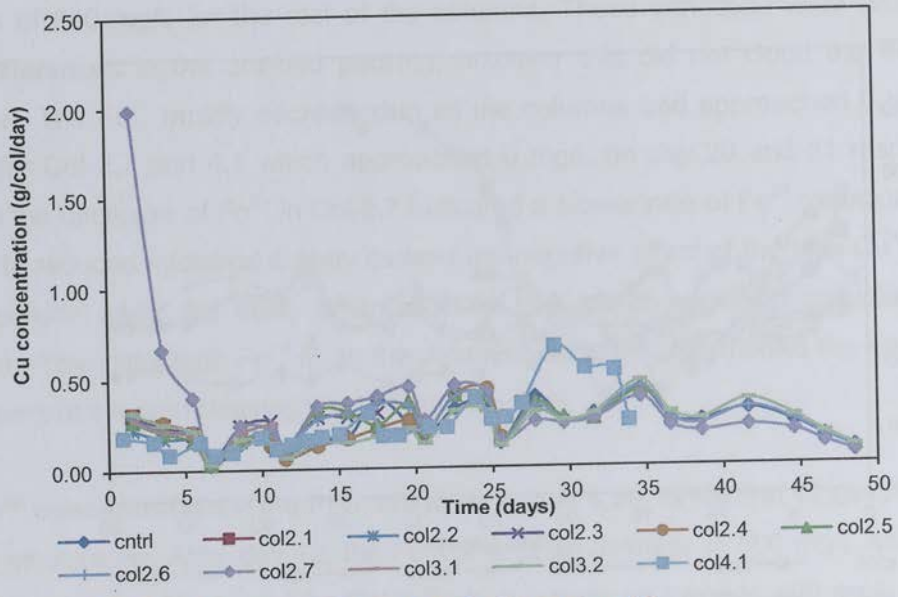


Figure 4. 21: Total Cu measured in the PLS of Experiment 2

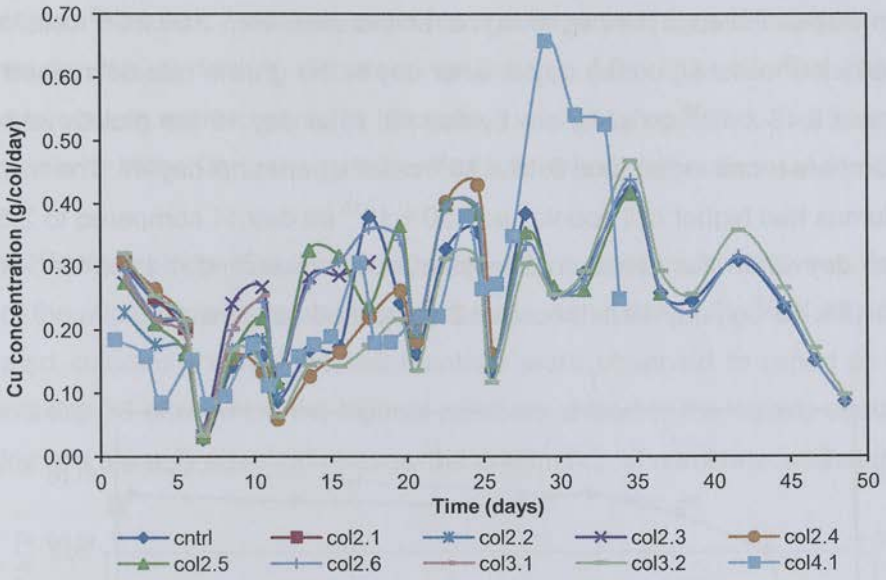


Figure 4. 22: Total Cu measured in the PLS of Experiment 2 (minus Col 2.7)

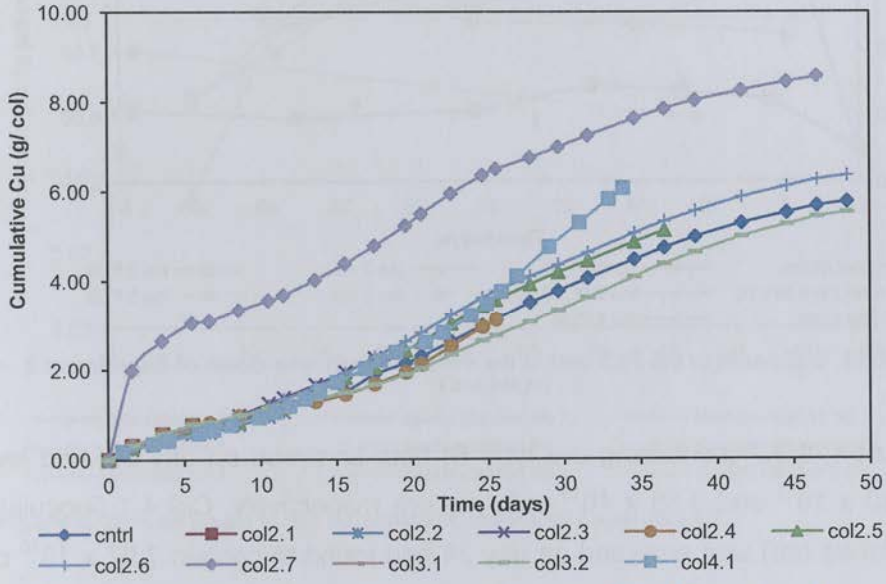


Figure 4. 23: Cumulative extracted Cu measured in the PLS of Experiment 2

4.3.2 Cell counts

Microbial cell counts were carried out on the PLS and on the cells detached from the ore at column take-down. The total cell numbers and the cell numbers in the different detachment phases are presented in logarithmic scale (to base 10) in Figures 4.24 and 4.25 respectively. All the columns were inoculated with 3.00×10^8 cells/ ton ore except for the Control column which was not inoculated and Col 4.1 which was inoculated with 3.00×10^9 cells/ ton ore.

The number of cells detached from the ore, as shown in Figure 4.24, exhibited a general growth curve typical of batch microbial cultures (Doran, 1995) similarly to Experiment 1. The

highest growth was exhibited in the beginning i.e. an increase from 3.00 x 10^5 cells/ kg ore at the start to 6.86 x 10^9 cells/ kg ore by day 6. After day 6, the growth rate decreased and cell numbers reached 5.43 x 10^{10} cells/ kg ore by day 18. After day 18 the growth was minimal and the cell numbers remained around 5.19 x 10^{10} cells/ kg ore until day 49. The Inoculum 3-inoculated columns had higher cell counts i.e. 3.90 x 10^{10} on day 11 compared to 2.55 x 10^{10} cells/kg ore on day 12 in the Inoculum 2-inoculated columns, and 1.41 x 10^{11} at day 49 compared to 5.19 x 10^{10} on day 49 in Inoculum 2-inoculated columns.

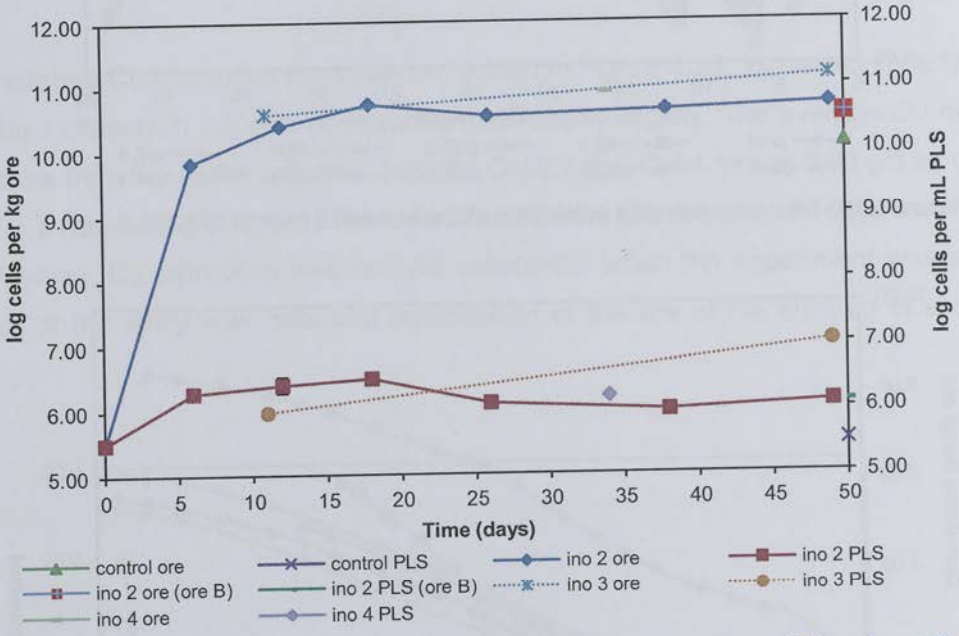


Figure 4. 24: Cell count in the PLS and in the ore at column take-down of Experiment 2

The Control and Col 2.7 (containing ore type B) both analysed on day 50, had lower cell counts i.e. 1.30 x 10^{10} and 3.56 x 10^{10} cells/ kg ore respectively. Col 4.1 (inoculated with 3.00 x 10^6 cells/ kg ore) was analysed on day 34 and found to contain 7.97 x 10^{10} cells/ kg ore. The cell numbers in the PLS, although much lower, had trends similar to the cells in the ore. The cell numbers in the PLS increased from 3.13 x 10^5 cells/mL at the start to 1.88 x 10^6 cells/mL on day 6 then to 3.13 x 10^6 on day 18. After day 18 the cell numbers decreased and finally reached 1.25 x 10^6 cells/mL by day 49. The PLS from columns inoculated with Inoculum 3 had 9.38 x 10^5 and 1.06 x 10^7 cells/mL by day 11 and day 49 respectively. The PLS from the Control and Col 2.7 had 3.13 x 10^5 and 1.25 x 10^6 cells/mL respectively, by day 50 whilst the PLS from Col 4.1 contained 1.56 x 10^6 cells/mL on day 34.

The batch-type growth curve indicated that the cell numbers attached to the ore reached a plateau although, as indicated in Section 4.3.3, the composition of the microbial community attached to the ore changed with time. These results indicated that Inoculum 3 colonised the

ore better than Inoculum 2 and this could have been caused by a difference in adaptation of these inocula; this is further discussed in Section 4.3.5. The Control column was not expected to contain micro-organisms because it was not inoculated. The possible source of micro-organisms in the Control was the ore as has already been discussed in Section 4.2.2.

The detachment protocol (Section 3.5.9) was used to distinguish between the detachment phases of the micro-organisms attached to the ore. As shown in Figure 4.25, in the Inoculum 2-inoculated columns the highest cell numbers were observed to report to the interstitial phase until day 38 after which the highest numbers shifted to the weakly attached phase. In the weakly and strongly attached phases, the domination of numbers oscillated until day 38.

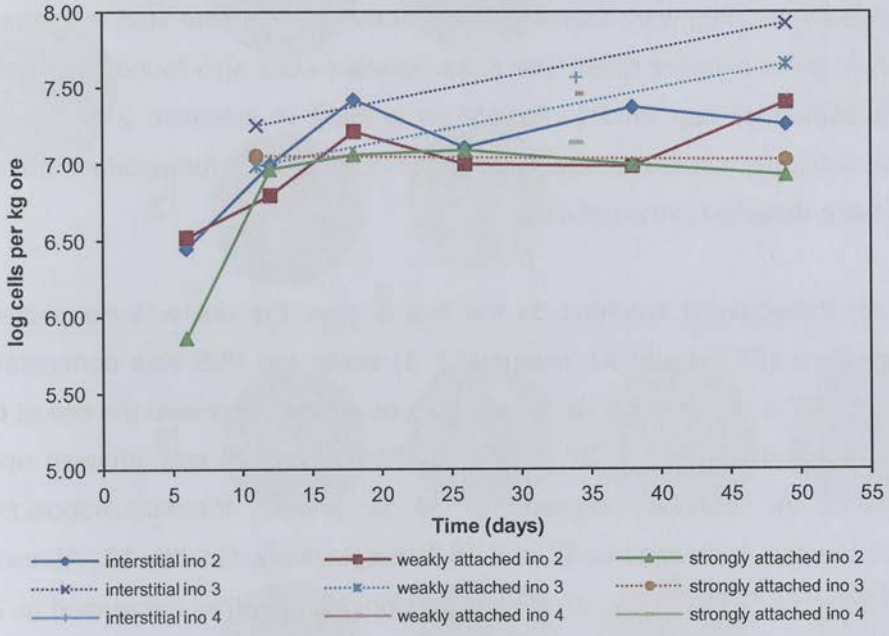


Figure 4. 25: Cell count in the detachment phases of Experiment 2

The shift of the highest numbers from the interstitial to the weakly attached phase could be an indication that the micro-organisms were attaching more to the ore with time. In the Inoculum 3-inoculated columns, initially i.e. at day 11, the highest cell numbers were in the interstitial phase. By day 49, the highest numbers were still in the interstitial phase, and the next highest were in the weakly attached and the least numbers in the strongly attached phase. In the Inoculum 4-inoculated column analysed at day 34 the highest cell numbers were in the interstitial phase, the next highest were in the weakly attached and the least numbers were in the strongly attached phase. Similar trends whereby the highest cell numbers were in the interstitial followed by the weakly attached and finally the strongly attached phase were also observed in other heap bioleaching experiments conducted at CeBER, as already discussed in Section 4.2.2.

4.3.3 qPCR analysis

The percentage composition of the microbial species detected in the inocula, the PLS and the ore using qPCR are in shown in Figure 4.26 and their genomic copy numbers are shown in Table B.8 (Appendix). As shown in Figure 4.26, Inoculum 2 was composed of *Acidithiomicrobium* spp. (74 %), *Metallosphaera* spp. (24 %); *A. cupricumulans* and *At. caldus* both at 1 %; and *Thermoplasmatales* spp. and *L. ferriphilum* both at < 1 %. Inoculum 3 was composed of *Metallosphaera* spp. (92 %), *Acidithiomicrobium* spp. (6 %); *A. cupricumulans* and *At. caldus* both at 1 %; and *Thermoplasmatales* spp. and *L. ferriphilum* both at < 1 %. Inoculum 4 was composed of *At. caldus* (55 %), *Acidithiomicrobium* spp. (44 %), *Metallosphaera* spp. (1 %) and *A. cupricumulans* (< 1 %). All the four major microbial species identified in Inoculum 2, 3 and 4 (*At. caldus, A. cupricumulans, Acidithiomicrobium* and *Metallosphaera* species) were found to colonise the ore from the start to the end of the experiment although at different rates. The same species were also found planktonic in the PLS. *Thermoplasmatales* spp. and *L. ferriphilum* present in Inoculum 2 and 3 were not detected in the columns, instead, *S. thermosulfidooxidans* and *At. ferrooxidans* not detected in the inocula were detected in the columns.

In the Inoculum 2-inoculated columns, in the first 6 days the ore was dominated by *S. thermosulfidooxidans* (58 %) and *At. caldus* (42 %) whilst the PLS was dominated by *At. caldus* at almost 100 %. On day 12, *At. caldus* took over and dominated the ore at 65 %, *S. thermosulfidooxidans* decreased to 31 % and *Acidithiomicrobium* spp. showed up at 4 % whilst in the PLS *At. caldus* decreased to 36 %, and *S. thermosulfidooxidans* and *Acidithiomicrobium* spp. increased to 48 and 16 % respectively. On day 18, *At. caldus* had further increased to 86 % in the ore, *S. thermosulfidooxidans* further decreased to 7 % and *Acidithiomicrobium* spp. increased to 7 %. In the PLS, *At. caldus* increased to 52 % whilst *S. thermosulfidooxidans* and *Acidithiomicrobium* spp. decreased to 40 and 8 % respectively. After this, *Acidithiomicrobium* spp. was not detected until the end. On day 26, *At. caldus* and *S. thermosulfidooxidans* dominated the ore, at 74 and 26 % and the PLS at 21 and 79 % respectively. On day 38, *At. caldus* continued to dominate the ore, at 65 %; *S. thermosulfidooxidans* was at 10 % and *Metallosphaera* spp. which was present at values < 1 % from the start increased to 25 % in the ore. In the PLS, *At. caldus* made up 84 % whilst *S. thermosulfidooxidans* and *Metallosphaera* spp. made up 11 and 5 % respectively. On day 49, *Metallosphaera* spp. took over and dominated the ore at 95 % whilst *S. thermosulfidooxidans* and *At. caldus* were 4 and 1 % respectively. *Metallosphaera* spp. also dominated the PLS at 95 %, and *Sulfobacillus* and *At. caldus* were 8 and 25 % respectively.

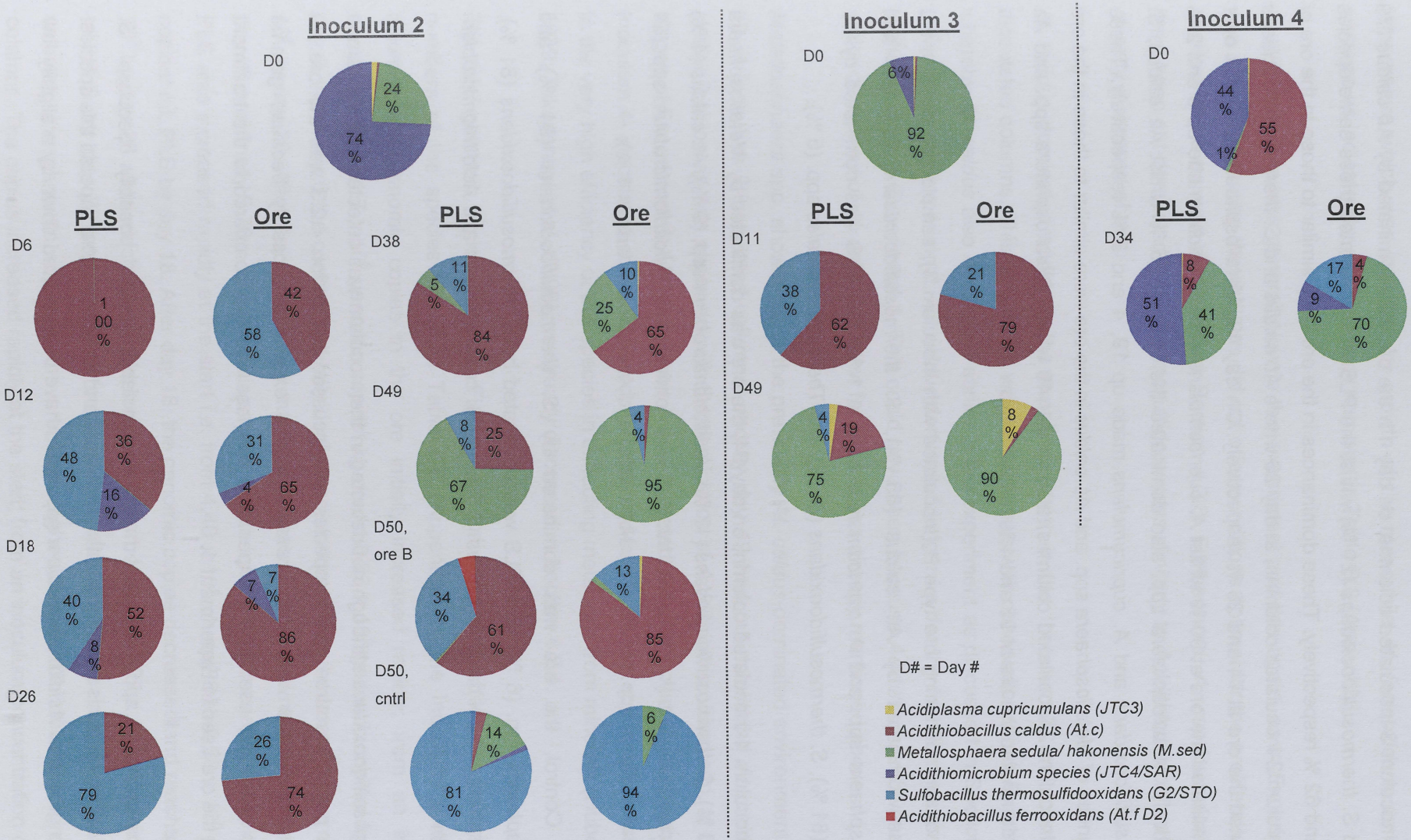


Figure 4. 26: Microbial composition of the inocula, the PLS and the ore in Experiment 2

In the Inoculum 3-inoculated columns, on day 11, the ore was dominated by *At. caldus* (79 %) and *S. thermosulfidooxidans* (21 %) whilst the PLS was dominated these same species at 38 and 62 % respectively. These dominances in the ore were similar to those in the ore of the Inoculum 2-inoculated columns, at day 26 where *At. caldus* and *S. thermosulfidooxidans* dominated the ore at 74 and 26 % respectively. On day 49, *Metallosphaera* spp. took over and dominated the ore at 90 % whilst *A. cupricumulans* and *At. caldus* made up 8 and 2 % respectively. *Metallosphaera* spp. also dominated the PLS, at 75 % whilst *At. caldus, S. thermosulfidooxidans* and *A. cupricumulans* made up 19, 4 and 2 % respectively. These dominances of *Metallosphaera* spp. and *At. caldus* in the ore were similar to those in the ore of the Inoculum 2-inoculated columns, also at day 49 where *Metallosphaera* spp. and *At. caldus* dominated the ore at 95 and 4 % respectively.

In the column containing ore type B (inoculated with Inoculum 3), sampled on day 50, the ore was dominated by *At. caldus* (85 %), *S. thermosulfidooxidans* (13 %); and *Metallosphaera* spp. and *A. cupricumulans* both at 1 %. The PLS was dominated by *At. caldus* (61 %), *S. thermosulfidooxidans* (34 %), and *Thermoplasmatales* spp. (5 %).

In the Inoculum 4-inoculated column, on day 34, the ore was dominated by *Metallosphaera* spp. (70 %), *S. thermosulfidooxidans* (17 %), *Acidithiomicrobium* spp. (9 %), *At. caldus* (4 %) and *A. cupricumulans* (< 1 %) whilst the PLS was dominated by *Acidithiomicrobium* spp. (51 %), *Metallosphaera* spp. (41 %), *At. caldus* (8 %) and *A. cupricumulans* (< 1 %).

In the Control, the ore was dominated by *S. thermosulfidooxidans* (94 %) and *Metallosphaera* spp. (6 %). The PLS was dominated by *S. thermosulfidooxidans* (81 %), *Metallosphaera* (14 %) and *At. caldus* (2 %), and *A. cupricumulans* and *Acidithiomicrobium* spp. both at 1 %.

S. thermosulfidooxidans although not detected in the inoculum was detected in the columns and the possible source of this species as discussed in Section 4.2.3 was the ore. *S. thermosulfidooxidans* was more prevalent in this experiment possibly because gamma radiation of the ore used in this experiment was less effective compared to the treatment done on the ore used in Experiment 1.

Acidithiomicrobium spp. decreased to undetectable levels possibly because *S. thermosulfidooxidans* is more metabolically diverse and therefore out-competed this species. Also, the growth of *Acidithiomicrobium* spp. could have been slowed down by a slightly low

temperature which was indicated by growth of the mesophile *At. ferrooxidans* during this instance as indicated by genomic copy numbers in Table B.8 (Appendix).

A. cupricumulans was generally low in this experiment and this again, as in Experiment 1, was probably because the columns did not offer conditions optimal for the proliferation of *A. cupricumulans* as was discussed in Section 4.2.3. Furthermore, *A. cupricumulans* could have been out-competed by *S. thermosulfidooxidans* similarly to *Acidithiomicrobium* spp. as already discussed above.

At. caldus dominated earlier in the experiment both in the Inoculum 2- and Inoculum 3-inoculated columns. This dominance was in consensus with observations from Experiment 1 whereby *At. caldus* also dominated earlier in the experiment as discussed in Section 4.2.4. However, in this experiment, *At. caldus* dominated longer i.e. for the first 38 days compared to the 25 days in Experiment 1. This could have been because *Acidithiomicrobium* spp. the major competitor of *At. caldus* was low for the reasons already mentioned above.

Metallosphaera spp. showed up in the previously *At. caldus*-dominated environment on day 39 of Inoculum 2-inoculated columns, and by day 49 had out-dominated *At. caldus* in a similar fashion as was observed with the dominance of *Acidithiomicrobium* spp. in the later stages of Experiment 1. *Metallosphaera* spp. dominated in the Inoculum 2-, Inoculum 3- and Inoculum 4- inoculated columns. The dominance of *Metallosphaera* spp. could be attributed to the very high efficiency of this species in extracting metal ions from minerals (Huber *et al.*, 1989; Takayanagi *et al.*, 1996), as already discussed in Section 4.2.4.

The total genomic copies, shown in Figure 4.27, were calculated from the genomic copy numbers of the species shown in Table B.8 (Appendix). In the Inoculum 2-inoculated columns, the genomic copies in the ore initially increased rapidly; from an inoculum concentration of 1.7×10^6 genomic copies/ kg ore to 8.83×10^9 genomic copies/ kg ore by day 6. After day 6 the copies gradually decreased until reaching the lowest value of 2.93×10^8 by day 26. After day 26 the copy numbers increased again and reached 5.84×10^8 by day 38 and finally 5.70×10^{10} genomic copies/ kg ore by day 49. The genomic copies in the PLS also increased rapidly at the start i.e. from 4.40×10^4 on day 6 to 2.39×10^6 genomic copies/ mL PLS by day 18. After day 18, the genomic copies decreased and reached 2.25×10^3 by day 26 after which the copies increased again, to reach 1.27×10^6 genomic copies/ mL PLS by day 38 and remained constant until day 49. In the Inoculum 3-inoculated columns, the genomic copies in the ore were higher than in the Inoculum 2-inoculated columns. The copies increased rapidly at the start; from an inoculum concentration of 8.39 x

10^5 genomic copies/ kg ore to 1.22 x 10^{11} genomic copies/ kg ore by day 11. After day 11 the increase was slower and the numbers reached 2.37 x 10^{11} genomic copies/ kg ore by day 49. In the PLS, the copy numbers decreased from 6.34 x 10^6 on day 11 to 3.44 x 10^5 genomic copies/ mL PLS at day 49. The cause of the decrease of genomic copies in the Inoculum 2-inoculated columns, from day 12 to day 25 is not known. Although these results could not be used to calculate the growth rates, they were useful in confirming the differences in the starting and final concentrations of total microbial communities between Inoculum 2 and Inoculum 3. These results supported the observations made from the cell counts, shown in Section 4.3.2, that Inoculum 3 colonised the ore more efficiently than Inoculum 2.

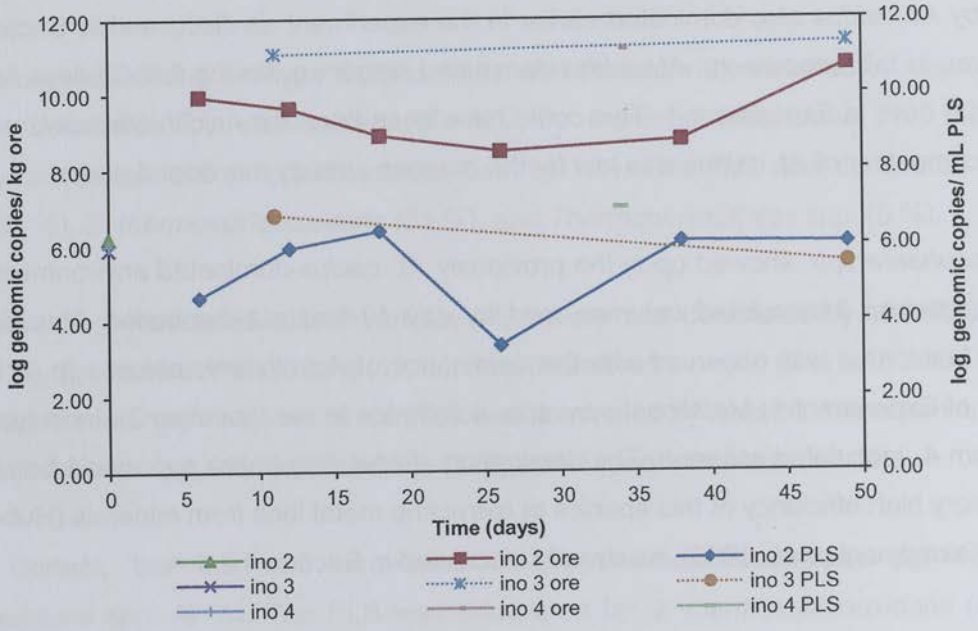


Figure 4. 27: Total genomic copies in Inoculum 3 and 4, the PLS and the ore in Experiment 2

4.3.4 The activity of the microbial community

An activity test was set-up as detailed in Section 3.6.4. In this experiment, samples were collected from Inoculum 3 and 4 at the start of the experiment, and from cells detached on day 6, 18, 49 and 50. Fe^{2+} oxidation rates for the inocula were measured over the first 4 days whilst for the detached cells it was measured over the entire 14 days of the activity test because the inocula depleted Fe^{2+} faster than the detached cells as shown in Figure 4.28. The biological Fe^{2+} oxidation rates of the different samples are shown in Figure 4.29. The starting Fe^{2+} concentration in the samples from day 49 and 50 was higher because of the higher leaching of Fe from the ore on these days compared to earlier days (day 6 and 18).

The activity test, as shown in Figure 4.29, showed that Inoculum 2 had the highest iron oxidation rate i.e. 0.644 g Fe^{2+}/L/day followed by Inoculum 3 at 0.389 g/L/day. The detached cells had lower oxidation rates. However, the oxidation rate was shown to increase with time i.e. 0.020, 0.076 and 0.188 g/L/day by day 6, 18 and 49 respectively for the Inoculum 2-inoculated columns containing ore type A. Inoculum 3-inoculated columns had a slightly higher oxidation rate i.e. 0.191 g/L/day on day 49 whilst the column containing ore type B (inoculated with Inoculum 2) had an oxidation rate of 0.161 g/L/day on day 50.

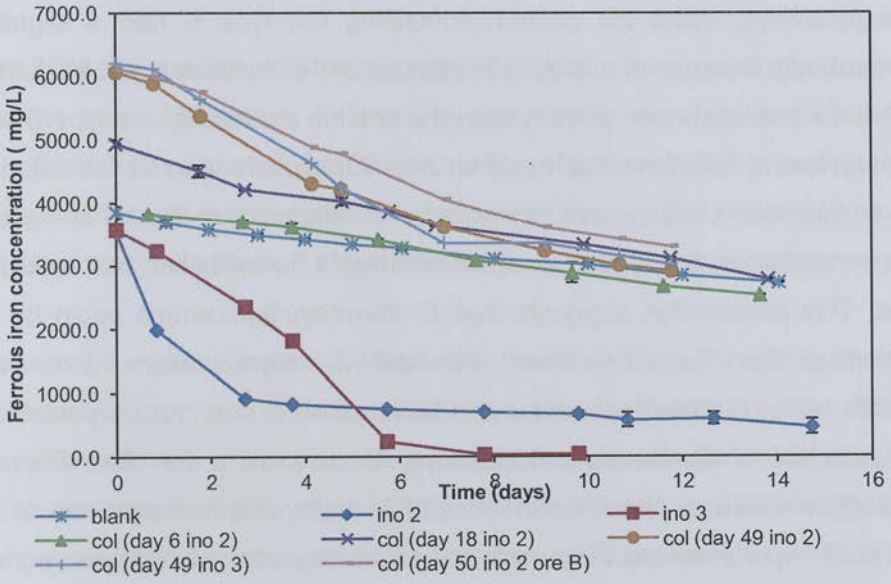


Figure 4. 28: Fe^{2+} depletion by different samples from Experiment 2

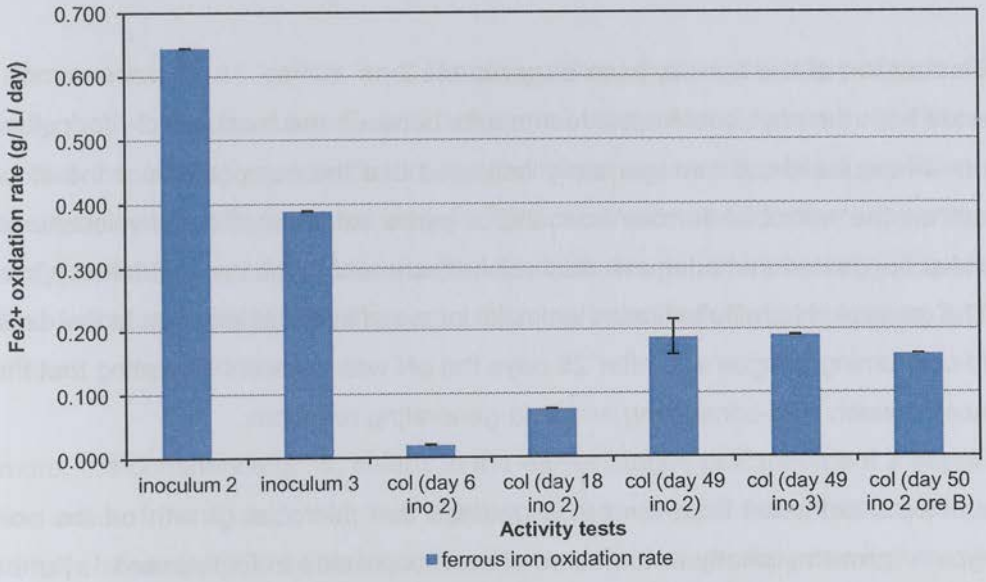


Figure 4. 29: Ferrous iron oxidation rates by the inocula and detached cells in Experiment 2

The increase in the Fe^{2+} oxidation rate by the detached samples could be indicative of either an increase in the composition of Fe-oxidisers or an increase in the specific activity of these Fe-oxidisers in the microbial community colonising the ore, with time. Inoculum 2 (composed of 74 % *Acidithiomicrobium* spp. and 24 % *Metallosphaera* spp.) performed better than Inoculum 3 (6 % *Acidithiomicrobium* spp. and 92 % *Metallosphaera* spp.) implying that *Acidithiomicrobium* spp. is a better Fe-oxidiser than *Metallosphaera* spp. Cells from the Inoculum 2- and Inoculum 3-inoculated columns had similar Fe^{2+} oxidation rates by the end of the experiment probably because they had a similar composition of Fe-oxidisers (i.e. 99 % and 98 % respectively), whilst the column containing ore type B had a slightly lower oxidation rate probably because of a lower composition of Fe-oxidisers (i.e. 15 %). However, considering that the oxidation rate of cells from the column containing ore type B was only slightly lower than that of cells from the Inoculum 2- and Inoculum 3-inoculated columns, and comparing their microbial compositions, it could be concluded that the 15 % Fe-oxidisers from the column containing ore type B were more efficient Fe-oxidisers than cells from the other columns. This observation suggests that *S. thermosulfidooxidans* could be a more efficient Fe-oxidiser than *Metallosphaera* spp. and *A. cupricumulans* under the test conditions of this study. Further test work would be required to confirm this postulation and evaluate the potential of *S. thermosulfidooxidans* for use as a Fe- and S-oxidiser in bioleaching. *Sulfobacillus* spp. have been thought to have only a minor role in mineral oxidation, and their importance has been assigned to the degradation of organic compounds which when accumulated would be toxic to other chemolithoautotrophs (Dopson *et al.*, 2003; Schippers, 2007).

4.3.5 Discussion of the results from Experiment 2

The trends from the physicochemical test results between the Inoculum 2-, Inoculum 3- and Inoculum 4-inoculated columns generally indicated that the composition of the inocula had no effect on the microbial ferrous iron and/or pyrite oxidation. The physicochemical test results also suggested that adequate microbial attachment to the ore had taken place after 6 days. The general pH profile indicated an initial increase in the acidity due to the depletion of the acid-consuming gangue and after 25 days the pH was constant indicating that there was a balance between acid-consuming and acid-generating reactions.

Counts of cells detached from the ore suggested that microbial growth on the ore was a batch-type of growth similarly to the same observation made in Experiment 1. Furthermore, the cell counts indicated that Inoculum 3 colonised the ore better than Inoculum 2. Again, similarly to Experiment 1, the Control column was contaminated and the contaminant micro-organisms were likely to have been indigenous to the ore.

Inoculum 3 performed better probably because it was more adapted to chalcopyrite and pyrite than Inoculum 2. Inoculum 3 was withdrawn from a stock culture that had been continuously running for at least 6 months at CeBER. On the other hand, Inoculum 2 was obtained fresh from BHP Billiton and lied dormant for 10 days only to be re-activated immediately before inoculating the columns. An inference can be made from these observations that in inocula containing similar microbial species, adaptation is more important than the microbial composition.

Again, similarly to Experiment 1, the highest cell numbers were generally observed to initially report to the interstitial phase, followed by the weakly attached and finally the strongly attached phase. Later i.e. after day 38, the numbers increased in the weakly attached phase suggesting that the micro-organisms were attaching more to the ore over time and/or that in the EPS there was multiplication of microbial cells already attached to the ore.

All the four major microbial species detected in the inocula i.e. *At. caldus, A. cupricumulans, Acidithiomicrobium* spp. and *Metallosphaera* spp., attached to the ore. The attachment of the micro-organisms to the ore was quick probably because the microbial species had been adapted to chalcopyrite and pyrite both of which were used as their energy sources in the stock culture, as already discussed in the summary of Experiment 1 results in Section 4.2.6. The total genomic copies similarly to the cell counts indicated that Inoculum 3 colonised the ore more efficiently than Inoculum 2.

Similar dominances of *At. caldus* and *Metallosphaera* spp. at the start and at the end respectively, both in the Inoculum 2- and Inoculum 3-inoculated columns in this experiment indicated that the starting composition of the inoculum was not important provided similar microbial species were present in the inocula. This observation was further supported by the similar early dominances of *At. caldus* observed in both this experiment, and in Experiment 1 which used Inoculum 1 containing the same microbial species found in Inoculum 2, 3 and 4 but at different compositions.

Furthermore, the dominance of *At. caldus* in the earlier stages of Experiment 2 supports the postulation made from Experiment 1 in Section 4.2.3 and 4.2.6 that in mixed cultures, S-oxidisers have a competitive advantage over Fe- and S-oxidisers in an environment where both Fe^{2+} and reduced sulphur substrates are available. This postulation was further supported by the fact that *At. caldus* also dominated in the column containing ore type B. Unlike in the columns that contained ore type A where *At. caldus* dominated in the early

stages, in the column containing ore type B, *At. caldus* was found still dominating at day 50. Unfortunately this was the only column containing ore type B out of all the columns run in this study therefore the microbial dynamics in ore type B could not be assayed earlier in the experiment. However, ore type B had a higher amount of the easily-leachable component than ore type A resulting in a higher Cu concentration at the beginning of the experiment, as shown and discussed in Section 4.3.1. The slightly initial higher Cu in this column possibly shocked the micro-organisms and generally slowed the microbial growth including the growth of *At. caldus* hence this species started colonising the ore late in the experiment.

Similar dominances of *At. caldus* in the ore on day 11 of the Inoculum 3-inoculated columns and on day 26 of the Inoculum 2-inoculated columns could be an indicator that Inoculum 3 performed better than Inoculum 2 i.e. *At. caldus* in Inoculum 3 colonised the ore faster than *At. caldus* in Inoculum 2. This was also supported by the total genomic copies of all the microbial species of interest which were higher in the Inoculum 3-inoculated columns than in the Inoculum 2-inoculated columns throughout the experiment as depicted in Figure 4.27.

The activity test, similarly to the activity test in Experiment 1, showed that the inocula had the highest Fe^{2+} oxidation rates. The detached cells had lower oxidation rates but the oxidation rate increased with time either due to an increase in the composition of Fe-oxidisers and/or due to increase of Fe-oxidisers already attached to the ore in capability to oxidise Fe^{2+}.

4.4 The effect of copper concentration on the growth of moderate thermophiles on whole ore

Experiment 3 was run to investigate the effect of irrigation copper concentration on the growth and dominance of microbial species ore in a heap leach environment. This experiment was run for 27 days and its set-up is described in Section 3.3. All the columns contained ore type A and were inoculated with 3 x 10^8 cells/ton ore of Inoculum 5 including the Control column. The composition of Inoculum 5, shown in Figure 4.30, was determined using qPCR analysis. Inoculum 5 was dominated by *Acidithiomicrobium* spp. (66 %) followed by *Metallosphaera* spp. (19 %), *A. cupricumulans* (8 %) *At. caldus* (6 %) and *Thermoplasmatales* spp. (1 %). Three columns (Col 5.2, 5.3 and 5.4) were irrigated with a solution containing 5.0 g/L Cu, another three (Col 5.5, 5.6 and 5.7) with 10.0 g/L and the other three (Col 5.8, 5.9 and 5.10) with 15.0 g/L Cu. The Control column (Col 5.1) was irrigated with a solution containing no copper and was sacrificed on day 27. One column from each of the Cu concentration regimes was sacrificed on day 7, 15 and 27.

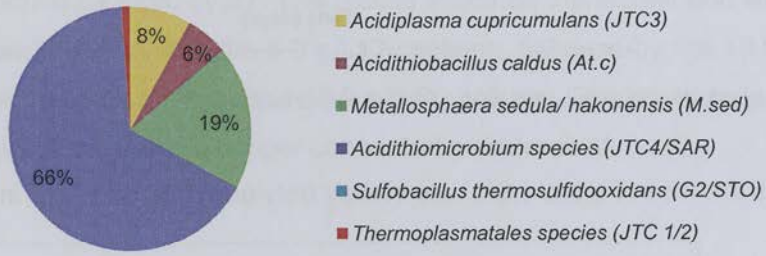


Figure 4. 30: The composition of Inoculum 5 used in Experiment 3

4.4.1 Physicochemical parameters

The Eh, pH, Fe^{2+}, Fe^{tot}, soluble Cu and Fe metal in the PLS from the columns, shown in Figures 4.31 to 4.35, were measured every second day. The physicochemical results from the three columns in each concentration regime were comparable therefore the results presented here are only for the measurements from the columns sacrificed on day 27.

The Eh, shown in Figure 4.31, started at an average of 575 mV, lagged for 4 days and thereafter started to increase in all the columns. The Eh increased the fastest and reached the highest in the Control i.e. reached 770 mV by day 15 and thereafter slowly increased until reaching 797 mV on day 27. In the copper-fed columns, the rate of Eh increase decreased with increasing Cu concentration. The Eh of the 5.0, 10.0 and 15.0 g/L Cu-columns reached 707, 651 and 613 mV by day 15, respectively. After day 15, the Eh increase slowed down in the 5.0 g/L column and reached 749 mV by day 27 whilst in the 10.0 g/L column the Eh continued to rise linearly and reached 719 mV by day 27. In the 15.0

g/L column the Eh continued to rise linearly and reached 657 mV by day 24 after which the Eh increased faster and finally reached 695 mV by day 27. The decreased rate of Eh increase with increasing Cu could be indicative of decreasing microbial activity as Cu concentration increased.

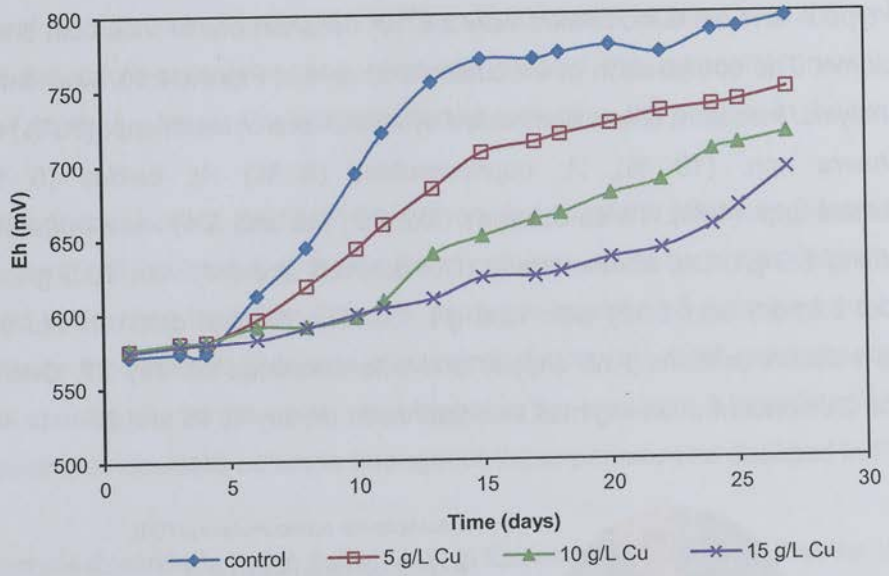


Figure 4. 31: Eh measured in the PLS of Experiment 3

The pH, as shown in Figure 4.32, was initially between 1.88 and 2.12 in all the columns.

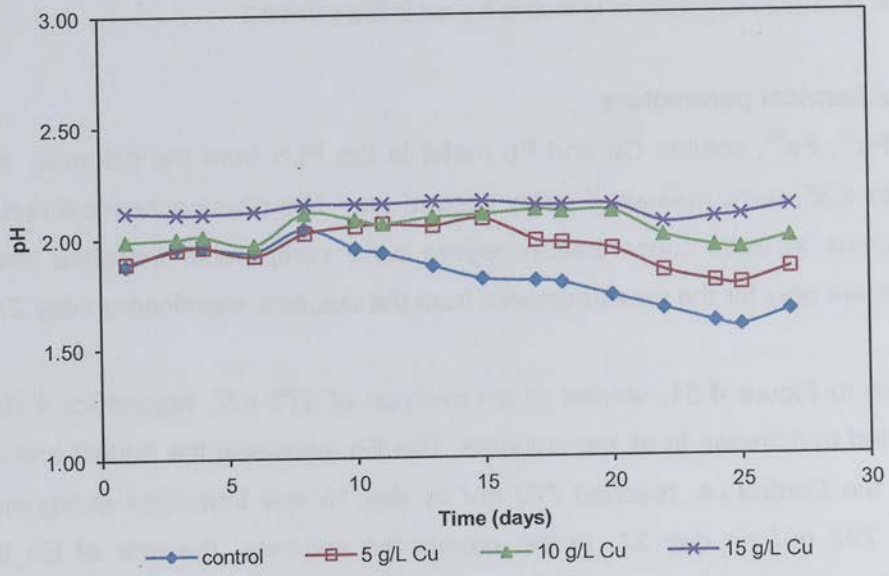


Figure 4. 32: pH measured in the PLS of Experiment 3

The pH decreased the fastest and reached the lowest value in the Control i.e. started at pH 1.88 and steadily decreased to pH 1.66 by day 27. The pH in the Cu-fed columns fluctuated during the 27 days of operation, ending at similar values after 27 days.

The Fe^{2+} concentration in the PLS, shown in Figure 4.33, included the amounts of Fe^{2+} continuously added with the feed i.e. 500 mg/L of Fe^{2+}. In the first 3 days the Fe^{2+} increased in all the columns and thereafter started to decrease. The Control and the 5.0 g/L column had similar Fe^{2+} profiles i.e. Fe^{2+} started at 455 and 565 mg/L and reached 1080 and 995 mg/L by day 3 respectively. After this, the Fe^{2+} in both these columns decreased and reached about 6.5 mg/L by day 17 after which the Fe^{2+} concentration stayed close to 0 mg/L until day 27. The Fe^{2+} concentration in the 10.0 and 15.0 g/L Cu columns was higher than in the Control after day 6 until the end of the experiment. In the 10.0 g/L column, the Fe^{2+} started at 670 mg/L and reached 865 mg/L by day 3. After day 3 the Fe^{2+} decreased and reached 6.5 mg/L by day 24 and thereafter stayed close to 0 until day 27. In the 15.0 g/L column, the Fe^{2+} started at 580 mg/L and reached 795 mg/L by day 3 and thereafter decreased and reached 84 mg/L by 27. The results indicated the fastest and similar rates of Fe^{2+}oxidation in the Control and in the 5.0 g/L Cu column, followed by the 10.0 g/L column and the slowest Fe^{2+} oxidation rate in the 15.0 g/L Cu column. This could be an indicator of decreasing microbial activity as the copper concentration increased.

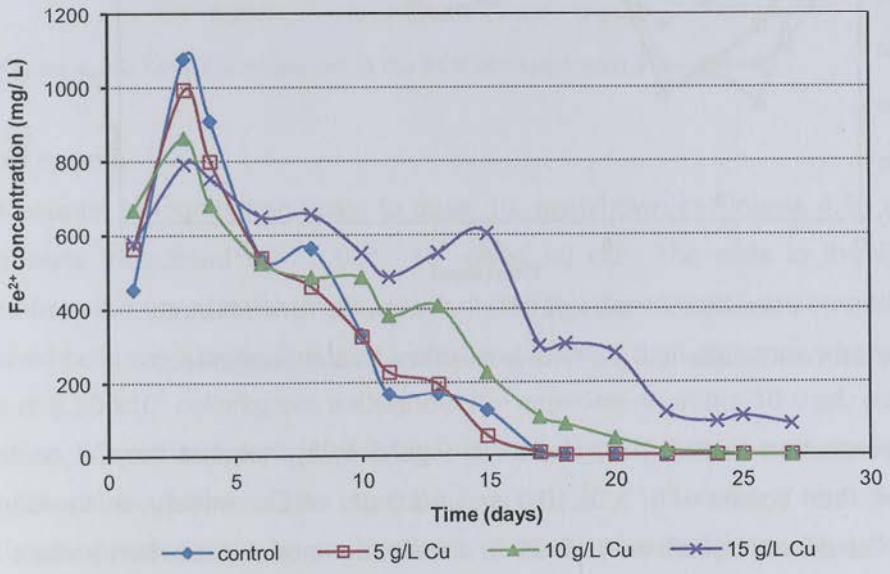


Figure 4. 33: Ferrous iron (Fe^{2+}) measured in the PLS of Experiment 3

The Fe^{tot} concentration in the PLS, shown in Figure 4.34, also included the Fe^{2+} continuously added with the feed i.e. 500 mg/L of Fe^{2+}. The Fe^{tot} started at an average of 655 mg/L in all the columns and decreased to reach 350, 274, 225 and 238 mg/L by day 8 in

the Control, 5.0 g/L-, 10.0 g/L- and 15.0 g/L-column respectively. After day 8, the Fe^{tot} rapidly increased and reached 499, 442 and 463 mg/L by day 10 and thereafter remained constant until day 25 in the 5.0 g/L-, 10.0 g/L- and 15.0 g/L- column respectively. In the Control, Fe^{tot} rapidly increased after day 8 and reached 696 mg/L by day 15 and thereafter remained constant until day 25. After day 8, the Fe^{tot} in the Cu fed-columns remained at values close to the feed concentration. This could be an indicator that in these columns the high Cu inhibited microbial pyrite oxidation or leaching of Fe. The increase of Fe^{tot} on day 15 in the Control coincided with the end of the exponential Eh increase in this column. The high Fe^{tot} was probably a result of increased microbial rate of pyrite oxidation owing to increased microbial colonisation of the ore. The initial decrease of Fe^{tot} in all the columns could have been a result of a hold-up of Fe inside the columns due to precipitation (Tupikina *et al.*, 2011). In an investigation of the effect of pH and acid stress in heap bioleaching, Tupikina *et al* (2011) observed that at a feed pH of 1.7 or higher, Fe precipitation occurred in the columns causing a decrease of Fe^{tot} in the PLS.

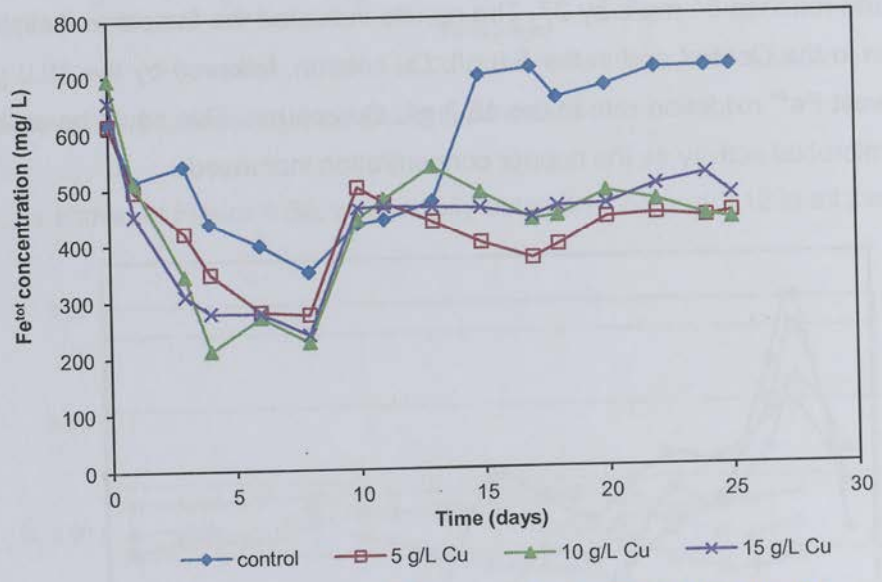


Figure 4. 34: Total soluble iron (Fe^{tot}) measured in the PLS of Experiment 3

The Cu concentration in the PLS, shown in Figure 4.35, included the Cu continuously added with the feed solution i.e. 5.0, 10.0 and 15.0 g/L of Cu. Initially, in the copper-fed columns the Cu concentration was close to the feed amount but after 6 days the Cu concentration decreased. The Cu concentration from the 15.0, 10.0 and 5.0 g/L column decreased to 8.9, 6.8 and 3.4 g/L by day 8 and thereafter remained constant until the end of the experiment. The Control (as indicated on the secondary vertical axis in Figure 4.35) had the lowest Cu concentration, starting at 0.17 g/L and quickly decreasing to reach 0.07 and 0.06 g/ L by day 1 and 8 respectively. After day 8 the Cu in the Control increased and

reached 0.12 g/L by day 17 and thereafter gradually decreased finally reaching 0.10 g/L by day 25. The decrease of Cu in the Cu-fed columns could have been due to precipitation and hold-up of Cu in the columns in areas not accessible to the flowing liquid. At column take-down it was noted that blue crystals, likely to be copper sulphate or some cupric salt, had been formed and held up in the columns, however these crystals were not characterised or quantified. In the Control, the initial decrease could have been due to the depletion of the easily-leachable component of the ore, and the increase after day 8 could have been the result of increasing microbial activity.

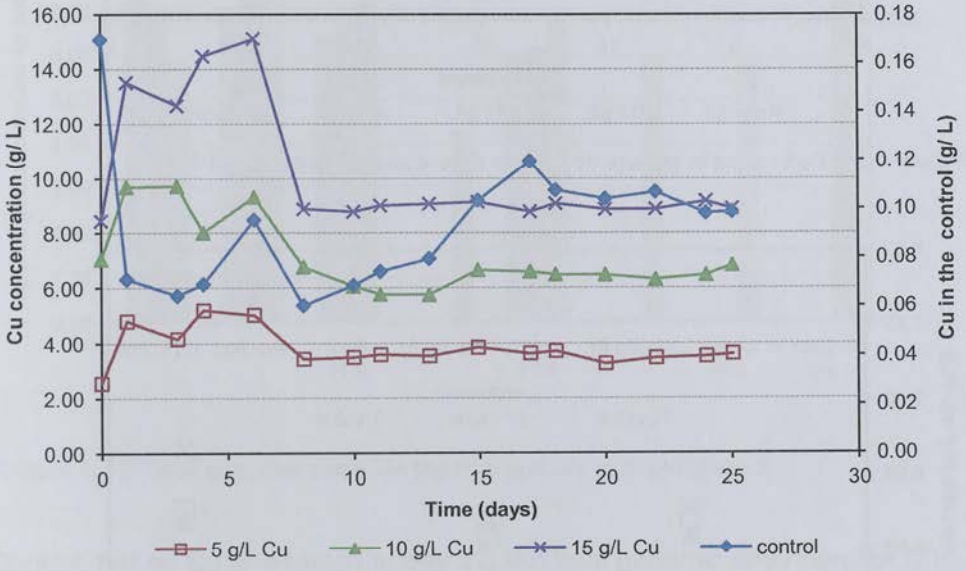


Figure 4. 35: Total Cu measured in the PLS of Experiment 3

4.4.2 Cell counts

The cell counts, in logarithmic scale to base 10, are shown in Figures 4.36 and 4.37. The columns were inoculated with 3.00 x 10^5 cells/ kg ore. The cells in the PLS and cells detached from the ore at column take-down during the experiment were counted. Cell counts of detached cells were highest in the Control and in the 5.0 g/L columns where there was an average of 8.30 x10^9 cells/kg ore throughout the experiment. In the 10.0 g/L columns the cell counts were low; at an average of 3.00 x 10^6 throughout the experiment. In the 15.0 g/L columns the cell counts were also at an average of 3.90 x 10^6 on day 7 and 15 but by day 27 the cell counts had drastically decreased to 6.40 x 10^4 cells/kg ore. The similarity of cell numbers between the 5.0 g/L column and the Control at the end of the experiment could be an indicator that microbial growth was not adversely affected by the 5 g/L Cu concentration. The low cell numbers in the 10 and 15 g/L was an indicator that the concentration of at least 10 g/L Cu was detrimental to microbial growth.

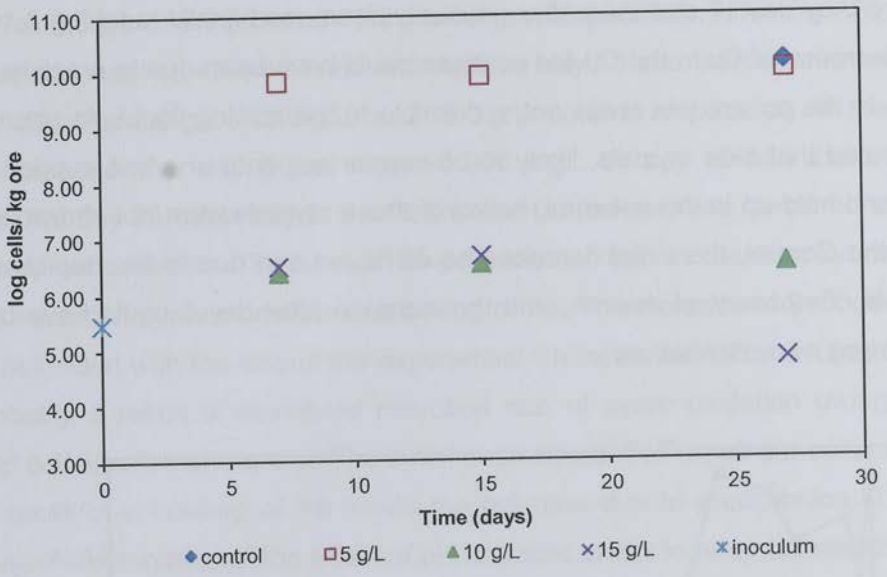


Figure 4. 36: Cell count in the ore at column take-down of Experiment 3

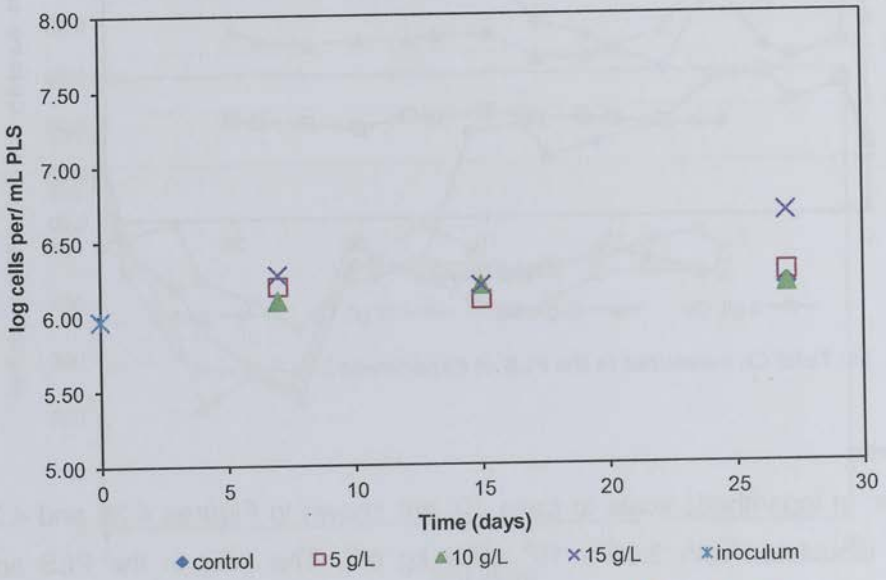


Figure 4. 37: Cell count in the PLS at column take-down of Experiment 3

In the PLS, the cell numbers were similar in the Control, the 5 g/L and the 10 g/L Cu-columns where there were an average of 1.50×10^6 cells/mL throughout the experiment. In the 15 g/L Cu-columns, the cell numbers were 1.70×10^6 on day 7 and 15 but increased to 4.70×10^6 cells/mL by the end of the experiment. The increase of cells in the PLS of the 15 g/L Cu-columns coincided with the decrease of cell numbers in the ore indicating that micro-organisms failed to attach to the ore at higher Cu concentration and were washed down with the flowing liquid.

4.4.3 qPCR analysis

A column was analysed from each Cu concentration on day 7, 15 and 27. The microbial composition was assayed using qPCR. Total genomic copy numbers of the species of interest in the PLS and the ore are shown in Figure 4.38. The percentage composition and the genomic copies of individual species are shown in Figures 4.39 and 4.40 respectively.

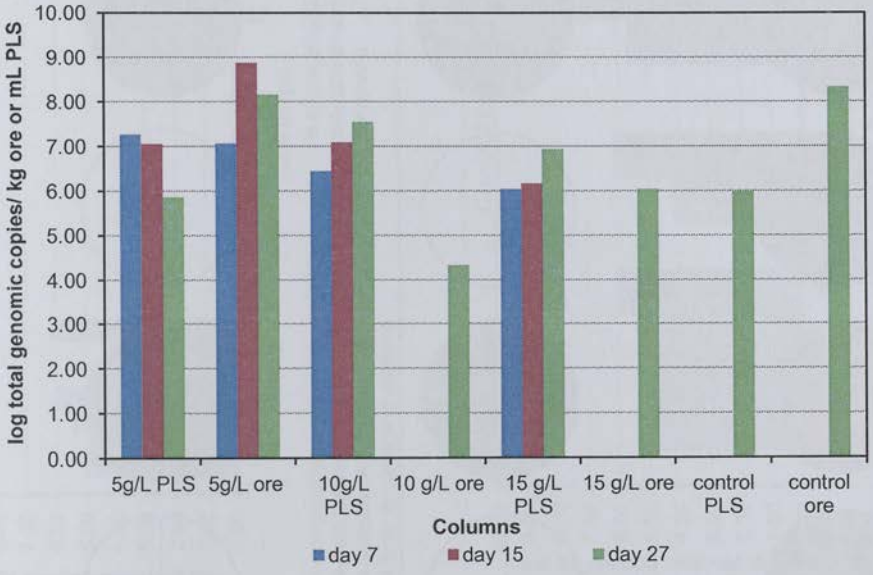


Figure 4. 38: Total genomic copies in the PLS and ore in Experiment 3

In the Control, fed no Cu and tested at day 27, the total genomic copy number (Figure 4.38) in the PLS was 9.71 x 10^5 copies/mL PLS and on the ore was 2.14 x 10^8 copies/kg ore. In the 5 g/L Cu columns, the total copy number in the PLS decreased i.e. 1.86 x 10^7, 1.13 x 10^7 and 7.28 x 10^5 copies/mL on day 7, 15 and 27 respectively. In the ore, the copy number increased from 1.17 x 10^7 on day 7 to 7.63 x 10^8 copies/kg ore on day 15 and thereafter decreased to 1.46 x 10^8 copies/kg ore on day 27. In 10 g/L, the copies in the PLS increased i.e. 2.78 x 10^6, 1.23 x 10^7 and 3.58 x 10^7 copies/mL on day 7, 15 and 27 respectively. On the ore, no gDNA was detected on day 7 and 15. The copy number on day 27 was 2.20 x 10^4 copies/kg ore. In 15 g/L, the total copy number in the PLS increased i.e. 1.11 x 10^6, 1.47 x 10^6 and 8.74 x 10^6 copies/mL on day 7, 15 and 27 respectively. In the ore, again no gDNA was detected on day 7 and 15, and the copy number on day 27 was 1.11 x 10^6 copies/kg ore. The similar copy numbers between 5 g/L Cu and the Control on day 27 could indicate that microbial growth was not adversely affected by 5 g/L Cu whilst the low copy numbers in 10 and 15 g/L indicated that at least 10 g/L Cu was detrimental to microbial growth. The increase of copies in the PLS in 10 and 15 g/L could be an indicator that micro-organisms failed to attach to the ore and were deported into the PLS in high Cu concentration.

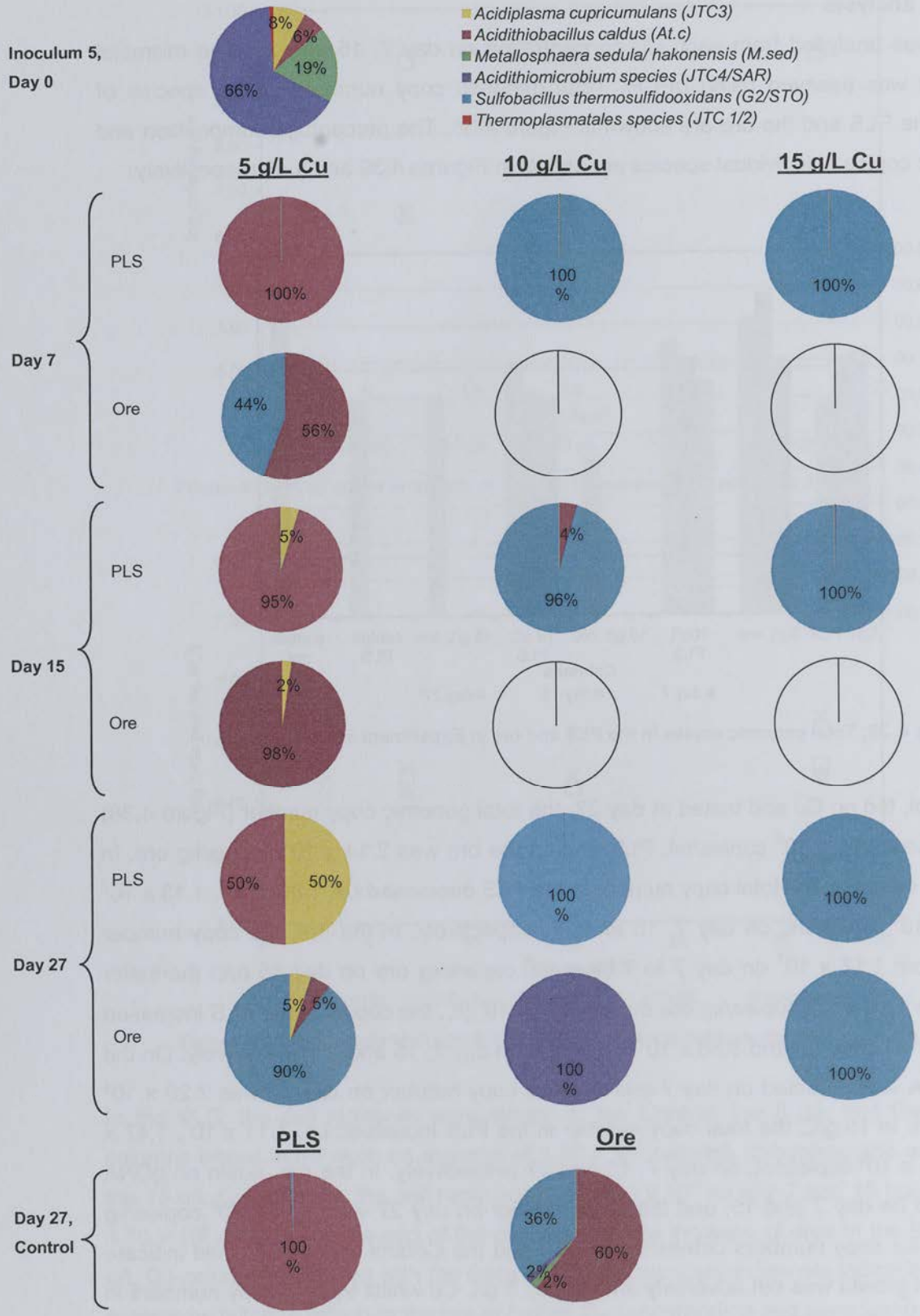


Figure 4. 39: Microbial composition in the PLS and ore at column take-down of Experiment 3

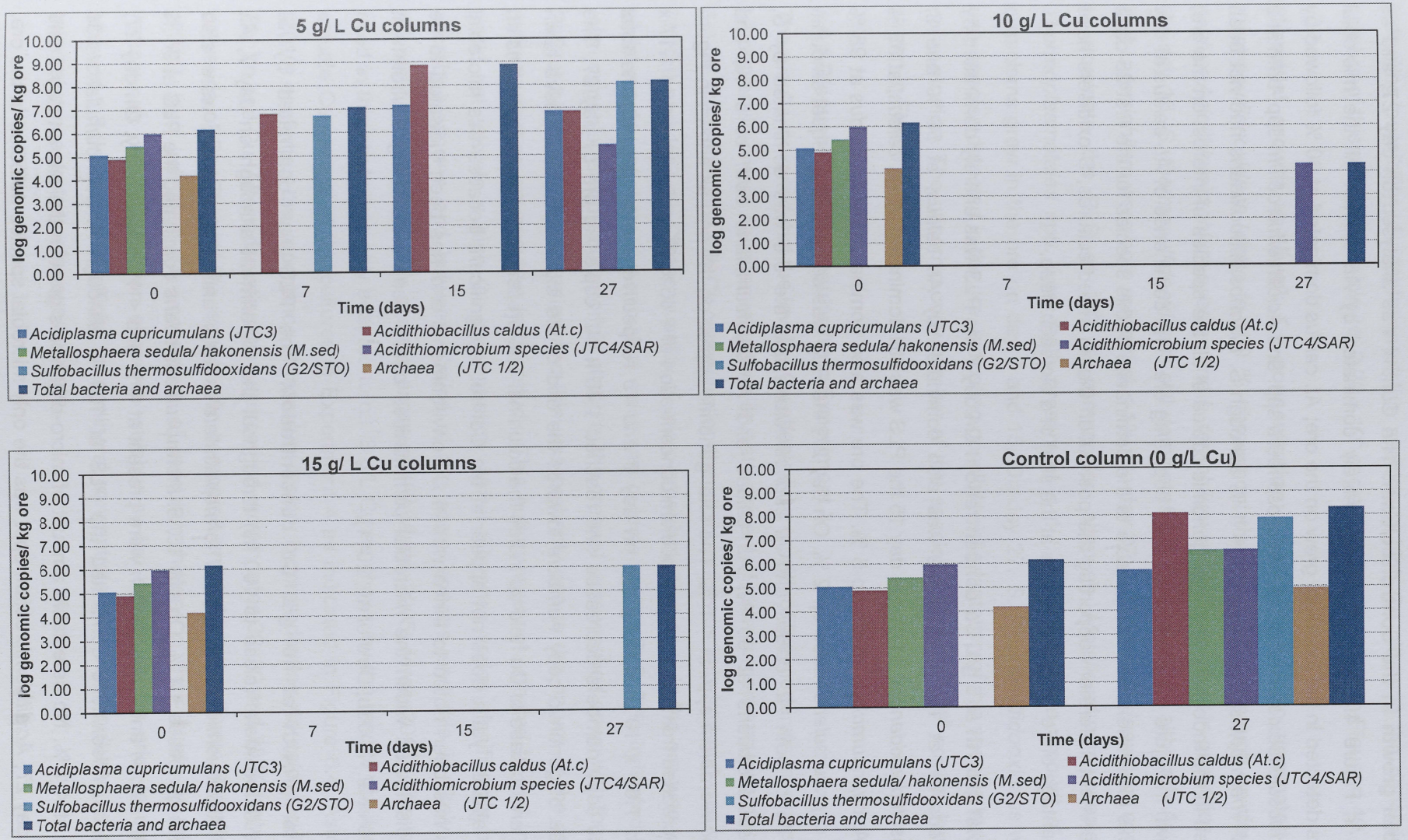


Figure 4. 40: The genomic copy numbers of individual microbial species in the ore at column take-down of Experiment 3

Microbial growth in the control column: The Control was analysed on day 27, and as shown in Figure 4.39, the PLS was entirely dominated by *At. caldus*. All the microbial species detected in the inoculum grew on the ore; *At. caldus* dominated at 60 % followed by *S. thermosulfidooxidans* at 36 %, *Metallosphaera* spp. and *Acidithiomicrobium* spp. both at 2 %, and *Thermoplasmatales* spp. at < 1 % although *S. thermosulfidooxidans* had not been detected in the inoculum. The total genomic copies of all the species of interest on the ore, as shown in Figure 4.40, was 2.14 x 10^8 copies/kg ore. The dominance of *At. caldus* at day 27 in this experiment was comparable to the dominance of this species for the first 25 days in Experiment 1, further supporting the postulation that in mixed cultures, S-oxidisers have an advantage of Fe- and S-oxidisers at the start of a bioleach heap.

Microbial growth at 5 g/L Cu concentration: On day 7 the PLS was entirely dominated by *At. caldus* i.e. at 100 %. The ore was also dominated by *At. caldus,* at 56 % and *S. thermosulfidooxidans* at 44 %. On day 15 the PLS was still dominated by *At. caldus,* at 95 % whilst *A. cupricumulans* made up 5 %. The ore was also dominated by *At. caldus* at 98 % and *A. cupricumulans* made up 2 %. On day 27, the PLS was composed of equal amounts i.e. 50 % of both *At. caldus* and *A. cupricumulans* and the ore was dominated by *S. thermosulfidooxidans* at 90 %, *At. caldus* and *A. cupricumulans* both at 5 %, and *Acidithiomicrobium* spp. at < 1 %. The total genomic copies on the ore, as shown in Figure 4.40, increased from the starting inoculum concentration of 1.45 x10^6 copies/kg ore to 1.17 x 10^7 and 7.63 x 10^8 copies/kg ore on day 7 and 15 respectively. After day 15, the total genomic copy numbers decreased and reached 1.46 x 10^8 copies/kg ore by day 27. The decrease of genomic copy numbers towards the end of the experiment could have been caused an increase of Cu held up inside the columns. The presence of *A. cupricumulans* on day 15 and 27 yet it does not appear in the Control is significant because this microbial species has been noted to thrive well in Cu environments and has been indicated to be widely distributed within the microbial communities of the heap bioleaching environment (Hawkes *et al.*, 2006b; Dew *et al.*, 2011).

Microbial growth at 10 g/L Cu concentration: The PLS was dominated by *S. thermosulfidooxidans* (close to 100 %) throughout the experiment although on day 7, *At. caldus, Acidithiomicrobium* spp., *Thermoplasmatales* spp. and *L. ferriphilum* were also present in quantities < 1 %. On day 15, *S. thermosulfidooxidans* dominated the PLS at 96 % whilst *At. caldus* and *L. ferriphilum* were present at 4 % and < 1% respectively. On day 27, *S. thermosulfidooxidans* dominated the PLS at 100 % although *A. cupricumulans* was present at < 1 %. No detectable amount of micro-organisms grew in the ore on day 7 and 15. On day 27, *Acidithiomicrobium* spp. was the only microbial species detected in the ore,

indicating possibility of adaptation of this species to high Cu although the genomic copy number of this species was low i.e. 2.20 x 10^4 copies/ kg ore as shown in Figure 4.40.

Microbial growth at 15 g/L Cu concentration: The PLS was also dominated by *S. thermosulfidooxidans* (close to 100 %) throughout the experiment although on day 7 *At. caldus*, *Acidithiomicrobium* species and *L. ferriphilum* were also present in quantities < 1 %. On day 15, *S. thermosulfidooxidans* still dominated the PLS at 100 % although *At. caldus* and *L. ferriphilum* were both present at < 1%. On day 27, *S. thermosulfidooxidans* continued to dominate the PLS at 100 % composition although *Metallosphaera* species and *Thermoplasmatales* spp. were both present at < 1 %. No detectable amount of micro-organisms grew in the ore on day 7 and 15. On day 27, *S. thermosulfidooxidans* was the only species detected in the ore, indicating possibility of adaptation of this species to high Cu concentration. The genomic copy number of this species was 1.11 x 10^6 copies/ kg ore as shown in Figure 4.40.

4.4.4 Discussion of the results from Experiment 3

The results indicated that the level of Cu affected the growth of the moderate thermophiles in a heap environment. Substantial growth and attachment of micro-organisms took place up to 5.0 g/L Cu but beyond this Cu concentration the attachment was impaired and was minimal. At 10.0 and 15.0 g/L Cu the quantity of micro-organisms that grew in the ore was very low whilst a substantial amount microbial mass was detected in the PLS. This indicated that the micro-organisms could not strongly attach to the ore and were washed down with the flowing PLS at higher Cu concentration and this was probably due to the nature of the microbial culture used as an inoculum in this study. This culture was not adapted to and therefore could not tolerate a high Cu concentration. Metal tolerance varies significantly between microbial species and between strains of the same species and this is likely due to adaptation to different levels of metal exposure (Rawlings, 2005; Jerez, 2011). The inoculum used in this study was only adapted to ca. 3 g/L Cu as shown by the results obtained from soluble Cu assays of the cultures in Experiment 4 as indicated in Figure 6.4. Aston *et al* (2010) confirmed the importance of inoculum history by pre-adapting *At. caldus* cultures in Pb, Zn and Cu. After adaptation the cultures had growth rates 39, 32 and 28 % higher in the presence of Pb, Zn, and Cu respectively, compared to unadapted cultures. Bromfield *et al* (2011) also indicated the importance of inoculum history in their study in which *M. hakonensis* cells cultured on elemental sulphur as the energy source prior to contacting showed 1.3 times greater affinity for a mineral concentrate than those cultured on sulphide mineral concentrates or ferrous sulphate.

By day 27 of the experiment the copy numbers of *Acidithiomicrobium* spp. on the ore in 5.0, 10.0 and 15.0 g/L Cu were 7.2 %, 0.6 % and 0 % of the genomic copy numbers in the Control, respectively. The observations from this experiment generally agree with observations made in Experiment 5 (Section 6.3) which tested the effect of Cu concentration on microbial growth in liquid culture using an inoculum with similar microbial species as the inoculum used in this experiment. The results from Experiment 5 also showed that Cu concentration up to 5 g/L (i.e. 2.5 and 5 g/L) had a lower negative effect on overall cell counts and overall capacity of iron oxidation of the culture compared to 7.5 g/L Cu, although the culture at 7.5 g/L later recovered over time probably due to adaptation.

S. thermosulfidooxidans was prevalent in this experiment; being detected in all the columns. This microbial species was not detected in the inoculum and could have either been present in the inoculum at low undetectable quantities or could have been inherent in the ore. This microbial species was detected in the columns of Experiment 1 and 2 although not originally detected in the inocula used and, as has already been discussed in Section 4.2.3, the likely source of *S. thermosulfidooxidans* could be the ore because this species was found to dominate in the Control columns that were not inoculated. *S. thermosulfidooxidans* is metal-tolerant (Dopson *et al.*, 2003; Schippers, 2007) and that could be the reason why it thrived in the high Cu environment.

Again, *At. caldus*, similarly to Experiment 1 and 2, was shown to dominate the early stages of the experiment (in the Control and in the 5 g/L Cu-columns), further supporting the postulation that in mixed cultures, S-oxidisers have an advantage of Fe- and S-oxidisers at the start of a bioleach heap as already discussed in Sections 4.2.7 and 4.3.5.

5. MATERIALS AND METHODS II: Microbial growth kinetics in liquid culture

This chapter presents a description of the materials, the reactor set-ups, the experimental protocols and analytical procedures used in the investigation carried out in liquid cultures in stirred tank reactors (STRs).

5.1 Reactor set-up

Jacketed, baffled, glass STRs with a working volume of 1 L were used in the investigation of the effect on CO_2 concentration and agitation speed, and copper concentration, on the growth of moderate thermophiles in mixed liquid cultures. Each STR had an internal diameter of 110 mm and was stirred with a pitched four-blade impeller with a diameter of 55 mm. The STRs were operated at 50 °C by circulating water through their jackets from a water bath set at 50°C. A shake flask, a 1 L Erlenmeyer flask operated at 500 mL and 50°C, was also used. The STRs, a schematic representation of a single STR and the shake flask are shown in Figures 5.1, 5.2 and 5.3 respectively.

A- overhead stirrer, B- condenser, C- STR, D- air flow meter, E- water bath

Figure 5. 1 The STR set-up for experiments at 50 °C

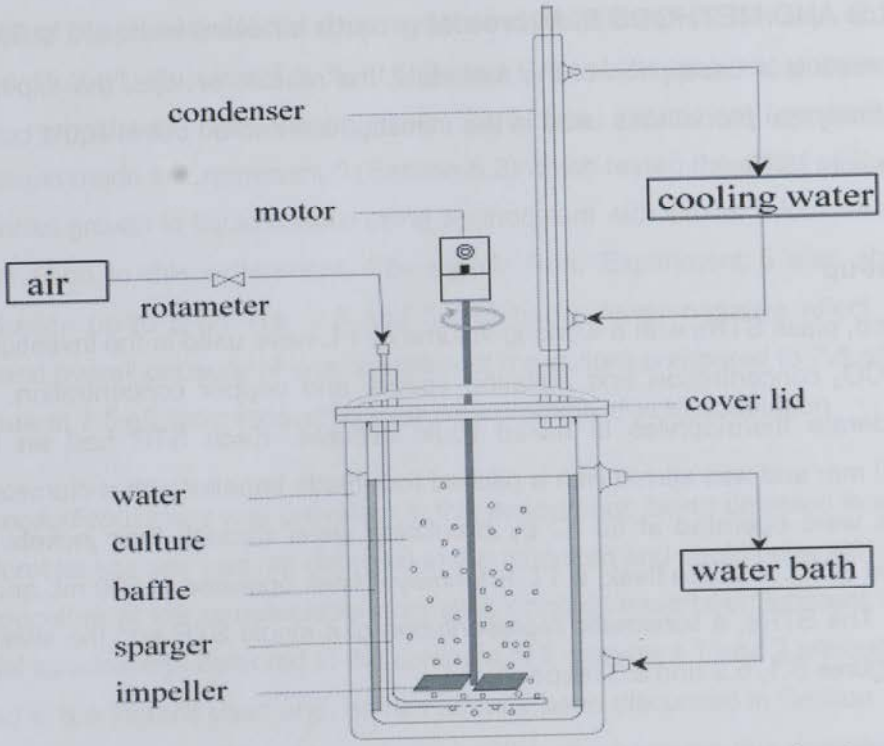


Figure 5. 2: The schematic diagram of a single STR set-up for the experiments at 50 °C

A- Erlenmeyer flask, B- culture, C- cotton wool plug, D- shaking platform

Figure 5. 3: The picture of a shake flask used in the experiment at 50 °C

5.2 Media

The cultures were prepared in a mineral salts medium modified from Norris *et al.* (1996) containing 0.4 g/L $(NH_4)_2SO_4$, 0.3 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$ and 0.1 g/L KCl in water, with the pH adjusted to 1.7 using H_2SO_4. Additionally, the feed medium for investigating the effect of Cu had varying Cu concentrations i.e. 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 g/L added as $CuSO_4.5H_2O$. The medium was sterilised by autoclaving before adding copper. Copper solutions were prepared separately and sterilised by filtering through a 0.22 µm membrane. The 0K and the activity test media were prepared as described in Section 3.4. All the cultures were supplemented with 1 % w/v chalcopyrite and 0.3 % w/v pyrite concentrates as energy sources.

5.3 Experimental protocol

The STRs were each inoculated with 900 mL of the 50°C stock culture and topped up to 1 L with the feed medium described above. The shake flask was inoculated with 450 mL of the stock culture and topped up to 500 mL with the feed medium.

All the cultures were sub-cultured twice every week to allow the micro-organisms to remain active in the exponential phase of their growth. This was done by withdrawing 150 mL culture from the STRs and adding 150 mL fresh feed medium, 1.45 g chalcopyrite and 0.45 g pyrite concentrate. In the shake flask, 75 mL of the culture was withdrawn and 75 mL of the medium, 0.725 g chalcopyrite and 0.225 g pyrite added. The STRs in Experiment 5 were sub-cultured with a medium containing Cu concentrations indicated in Section 5.2.

The agitation speed on the STRs was controlled by overhead stirrers set using a tachometer. A standard agitation rate of 550 rpm and an experimental value of 250 rpm were used. The agitation speed of 250 rpm was chosen to assess whether hydrodynamic stress was found at 550 rpm used to maintain bioleaching cultures at CeBER. The gas flow rates were controlled using rotameters. In Experiment 4, two STRs were sparged with air enriched to 1.0 % v/v CO_2 and one STR was sparged with normal air. One of the STRs sparged with enriched air and the STR sparged with normal air were stirred at 250 rpm whilst the other STR was stirred at 550 rpm. A shake flask was mixed on an orbital shaking platform at 180 rpm. In Experiment 5, four STRs were sparged with air enriched 1.0 % v/v CO_2 and stirred at 250 rpm. The Control (STR 1) contained no additional copper whilst STR 2 was fed with a medium containing an additional 2.5 g/L Cu, STR 3 with 5.0 g/L Cu and STR 4 with 7.5 g/L Cu. The Cu concentration in STRs 3 and 4 was gradually increased to an additional 10.0 g/L and 15.0 g/L respectively.

The reactors were monitored daily for temperature, agitation speed, gas flow, leakages, blockages and culture volume. To maintain a constant volume, the STRs were topped up to 1 L and the shake flask to 500 mL with tap water every time before sampling or sub-culturing. Sample volumes were taken from the 150 mL (or 75 mL) sub-cultured bi-weekly.

At the beginning of the experiment, the inoculum was sampled and assayed for Eh, pH, cell count, microbial composition using qPCR analysis and ferrous oxidation rate (activity test). On sub-culturing, the cultures were assayed for Eh, pH, Fe, Cu and cell count. Weekly, additional samples were collected for extraction of gDNA and subsequent qPCR analysis described in Section 3.6.3, and for the activity test.

5.4 Analytical procedures

The analytical procedures used in this part of the study included measurement of Eh, pH, Cu and Fe concentration, cell counts, qPCR analysis and activity tests. These procedures have been described in Section 3.6.

6. RESULTS AND DISCUSSION II: Microbial growth in liquid culture

6.1 Introduction

Two experiments were conducted to investigate the growth of moderate thermophiles in the liquid stock culture paying particular attention to *Acidithiomicrobium* species, a key iron- and sulphur-oxidising moderate thermophile. The first experiment investigated the effect of CO_2 supply and agitation speed. The second experiment investigated the effect copper concentration on the growth and dominance of the microbial species in liquid culture.

This study of the liquid culture was motivated by the changing composition of the bioleaching stock culture maintained at 50 °C at CeBER. This resulted in varying compositions of *Acidithiomicrobium* species under the same temperature, as shown in Figure 4.16. It was desired to grow a consistent culture with a high concentration of *Acidithiomicrobium* spp., for heap inoculation. Therefore, the effect of physicochemical parameters on the concentration of *Acidithiomicrobium* spp. in the stock culture was investigated. Additionally, the effect of Cu concentration in liquid culture was investigated to inform the observations made on the effect of Cu concentration in a whole ore environment presented in Section 4.4. The results of the studies are presented and discussed according to four categories: physicochemical parameters, cell counts, qPCR analysis and ferrous iron oxidation rates (activity tests).

6.2 The effect of carbon-dioxide and agitation speed on the growth of moderate thermophiles in liquid culture

Experiment 4 was conducted to investigate the effect of CO_2 supply and agitation speed on microbial growth in liquid culture. The experimental set up is described in Sections 5.1 and 5.3. Three stirred tank reactors (STRs) were inoculated with 900 mL of a mixed culture of moderate thermophiles (Inoculum 6) and topped up to 1 L with feed medium (Section 5.2). A shake flask was inoculated with 450 mL Inoculum 6 and topped up to 500 mL with the feed medium. All the cultures were sub-cultured twice every week and monitored for 98 days. The composition of culture, shown in Table 6.1, was determined using qPCR analysis.

Table 6. 1: Composition of Inoculum 6 used in Experiment 4

Micro-organism	Percentage composition in the inoculum
Acidiplasma cupricumulans (JTC4)	26
Acidithiobacillus caldus (At.c)	27
Metallosphaera sedula/hakonensis (M.sed)	35
Acidithiomicrobium species (JTC3/SAR)	12
Archaea (JTC 1/2)	<1

6.2.1 Physicochemical parameters

Twice weekly, just before sub-culturing, the cultures were assayed for Eh, pH, total soluble iron and soluble copper. The results of these assays are shown in Figures 6.1 to 6.4.

The Eh, shown in Figure 6.1, showed a similar trend in all the four reactors, ranging between 860 and 920 mV. This high Eh indicated the presence of active iron-oxidisers in the cultures although it was noted that the Eh was lowest in the shake flask for the first 70 days. The Eh in all the cultures started at an average of 860 mV and quickly increased in the first 7 days reaching 893, 905, 903 and 890 in STR 1, STR 2, STR 3 and the shake flask respectively. After this, the Eh increased slowly in the three STRs throughout the experiment finally reaching 908, 908 and 915 mV on day 98 the last day of the experiment respectively.

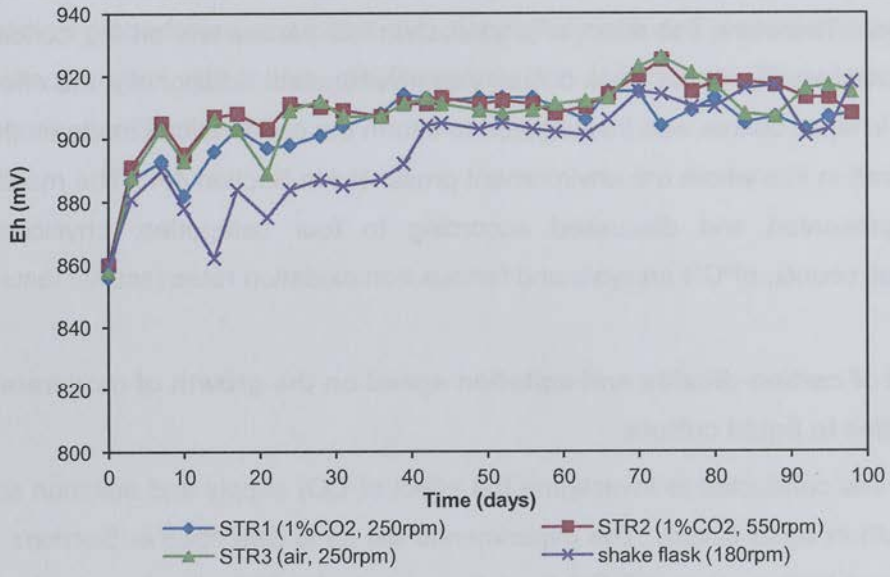


Figure 6.1: Eh measured in the four cultures in Experiment 4

The Eh in the shake flask decreased after 7 days and reached 884 mV by day 24 after which it started to increase and finally reached 904 mV by day 42. Thereafter, the Eh continued to slowly increase and reached similar values as the Eh of the STRs on day 70 and continued to increase gradually until reaching 918 mV by day 98. The initial lower Eh in the shake flask during the first 42 days could indicate low microbial activity due to low CO_2 availability caused by mass transfer limitations at low agitation speed. After 42 days the microbial community in the shake flask had adapted to the conditions present and microbial activity increased. In the first 24 days of the experiment the Eh in all the reactors was not stable, probably due to the frequent sub-culturing. After 24 days, the Eh stabilised in all reactors; this was attributed to increased microbial concentration, enabling the culture to efficiently oxidise the mineral concentrates supplied as energy source.

The pH, shown in Figure 6.2, showed a similar trend across all reactors and was in the range pH 1.2 - 1.7. The pH was highest in the shake flask for the first 73 days. This pH range was suitable for the bioleaching acidophiles (Brierley, 2001; Rawlings, 2007).

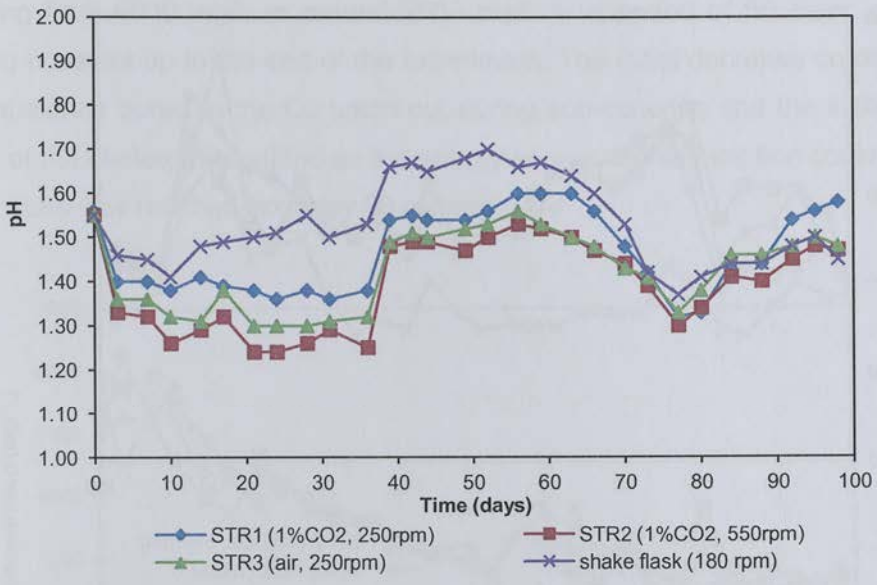


Figure 6. 2: pH measured in the four cultures in Experiment 4

All the cultures started at pH 1.55 and decreased quickly in the first 3 days reaching 1.40, 1.33, 1.36 and 1.46 in STR 1, STR 2, STR 3 and the shake flask respectively. After this, the pH decreased slowly in the STRs, reaching 1.38, 1.25 and 1.32 on day 36 respectively whilst the pH slowly increased in the shake flask reaching 1.53 on day 36. After this, the pH quickly increased again in all the four reactors to reach 1.54, 1.48, 1.49 and 1.66 by day 39 respectively. After day 39 the pH increased slowly in all the four reactors finally reaching 1.60, 1.53 and 1.56 on day 56 in the three STRs respectively whilst the pH reached 1.66 on day 52 in the shake flask. After this the pH quickly decreased reaching about 1.40 in all the four reactors by day 73 and continued to decrease and reached 1.32, 1.30, 1.33 and 1.37 by day 77 in STR 1, STR 2, STR 3 and shake flask respectively. After day 77, the pH increased again in the four reactors finally reaching 1.58, 1.47, 1.48 and 1.45 by day 98 respectively. The initial high pH in the shake flask could indicate low microbial activity due to low substrate availability caused by mass transfer limitations at low agitation speed. However, the microbial community could have adapted to the conditions in the shake flask and hence microbial activity increased as the experiment proceeded, and the pH reached values similar to the pH in the STRs around day 70. This was in consensus with the Eh measured in the same shake flask, which also synchronised with the rest of the reactors around day 70 as indicated in Figure 6.1.

Soluble Fe, shown in Figure 6.3, had an average of 990 mg/L at the start of the experiment. Similar trends across all the reactors were found although the soluble Fe in the shake flask was the lowest for the first 77 days.

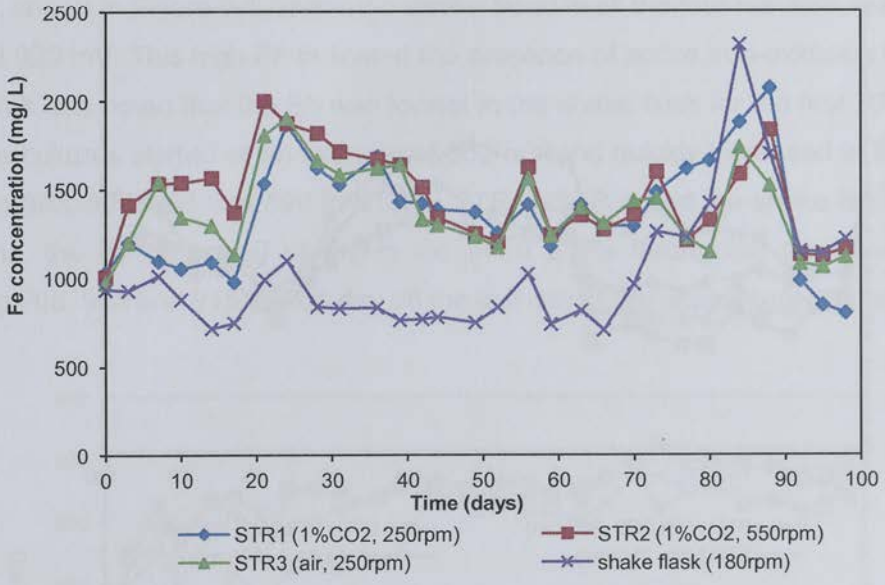


Figure 6. 3: Soluble iron measured in the four cultures in Experiment 4

In the first 17 days, Fe in all the reactors varied. STRs 2 and 3 quickly increased from the start both reaching an average of 1540 mg/L by day 7 after which that in STR 3 quickly decreased to reach 983 mg/L by day 17 whilst little change was observed in STR 2. The Fe concentration in STR 1 stayed almost constant across days 1 to 17, reaching 983 mg/L by day 17. The Fe in solution in the shake flask decreased to 749 mg/L by day 17. Thereafter, the Fe in all the four reactors increased quickly. The three STRs reached an average of 1880 mg Fe/L, whilst the shake flask reached 1100 mg/L by day 24. After day 24, the Fe in the three STRs was similar until day 73 whilst the Fe in the shake flask continued being the lowest. After day 24, the Fe in the STRs decreased from 1880 mg/L reaching an average of 1220 mg/L by day 59 after which the Fe increased again and reached an average of 1510 mg/L by day 73. After day 73, the Fe in STR 1 continued to increase until reaching a maximum of 2067 mg/L by day 88. Thereafter it decreased rapidly to 802 mg/L by day 98. The Fe in solution in STR 2 and 3 fluctuated but reached a maximum of 1832 and 1737 mg/L by day 88 and 84 respectively, thereafter decreasing to reach an average of 1140 mg/L by day 98. After day 24, the Fe in the shake flask decreased from 749 mg/L and reached 708 mg/L by day 66 after which the Fe rapidly increased and reached a maximum of 2313 mg/L by day 84. After day 84, the Fe in the shake flask rapidly decreased reaching values similar to STR 2 and 3 from day 92 until day 98. The initial lower Fe in the shake flask could

indicate low microbial activity due to low substrate availability caused by mass transfer limitations at low agitation speed.

Soluble copper, shown in Figure 6.4, had a similar trend in all the four reactors; gradually decreasing from 6000 mg/L to around 2500 mg/L in a period of 50 days, and thereafter remaining constant up to the end of the experiment. The initial decrease could be attributed to the imbalance between the Cu taken out during sub-culturing and the initial slow rate of leaching of the chalcopyrite added as the energy source. An assumption could be made that a steady state was reached from day 50 onwards.

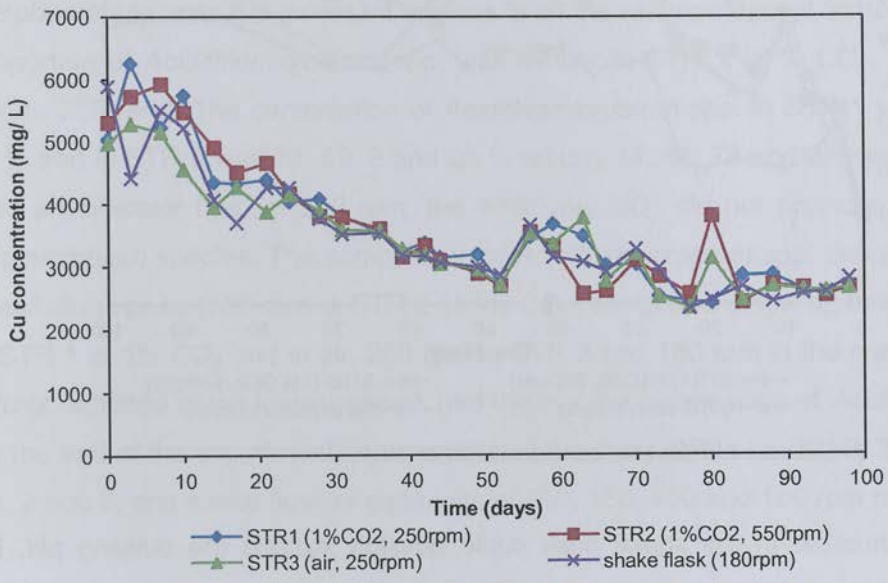


Figure 6. 4: Soluble copper measured in the four cultures in Experiment 4

6.2.2 Cell count

Microbial cell counts were initially done twice a week until day 56, weekly from day 56 to day 70, thereafter fortnightly. The trend in cell numbers, expressed in logarithm to base 10, is shown in Figure 6.5. The cell number in the three STRs quickly increased from 1.55×10^8 at the start and reached 6.60×10^8, 6.00×10^8 and 5.60×10^8 cells/mL on day 28 in STRs 1, 2 and 3 respectively. The initial fastest growth i.e. from the start to day 28 was observed in STR 1 (1% CO_2, 250 rpm) whilst the growth in STR 2 (1% CO_2, 550 rpm) and STR 3 (air, 250 rpm) was similar. After day 28, the cell numbers in STR 1 and 2 were similar and constant at an average of 6.00×10^8 cells/mL up to the end of the experiment. The cell numbers in STR 3 were also constant at an average of 6.00×10^8 cells/mL after day 28, until day 52. After day 52, the cell numbers in STR 3 decreased and reached 3.65×10^8 cells/mL by day 63 and thereafter gradually increased and finally reached same levels as the other STRs by the end of the experiment. The cell number in the shake flask was the lowest of all

the reactors from day 7 until the end of the experiment. Rapid growth in the shake flask took place in the first 3 days i.e. an increase from 1.55 x 10^8 to 2.50 x 10^8 cells/mL, and thereafter the cell count decreased to 2.15 x 10^8 cells/mL by day 7 and stayed almost constant until day 24. After day 24, the cells in the shake flask exponentially increased again, and reached 3.60 x 10^8 cells/mL by day 28, and thereafter gradually and steadily increased until reaching 4.40 x 10^8 cells/mL by day 98.

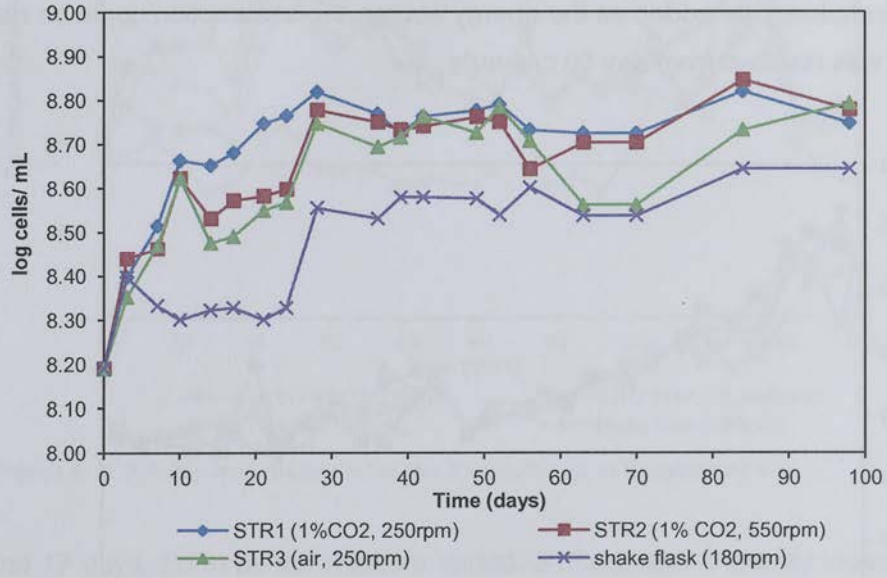


Figure 6. 5: Cell count of the four cultures in Experiment 4

The low cell number in the shake flask could possibly explain the outlying pH, Eh and soluble Fe in this reactor compared to the STRs in the first 70 days, as shown in Figures 6.1 to 6.3. After 70 days, the Eh, pH and the Fe reached, or surpassed, those in the STRs indicating increased microbial activity in the shake flask. This increased microbial activity could be due to an increase in cell numbers and/or adaptation of the cells to the conditions in the shake flask.

6.2.3 qPCR analysis

The microbial composition of the inoculum was assayed at the start of the experiment and that of the STRs was assayed weekly using qPCR. The composition of the inoculum and the cultures is shown in Figure 6.6. The growth curves of the two main competitors *Acidithiomicrobium* and *Metallosphaera* species are shown in Figure 6.7 whilst the genomic copy numbers of all the microbial species of interest in the inoculum and in the cultures throughout the experiment, are shown in Figure 6.8 and in Table B.5 in the Appendix.

The starting inoculum (Figure 6.6), was dominated by *Metallosphaera* spp. (35 %), followed by *A. caldus* (35 %), *A. cupricumulans* (26 %), *Acidithiomicrobium* spp. (12 %) and *Thermoplasmatales* spp. (<1 %). Generally, the dominating and competing species in the four reactors were *Metallosphaera* and *Acidithiomicrobium*. The other species originally present in the inoculum also persisted, throughout the experiment, at low proportions. The total genomic copy number of *Metallosphaera* and *Acidithiomicrobium* species were almost equal to the total genomic copies of all the species in the cultures, as indicated in Figure B.1 (Appendix). On day 14, *Acidithiomicrobium* spp. had increased from the initial 12 % to be the dominant species in all the reactors. By day 42, *Acidithiomicrobium* spp. started to decrease and *Metallosphaera* spp. increased such that from day 70 until the end of the experiment *Metallosphaera* spp. was the dominant species in all the reactors except for the shake flask. The proportion of *Acidithiomicrobium* spp. was similar in STR 1 (1 % CO_2, 250 rpm) and STR 3 (air, 250 rpm). The composition of *Acidithiomicrobium* spp. in STR 1 was 84, 74, 14 and 22 % and in STR 3 was 83, 59, 9 and 25 % on day 14, 42, 70 and 98 respectively. This could be an indicator that, at 250 rpm, the additional CO_2 did not promote the growth of *Acidithiomicrobium* species. The composition of *Acidithiomicrobium* spp. decreased faster in higher agitation speed (550 rpm in STR 2 at 1% CO_2) compared to low agitation speed (250 rpm in STR 1 at 1% CO_2 and in air, 250 rpm in STR 3 and 180 rpm in the shake flask). The shake flask, agitated at the lowest speed, had the highest composition of *Acidithiomicrobium* spp. by the end of the experiment compared to all the three STRs i.e. 22, 9, 25 and 54 % in STRs 1, 2 and 3, and shake flask at agitations of 250, 550, 250 and 180 rpm respectively.

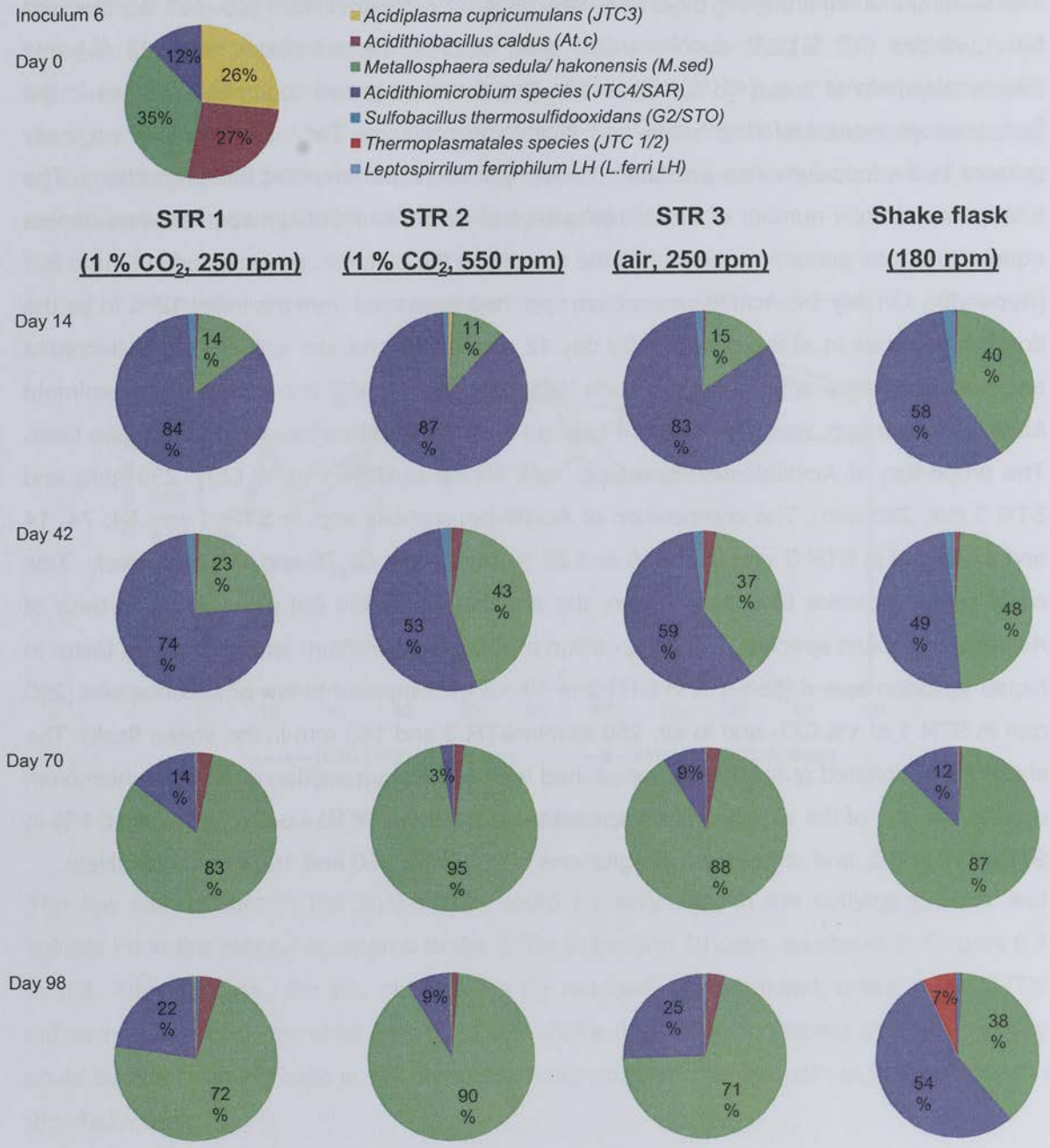


Figure 6. 6: Composition of Inoculum 6 and the four cultures in Experiment 4

A comparison of genomic copy numbers of *Acidithiomicrobium* and *Metallosphaera* species (expressed as logarithms to base 10) is shown in Figure 6.7. *Metallosphaera* spp. generally maintained numbers between 1.10 x 10^8 and 7.40 x 10^8 genomic copies/ mL throughout whilst *Acidithiomicrobium* spp. initially increased then decreased. *Metallosphaera* spp. started at 1.08 x 10^8 and ended up at 1.26 x 10^8, 2.00 x 10^8, 1.73 x 10^8 and 5.22 x 10^7 genomic copies/mL in STRs 1, 2 and 3, and the shake flask respectively, on day 98. *Acidithiomicrobium* spp. started at 3.74 x 10^7 and increased to an average of 2.08 x 10^9

copies/ mL in the STRs 1, 2 and 3, and 3.58 x 10^8 copies/mL in the shake flask, by day 14. After this, copies of *Acidithiomicrobium* spp. gradually decreased in all the reactors until reaching 3.87 x 10^7, 1.90 x 10^7, 6.10 x 10^7 and 7.46 x 10^7 genomic copies/mL in STRs 1, 2 and 3, and the shake flask respectively, by day 98. After day 14, the number of *Acidithiomicrobium* genomic copies was similar between STRs 1 and 3, both agitated at 250 rpm. In both STR 1 and 3, *Metallosphaera* spp. out-dominated *Acidithiomicrobium* spp. by day 56. In STR 2 (agitated at 550 rpm), the *Acidithiomicrobium* spp. decreased at a faster rate and was out-dominated by the *Metallosphaera* spp. earlier i.e. on day 42.

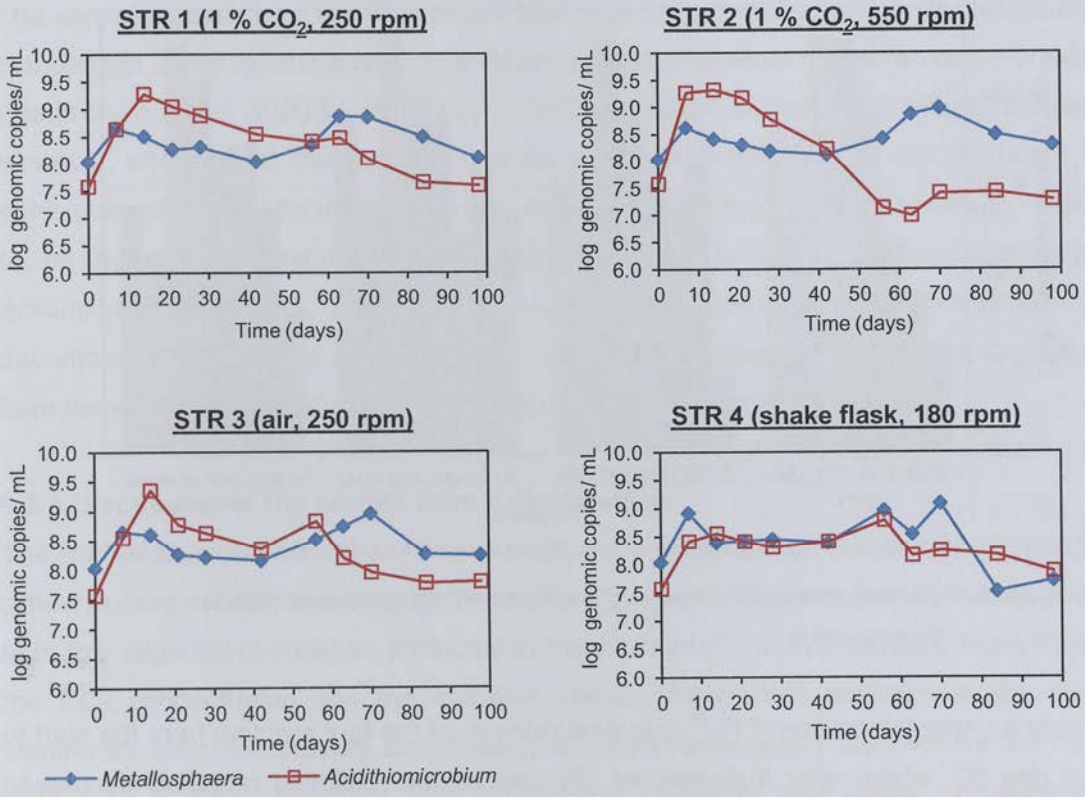


Figure 6. 7: Genomic copy numbers of *Acidithiomicrobium* spp. and *Metallosphaera* spp. in STRs 1 to 3 and in the shake flask in Experiment 4

On day 98, the last day of the experiment, STR 2 (1 % CO_2, 550 rpm) had the largest difference between the genomic copy numbers of *Metallosphaera* spp. and *Acidithiomicrobium* spp. i.e. 2.00 x 10^8 and 1.90 x 10^7 respectively. The shake flask (not sparged, 180 rpm) had the smallest difference i.e. 5.22 x 10^7 and 7.46 x 10^7 respectively. In STR 1 and 3 the difference was similar. STR 1 (1 % CO_2, 250 rpm) had 1.26 x 10^8 and 3.87 x 10^7 copies/mL of *Metallosphaera* spp. and *Acidithiomicrobium* spp. respectively whilst STR 3 (air, 250 rpm) had 1.73 x 10^8 and 6.10 x 10^7 copies/mL respectively. These differences in the final genomic copy numbers of *Metallosphaera* and *Acidithiomicrobium* species clearly

indicated the negative effect of high agitation speed on the growth of *Acidithiomicrobium* spp. This is further discussed in Section 6.2.5.

6.2.4 Activity of the microbial community

An activity test was set-up as detailed in Section 3.6.4. In this experiment, samples were collected weekly in the first month of the experiment and thereafter fortnightly. The rate of Fe^{2+} oxidation for all the samples was measured over the first 6 days of the activity test. The biological Fe^{2+} oxidation rates of the different samples are shown in Figure 6.8.

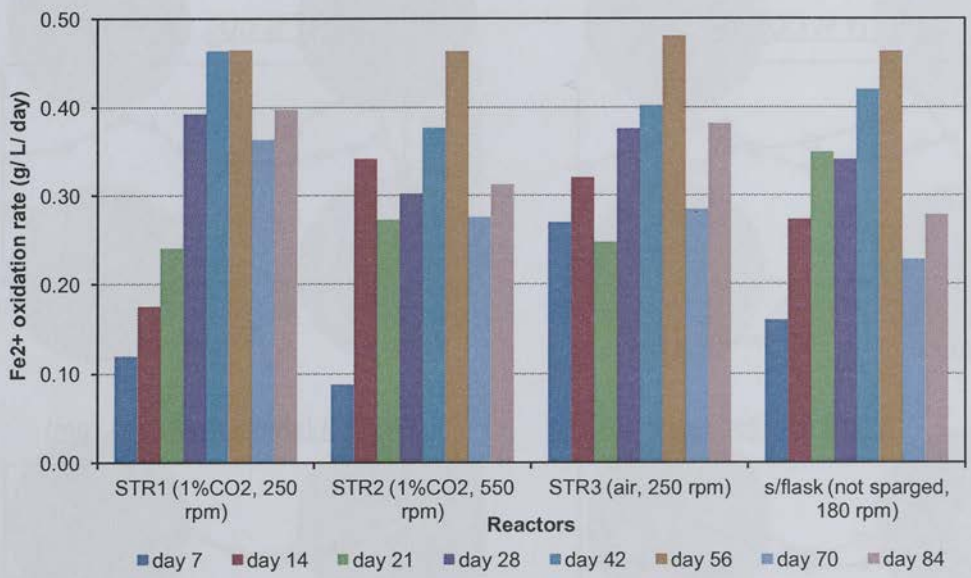


Figure 6. 8: Ferrous iron oxidation rates of the four cultures at different days in Experiment 4

There was a general increase of Fe^{2+} oxidation rates in all the four reactors from the start to peak at day 56; where after it decreased. By day 70 the oxidation rate had decreased although at day 84, the last sampling point assayed, the oxidation rate started to increase again in all the reactors. In STR 1 (1 % CO_2, 250 rpm), the oxidation rate started at 0.120 g Fe^{2+}/L/day on day 7 and steadily increased to 0.464 and 0.465 g/L/day on day 42 and 56 respectively. The oxidation rate decreased to 0.364 on day 70 and then slightly increased to 0.398 g/L/day on day 84. In STR 2 (1 % CO_2, 550 rpm), the oxidation rate started at the lowest starting point compared to the other reactors i.e. 0.088 g/L/day on day 7. The oxidation rate in STR 2 steadily increased to 0.464 g/L/day on day 56, although on day 14 the oxidation rate reached 0.342 g/L/day, a high value which seemed to be an outlier considering the general trend. The oxidation rate decreased after day 56 and reached 0.276 on day 70 and then slightly increased to 0.313 g/L/day on day 84. In STR 3 (air, 250 rpm), the oxidation rate started at the highest starting point compared to the other reactors i.e.

0.270 g/L/day on day 7. The oxidation rate in STR 3 steadily increased to 0.481 g/L/day, the highest peak compared to the other reactors, on day 56. In spite of the steady increase from the start, on day 21 the oxidation rate reached 0.248 g/L/day, a low value which seemed to be an outlier considering the general trend. The oxidation rate decreased after day 56 and reached 0.285 on day 70 and then slightly increased to 0.382 g/L/day on day 84. In the shake flask (not sparged, 180 rpm), the oxidation rate started at 0.161 g/L/day on day 7 and steadily increased to 0.464 g/L/day on day 56. The oxidation rate decreased to 0.228 on day 70 and the slightly increased to 0.278 g/L/day on day 84.

The same inoculum size of 10^9 cells per flask was used in all the activity tests, allowing the comparison of the oxidation rates to be standardised. Therefore the increasing iron oxidation rate in the cultures could be attributed to the increasing composition of *Metallosphaera* spp. However, after day 56, the oxidation rate decreased in all the reactors and in STRs 1, 2 and 4 this coincided with *Metallosphaera* spp. out-dominating *Acidithiomicrobium* spp. This could be an indicator of a symbiotic relationship or synergy between *Metallosphaera* spp. and *Acidithiomicrobium* spp. such that when the composition of *Acidithiomicrobium* spp. decreases, the activity of *Metallosphaera* spp. also decreases. Alternatively, it could result from the decreased metabolic activity of the *Metallosphaera* with time.

6.2.5 Discussion of the results from Experiment 4

The similar physicochemical trends observed in the four reactors over the 98 days indicated similar culture conditions across all the reactors and hence the microbial dynamics observed from this experiment could be attributed to the effect of the two parameters being tested i.e. the CO_2 concentration and the agitation speed. Quantitative analyses of the microbial community indicated varying dominances of *Acidithiomicrobium* spp. across the reactors. STR 1 (1 % CO_2, 250 rpm) and STR 3 (air, 250 rpm) had similar concentrations of *Acidithiomicrobium* spp. i.e. 3.87×10^7 and 6.10×10^7 copies/mL respectively, whilst the shake flask (not sparged, 180 rpm) had the highest concentration of *Acidithiomicrobium* spp. i.e. 7.46×10^7 copies/mL by the end of the experiment. This indicated that at low agitation speed ($\leq$ 250 rpm), CO_2 concentration was not a limitation and did not affect the dominance of *Acidithiomicrobium* spp. regardless of CO_2 content in the air reaching the culture. However, further test work, particularly the measurement of dissolved CO_2, is required to confirm this conclusion. To date, there has been no consensus on either the lower limit where CO_2 becomes a limiting substrate or the upper limit where CO_2 becomes inhibitory. Different literature reports indicate that CO_2 demand in bioleaching varies with different cultures, bacterial adaptation and the feed material (Witne and Phillips, 2000).

The data suggested that the agitation speed in the stirred tank reactor was vital in determining the culture composition. STR 2 (1 % CO_2, 550 rpm) had the lowest concentration of *Acidithiomicrobium* spp. from day 42 until the end of the experiment. At the instance when *Acidithiomicrobium* spp. was lowest in STR 2 i.e. at day 63, the amount of *Acidithiomicrobium* spp. in this reactor was 97 % less than the amount of *Acidithiomicrobium* spp. in STR 1 (1 % CO_2, 250 rpm), indicating that high agitation speed was detrimental to the growth of this microbial species. In all cases, the concentration of *Metallosphaera* remained little changed. Two possible causes of the observed effects of high agitation speed could be (i) increased mass transfer and/or (ii) shear stress. Increasing the agitation speed both increased the liquid phase mass transfer and the gas-liquid transfer of CO_2 increasing substrate availability to the micro-organisms. The results of CO_2 enrichment, it showed that CO_2 provision was not the controlling factor in this environment. Increasing the agitation speed could have also triggered the decrease in cell numbers of *Acidithiomicrobium* spp. through shear stress. Shear stress is recognised to be related to the impeller tip speed and the turbulent eddies generated (Chisti, 2001). Further, attrition and grinding are a function of both the impeller speed and the solids loading (Lee and Lee, 2003). Typically it is expected that the impact of shear stress is a function of the resilience of the cell envelope with the less resilient cell envelope more strongly impacted by shear stress. However, *Acidithiomicrobium* spp. is a Gram positive bacterium with a thick peptidoglycan-containing cell wall. Conversely, *Metallosphaera* spp. are thermophilic archaea that lack a rigid peptidoglycan cell wall (Nemati and Harrison, 2000). Therefore, *Metallosphaera* species are expected to be more susceptible to shear than *Acidithiomicrobium* spp. Attrition and grinding also seem unlikely because the pulp density of the culture was low (ca. 2 to 3 % w/v) and at such low pulp density, hydrodynamic stress is unlikely (Rawlings *et al.*, 2003). This dismissal is supported by Nemati and Harrison (2000) who carried out tests using the thermophilic archaeon *Sulfobacillus metallicus*. In the 3 - 9 % solids region, the pulp density did not influence the iron solubilisation rate indicating that the cells were viable. At a pulp density of 18 %, the iron solubilisation rate was significantly reduced because increasing agitation rate to 500 rpm resulted in the disruption of microbial cells and a subsequent reduction in cell count.

The activity test indicated an increasing Fe^{2+} oxidation by the culture over time. Furthermore, the highest sustained oxidation rate was maintained in STR 1 and STR 3 with a marked reduction in STR 2. It is difficult to assign any individual microbial species as responsible for the increasing oxidation rate since the micro-organisms were in a mixed culture; however, the *Metallosphaera* numbers remained little changed across the reactors while the *Acidithiomicrobium* cell numbers mimicked the trend in Fe^{2+} iron oxidation rate. Further test work would be valuable to ascertain which microbial species dominant Fe-oxidisers.

6.3 The effect of copper concentration on the growth of moderate thermophiles in liquid culture

Experiment 5 was run to investigate the effect of Cu concentration on microbial growth in liquid culture. This set up was described in Sections 5.1 and 5.3. One litre of the mixed culture of moderate thermophiles (Inoculum 7) was placed into each of four STRs and the cultures were sub-cultured twice every week. Inoculum 7 was assayed using qPCR and found to have a microbial composition shown in Table 6.2. The Control (STR 1) was run until day 98. STR 2, operated at 2.5 g/L Cu, was run until day 54. STR 3, initially fed with 5 g/L Cu, was taken up to 7.5 g/L Cu on day 54, then to 10.0 g/L on day 78 and was run until day 98. STR 4, initially fed with 7.5 g/L Cu, was taken up to 10.0 g/L on day 54, then to 12.5 g/L on day 78, and to 15.0 g/L Cu on day 113 and was run until day 127.

Table 6. 2: Composition of Inoculum 7 used in Experiment 5

Micro-organism	Percentage composition in the inoculum
Acidiplasma cupricumulans (JTC4)	<1
Acidithiobacillus caldus (At.c)	<1
Metallosphaera sedula/hakonensis (M.sed)	72
Acidithiomicrobium species (JTC3/SAR)	24
Archaea (JTC 1/2)	2
Leptospirillum ferriphilum (L.ferri)	<1

6.3.1 Physicochemical parameters

Twice every week, before sub-culturing, the cultures were assayed for Eh, pH, soluble iron and soluble copper. The results of these assays are shown in Figures 6.9 to 6.14.

The Eh in all the STRs, shown in Figure 6.9, started at an average of 904 mV. The Eh of the Control (STR 1 fed with no additional Cu) remained constant around 906 mV until day 98 when it was stopped. The Eh in STR 2 (fed with an additional 2.5 g/L Cu) had similar values to STR 1 until day 54 when it was stopped. The Eh in STR 3 (initially fed with an additional 5.0 g/L Cu) was slightly lower than that of the Control, and the Eh in STR 4 (initially fed with an additional 7.5 g/L Cu) was the lowest of all the STRs. The Eh profiles of STRs 3 and 4 are clearly shown in Figures 6.10 and 6.11 respectively.

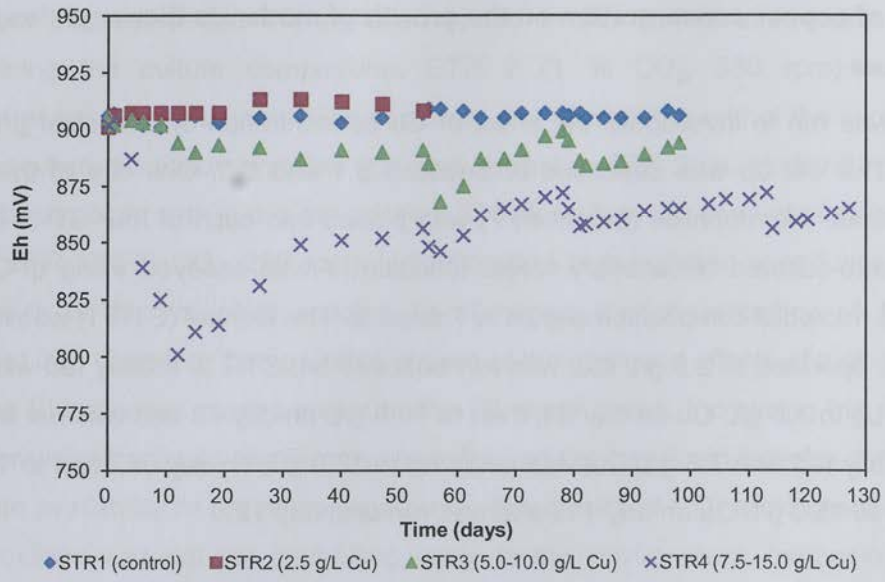


Figure 6. 9: Eh measured in the four cultures in Experiment 5

The Eh in STR 3, as shown in Figure 6.10, gradually decreased from 905 mV at the start and reached 887 mV by day 33 and thereafter started to increase gradually until reaching 891 mV on day 54. After increasing the Cu to 7.5 g/L on day 54, the Eh sharply decreased and reached 868 mV by day 57 and thereafter started to increase again until reaching 899 mV on day 78. After the Cu was taken up to 10.0 g/L on day 78, the Eh decreased again and reached 884 mV by day 84 and thereafter started to increase again until reaching 894 mV on day 98 when this STR was stopped. The Eh in STR 4, as shown in Figure 6.11, sharply decreased from 902 mV to reach 801 mV on day 12 following addition of 7.5 g/L Cu and thereafter started to increase until reaching 856 mV on day 54. Thereafter the Eh profile of this STR had a trend similar to that observed in STR 3 as the Cu concentration was increased. After the Cu was taken up to 10.0 g/L on day 54, the Eh decreased and reached 846 mV on day 57 after-which the Eh started to increase finally reaching 872 mV on day 78. After the Cu was taken up to 12.5 g/L on day 78, the Eh decreased sharply and reached 857 mV by day 81 and thereafter started to increase and again reached 872 mV, on day 113. After the Cu was taken up to 15.0 g/L on day 113, the Eh decreased sharply again and reached 856 mV by day 114 and thereafter started to increase gradually again until reaching 865 mV on day 127 when this STR was stopped.

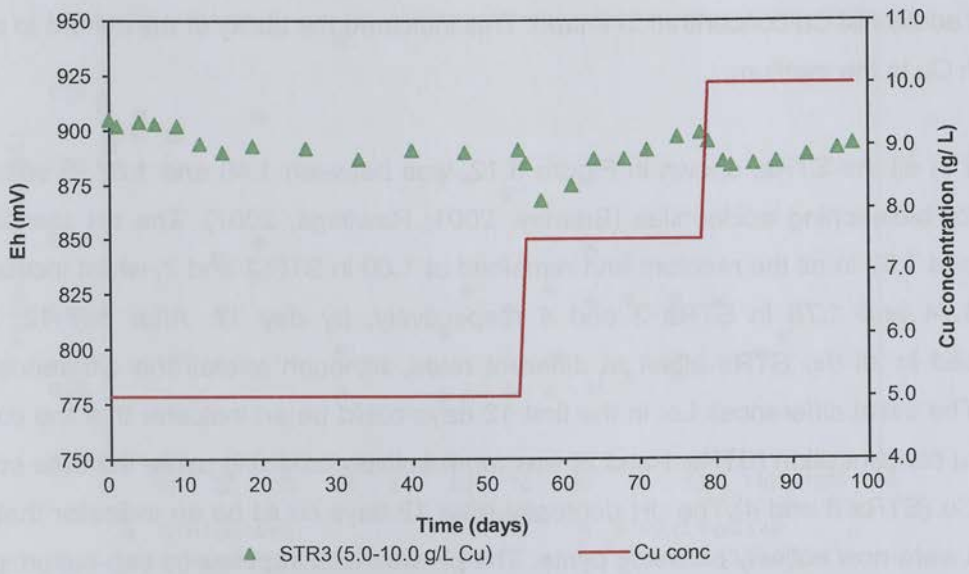


Figure 6. 10: Eh of Culture 3 in the additional Cu concentration of 5.0 up to 10.0 g/L

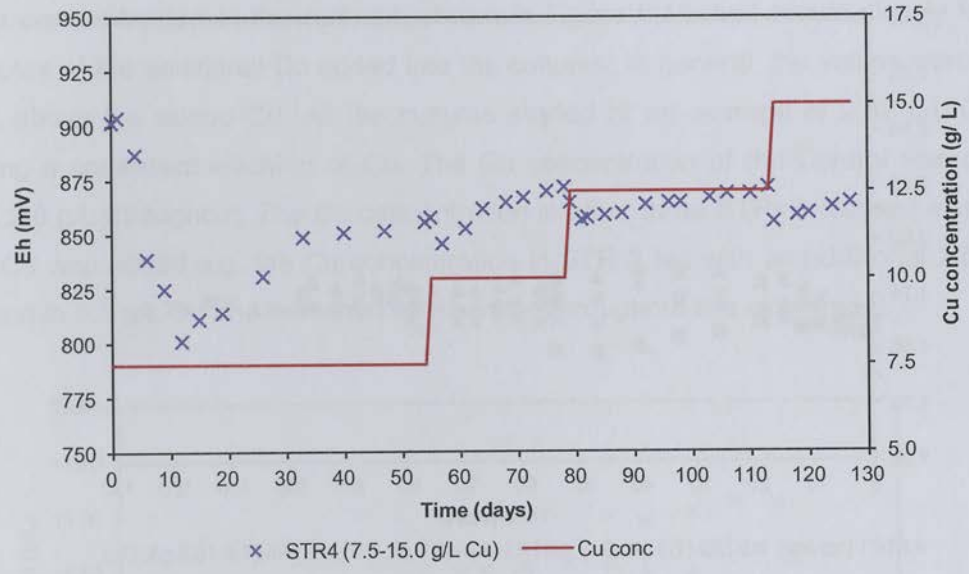


Figure 6. 11: Eh of Culture 4 in the additional Cu concentration of 7.5 up to 15.0 g/L

The similarity of the Eh values between STR 1 and STR 2 indicated that an additional 2.5 g/L Cu had minimal effect on the overall iron oxidising capability of the mixed culture. The Eh profiles of STRs 3 and 4 indicated that a Cu concentration of both 5.0 and 7.5 g/L decreased the overall capability of the culture to oxidise iron. The initial Cu concentration of 7.5 g/L had the higher negative effect, causing a decrease in the Eh by about 100 units in 12 days, probably because the micro-organisms were shocked by the sudden increase of Cu concentration. Although the Eh initially decreased on increasing the Cu concentration, it gradually increased back to 875 – 900 mV after every Cu increase, up to 15.0 g/L, the

highest additional Cu concentration tested. This indicated the ability of the culture to adapt to the high Cu in the medium.

The pH in all the STRs, shown in Figure 6.12, was between 1.49 and 1.87, a suitable pH range for bioleaching acidophiles (Brierley, 2001; Rawlings, 2007). The pH started at an average of 1.60 in all the reactors and remained at 1.60 in STR 1 and 2, whilst increasing to reach 1.74 and 1.78 in STRs 3 and 4 respectively, by day 12. After day 12, the pH decreased in all the STRs albeit at different rates, although overall the differences were minor. The initial differences i.e. in the first 12 days could be an indicator that the culture at lower Cu concentration (STRs 1 and 2) was more actively oxidising pyrite than the culture in higher Cu (STRs 3 and 4).The pH decrease after 12 days could be an indicator that all the cultures were now actively oxidising pyrite. The pH was also kept low by sub-culturing which introduced a fresh medium at pH 1.7 twice every week.

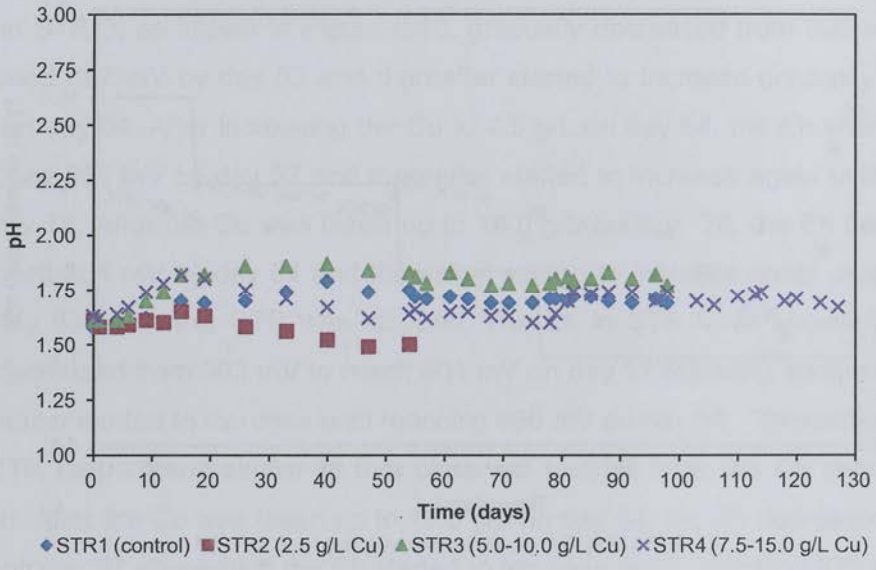


Figure 6. 12: pH measured in the four cultures in Experiment 5

The total Fe concentration in all the four STRs, shown in Figure 6.13, was between 0.17 and 1.43 g/L throughout the experiment. The Fe started at an average of 1.24 g/L in all the STRs and decreased for the first 19 days reaching 0.50, 0.68, 0.34 and 0.31 g/L in STRs 1, 2, 3 and 4 respectively by day 19. Thereafter, the Fe increased in all the STRs at different rates. However, Fe in STR 4 decreased again after day 78 and this coincided with an increase of Cu from 10.0 to 12.5 g/L although the Fe started to increase again after day 110 indicating a possible adaptation of the culture to high Cu.

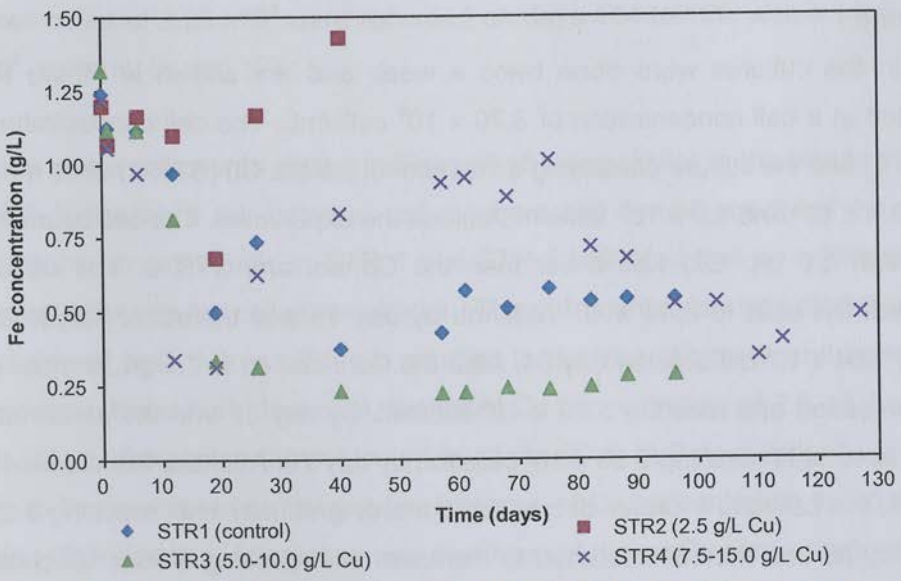


Figure 6. 13: Total soluble iron measured in the four cultures in Experiment 5

The Cu concentration in the cultures, shown in Figure 6.14, had trends closely following the amount of the additional Cu added into the cultures. In general, the values were around 2.8 g/L above the added Cu. All the cultures started at an average of 2.82 g/L Cu, thus indicating a consistent leaching of Cu. The Cu concentration of the Control was constant around 2.8 g/L throughout. The Cu concentration in other three STRs increased accordingly as the Cu was added e.g. the Cu concentration in STR 2 fed with an additional 2.5 g/L Cu increased to 5.1 g/L Cu and remained at this value throughout this experiment.

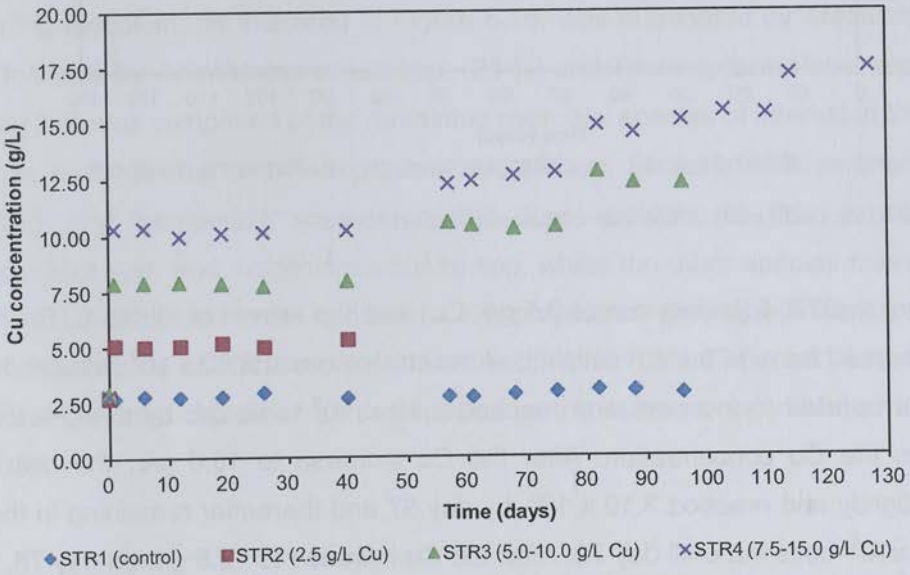


Figure 6. 14: Soluble copper measured in the four cultures in Experiment 5

6.3.2 Cell count

Cell counts in the cultures were done twice a week and are shown in Figure 6.15. All cultures started at a cell concentration of 3.70 x 10^8 cells/mL. The cell concentration in the Control (STR 1) and the culture containing an additional 2.5 g/L Cu (STR 2) were maintained in the range 3.7 x 10^8 and 3.9 x 10^8 cells/mL across the experiment. The cell count in STR 3 (initially fed with 5.0 g/L Cu) was lower than the Control and STR 2. The cell number decreased from the start to 3.44 x 10^8 cells/mL by day 15 and thereafter stayed constant until reaching 3.54 x 10^8 cells/mL by day 54. After the Cu increase to 7.5 g/L, the cell number drastically decreased and reached 3.28 x 10^8 cells/mL by day 57 and thereafter started to gradually increase until reaching 3.39 x 10^8 cells/mL by day 78. After the Cu increase to 10.0 g/L on day 78, the cell count further decreased but only gradually until reaching 3.26 x 10^8 cells/mL on day 85 and thereafter started to increase until reaching 3.36 x 10^8 cells/mL by day 98 when it was stopped.

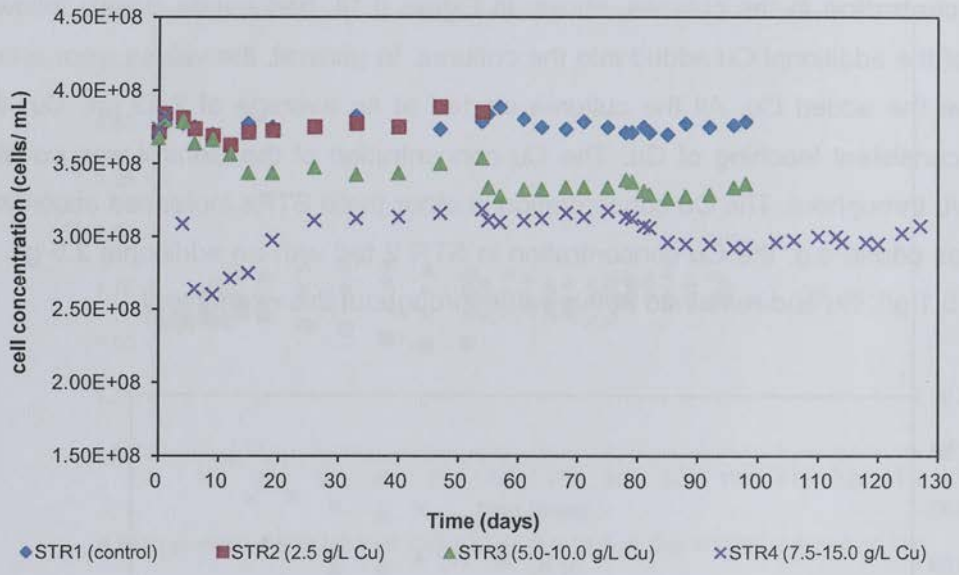


Figure 6. 15: Cell count of the four cultures in Experiment 5

The cell count in STR 4 (initially run at 7.5 g/L Cu) was the lowest of all the STRs. The cell number decreased from 3.70 x 10^8 cells/mL at the start to reach 2.60 x 10^8 cells/mL by day 9 and thereafter started to increase and reached 3.19 x 10^8 cells/mL by day 54, indicating adaptation to the Cu concentration. After the Cu increase to 10.0 g/L, the cell number decreased slightly and reached 3.10 x 10^8 by day 57 and thereafter remaining in the range 3.10 to 3.14 x10^8 cells/mL until day 78. After the Cu increase to 12.5 g/L on day 78, the cell count further decreased gradually to 2.96 x10^8 on day 85 and thereafter remaining in the range 2.95 to 3.00 x10^8 cells/mL by day 113. After the final Cu increase to 15.0 g/L on day

113, a cell count of 2.95 $x10^8$ were recorded on days 114 to 120, with a small increase to 3.08 $x10^8$ cells/mL by day 127.

The trends of the cell counts shown in Figure 6.15 were similar to the trends of Eh shown in Figure 6.9, indicating a link between cell numbers and overall activity of the cultures. The similarity between cell counts in STR 1 and STR 2 indicated that an additional 2.5 g/L Cu had minimal effect on the cell concentration. The cell counts also indicated that an initial Cu concentration of both 5.0 and 7.5 g/L resulted in decreased growth and hence decreased cell numbers in the culture. However, the initial Cu concentration of 7.5 g/L had the highest negative effect, decreasing the cell count by about 30 % in 9 days, prior to recovery of cell numbers to those attained on the gradual increase of Cu concentration to 7.5 g/L, indicating adaptation to Cu loading. Adaptation was also seen at higher Cu concentrations.

6.3.3 qPCR analysis

The microbial composition of the inoculum was assayed at the start and that of the STRs was assayed on day 6, 12, 19, 40, 54, 68, 82, 96, 110 and 127, using qPCR. The composition of the inoculum and the cultures at day 6, 19, 40, 68 and 96 is shown in Figure 6.16. The growth curves of the two main competitors *Acidithiomicrobium* and *Metallosphaera* species are shown in Figure 6.19 and the genomic copy numbers of all the microbial species of interest in the inoculum and in the cultures during the experiment are shown in Figure B.2 (Appendix).

The starting inoculum, as indicated in Figure 6.16, was dominated by *Metallosphaera* spp. (72 %), followed by *Acidithiomicrobium* spp. (24 %) and *Thermoplasmatales* spp. (2 %). The remaining 2 % was composed of the remaining microbial species of interest in the culture i.e. *At. caldus*, *L. ferriphilum* and *Ferroplasma acidophilum*. Similarly to Experiment 4, the two dominating and competing species in the four reactors of this experiment were *Metallosphaera* spp. and *Acidithiomicrobium* spp. whilst the other species originally present in the inoculum persisted in the cultures at low proportions throughout the experiment. The total genomic copies of *Metallosphaera* and *Acidithiomicrobium* species were almost equal to the total genomic copies of all the species in the cultures, as indicated in Figure B.2 (Appendix).

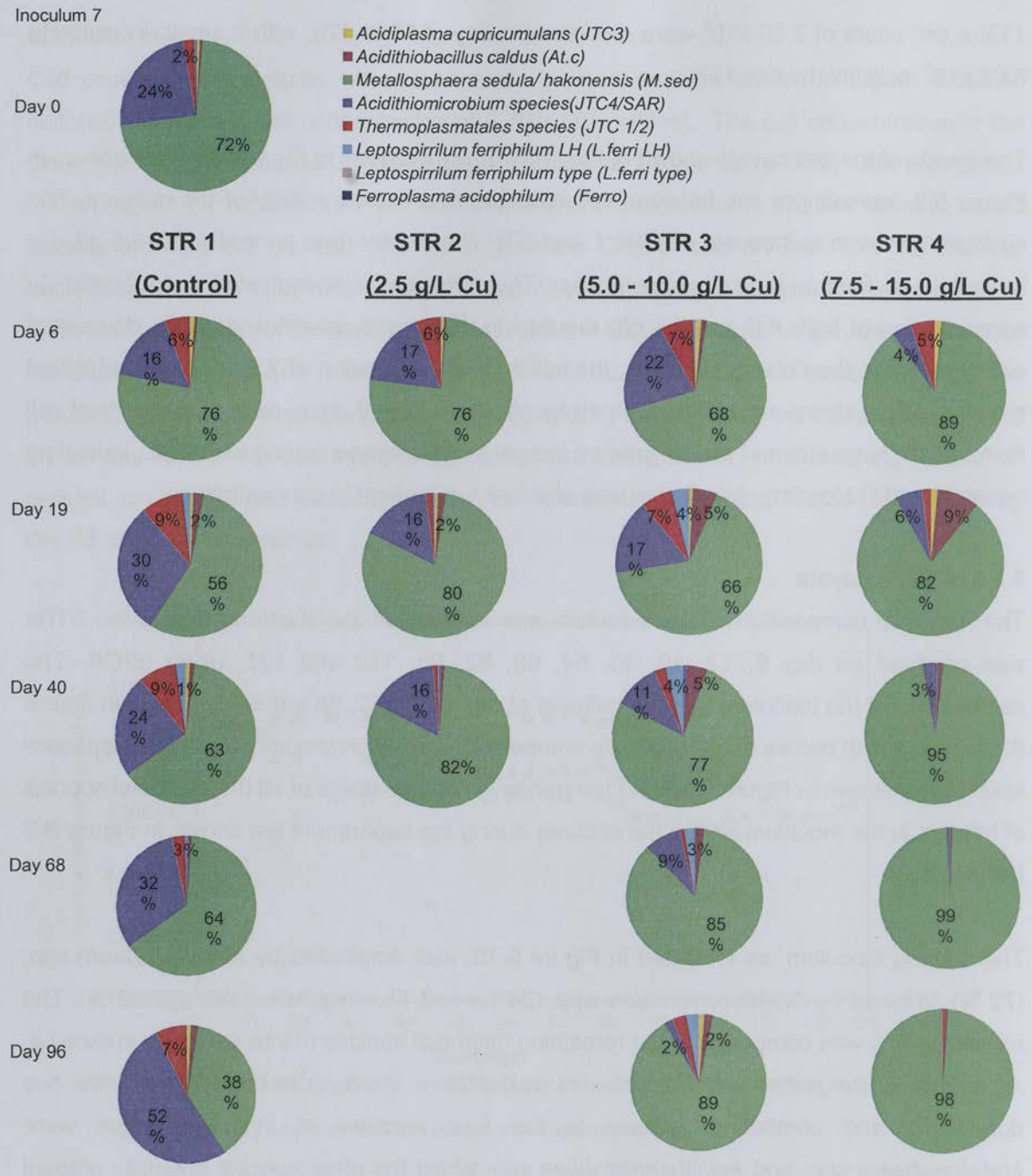


Figure 6. 16: Composition of Inoculum 7 and the four cultures in Experiment 5

In STR 1 (the Control with no additional Cu), *Metallosphaera* spp. increased to 76 % by day 6, whilst *Acidithiomicrobium* spp. decreased to 16 % and *Thermoplasmatales* spp. increased to 6 %. Thereafter, the composition of *Acidithiomicrobium* spp. increased and reached 30, 24, 32 and 52 % by day 19, 40, 68 and 96 respectively. *Metallosphaera* spp. decreased and reached 56, 63, 64 and 38 % by day 19, 40, 75 and 96 respectively. In STR 2 (fed with 2.5 g/L Cu), the microbial composition on day 6 was similar to that of the Control on the same day i.e. 76, 17 and 6 % of *Metallosphaera* spp., *Acidithiomicrobium* spp. and

Thermoplasmatales spp. respectively. By day 19, *Metallosphaera* species increased to 80 % and *Acidithiomicrobium* spp. slightly decreased to 16 %. By day 40, *Metallosphaera* spp. increased slightly again, to 82 % whilst *Acidithiomicrobium* spp. remained at 16 %. In STR 3 (initially at 5.0 g/L Cu, increasing to 7.5 g/L at day 54and 10 g/L at day 78), *Acidithiomicrobium* spp. decreased from 22 % on day 6 to 17 %, 11 %, 9 % and 2 % on day 19, 40, 68 and 96 respectively. *Metallosphaera* spp. generally increased from 68 % on day 6 to 66 %, 77 %, 85 % and 89 % on day 19, 40, 68 and 96 respectively. In STR 4 (initially at 7.5 g/L Cu, increasing to 10 g/L at day 54, 12.5 g/L at day and 15 g/L at day 113), *Acidithiomicrobium* spp. generally decreased and its composition was 4, 6 and 3 % on day 6, 19 and 40 respectively and < 1 % for the rest of the experiment. On the other hand, *Metallosphaera* spp. increased and its composition was 89, 82, 95, 99 and 98 % on day 6, 19, 40, 68 and 96 respectively.

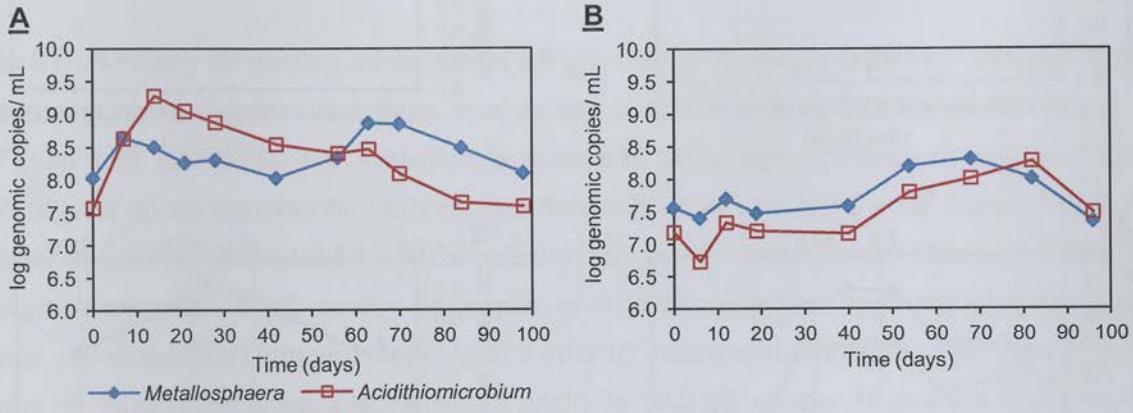


Figure 6. 17: Genomic copy numbers of *Acidithiomicrobium* and *Metallosphaera* species in STR 1 (1 % CO_2, 250 rpm) in Experiment 4 (A) and in Experiment 5 (B)

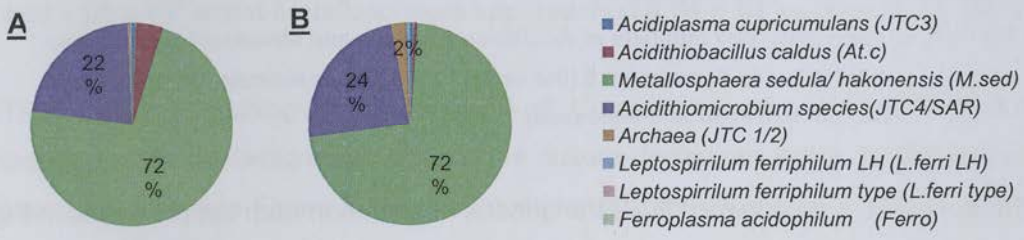


Figure 6. 18: The composition of the culture in STR 1 (1 % CO_2, 250 rpm) on the last day of Experiment 4 (A), and on the first day (Inoculum 7) of Experiment 5 (B)

The bioleaching stock culture maintained at 50 °C at CeBER, as discussed in Section 4.3, was dynamic, with variations in microbial composition. Also, as discussed in Section 6.2.3 and as shown in Figure 6.17, the composition of *Acidithiomicrobium* spp. in Inoculum 6 i.e.

STR 1 sparged with 1 % CO_2 and stirred at 250 rpm in Experiment 4, decreased from day 14 until the end of the experiment. On the last day of Experiment 4, as shown in Figure 6.18, Inoculum 6 was composed of 22 % *Acidithiomicrobium* spp. and 72 % *Metallosphaera* spp. This culture was used as Inoculum 7 for Experiment 5 and was also maintained as the Control in STR 1 fed with 1 % CO_2 and stirred at 250 rpm. Inoculum 7 was composed of 24 % *Acidithiomicrobium* spp. and 72 % *Metallosphaera* spp., a composition similar to that of Inoculum 6 at the end of Experiment 4; however, the total genomic copies of both these microbial species were lower in Inoculum 7.

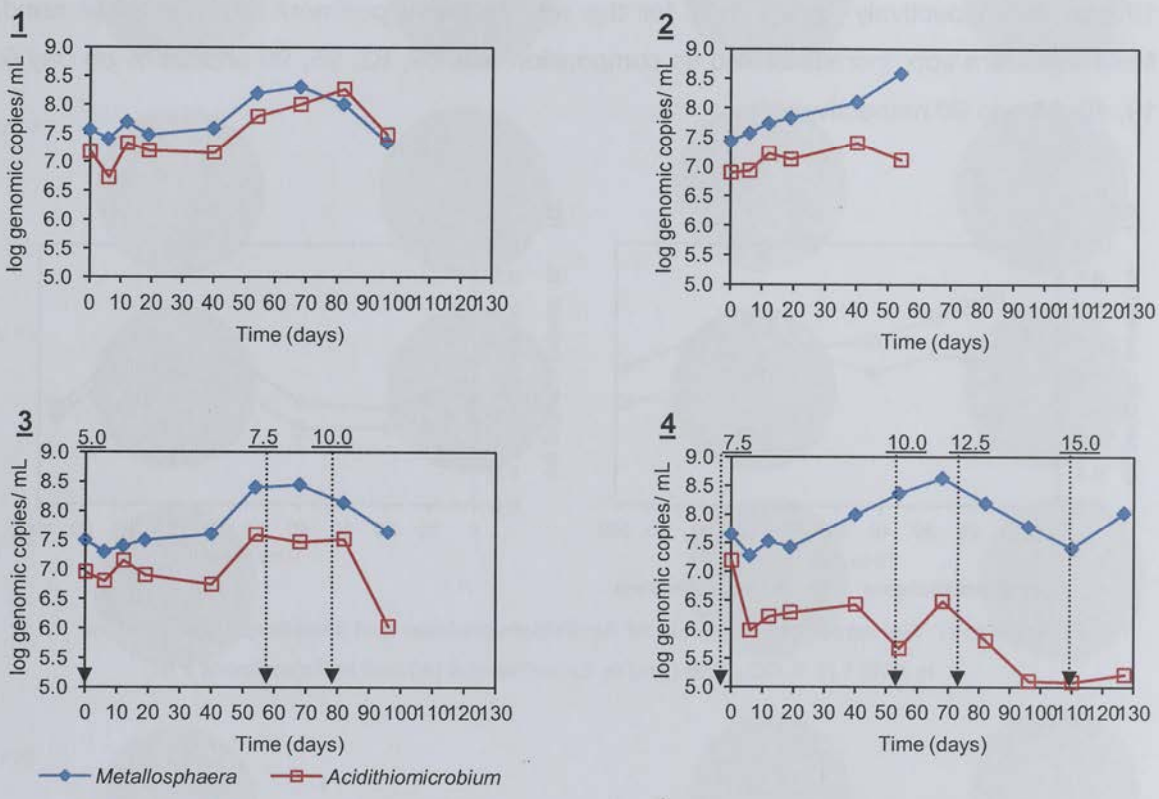


Figure 6. 19: Genomic copy numbers of *Acidithiomicrobium* and *Metallosphaera* species in STR 1 - 4 in Experiment 5 (the vertical dotted lines indicate the days when Cu concentration was increased)

In the Control (STR 1, no additional Cu) the general observation from the pie charts in Figure 6.16 was that the composition of *Acidithiomicrobium* spp. increased. However, the genomic copy numbers of *Acidithiomicrobium* spp. in STR 1, as indicated in Figure 6.17, compared to the genomic copy numbers in the previous Inoculum 6 were low for the first 40 days. *Metallosphaera* spp., although also low in numbers, dominated the Control. After day 40, the genomic copies of both *Acidithiomicrobium* spp. and *Metallosphaera* spp. in the Control started to increase and by day 84 *Acidithiomicrobium* spp. out-dominated *Metallosphaera*

spp. until the end of the experiment. However, the copy numbers of both species decreased again towards the end of the experiment.

In STR 2 fed with an additional 2.5 g/L Cu, the composition of *Acidithiomicrobium* spp. in the culture slowly decreased while *Metallosphaera* spp. slowly increased as indicated in Figure 6.16. Comparison of these two species as indicated in Figure 6.19 revealed that the genomic copies of both species increased although *Metallosphaera* spp. increased at a faster rate than *Acidithiomicrobium* spp. *Acidithiomicrobium* and *Metallosphaera* species started at 7.83 x 10^6 and 2.73 x 10^7 copies/mL, and reached 1.29 x 10^7 and 3.87 x 10^8copies/mL respectively, by day 54. Comparing the genomic copy numbers in STR 2 and in the Control indicated a possibility that the additional 2.5 g/L Cu was detrimental to the growth of *Acidithiomicrobium* spp., however, this was not consistent with STR 3 where addition of 5 g/L Cu did not affect *Acidithiomicrobium* spp. copy numbers.

In STR 3 initially containing an additional 5.0 g/L Cu and gradually raised to 10.0 g/L Cu, the composition of *Acidithiomicrobium* spp. in the culture gradually decreased as shown in Figure 6.16. Comparison of *Acidithiomicrobium* and *Metallosphaera* copy numbers in Figure 6.19 showed an increase for both species from 8.99 x 10^6 and 3.15 x 10^7 copies/mL at the start to 3.87 x 10^7 and 2.54 x 10^8 copies/mL at day 54 respectively. After increasing Cu concentration to 7.5 g/L on day 54, copies of *Acidithiomicrobium* spp. and *Metallosphaera* spp. remained little changed, reporting at 2.89 x 10^7 copies/mL and 2.80 x 10^8 copies/mL by day 68. Cu concentration was increased again, to 10.0 g/L on day 78 and this resulted in a decrease of copies of both species. *Acidithiomicrobium* spp. decreased at a more rapid rate compared to *Metallosphaera* spp. indicating that *Acidithiomicrobium* spp. was more sensitive to the high Cu concentration compared to *Metallosphaera* spp. *Acidithiomicrobium* spp. reached 1.08 x 10^6 whilst *Metallosphaera* spp. reached 4.34 x 10^7 copies/mL by day 98.

In STR 4, initially containing an additional 7.5 g/L Cu and gradually raised to 15.0 g/L Cu, the composition of *Acidithiomicrobium* spp. in the culture decreased whilst *Metallosphaera* spp. increased as shown in Figure 6.16. A comparison of genomic copy numbers of these two species, shown in Figure 6.19, revealed that the starting Cu concentration of 7.5 g/L drastically reduced the gDNA concentration of *Acidithiomicrobium* spp. whilst *Metallosphaera* spp. flourished in this Cu concentration. The genomic copies of *Acidithiomicrobium* spp. decreased from 1.54 x 10^7 at the start and reached 9.48 x 10^5 copies/mL by day 6. After day 6, ignoring the sudden decrease on day 54 which was possibly an outlier (see explanation below), the genomic copies of *Acidithiomicrobium* spp. gradually increased until reaching 3.04 x 10^6 copies/mL on day 68. The increase of Cu

concentration to 10.0 g/L on day 54 did not deter the gradual increase of *Acidithiomicrobium* spp. indicating a possible adaptation of this microbial species to an increasing Cu concentration. (It should be noted that on day 54, the Cu was increased after a sample for qPCR analysis had already been collected. Therefore the sudden decrease of *Acidithiomicrobium* spp. on day 54 occurred in the 7.5 g/L Cu concentration and this decrease was possibly an outlier considering the general trend). On day 78, the Cu was increased to 12.5 g/L and this resulted in a further drastic decrease of *Acidithiomicrobium* spp. copy numbers to 1.27 x 10^5 copies/mL on day 96 and thereafter the genomic copies of *Acidithiomicrobium* spp. stayed low until the end of the experiment.

6.3.4 Discussion of the results from Experiment 5

Overall, the results indicated that *Acidithiomicrobium* spp. was sensitive to an additional 2.5 g/L Cu although a Cu concentration up to 5.0 g/L had a lower negative effect compared to 7.5 g/L Cu. Other test work done on *Acidithiomicrobium* spp. indicate that this species is generally more sensitive to Cu compared to other bioleaching acidophiles (Dew, 2012 unpublished data). On day 20 of the experiment the amount of *Acidithiomicrobium* spp. in initial Cu concentration of 2.5, 5.0 and 7.5 g/L was shown to have been reduced by 17 %, 50 % and 88 %, respectively. The amount of *Acidithiomicrobium* spp. was observed to recover, up to 5 g/L Cu but at higher Cu concentration this species was not shown to recover until the experiment was stopped. *Metallosphaera* spp. generally thrived well in the starting Cu concentration of 7.5 g/L probably because it is a metal tolerant species (Huber *et al.*, 1989; Dopson *et al.*, 2003; Schippers, 2007).

The similarity of physicochemical parameters (particularly Eh and pH) and the similarity of cell counts between STR 1 (the Control fed no additional Cu) and STR 2 (fed with additional 2.5 g/L Cu) indicated that the additional 2.5 g/L of Cu had a minimal effect on the overall cell numbers and overall iron oxidising capability of the mixed culture. The Eh, pH and cell counts in STR 3 (initially at 5.0 g/L and gradually raised to 10.0 g/L Cu) and in STR 4 (initially at 7.5 g/L and gradually raised to 15.0 g/L Cu) indicated that an initial Cu concentration of both 5.0 and 7.5 g/L negatively affected the culture. The initial Cu concentration of 7.5 g/L was shown to have the highest negative effect with large reductions in the cell counts and the Eh, probably because the micro-organisms were shocked by the sudden increase of Cu. However, after increasing the Cu concentration, the Eh recovered and cell counts increased towards their original values, up to 15.0 g/L, the highest additional Cu concentration tested. This could be an indicator that the culture was adapting to the increasing Cu in the medium although qPCR indicated that not all microbial species in the culture adapted. Soluble Fe measurements also indicated that the cultures in lower Cu

concentration (STRs 1 and 2) were more actively oxidising pyrite and/or chalcopyrite concentrates in the medium than the cultures in higher Cu (STRs 3 and 4). The evidence of adaptation is supported by the fact that, often, microbial cultures are adapted to a particular ore feed in a laboratory or pilot plant so that potentially inhibitory agents in the feed can select for organisms able to oxidise Fe and sulphur in the presence of these agents e.g. the tolerance to arsenic has been increased under selective pressure in CSTRs and mercuric ions select for strains that volatilize mercury by mercuric reductase (Olson *et al.*, 1982). Aston *et al* (2010) also confirmed the possibility of adaptation by pre-adapting *At. caldus* cultures in Pb, Zn and Cu. After adaptation, the cultures respectively had growth rates 39, 32 and 28 % higher in the presence of Pb, Zn, and Cu compared to unadapted cultures.

Metal tolerance varies significantly between microbial species and between strains of the same species and this is likely due to adaptation to different levels of metal exposure (Rawlings, 2005; Jerez, 2011). Microbial analysis also revealed that the quantity of the dominating microbial species influence the overall growth curve of a mixed culture. Therefore when a mixed microbial culture seems to be adapting to a certain physicochemical pressure, not all the species in the culture necessarily adapt similarly but a one or more species may be selected and dominate the culture.

The observations from this experiment generally agree with observations made in Experiment 3 (Section 4.4) which tested the effect of Cu concentration on microbial growth on whole ore. The inoculum used in this experiment had a similar microbial composition to the inoculum used in Experiment 3. Microbial analysis from Experiment 3 showed that Cu concentration up to 5 g/L negatively affected cell numbers to a lower extent compared to 10.0 and 15.0 g/L Cu. By day 27 of Experiment 3 the copy numbers of *Acidithiomicrobium* spp. on the ore in 5.0, 10.0 and 15.0 g/L Cu were 7.2 %, 0.6 % and 0 % of the genomic copy numbers in the Control, respectively.

7. CONCLUSIONS AND RECOMMENDATIONS

This chapter presents the conclusions from the two parts of this study in light of the key questions raised during literature review, and in light of the objectives and hypotheses of the study. Recommendations for future research related to this study are also presented in this chapter in light of the challenges encountered during this study and/or the short-comings of the experimental set-ups in this study.

7.1 Conclusions

The combination of cell counts and qPCR analysis were shown to be sufficient as tools for investigating microbial community dynamics in both heap and tank bioleaching because the total gDNA extracted from microbial cells was proportional to cell numbers in the samples.

Microbial growth on the ore was observed to be a batch-type of growth. The cell numbers in the ore reached a plateau but the composition of the microbial community attached to the ore changed over time because the microbial species were competing, some becoming dominant and others receding as environmental conditions were altered. In the column bioleach environment on whole, agglomerated low grade ore, the highest ore-associated cell numbers were initially observed in the interstitial phase but, over time, more micro-organisms attached firmly to the ore and/or the microbial cells already attached to the ore multiplied in the EPS. Therefore, with time, cell numbers increased in the weakly and strongly attached phases. Substantial attachment of the major microbial species to the ore had taken place within 6 days of inoculation. Attachment was quick because the microbial species had been adapted to chalcopyrite and pyrite, the major sulphide components of the ore used in this study. Microbial growth was promoted because the environmental conditions provided in these columns were representative of the moderate temperature range of the heap and allowed the proliferation of the moderately thermophilic microbial species selected for inoculation. However, these conditions were not optimal for all the species, hence different growth rates were observed between the microbial species. Ferrous iron oxidation rates by the attached cells increased over time owing to an increased composition of iron-oxidisers in the microbial community colonising the ore.

In order to guide and evaluate the study, the following set of hypotheses was formulated:

1. The growth rates and dominance of the microbial species on whole ore are not affected by using different compositions of inocula, owing to these being influenced most strongly by the physicochemical environment.

2. The changes in the physicochemical environment of the culture affect the microbial species to different extents such that the dominance changes, owing to the differing optimal physicochemical conditions required for different species.

3. Different copper concentrations affect the microbial species to different extents such that the dominance changes in both the whole ore and the liquid culture, owing to the differing critical inhibition concentrations for different species.

4. Similar copper concentrations affect the dominance of microbial species similarly in both the whole ore and the liquid culture, owing to the same critical concentrations for the same species regardless of the bioleach system.

The conclusions drawn from the study are presented in light of the key questions and the under-lying hypotheses below.

7.1.1 Selective attachment and growth of microbial species to the ore at 50 °C

All the four major microbial species detected in the inoculum i.e. *Acidithiobacillus caldus, Acidiplasma cupricumulans, Acidithiomicrobium* spp. and *Metallosphaera* spp., attached to the ore albeit at different rates. *At. caldus* dominated the columns in the earlier stages of the experiment, suggesting that in mixed cultures in an environment with both ferrous iron and reduced sulphur substrates, sulphur-oxidisers have a competitive advantage over iron-oxidisers. However, *Acidithiomicrobium* and *Metallosphaera* species took over later and dominated the columns indicating that microbial species that are both iron- and sulphur-oxidisers finally become dominant over the sulphur-oxidisers in the bioleach heap system over time.

The different growth rates of the microbial species determined the dominances in the ore. The conditions in the columns affected the growth rates of the microbial species differently because the conditions were optimal for some species giving them an advantage over the species whose optimal conditions were not met. The metabolic characteristics of the individual microbial species also affected the growth rates and dominance. The growth rates of individual microbial species were not constant throughout the experiment but differed according to the response of the micro-organisms to the changing physicochemical and biological environment in the ore. The maximum specific growth rates obtained in this study (0.0177 h^{-1}, 0.0453 h^{-1}, 0.0413 h^{-1} and0.0248 h^{-1} for *A. cupricumulans, At. caldus, Metallosphaera* spp. and *Acidithiomicrobium* spp. respectively) were an under-estimation because of transport of microbial cells from the ore to the PLS. These growth rates could be

not compared with literature values because of the scarcity of published data on growth rates in the whole ore environment typical of bioleach heap environments.

7.1.2 Dependence of the growth rates and dominance of the attached micro-organisms on inoculum composition used

Different compositions of the inoculum were shown not to affect the resultant dominance on the whole ore, provided the microbial species present in the different inocula were similar. This suggests that ore-associated communities develop based on the conditions within the ore-bed rather than being controlled by direct attachment of the community provided. Furthermore, it was shown that, in inocula containing similar microbial species, adaptation of the inoculum prior to addition to the ore-bed is more important than the numbers of the individual species added. The state of adaptation of the inocula affects the growth rates of the individual microbial species and therefore alters the dynamics of the resultant community and associated dominances.

7.1.3 The effect of copper concentration in solution on the growth rates and dominance of the attached micro-organisms in the ore-bed and those in liquid culture

Substantial growth and attachment of micro-organisms took place in the column experiments where the irrigation solution contained up to 5.0 g/L Cu. At concentrations above 5.0 g/L Cu, the attachment was impaired and was minimal; the micro-organisms could not attach to the ore nor did their concentration build up in the interstitial fluid. Instead, these micro-organisms were largely washed out with the flowing PLS. It is noted that the inoculum used in this study was pre-adapted to growth on pyrite and chalcopyrite, but not pre-adapted to Cu and therefore could not tolerate a high Cu concentration.

To better understand the effect of Cu in both heap leaching and in the preparation of inocula, the effect was studied in submerged culture in a stirred tank reactor. *Acidithiomicrobium* spp. was shown to be sensitive to an additional 2.5 g/L Cu although a Cu concentration up to 5.0 g/L had a lower negative effect compared to 7.5 g/L Cu. On day 20 of the experiment, the cell number of *Acidithiomicrobium* spp. at a Cu concentration of 2.5, 5.0 and 7.5 g/L was shown to have been reduced by 17 %, 50 % and 88 %, respectively. The *Acidithiomicrobium* spp. copy number was observed to recover its original value, up to 5 g/L Cu. At higher Cu concentrations, this species did not recover the copy number prior to Cu addition, over the duration of the experiment. Also, the Cu concentration up to 5.0 g/L was shown to have a lower negative effect on the overall cell numbers and overall iron oxidising capability of the mixed culture compared to an initial additional Cu concentration of 7.5 g/L. The concentration of 7.5 g/L Cu was detrimental to the culture, decreasing the cell count by

about 30 % in 9 days probably because the micro-organisms were shocked by the sudden increase of Cu. On increasing the Cu concentration, up to 15.0 g/L, the final counts in mixed culture was reduced only slightly compared with 7.5 g/L, probably due to adaption to the increasing Cu. Microbial analysis revealed that the quantity of the dominating microbial species influenced the overall growth curve of a mixed culture. Therefore the adaptation of a culture could be due to some species which were selected for and grew well to the point of dominating the cultures.

The inocula used to investigate the effect of copper concentration had similar microbial species present for the whole ore and the liquid culture experiments. In both experiments a Cu concentration up to 5 g/L was shown to have a lower negative effect on the overall cell counts and oxidation capability of the micro-organisms whilst a concentration beyond 5.0 g/L Cu was detrimental to the overall performance of the micro-organisms. However, the liquid culture was able to adapt to the high Cu probably because of agitation and sparging which generally improve mass transfer of substrates thereby improving growth rates of microbial species. Also, the column is an open flow through system, therefore cells could be washed out quickly if not attached whereas in the tank, only a small amount of cells is removed when sub-culturing allowing the culture to recover and adapt much more easily.

7.1.4 Response of *Acidithiomicrobium* species, a key Fe- and S-oxidising moderate thermophile, to availability of carbon dioxide and to agitation speed in liquid culture

The carbon dioxide concentration in normal air was shown not to be limiting to the growth of *Acidithiomicrobium* species in a 1-litre tank at an agitation speed of 250 rpm i.e. sparging with air enriched to 1 % CO_2 was not beneficial at low agitation speed. However, further test work is required, particularly the determination of the critical dissolved CO_2 concentration required in the culture to support the maximum growth rate, to confirm this conclusion.

Higher agitation speed (i.e. 550 rpm in the 1-litre tank) was detrimental to *Acidithiomicrobium* spp. in the mixed culture, leading to decreased copy numbers of *Acidithiomicrobium* spp. while growth of *Metallosphaera* spp. was supported. This may be attributed either to shear, attrition or because high agitation improved mass transfer thereby availing certain substrates to other microbial species which were more efficient in their use than *Acidithiomicrobium* spp. e.g. *Metallosphaera* spp. Agitation was thus recognised as an important criterion for design of inoculum development processes. To optimise this, additional studies to clarify the driving mechanism through which agitation influences the growth of *Acidithiomicrobium* spp. is recommended.

7.2 Recommendations

For future research related to this study, the recommendations are:

1. Temperature should be more strictly controlled such that there is a uniform temperature profile in the columns. This can be achieved by either designing new columns or by improving the operation of the current columns. Some of the suggestions would be to heat the irrigation solution for example in a water bath to 50 °C, pack the columns with less ore, use heating jackets that cover the whole of each column and also use a different material that is more heat insulating than marbles in the column packing.

2. Ore samples must be collected more frequently so that important data points may not be missed. This is necessary because the logistics do not allow for the samples to always be tested on the day of sampling, and therefore qPCR results are obtained later hence there is no on-time tracking of the microbial community changes taking place in the columns. Since in the column test work it is not desirable to disturb the ore bed in the duration of the experiment; this suggestion can only be implemented by setting up more columns to take down during the duration of the experiment.

3. An experiment similar to Experiment 3 should be run with Cu concentrations at closer intervals e.g. with differences of 2.5 g/L similar to the intervals used in Experiment 5. This would enable a clearer determination of the inhibitory copper concentration in the bioheap system. Such an experiment should also be run longer so as to assess whether micro-organisms in the ore adapt to the increased Cu concentration.

4. An experiment in the liquid culture should be run similarly to Experiment 4 and 5 but without sub-culturing. This would enable calculation of growth rates of microbial species in the mixed liquid culture, and also allow the culture to reach stationary phase hence allowing for an assessment of which microbial species are better competitors in the batch type of set-up.

In addition to improving the experimental design, the findings from the study have raised a new selection of questions. To address these, the following recommendations are put forward:

1. While it is clear that CO_2 is not limiting in the tank system as operated in these experiments, it would be useful to determine the critical dissolved CO_2 concentration

required to support growth in this system and to investigate whether this varies across species, as demonstrated for *At. ferrooxidans* and *L. ferriphilum* (Bryan *et al.*, 2012).

2. Bio-stimulation by adding some yeast extract into the bioleach system should be considered to boost the growth of mixotrophs such as *Acidithiomicrobium* spp. However, this should be weighed up against the possibility of the yeast extract becoming detrimental to the growth of other microbial species that are sensitive to organics.

3. Moderate thermophiles operate at a temperature range of 40 to 60 °C, however it is noted at 50 to 55 °C there is less biodiversity and activity of acidophiles (Franzmann *et al.*, 2005; du Plessis *et al.*, 2007). It would be useful to determine how a temperature slightly higher than 55 °C would influence the outcome of the study.

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APPENDICES

Appendix A: Analytical procedures

A1. Colometric assay of ferrous iron concentration

A1.1 Reagents

Ammonium acetate buffer solution (AABS): This solution was prepared by dissolving 250 g of ammonium acetate in 150 mL de-ionised water and adding 700 mL glacial acetic acid.

1,10-phenanthroline indicator: This indicator was prepared by adding 2127.708 mg of 1,10-phenathroline monohydrate into 800 mL of de-ionised water and leaving stirring overnight to dissolve then making up to 1000 mL with de-ionised water. This gave a phenanthroline concentration in excess of the stoichiometric requirement.

A1.2 The iron/absorbance standard curve

A series of standard solutions i.e. 5, 10 and 20 mg/L, was prepared by diluting the 1000 mg/L Fe stock solution (Merck, South Africa) with de-ionised water. The standard solutions were treated as described in Section 3.6.1.3 for Fe^{2+} assay except that a scoop of hydroxylamine was added into the test tubes right from the start. The test-tubes were vortexed and left to stand for 5 minutes before measuring the absorbance. A standard curve shown in Figure A.1 was constructed from the measured absorbance.

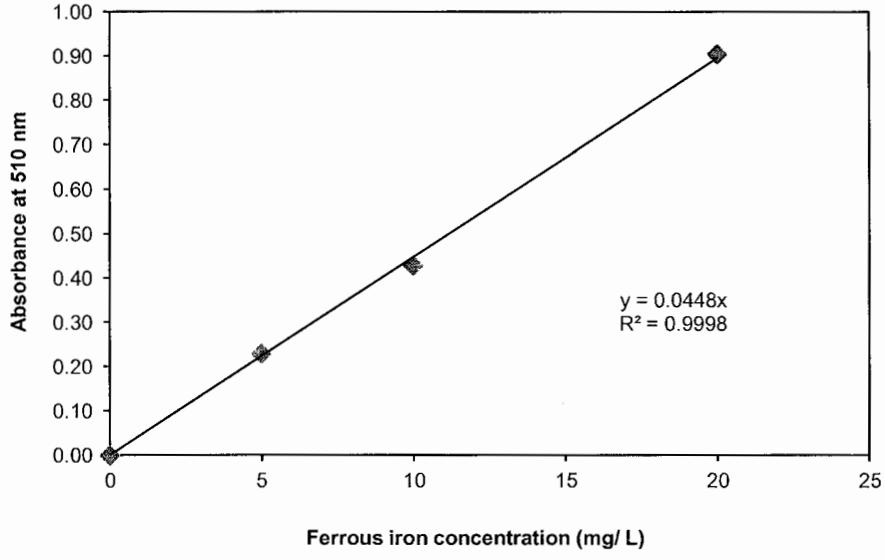

Figure A. 1: The standard curve for the 1,10-phenanthroline assay of Fe^{2+} and Fe^{tot}

A1.3 Calculation of Fe^{2+} concentration in the PLS

The Fe^{2+} concentration in the samples was calculated from the standard curve (Figure A.1) e.g. if a sample that was diluted 100 times had an absorbance of 0.972;

$$\text{The } Fe^{2+} \text{ concentration} = \frac{0.972}{0.0448} \times 100$$

$$= 2170 \text{ mg/L}$$

A1.4 Calculation of metal (Cu and Fe) extracted from the ore

The amount of Cu and Fe metal extracted from the ore was calculated from the concentration of the metal in the PLS, obtained from the AAS assay e.g.

$[Cu]_{PLS}$ from the AAS assay = 340 ppm

Volume of the overnight PLS = 2.00 L

$$\text{[Cu] per 5 kg ore} = \frac{340 \text{ mg}}{\text{L}} \times 2.00 \text{ L}$$

$$= 680 \text{ mg (0.68 g)}$$

A2. Cell count

A2.1 Cell count calculations

Depth of THOMA chamber = 0.02 mm

Width of one small square = 0.05 mm

Volume of one small square = depth x area = 0.02 mm x (0.05 mm x 0.05 mm)

$$= 5 \times 10^{-5} \text{ mm}^3 \ (5 \times 10^{-8} \text{ mL})$$

Number of small squares counted = 64

Volume of small squares counted = 64 x (5×10^{-8} mL) = 0.0000032 mL

Number of cells counted in the 64 squares = n

$$\text{Cell concentration (cells/mL)} = \text{dilution factor} \times \frac{\text{n}}{0.0000032 \text{ mL}} \times 1 \text{ mL}$$

$$= \text{dilution factor x n x } 312500$$

A2.2 Calculation of the required amount of inoculum for the columns

Cell count of the inoculum = m cells/mL

Required amount of cells = 3.00E+09 cells/ton ore

Amount of ore per column = 5 kg

$$\text{Required amount of inoculum per column} = \frac{3.00\text{E+09 cells}}{\text{ton}} \times \frac{\text{ton}}{1000\text{kg}} \times \frac{1\text{mL}}{\text{m cells}} \times 5 \text{ kg}$$

$$= \frac{1.5\text{E+07}}{\text{m}} \text{ mL}$$

A3. Quantitative real-time polymerase chain reaction (qPCR)

A3.1 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 (inoculum, PLS, detached solution) filtered for DNA extraction = 100 mL

Amount of ore per column = 5 kg

% dry weight of the ore = 0.800

Amount of ore sample detached = 500 g

A3.1.1 Calculation of gDNA concentration

$$\text{Concentration of gDNA in the sample} = \frac{\beta \frac{\text{ng}}{\mu\text{L}} \text{ x } 50\mu\text{L}}{100 \text{ mL}}$$

$$= 0.5\beta \text{ ng/mL sample}$$

$$\text{Concentration of gDNA in the ore} = \frac{\beta \frac{\text{ng}}{\mu\text{L}} \text{ x } 50\mu\text{L}}{500\text{g x } 0.800} \text{ x } \frac{1000\text{g}}{\text{kg}}$$

$$= 125\beta \text{ ng/kg dry ore}$$

A3.1.2 Calculation of genomic copy numbers

If for an example, for *Acidithiobacillus caldus* (Atc);

Number of 16S rRNA genes of Atc per ω ng of gDNA analysed = 5.00E+06 genes

Gene dosage of *Acidithiobacillus caldus* = 1 gene per genomic copy

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

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

$$\text{Genomic copy number of Atc in the ore} = \frac{5.00\text{E+06 genes x } 125\beta \frac{\text{ng}}{\text{kg dry ore}}}{\omega \text{ ng x } \frac{1 \text{ gene}}{\text{genomic copy}}}$$

$$= \frac{2.50\text{E+08 } \beta}{\omega} \text{ genomic copies/kg dry ore}$$

Appendix B: Raw data

Table B. 1: pH, redox potential and Eh measurements in the PLS of Experiment 1

Date	Time	Days	pH									Redox potential (mV)									Eh (mV)								
			2	3	4	5	6	7	8	9	10	2	3	4	5	6	7	8	9	10	2	3	4	5	6	7	8	9	10
2011-04-05	7:45	0.00																											
2011-04-06	8:00	1.01	1.935	1.850	1.950	2.045	2.038	1.981	1.947	1.968	2.013	363	356	363	358	357	359	354	354	358	570	563	570	565	564	566	561	561	565
2011-04-07	7:30	1.99	1.930	2.159	1.970	2.054	2.022	1.973	1.935	1.952	2.003	364	353	361	358	362	361	365	357	361	571	560	568	565	569	568	572	564	568
2011-04-08	7:30	2.99	1.671	1.766	1.728	1.827	1.820	1.809	1.804	1.890	1.833	366	358	362	361	363	364	366	363	362	573	565	569	568	570	571	573	570	569
2011-04-09	10:30	4.11	1.841	1.947	1.855	1.948	1.904	1.843	1.821	1.865	1.911	370	363	368	365	368	365	370	374	367	577	570	575	572	575	572	577	581	574
2011-04-11	7:45	6.00	1.827	1.911	1.832	1.910	1.865	1.808	1.786	1.824	1.867	366	360	382	393	370	366	421	389	370	573	567	589	600	577	573	628	596	577
2011-04-12	7:00	6.97	1.858	1.919	1.888	1.930	1.935	1.872	1.874	1.906	1.908	368	368	427	430	411	407	463	436	388	575	575	634	637	618	614	670	643	595
2011-04-13	7:45	8.00	1.864	1.871	1.887		1.995	2.004	1.89	1.884	1.891	371	388	447		454	470	483	473	406	578	595	654		661	677	690	680	613
2011-04-14	9:15	9.06	1.796	1.817	1.785		1.824	1.905	1.927	1.770	1.799	379	425	472		484	504	508	498	423	586	632	679		691	711	715	705	630
2011-04-15	7:00	9.97	1.968	2.006	1.930		1.949	2.031	2.068	2.000	1.968	393	448	503		504	516	520	509	436	600	655	710		711	723	727	716	643
2011-04-16	10:30	11.11	1.821	1.876			1.743	1.871	1.920	1.896	1.845	409	465			521	524	532	516	446	616	672			728	731	739	723	653
2011-04-18	7:00	12.97	1.856	1.881			1.692	1.831	1.863	1.871	1.835	443	492			536	532	552	524	457	650	699			743	739	759	731	664
2011-04-19	7:40	14.00	1.883	1.885			1.695	1.833		1.881	1.857	467	509			539	538		532	461	674	716			746	745		739	668
2011-04-20	6:40	14.95	1.958	1.951			1.671	1.909		1.940	1.959	488	521			541	556		538	468	695	728			748	763		745	675
2011-04-21	7:40	16.00	1.877	1.819			1.647			1.814	1.854	483	524			543			545	476	690	731			750			752	683
2011-04-23	11:00	18.14	1.935	1.861			1.691			1.914	1.845	528	539			556			539	535	735	746			763			746	742
2011-04-25	10:30	20.11	1.835	1.927			1.751			1.787	1.800	605	532			592			560	567	812	739			799			767	774
2011-04-27	8:30	22.03	1.746	1.878						1.743	1.732	605	544						569	588	812	751						776	795
2011-04-28	7:00	22.97	1.811	1.963						1.820	1.814	604	554						575	595	811	761						782	802
2011-04-30	8:30	25.03	1.711	1.868						1.714	1.699	612	582						606	613	819	789						813	820
2011-05-02	10:30	27.11	1.689	1.782						1.700	-	607	578						588		814	785						795	
2011-05-03	8:00	28.01	1.708	1.762						1.702	-	606	585						584		813	792						791	
2011-05-04	8:00	29.01	1.681	1.741						1.679	-	612	593						585		819	800						792	
2011-05-05	7:30	29.99	1.734	1.750						1.682	-	610	609						593		817	816						800	
2011-05-06	7:45	31.00	1.755							1.697	-	606							602		813							809	
2011-05-08	8:45	33.04	1.764							1.703	-	613							618		820							825	
2011-05-09	7:25	33.99	1.749							1.739	-	607							625		814							832	

Table B. 2: Fe^{2+} and Fe^{tot} measurements in the PLS of Experiment 1

Days	Abs (for Fe^{2+})									Fe^{2+} (mg/L)									$Fe^{tot.}$ AAS (mg/L)								
	2	3	4	5	6	7	8	9	10	2	3	4	5	6	7	8	9	10	2	3	4	5	6	7	8	9	10
0.00																											
1.01	0.511	0.408	0.481	0.476	0.513	0.511	0.539	0.511	0.529	567.8	453.3	534.4	528.9	570.0	567.8	598.9	567.8	587.8	565.6	534.3	568.6	569.6	579.7	590.9	633.3	596.9	589.8
1.99	0.533	0.568	0.532	0.568	0.568	0.600	0.614	0.568	0.589	592.2	631.1	591.1	631.1	631.1	666.7	682.2	631.1	654.4	706.0	650.4	642.4	655.2	688.8	693.9	705.0	678.7	712.1
2.99	0.555	0.600	0.541	0.606	0.617	0.629	0.620	0.593	0.613	616.7	666.7	601.1	673.3	685.6	698.9	688.9	658.9	681.1	723.2	761.5	677.7	725.2	746.4	821.1	826.2	746.4	766.6
4.11	0.540	0.520	0.554	0.565	0.610	0.628	0.601	0.571	0.565	600.0	577.8	615.6	627.8	677.8	697.8	667.8	644.1	627.8	642.4	637.3	602.0	653.5	717.1	756.5	687.8	677.7	626.2
6.00	0.477	0.487	0.494	0.469	0.577	0.563	0.402	0.567	0.500	530.0	541.1	548.9	521.1	641.1	625.6	446.7	630.0	555.6	612.1	566.6	590.9	607.0	668.6	722.2	529.2	736.3	598.9
6.97	0.457	0.434	0.390	0.341	0.488	0.470	0.211	0.400	0.397	507.8	482.2	433.3	378.9	542.2	522.2	234.4	444.4	441.1	589.8	581.8	635.3	331.3	722.2	672.7	633.3	733.3	601.0
8.00	0.504	0.496	0.340	-	0.301	0.151	0.098	0.180	0.436	560.0	551.1	377.8		334.4	167.8	108.9	200.0	484.4	605.0	610.0	639.3		609.0	548.4	544.4	578.7	506.0
9.06	0.528	0.420	0.209	-	0.130	0.062	0.051	0.081	0.406	586.7	466.7	232.2		144.4	68.9	56.7	90.0	451.1	605.0	614.1	604.0		559.5	425.2	494.9	510.1	565.6
9.97	0.486	0.308	0.065	-	0.070	0.041	0.026	0.044	0.341	540.0	342.2	72.2		77.8	45.6	28.9	48.9	378.9	664.6	645.4	561.6		566.6	615.1	451.5	492.9	608.0
11.11	0.433	0.217	-	-	0.067	0.160	0.161	0.259	0.307	481.1	241.1			74.4	177.8	178.9	287.8	341.1	643.4	637.3			657.5	584.8	471.7	522.2	647.4
12.97	0.284	0.098	-	-	0.035	0.034	0.027	0.033	0.243	315.6	108.9			38.9	37.8	30.0	36.7	270.0	593.9	616.1			788.8	641.4	532.3	544.4	609.0
14.00	0.221	0.049	-	-	0.057	0.032	-	0.028	0.220	245.6	54.4			63.3	35.6		31.1	244.4	532.3	559.5			909.0	692.9		581.8	682.8
14.95	0.043	0.016	-	-	0.019	0.009	-	0.015	0.021	47.8	17.8			21.1	10.0		16.7	23.3	557.5	559.5			880.2	717.1		587.8	628.2
16.00	0.139	0.026	-	-	0.019	-	-	0.023	0.207	154.4	28.9			21.1			25.6	230.0	572.7	402.0			862.5			505.0	657.5
18.14	0.038	0.025	-	-	0.021	-	-	0.037	0.034	42.2	27.8			23.3			41.1	37.8	592.9	493.9			673.7			537.3	710.0
20.11	0.007	0.122	-	-	0.258	-	-	0.318	0.006	7.8	135.6			286.7			353.3	6.7	506.0	522.2			621.2			876.7	592.9
22.03	0.012	0.018	-	-	-	-	-	0.020	0.008	13.3	20.0						22.2	8.9	544.4	483.8						966.6	542.4
22.97	0.000	0.000	-	-	-	-	-	0.000	0.000	0.0	0.0						0.0	0.0	645.4	429.3						927.2	584.8
25.03	0.005	0.000	-	-	-	-	-	0.004	0.004	5.6	0.0						4.4	4.4	666.6	402.0						838.3	695.9
27.11	0.011	0.024	-	-	-	-	-	0.064	-	12.2	26.7						71.1		651.5	589.8						882.7	
28.01	0.008	0.010	-	-	-	-	-	0.013	-	8.9	11.1						14.4		637.3	794.9						956.5	
29.01	0.004	0.021	-	-	-	-	-	0.013	-	4.4	23.3						14.4		633.3	861.5						970.6	
29.99	0.002	0.012	-	-	-	-	-	0.010	-	2.2	13.3						11.1		669.6	768.6						950.4	
31.00	0.020	-	-	-	-	-	-	0.013	-	22.2							14.4		863.6							968.6	
33.04	0.020	-	-	-	-	-	-	0.012	-	22.2							13.3		753.5							796.9	
33.99	0.000	-	-	-	-	-	-	0.057	-	0.0							63.3		725.2							780.7	

Table B. 3: Effluent volume and Cu measurements in the PLS of Experiment 1

Days	PLS volume (L)									Cu, AAS (mg/L)									Cu (g/5 kg ore)								
	2	3	4	5	6	7	8	9	10	2	3	4	5	6	7	8	9	10	2	3	4	5	6	7	8	9	10
0.00																											
1.01	1.00	1.04	0.85	1.10	0.79	0.90	0.93	1.00	0.90	143.0	139.0	180.8	154.5	165.4	178.4	182.3	166.2	175.8	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.2
1.99	1.23	1.26	1.34	1.02	0.98	1.11	1.15	1.25	1.25	8.3	72.7	69.7	90.3	86.8	84.8	83.1	80.0	79.5	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
2.99	1.24	1.25	1.33	1.02	0.97	1.10	1.15	1.24	1.24	57.2	66.7	62.2	76.9	72.2	71.4	70.2	66.7	62.7	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
4.11	1.38	1.40	1.50	1.12	0.99	1.22	1.26	0.15	1.37	47.0	53.0	50.2	61.8	63.1	59.4	55.9	50.4	53.4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1
6.00	2.29	2.13	2.26	1.85	1.65	2.05	2.09	2.05	2.30	42.1	48.2	48.8	57.9	61.7	58.6	57.0	75.7	49.4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1
6.97	1.16	1.17	1.20	0.94	0.81	1.03	1.07	1.08	1.15	52.2	60.0	68.0	46.2	91.6	76.4	75.5	91.3	60.4	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1
8.00	1.42	1.39	1.44		0.91	1.28	0.64	1.33	1.42	54.8	66.2	104.2		148.6	116.6	103.0	122.6	55.9	0.1	0.1	0.2		0.1	0.1	0.1	0.2	0.1
9.06	1.35	1.30	1.02		0.72	1.21	0.91	1.25	1.34	45.9	57.0	88.4		167.6	126.5	185.4	136.0	58.9	0.1	0.1	0.1		0.1	0.2	0.2	0.2	0.1
9.97	1.15	1.03	1.24		0.63	1.01	0.80	1.05	1.14	51.3	75.8	119.8		177.1	135.6	150.9	121.6	31.7	0.1	0.1	0.1		0.1	0.1	0.1	0.1	0.0
11.11	1.37	1.34			0.79	1.22	0.96	1.27	1.37	50.8	72.5			215.9	190.6	149.9	169.0	63.8	0.1	0.1			0.2	0.2	0.1	0.2	0.1
12.97	2.21	2.18			1.38	1.97	1.56	2.07	2.23	53.9	73.9			207.5	148.5	171.6	138.9	59.8	0.1	0.2			0.3	0.3	0.3	0.3	0.1
14.00	1.37	1.35			0.96	1.24		1.28	1.36	25.2	42.0			213.0	146.0		146.7	56.9	0.0	0.1			0.2	0.2		0.2	0.1
14.95	1.29	1.28			1.00	1.17		1.21	1.29	50.3	70.2			210.0	147.0		133.4	60.9	0.1	0.1			0.2	0.2		0.2	0.1
16.00	1.37	1.41			1.71			1.32	1.44	62.2	81.1			148.0			171.0	117.8	0.1	0.1			0.3			0.2	0.2
18.14	2.79	2.70			2.95			2.27	2.80	90.4	103.4			122.0			168.2	140.7	0.3	0.3			0.4			0.4	0.4
20.11	2.42	2.67			2.43			2.09	2.41	103.1	96.7			118.1			220.6	155.0	0.2	0.3			0.3			0.5	0.4
22.03	2.17	2.13						1.97	2.17	134.6	106.4						190.1	166.3	0.3	0.2						0.4	0.4
22.97	1.14	1.11						1.03	1.14	145.2	106.7						177.5	106.5	0.2	0.1						0.2	0.1
25.03	2.50	2.43						2.26	2.50	175.1	168.2						141.8	116.8	0.4	0.4						0.3	0.3
27.11	2.52	2.47						2.29		138.4	141.0						156.2		0.3	0.3						0.4	
28.01	1.24	1.22						1.13		118.7	175.8						141.0		0.1	0.2						0.2	
29.01	1.38	1.35						1.27		120.4	189.3						142.7		0.2	0.3						0.2	
29.99	1.20	1.17						1.07		119.4	203.7						143.6		0.1	0.2						0.2	
31.00	1.02							1.26		149.9							128.2		0.2							0.2	
33.04	2.46							2.25		124.7							121.7		0.3							0.3	
33.99	1.29							1.22		120.2							122.6		0.2							0.1	

Table B. 4: Cell count in the PLS at column take-down of Experiment 1

Days	7	10	13	15	20	25	30	34	34*
Cells/mL PLS	3.75E+06	2.50E+06	5.63E+06	4.69E+06	1.25E+06	1.25E+06	9.38E+06	9.69E+06	3.75E+06

*column inoculated with inoculum 2 whilst the rest were inoculated with inoculum 1

Table B. 5: Cell counts of cells detached from the ore at column take-down of Experiment 1

		Days								
	Inoculum	7	10	13	15	20	25	30	34	34*
Phases		Cells per kg of ore								
Interstitial		9.15E+08	5.39E+09	4.89E+09	9.81E+09	7.92E+09	1.04E+10	8.78E+09	1.84E+10	1.02E+10
Weakly attached		4.25E+08	3.80E+09	4.00E+09	4.66E+09	6.18E+09	4.87E+09	5.73E+09	1.01E+10	7.96E+09
Strongly attached		6.29E+08	2.15E+09	2.35E+09	2.67E+09	2.55E+09	2.79E+09	9.27E+09	1.23E+10	3.82E+09
TOTAL	3.00E+05	1.97E+09	1.13E+10	1.12E+10	1.71E+10	1.67E+10	1.80E+10	2.38E+10	4.08E+10	2.20E+10

*column inoculated with inoculum 2 whilst the rest were inoculated with inoculum 1

Table B. 6: Growth rates and standard deviations calculated from genomic copies of detached cells in Experiment 1 (only *Acidithiomicrobium* spp. is shown here)

Acidithicmicrobium species (JTC4/SAR)																					
		PLS		ore1	ore2	ore ave	ore								growth rates				doubling times			
Day	h	copies per mL	log copies per mL	copies per dry kg	copies per dry kg	copies per dry kg	log copies per dry kg1	log copies per dry kg2	log copies/ kg (ave)	log copies (stdev)	ln copies per dry kg1	ln copies per dry kg2	ln copies per kg ave	days	μ, per hour1	μ, per hour2	μ, per h (ave)	μ, (stdev)	td 1 (h)	td 2(h)	td ave(h)	td (stdev)
0	0			5.46E+05	5.46E+05	5.46E+05	5.74	5.74	5.74	0.00000	13.21	13.21	13.21									
7	168	0.00E+00		3.79E+06	3.79E+06	3.79E+06	6.58	6.58	6.58	0.00000	15.15	15.15	15.15	0 - 7	0.01153	0.01153	0.01153	0.00000	60.1	60.1	60.1	0.0
10	240	2.93E+03	3.47	3.80E+06	3.80E+06	3.80E+06	6.58	6.58	6.58	0.00000	15.15	15.15	15.15									
13	312	1.33E+03	3.12	7.15E+06	8.47E+06	7.81E+06	6.85	6.93	6.89	0.05203	15.78	15.95	15.87	7 - 13	0.00441	0.00558	0.00502	0.00083	157.2	124.1	138.0	23.4
15	360	8.11E+02	2.91	1.33E+07	3.56E+07	2.45E+07	7.12	7.55	7.39	0.30236	16.40	17.39	17.01									
20	480	3.76E+03	3.58	1.68E+08	1.16E+09	6.64E+08	8.23	9.06	8.82	0.59337	18.94	20.87	20.31									
25	600	1.39E+03	3.14	5.75E+06	1.66E+06	3.71E+06	6.76	6.22	6.57	0.38153	15.56	14.32	15.13									
30	720	8.70E+06	6.94	3.37E+10	8.33E+10	5.85E+10	10.53	10.92	10.77	0.27790	24.24	25.15	24.79	13 - 30	**0.02349**	**0.02554**	**0.02478**	**0.00144**	29.5	27.1	**28.0**	1.7
34	816	6.57E+06	6.82	9.01E+10	2.23E+11	1.56E+11	10.95	11.35	11.19	0.27790	25.22	26.13	25.78	13 - 34	0.01024	0.01024	0.01024	0.00000	67.7	67.7	67.7	0.0

Table B. 7: Activity test measurements (Eh, pH and Fe^{2+}) in shake flasks of Experiment 1 (only the blank and inoculum 1 are shown here)

Blank											inoculum 1										
Days	[Fe2+], mg/L				Eh			pH			Days	[Fe2+], mg/L				Eh			pH		
	1	2	ave	stdev	1	2	ave	1	2	ave		1	2	ave	stdev	1	2	ave	1	2	ave
0.00	3786.7	3920.0	3853.3	94.3	564	561	562.5	1.54	1.54	1.54	0.00	3720.0	3697.8	3708.9	15.7	549	548	548.5	1.67	1.68	1.68
1.06	3651.1	3724.4	3687.8	51.9	601	599	600.0	1.54	1.54	1.54	1.17	2995.6	2995.6	2995.6	0.0	630	630	630.0	1.94	1.94	1.94
1.96	3571.1	3577.8	3574.4	4.7	611	610	610.5	1.55	1.56	1.55	2.90	2371.1	2320.0	2345.6	36.1	646	647	646.5	2.00	2.02	2.01
3.00	3466.7	3504.4	3485.6	26.7	619	618	618.5	1.57	1.57	1.57	4.16	2171.1	1988.9	2080.0	128.9	655	657	656.0	1.99	2.02	2.01
4.00	3397.8	3428.9	3413.3	22.0	623	621	622.0	1.55	1.56	1.56	5.94	1757.8	1537.8	1647.8	155.6	664	665	664.5	1.99	2.03	2.01
5.00	3337.8	3337.8	3337.8	0.0	629	629	629.0	1.54	1.56	1.55	6.93	1537.8	1477.8	1507.8	42.4	667	667	667.0	1.99	2.04	2.02
6.05	3260.0	3302.2	3281.1	29.9	631	630	630.5	1.55	1.55	1.55	8.94	1146.7	1051.1	1098.9	67.6	673	674	673.5	2.06	2.07	2.06
8.02	3106.7	3108.9	3107.8	1.6	634	634	634.0	1.60	1.58	1.59	10.05	1064.4	913.3	988.9	106.9	679	680	679.5	1.96	2.04	2.00
10.00	3013.3	3031.1	3022.2	12.6	640	640	640.0	1.62	1.61	1.61	11.22	855.6	795.6	825.6	42.4	686	688	687.0	1.96	2.04	2.00
12.00	2844.4	2851.1	2847.8	4.7	645	644	644.5	1.60	1.61	1.60	12.99	677.8	671.1	674.4	4.7	694	697	695.5	1.97	2.05	2.01
14.04	2726.7	2735.6	2731.1	6.3	648	646	647.0	1.62	1.60	1.61	13.94	633.3	537.8	585.6	67.6	696	698	697.0	1.94	2.04	1.99

Table B. 8: Genomic copy numbers of microbial species in the inocula (2, 3 and 4), in the PLS and in the ore in Experiment 2

	Acidiplasma cupricumulans (JTC4)		*Acidithiobacillus caldus* (At.c)		*Metallosphaera sedula/ hakonensis* (M.sed)		*Acidithiomicrobium* species (JTC3/SAR)		*Sulfobacillus thermosulfidooxidans* (G2/STO)*		*Acidithiobacillus ferrooxidans* (Atf D2)**	
	PLS	Ore	PLS	Ore	PLS	Ore	PLS	Ore	PLS	Ore	PLS	Ore
Day	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg
0[a]		4.27E+03		4.21E+03		7.76E+05		5.34E+04		9.87E+02*		3.67E+01*
6[b]	-	-	4.40E+04	3.71E+09	3.34E+01	5.52E+04	-	-	-	5.12E+09	-	1.40E+06
12[b]	-	-	3.16E+05	2.76E+09	1.72E+00	3.83E+05	1.36E+05	1.55E+08	4.17E+05	1.30E+09	8.96E+01	5.38E+05
18[b]	-	-	1.24E+06	6.68E+08	-	-	1.87E+05	5.57E+07	9.68E+05	4.96E+07	3.61E+02	1.44E+05
26[b]	-	2.57E+05	4.69E+02	2.16E+08	-	-	-	-	1.78E+03	7.69E+07	-	8.61E+04
38[b]	1.06E+03	2.45E+06	1.07E+06	3.77E+08	5.78E+04	1.45E+08	-	-	1.38E+05	5.93E+07	2.71E+02	-
49[b]	-	9.18E+07	2.85E+05	7.91E+08	7.52E+05	5.38E+10	-	-	9.22E+04	2.31E+09	9.28E+01	-
50[b,c]	8.06E+02	2.43E+05	7.47E+05	2.80E+07	5.75E+03	5.24E+05	-	-	4.10E+05	4.34E+06	5.76E+04	-
0[d]		2.52E+04		1.00E+04		4.04E+05		1.26E+06		1.32E+03*		1.68E+02*
11[e]	-	-	3.90E+06	9.69E+10	-	4.31E+06	-	-	2.44E+06	2.51E+10	8.92E+02	1.90E+07
49[e]	8.33E+03	1.94E+10	6.42E+04	5.25E+09	2.57E+05	2.12E+11	-	-	1.40E+04	-	-	-
0[f]		4.47E+05		5.82E+05		1.18E+04		2.21E+04		-		-
34[g]	2.28E+04	3.66E+07	7.59E+05	6.70E+09	3.86E+06	1.08E+11	4.86E+06	1.56E+10	-	2.54E+10	-	-
50[h]	2.19E+03	-	4.70E+03	-	2.19E+04	5.22E+04	2.11E+03	-	1.30E+05	7.61E+05	-	-

a- Inoculum 2; *b*- columns inoculated with Inoculum 2, *c*- column containing ore type B; *d*- Inoculum 3; *d, e*- columns inoculated with Inoculum 3, *f*- Inoculum 4, *g*- column inoculated with Inoculum 4, *h*- Control column

*It was *Thermoplasmatales* spp. (JTC 1/2) detected in both inocula and not *Sulfobacillus thermosulfidooxidans* (G2/STO)

**It was *Leptospirillum ferriphilum* (Lferri LH) detected in both inocula and not *Acidithiobacillus ferrooxidans* (Atf D2)

Table B. 9: Genomic copy numbers of microbial species in Inoculum 5, in the PLS and in the ore in Experiment 3

		Acidiplasma cupricumulans (JTC4)		*Acidithiobacillus caldus* (At.c)		*Metallosphaera sedula/ hakonensis* (M.sed)		*Acidithiomicrobium* species (JTC3/SAR)		*Sulfobacillus thermosulfidooxidans* (G2/STO)		Archaea (JTC 1/2)		*Leptospirillum ferriphilum* (L.ferri LH)	
		PLS	ore	PLS	ore	PLS	ore	PLS	ore	PLS	ore	PLS	ore	PLS	ore
Day	Feed Cu, g/ L	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg	copies per mL	copies per kg
0	inoculum		1.18E+05		8.14E+04		2.77E+05		9.54E+05				1.59E+04		
7	5	-	-	1.86E+07	6.53E+06	1.56E+02	-	-	-	-	5.21E+06	-	-	-	-
	10	-	-	5.44E+03	-	-	-	5.79E+02	-	2.78E+06	-	8.05E+01	-	3.68E+02	-
	15	-	-	1.34E+03	-	-	-	1.27E+02	-	1.11E+06	-	-	-	1.32E+02	-
15	5	5.42E+05	1.46E+07	1.08E+07	7.48E+08	1.04E+02	-	-	-	-	-	-	-	-	-
	10	-	-	5.32E+05	-	-	-	-	-	1.17E+07	-	-	-	1.23E+03	-
	15	-	-	3.61E+03	-	-	-	-	-	1.47E+06	-	-	-	2.00E+02	-
27	5	3.64E+05	7.51E+06	3.64E+05	7.51E+06	2.70E+02	-	-	2.69E+05	-	1.31E+08	-	-	-	-
	10	4.20E+03	-	-	-	-	-	-	2.20E+04	3.58E+07	-	-	-	-	-
	15	-	-	-	-	1.43E+02	-	-	-	8.74E+06	1.11E+06	3.98E+02	-	-	-
	Control	-	5.27E+05	9.65E+05	1.29E+08	2.24E+03	3.52E+06	1.39E+03	3.71E+06	1.78E+03	7.69E+07	-	8.61E+04	-	-

Table B. 10: Genomic copy numbers of microbial species in Inoculum 6 and in the cultures of Experiment 4 (1 of 2)

Reactor	Day	*Ferroplasma cupricumulans* (JTC4)	*Acidithiobacillus caldus* (At.c)	*Metallosphaera sedula/ hakonensis* (M.sed)	*Acidithiomicrobium species* (JTC3/SAR)	*Sulfobacillus thermosulfidooxidans* (G2/STO)	Archaea (JTC 1/2)	*Leptospirillum ferriphilum* (L.ferri LH)	*Leptospirillum ferriphilum type* (L.ferri type)	*Acidithiobacillus ferrooxidans* D2 (At.f D2)
		copies per mL culture								
inoclm	0	8.28E+07	8.28E+07	1.08E+08	3.74E+07	-	4.26E+05	1.19E+04	-	-
STR 1	7	1.72E+08	1.72E+08	4.45E+08	4.29E+08	-	4.56E+06	-	-	-
STR 2		2.09E+08	2.09E+08	4.20E+08	1.84E+09	-	1.75E+07	-	-	-
STR 3		1.67E+08	1.67E+08	4.50E+08	3.55E+08	-	8.57E+06	-	-	-
S/flask		1.44E+08	1.44E+08	8.30E+08	2.58E+08	-	4.95E+06	-	-	-
STR 1	14	7.24E+06	1.05E+07	3.19E+08	1.89E+09	2.97E+07	-	1.74E+04	-	-
STR 2		1.77E+07	4.78E+06	2.60E+08	2.08E+09	2.54E+07	-	4.10E+05	-	-
STR 3		3.30E+06	1.33E+07	4.06E+08	2.27E+09	4.11E+07	-	3.45E+04	-	-
S/flask		1.16E+06	2.16E+06	2.45E+08	3.58E+08	1.44E+07	-	3.14E+03	-	-
STR 1	21	1.28E+06	5.21E+06	1.84E+08	1.11E+09	2.24E+07	-	1.44E+05	-	-
STR 2		4.90E+06	3.03E+06	2.04E+08	1.49E+09	2.44E+07	-	1.32E+06	-	-
STR 3		1.39E+06	9.37E+06	1.83E+08	5.95E+08	1.43E+07	-	2.71E+04	-	-
S/flask		3.90E+05	1.03E+06	2.64E+08	2.57E+08	1.00E+07	-	2.28E+03	-	-
STR 1	28	5.07E+05	6.61E+06	2.03E+08	7.58E+08	1.41E+07	-	2.48E+05	-	-
STR 2		1.33E+07	3.67E+06	1.54E+08	5.90E+08	1.24E+07	-	7.68E+05	-	-
STR 3		4.02E+05	1.66E+07	1.64E+08	4.26E+08	1.05E+07	-	3.71E+04	-	-
S/flask		5.05E+04	2.29E+06	2.83E+08	2.01E+08	1.19E+07	-	1.88E+03	-	-
STR 1	42	3.86E+05	3.04E+06	1.08E+08	3.47E+08	8.25E+06	-	6.08E+04	-	-
STR 2		3.28E+05	7.22E+06	1.34E+08	1.68E+08	6.61E+06	-	7.42E+04	-	-
STR 3		3.00E+05	9.83E+06	1.44E+08	2.29E+08	7.34E+06	5.55E+05	3.26E+04	-	-
S/flask		2.74E+05	6.18E+06	2.46E+08	2.56E+08	1.04E+07	-	2.36E+04	-	-

Table B. 11: Genomic copy numbers of microbial species in Inoculum 6 and in the cultures of Experiment 4 (2 of 2)

		Ferroplasma cupricumulans (JTC4)	*Acidithiobacillus caldus* (At.c)	*Metallosphaera sedula/ hakonensis* (M.sed)	*Acidithiomicrobium species* (JTC3/SAR)	*Sulfobacillus thermosulfidooxidans* (G2/STO)	Archaea (JTC 1/2)	*Leptospirillum ferriphilum* (L.ferri LH)	*Leptospirillum ferriphilum type* (L.ferri type)	*Acidithiobacillus ferrooxidans* D2 (At.f D2)
Reactor	Day	copies per mL culture								
STR 1	56	1.03E+06	2.68E+06	2.20E+08	2.53E+08	1.02E+07	-	3.40E+04	-	-
STR 2		6.29E+05	8.47E+06	2.64E+08	1.36E+07	1.00E+07	7.22E+04	1.66E+05	-	-
STR 3		2.51E+05	1.53E+07	3.29E+08	6.72E+08	-	2.51E+05	2.15E+05	-	-
S/flask		3.08E+05	4.11E+06	9.22E+08	6.19E+08	-	3.08E+05	1.83E+04	-	-
STR 1	63	1.80E+06	1.66E+07	7.38E+08	2.93E+08	-	6.57E+05	2.17E+05	4.02E+05	1.35E+06
STR 2		2.48E+05	5.67E+06	7.33E+08	9.76E+06	-	2.00E+05	7.02E+05	1.39E+04	1.49E+05
STR 3		1.15E+06	9.63E+06	5.47E+08	1.56E+08	-	6.77E+05	4.69E+05	2.36E+05	6.88E+05
S/flask		5.93E+05	2.12E+06	3.48E+08	1.43E+08	-	4.62E+05	3.74E+04	1.84E+05	0.00E+00
STR 1	70	1.16E+06	2.71E+07	7.13E+08	1.23E+08	-	7.91E+05	1.24E+05	2.08E+05	8.79E+05
STR 2		4.80E+05	1.91E+07	9.54E+08	2.51E+07	-	5.09E+05	4.52E+05	7.00E+04	5.80E+05
STR 3		6.55E+05	2.83E+07	9.12E+08	8.88E+07	-	8.14E+05	6.69E+05	1.22E+05	5.19E+05
S/flask		1.33E+06	8.05E+06	1.24E+09	1.77E+08	-	1.55E+06	4.33E+04	2.43E+05	5.96E+05
STR 1	84	6.33E+05	2.43E+06	3.09E+08	4.42E+07	-	6.10E+05	1.14E+05	6.08E+04	1.97E+05
STR 2		4.02E+05	2.81E+06	3.10E+08	2.62E+07	-	3.06E+05	2.05E+05	3.76E+04	1.41E+05
STR 3		3.20E+05	1.20E+07	1.82E+08	5.86E+07	-	1.54E+05	3.85E+05	8.73E+04	4.04E+05
S/flask		5.92E+05	1.06E+06	3.34E+07	1.46E+08	-	2.90E+06	9.68E+05	2.42E+05	
STR 1	98	1.14E+06	7.60E+06	1.26E+08	3.87E+07	-	4.39E+05	7.09E+04	6.14E+04	3.76E+05
STR 2		1.98E+05	2.72E+06	2.00E+08	1.90E+07	-	3.88E+05	1.07E+05	3.04E+04	1.37E+05
STR 3		6.00E+05	8.92E+06	1.73E+08	6.10E+07	-	4.02E+05	1.76E+05	9.90E+04	4.02E+05
S/flask		8.00E+05	5.07E+05	5.22E+07	7.46E+07	-	9.08E+06	3.43E+05	-	-

Table B. 11: Genomic copy numbers of microbial species in Inoculum 6 and in the cultures of Experiment 4 (2 of 2)

Reactor	Day	*Ferroplasma cupricumulans* (JTC4)	*Acidithiobacillus caldus* (At.c)	*Metallosphaera sedula/ hakonensis* (M.sed)	*Acidithiomicrobium species* (JTC3/SAR)	*Sulfobacillus thermosulfidooxidans* (G2/STO)	Archaea (JTC 1/2)	*Leptospirillum ferriphilum* (L.ferri LH)	*Leptospirillum ferriphilum type* (L.ferri type)	*Acidithiobacillus ferrooxidans* D2 (At.f D2)
		copies per mL culture								
STR 1	56	1.03E+06	2.68E+06	2.20E+08	2.53E+08	1.02E+07	-	3.40E+04	-	-
STR 2		6.29E+05	8.47E+06	2.64E+08	1.36E+07	1.00E+07	7.22E+04	1.66E+05	-	-
STR 3		2.51E+05	1.53E+07	3.29E+08	6.72E+08	-	2.51E+05	2.15E+05	-	-
S/flask		3.08E+05	4.11E+06	9.22E+08	6.19E+08	-	3.08E+05	1.83E+04	-	-
STR 1	63	1.80E+06	1.66E+07	7.38E+08	2.93E+08	-	6.57E+05	2.17E+05	4.02E+05	1.35E+06
STR 2		2.48E+05	5.67E+06	7.33E+08	9.76E+06	-	2.00E+05	7.02E+05	1.39E+04	1.49E+05
STR 3		1.15E+06	9.63E+06	5.47E+08	1.56E+08	-	6.77E+05	4.69E+05	2.36E+05	6.88E+05
S/flask		5.93E+05	2.12E+06	3.48E+08	1.43E+08	-	4.62E+05	3.74E+04	1.84E+05	0.00E+00
STR 1	70	1.16E+06	2.71E+07	7.13E+08	1.23E+08	-	7.91E+05	1.24E+05	2.08E+05	8.79E+05
STR 2		4.80E+05	1.91E+07	9.54E+08	2.51E+07	-	5.09E+05	4.52E+05	7.00E+04	5.80E+05
STR 3		6.55E+05	2.83E+07	9.12E+08	8.88E+07	-	8.14E+05	6.69E+05	1.22E+05	5.19E+05
S/flask		1.33E+06	8.05E+06	1.24E+09	1.77E+08	-	1.55E+06	4.33E+04	2.43E+05	5.96E+05
STR 1	84	6.33E+05	2.43E+06	3.09E+08	4.42E+07	-	6.10E+05	1.14E+05	6.08E+04	1.97E+05
STR 2		4.02E+05	2.81E+06	3.10E+08	2.62E+07	-	3.06E+05	2.05E+05	3.76E+04	1.41E+05
STR 3		3.20E+05	1.20E+07	1.82E+08	5.86E+07	-	1.54E+05	3.85E+05	8.73E+04	4.04E+05
S/flask		5.92E+05	1.06E+06	3.34E+07	1.46E+08	-	2.90E+06	9.68E+05	2.42E+05	
STR 1	98	1.14E+06	7.60E+06	1.26E+08	3.87E+07	-	4.39E+05	7.09E+04	6.14E+04	3.76E+05
STR 2		1.98E+05	2.72E+06	2.00E+08	1.90E+07	-	3.88E+05	1.07E+05	3.04E+04	1.37E+05
STR 3		6.00E+05	8.92E+06	1.73E+08	6.10E+07	-	4.02E+05	1.76E+05	9.90E+04	4.02E+05
S/flask		8.00E+05	5.07E+05	5.22E+07	7.46E+07	-	9.08E+06	3.43E+05	-	-

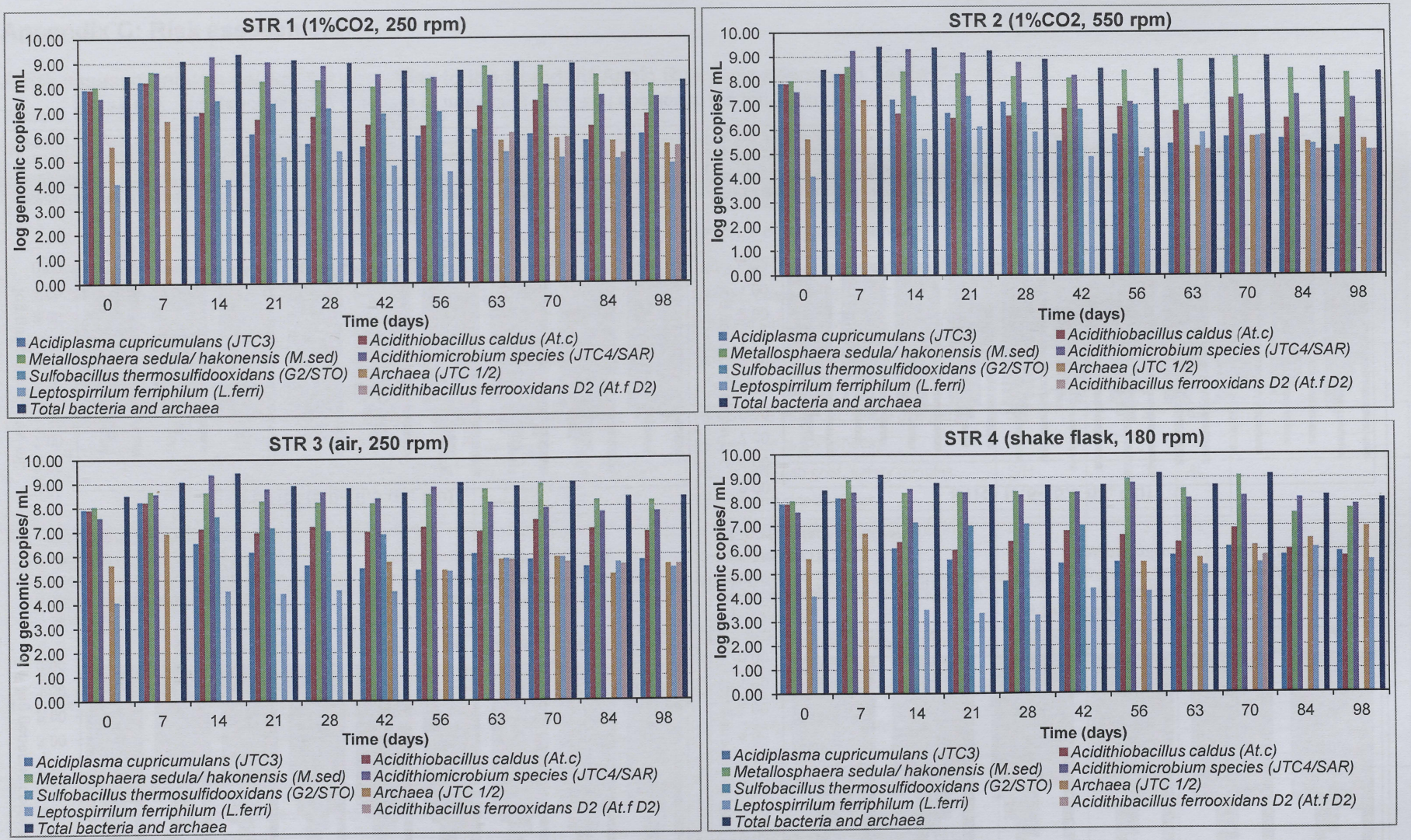

Figure B. 1: The genomic copy numbers of individual microbial species in the four cultures in Experiment 4

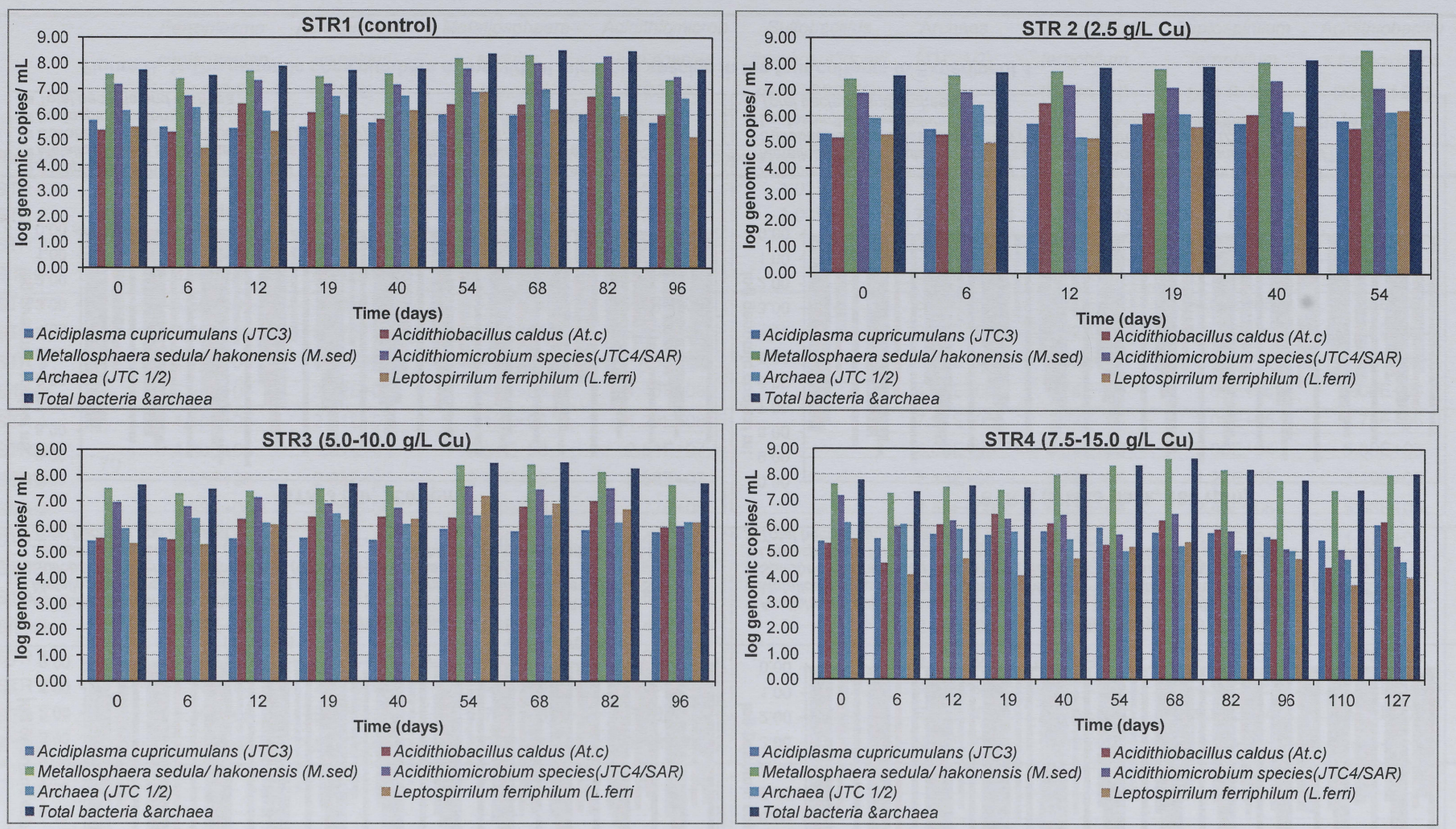

Figure B. 2: The genomic copy numbers of individual microbial species in the four cultures in Experiment 5

Appendix C: Risk assessment

A risk assessment was carried out according to the Standard Anglo Risk Matrix shown in Table C.1 and C.2.

Table C. 1: Standard Anglo Risk Matrix, 2011 (1 of 2)

No	Sub System	Hazard - "what if"	Risk	Current Controls	Loss Type	C	L	R	Recommended Additional Controls
1	Splitting, blending and agglomeration of the ore. Loading and take-down of columns.	Dust produced when splitting and blending could be inhaled	Injury to lungs or irritation of the respiratory system	Wear personal protective equipment (PPE), a dust musk in this case	S/H	1	3	4L	
		Sulphuric acid used for agglomeration could come into contact	Skin irritation, burns, tissue damage	Wear PPE (lab coat, safety goggles, gloves, closed shoes) Use the safety shower	S/H	2	3	8M	
		The ore and the glass columns could be heavy	Physical strain and back ache	Lift in smaller portions. Get help from another person	S/H	1	3	4L	
2	Preparation of the media	Sulphuric acid used for the media could come into contact	Skin irritation, burns, tissue damage	Wear PPE (lab coat, safety goggles, gloves and closed shoes)	S/H	2	3	8M	
		The inorganic salts for the feed could come into contact	Respiratory tract or skin irritation	Wear PPE (lab coat, safety goggles, gloves and closed shoes)	S/H	1	3	4L	
3	Inoculation, irrigation and sample analysis	Micro-organisms could contaminate the environment	Environmental contamination	Dispose of contaminated material in biohazard bins	EI	1	3	4L	
		The flame used for sterilisation could cause fire	Fire	Never leave a flame unattended. Use a fire extinguisher	BI/MD S/H	2	3	8M	
		Acetic acid used for iron assay reagents could come into contact	Irritation, burns, tissue damage	Wear PPE (lab coat, safety goggles, gloves and closed shoes)	S/H	2	3	8M	
4	Electricity	Incorrect connections could cause fire or electric shock	Injury and fire	Installation and/ or repair of electrical equipment, wires and connections should be done by certified personnel	S/H	1	3	4L	
5	Chemical spills and broken glassware	Spills could occur from the column or reactor connections and lab glassware could break	Cuts, slipping	Immediately wipe spills. Carefully handle glassware and dispose of broken glassware in designated bins	S/H	1	3	4L	

Table C. 2: Standard Anglo Risk Matrix, 2011 (2 of 2)

		Hazard Effect / Consequence				
Loss Type (Additional 'Loss Types' may exist for an event; identify & rate accordingly)		1 Insignificant	2 Minor	3 Moderate	4 Major	5 Catastrophic
(S/H) Harm to People (Safety / Health)		First aid case / Exposure to minor health risk	Medical treatment / Exposure to major health risk	Loss time injury / Reversible impact on health	Single fatality or loss of quality of life / Irreversible impact on health	Multiple fatalities / Impact on health ultimately fatal
(EI) Environmental Impact		Minimal environmental harm - L1 incident	Material environmental harm - L2 incident remediable short term	Serious environmental harm - L2 incident remediable within LOM	Major environmental harm - L2 incident remediable post LOM	Extreme environmental harm - L3 incident irreversible
(BI/MD) Business Disruption / Material Damage & Other Consequential Losses		No disruption to operation / R500k to less than R5m	Brief disruption to operation / R5m to less than R50m	Partial shutdown / R50m to less than R500m	Partial loss of operation R500m to less than R5bn	Substantial or total loss of R5bn and more
(L&R) Legal & Regulatory		Low level legal issue	Minor legal issue; non compliance and breaches of the law	Serious breach of law; investigation/report to authority, prosecution and/or moderate penalty possible	Major breech of the law; considerable prosecution and penalties	Very considerable penalties & prosecutions. Multiple law suits & jail terms
(R/S/C) Impact on Reputation/Social/Community		Slight impact - public awareness may exist but no public concern	Limited impact - local public concern	Considerable impact - regional public concern	National impact - national public concern	International impact - international public attention
Likelihood	Examples	Risk Rating				
5 Almost Certain	The unwanted event has occurred frequently; occurs in order of one/ more times per year & is likely to reoccur within 1 year	11 (M)	16 (H)	20 (H)	23 (Ex)	25 (Ex)
4 Likely	The unwanted event has occurred infrequently; occurs in order of less than once per year & is likely to recur within 5 years	7 (M)	12 (M)	17 (H)	21 (Ex)	24 (Ex)
3 Possible	The unwanted event could well have occurred in the business at some point within 10 years	4 (L)	8 (M)	13 (H)	18 (H)	22 (Ex)
2 Unlikely	The unwanted event has happened in the business at some time; or could happen within 20 years	2 (L)	5 (L)	9 (M)	14 (H)	19 (H)
1 Rare	The unwanted event has never been known to occur in the business; or is highly unlikely to ever occur beyond 20 years	1 (L)	3 (L)	6 (M)	10 (M)	15 (H)

Interpretation of Risk Level

Risk rating	Risk Level	Guidelines for Risk Matrix
21 to 25	(Ex) – Extreme	Eliminate, avoid, implement specific action plans / procedures to manage & monitor
13 to 20	(H) – High	Proactively manage
6 to 12	(M) – Medium	Actively manage