# Factors affecting the attachment of *Metallosphaera hakonensis* during the colonisation of low grade mineral sulphide heaps





**By**

**Lucinda Bromfield**

**Thesis presented for the Degree of**

**Master of Applied Science**

**Centre for Bioprocess Engineering Research**

**Department of Chemical Engineering**

**University of Cape Town**

**June 2011**

# Declaration

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

11th February 2011

Lucinda Valerie Bromfield

---

# Acknowledgements

I would like to thank my supervisor, Dr R. P. van Hille, and my co-supervisor, Prof S. T. L. Harrison for their help and guidance throughout the duration of my postgraduate studies. They provided valuable input and played key roles in both the experimental and writing components of my thesis.

Thank you to all the members of the Centre for Bioprocess Engineering Research for all the support they provided as well as the friendships that were developed. A special thank you to Mrs F. Pocock for her assistance in the laboratory and always ensuring that the laboratory runs smoothly. Thanks to Ms B. Mc Fadzean for help with the mineralogy analyses and Mrs H. Divey for her help with the analytical techniques. Thanks to Mrs S. Jobson for her assistance in ensuring that all administrative matters are effectively managed.

I would like to acknowledge the financial support of the National Research Foundation, BHP Billiton (South Africa), the K. W. Johnstone scholarship and UCT.

Finally, I would like to show my immense gratitude to my friends and family who have loved and supported me through my postgraduate studies.

# Synopsis

The global demand for copper has increased substantially over the past decade, driving the exploitation of lower grade and more refractory deposits. This thesis pertains to the extraction of copper via heap bioleaching, focussing specifically on the bioleaching of the mineral sulphide, chalcopyrite ($CuFeS_2$). Industrial heap bioleaching offers an attractive alternative to conventional extraction methods, such as smelting, for processing low grade ores. There remain a number of operational challenges associated with bioleaching heaps, such as the lag time before efficient extraction is achieved and ineffective heap inoculation, as well as the difficulty in controlling the conditions within the heap. Chalcopyrite bioleaching under ambient conditions is ineffective due slow kinetics and passivation of the mineral surface. These may be addressed by raising the temperature to the thermophilic (> 60°C) range.

Previous research has proven that the attachment of microorganisms enhances the rate of mineral solubilisation during heap bioleaching. However, attachment studies have predominantly been restricted to shake flask experiments with mesophilic cultures. As it becomes increasingly necessary to mine low grade deposits, due to diminishing high grade resources, research into high temperature bioleaching is becoming critical. The presence of thermophiles is important to sustain heap temperature in the range that minimises the impact of chalcopyrite passivation. Existing studies on the attachment of thermophiles have been conducted either at ambient or optimum growth conditions and the attachment behaviour of thermophilic microorganisms at intermediate temperatures is poorly understood. The agitated batch systems previously employed to investigate attachment do not adequately represent the hydrodynamic conditions within a heap and the applicability of the results may therefore be questionable.

This study addresses a number of areas where the gaps in the current knowledge have operational relevance in terms of enhancing copper extraction from chalcopyrite. The following key areas were investigated:

- Effect of temperature on the attachment of a thermophilic microorganism
- Effect of experimental set-up on the applicability of the results to a heap bioleaching system
- Effect of mineral substrate on the attachment of the thermophilic microorganism
- Effect of culture history on the attachment of the thermophilic microorganism

These factors were investigated using *Metallosphaera hakonensis,* a thermophilic archeae, adapted to four different substrates (elemental sulphur, ferrous iron, chalcopyrite concentrate and pyrite concentrate). Attachment to three mineral substrates (chalcopyrite concentrate, pyrite concentrate and a low grade ore) was investigated in both shake flask and flow-through column experiments at 25°C, 45°C and 65°C. Metabolic activity tests and characterisation of the microbial surface were conducted to enhance the value of the attachment studies.

The agitated batch studies were conducted using a 100 ml volume, in 250 ml Erlenmeyer flasks, at a 2% (w/v) substrate loading and a total cell inoculum of $2x10^9$ cells for the full range of temperatures and mineral types. There was a consistent trend of increasing levels of attachment with increasing temperature. The rate data provided a good fit to the Arrhenius equation, suggesting that the increase in the levels of attachment were due to the increased temperature and not intrinsic factors, such as metabolic activity. The cells cultured on elemental sulphur exhibited the greatest levels of attachment across the temperature range, for all substrates, and there was little variance between the level of attachment to the different substrates. Overall attachment of 39%, 63% and 83%, for cells cultured on elemental sulphur, was achieved onto a chalcopyrite concentrate at 25°C, 45°C and 65°C respectively. Attachment was greatest at 65°C for all culture histories, with 61%, 65% and 59% attachment to a chalcopyrite concentrate recorded for cells cultured on ferrous iron, chalcopyrite and pyrite respectively. The attachment trends were similar for all mineral substrates investigated and similar levels of attachment was achieved.

Studies conducted using gangue material (tailings from the flotation of the low grade ore) showed slightly lower, but significant levels of attachment of sulphur adapted cells. However, negligible attachment was observed to quartz at 65°C (~0%). The mineralogy of the low grade ore shows that ~53% of the gangue material is comprised of minerals other than quartz. This suggests that significant attachment to the non-quartz gangue minerals had occurred.

The hydrophobicity and zeta potential of the microbial surfaces were investigated to provide additional evidence for the trends observed. The hydrophobicity tests indicated that the cells adapted to chalcopyrite were the most hydrophobic, which is not consistent with the attachment results. Sulphide minerals are typically hydrophobic and the attachment data suggest sulphur adapted cells should be the most hydrophobic. Zeta potential was also measured as electrostatic interactions are important in the initial adhesion of cells. Measurements were taken over a pH range of 2 to 4, as dissolution of the acid consuming gangue material can result in an increase in pH during the initial stages of the heap. The

results showed that the zeta potential of the cells cultured on elemental sulphur were least affected by an increase in pH, remaining just below 0 mV. The surface charge of iron and mineral adapted cells decreased more significantly as pH increased, which would result in a greater repulsive force between these cells and the negatively charged solid substrate at higher pHs.

The packed columns were loaded with 300 mineral coated glass beads and solution fed from the bottom, creating a saturated column and ensuring that the effect of preferential flow was minimised. An inoculum of $1x10^9$ cells in 10 ml was fed into the column as a single pulse. The same trend of increasing attachment with increasing temperature, as seen in the shake flask studies, was observed and the cells cultured on elemental sulphur exhibited the greatest extents of attachment across all conditions investigated. The level of attachment was significantly lower, with the highest retention (43%) achieved using sulphur adapted cells and chalcopyrite coated beads at 65°C. Selective attachment to sulphide minerals was more pronounced in the column experiments, with the greatest extent of attachment to low grade ore coated particles being only 28%. The overall extent of attachment is affected by bed height and a number of columns in series could provide information on this trend.

To assess the potential effect of metabolic activity at increasing temperature, ferrous iron oxidation and oxygen utilisation tests were conducted. Both tests showed that under the experimental conditions employed, metabolic activity was negligible. In order to conclusively prove the independence of attachment on metabolic activity, experiments with dead or metabolically inactive cells should be conducted.

The difference in attachment between the two experimental set-ups could not be accounted for by microbe-mineral contact time, as the attachment equilibrium was achieved after 10 min in the shake flasks and the residence time of the columns was 60 min. A possible explanation is the dependence of attachment on the planktonic cell concentration. This is consistent with the Langmuir adsorption model which has been used to accurately depict the initial adhesion of microorganisms. When directly comparing the levels of attachment, the differences which between the experimental set-ups must be accounted for. The shake flask experiments had an initial cell concentration of $2 \times 10^7$ cells/ml ($2 \times 10^9$ cells total) and the columns were inoculated with a 10 ml pulse of ~$1 \times 10^8$ cells/ml ($1 \times 10^9$cells total). The agitated shake flask system provides good microbe-mineral contacting. The columns were fed from the bottom to create a fully saturated system in order to avoid channelling and improve the microbe-mineral contact that was achieved in the column, making the results more comparable to the results obtained in the shake flasks. This study illustrates the importance of developing new experimental

techniques, which more accurately mimic the conditions within a heap bioleaching system, thus providing more appropriate data.

This study has shown that temperature and growth history have a clear impact on attachment of thermophilic archeae to mineral substrates. The significant improvement in attachment efficiency between 25°C and 45°C provides evidence in support of a two-stage inoculation process. In addition, the study highlights the fact that shake flask studies may not accurately describe the conditions within a bioleaching heap as the extent of attachment observed differed and only general trends were comparable. Novel experimental configurations, such as flow-through column developed in this study are critical in furthering our understanding of bioleaching heaps.

# Table of Contents

# List of Figures

# List of Tables

# Abbreviations and Nomenclature

| | |
|---|---|
| AFM | Atomic force microscopy |
| B | Bacteria |
| BS | Irreversible attachment |
| $[BS]^*$ | Metastable complex (reversible attachment) |
| CSTR | Continuous stirred tank reactor |
| EDS | Energy dispersive X-ray spectroscopy |
| EELS | Electron energy-loss spectroscopy |
| EPS | Extra-cellular polymeric substances |
| FISH | Fluorescent in situ hybridisation |
| HAADF STEM | High angle annular dark field scanning transmission electron microscopy |
| PCR | Polymerase chain reaction |
| S | Surface sites involved in initial adhesion |
| SEM | Scanning electron microscopy |
| XDLVO | Extended Derjaguin Landau Verwey Overbeek theory |

# 1. Introduction

The decline in high grade copper ores, suitable for extraction by conventional methods, has fuelled the development of technologies to extract copper from low grade ores and waste from current operations. Chalcopyrite, a primary, refractory copper sulphide is the most abundant copper-containing mineral, but the economic viability of its extraction from low grade ores by conventional methods is questionable (Watling, 2006). Heap bioleaching is considered a potential alternative due to its comparatively low operating costs and reduced environmental impact (Watling, 2006; Pradhan *et al.*, 2008). The importance of this technology is likely to increase as demand drives the further exploitation of low-grade chalcopyrite ores.

The extraction of metals is catalysed by the presence of microorganisms during the bioleaching process. Chemical dissolution of the mineral takes place via the attack of ferric ions, protons or both. This process leads to the formation of ferrous ions and reduced sulphur species, which the microorganisms oxidise to obtain their energy. This process regenerates ferric iron and produces acidity, the reactants required for mineral dissolution. Heap bioleaching typically involves heaps of agglomerated ore. The heap is aerated from the base and an acidic solution is fed from the top of the heap and allowed to percolate through. The collected solution, containing the solubilised products, is called the pregnant leach liquor and is sent for metal recovery by conventional hydrometallurgical means. A portion of the treated raffinate may be recycled back to the heap. Inoculation of the heap is typically a once-off process which occurs at the start-up of the heap. While certain microorganisms are naturally found on the ore, inoculation ensures that a microbial flora that facilitate efficient bioleaching are present. Inoculation has been found to enhance the initial metal dissolution achieved. Despite inoculation, most heaps are characterised by a substantial lag period before adequate metal extraction is achieved (Watling, 2006).

There are still challenges associated with heap bioleaching to extract copper from chalcopyrite. At mesophilic temperatures, the extraction of copper is constrained by slow kinetics and the passivation of the surface. While there has been research devoted to understanding the passivation mechanism in the mesophilic temperature range (Yu *et al.,* 2008; Klauber, 2008), operation at above 60°C, where these constraints are largely overcome, is the favoured approach. Data from a set of bioreactors indicate that optimum copper dissolution from chalcopyrite is only achieved once temperatures in excess of 60°C are reached. In order to achieve these temperatures the presence of thermophilic iron and sulphur oxidisers within the heap is critical (Rodriguez *et al.,* 2003a; Gautier *et al.,* 2003).

Attachment of the microorganisms to the mineral surface has been shown to enhance the rate of mineral solubilisation during bioleaching (Gehrke et al., 1998; Fowler and Crundwell, 1999). van Loosdrecht *et al.* (1990) proposed a 4-step attachment mechanism that has been widely accepted. The four attachment steps are: transportation, initial adhesion, firm attachment and colonisation. During the firm attachment and colonisation stages, extracellular polysaccharide substances (EPS) are excreted by the cells. This provides a reaction space in which the chemical dissolution can occur. This ensures that the reactants, mineral and microbial catalysts are all in close proximity to each other and allows for efficient chemical attack to take place (Harneit *et al.,* 2006; Kinzler *et al.,* 2003).

The focus of this study is on the initial attachment of the cells to the mineral surface. This step is important as it impacts on the levels of attachment that can be achieved in the subsequent stages of attachment. The initial adhesion of the cells is a physicochemical process, controlled primarily by hydrophobic and electrostatic forces (van Loosdrecht et al., 1990; Devasia et al., 1993; Harneit et al., 2005). It follows that the rate and extent of attachment is affected by the surface properties of the microorganism and the mineral substrate (Yee et al., 2000), which in turn are affected by solution chemistry, growth history, temperature and mineral composition.

Bioleaching heaps are inoculated at temperatures often below 25°C and it can take up to a few months before the heap reaches optimum temperatures for microbial activity. Thermophiles have exhibited relatively poor attachment at ambient conditions (Africa, 2009). This has prompted the suggestion of a two step inoculation system, where the thermophiles are introduced to the heap once it has already reached the maximum temperature obtainable with only mesophiles (approximately 45°C) (Zou *et al.,* 2006). To assess the validity of this suggestion, the effect on attachment efficiency of raising the temperature to 45°C needs to be assessed. There have been numerous studies on the effect of temperature on the physiology of bioleaching organisms, but these have typically focused on iron and sulphur oxidising activity (Franzmann *et al.,* 2005 and Breed *et al.,* 1999).

Previous attachment studies have been done predominantly in well agitated shake flasks, where the mixing ensures suspension of the mineral ore particles and efficient transport of the cells to the mineral surface (Ghauri *et al.,* 2007; Rodriguez *et al.,* 2003b and Sampson *et al.*, 2000). In addition, the batch nature of the experiments ensures conservation of cells within the system so the equilibrium achieved is a function of the inoculum size. This type of experiment does not accurately represent the hydrodynamic conditions within a heap, where there is continuous flow of fluid through the system. The

dynamic nature of the flow through system and deviations from perfect plug flow suggest that the equilibria between planktonic and weakly attached cells differ from batch shake flask.

The objectives of the study are:

- To investigate the trends in attachment of *M. hakonensis* to pyrite and chalcopyrite concentrates as well as low grade ores with quartz as a control.
- To investigate the effect of culture history on the surface properties of *M. hakonensis* and the attachment of the cells to mineral concentrates and gangue.
- To investigate the effect of temperature (25°C to 65°C) and the possible role of metabolic activity on the attachment of *M. hakonensis* to mineral concentrates and gangue.
- To investigate the applicability of shake flask attachment results by comparing results from shake flask and column studies.

The literature review, Chapter 2, contains a brief description of the bioleaching process and the factors which affect the extraction efficiency. A key factor, attachment of the microorganisms to the mineral substrate, is reviewed and the important influencing factors discussed. The current literature is discussed and gaps in the current knowledge identified. All experimental methods, analytical techniques and equipment used in this study are described and the rationale behind each experiment explained in Chapter 3. Chapter 4 contains the results and discussion of the shake flask attachment study and microbial surface property tests conducted. Maximum levels of attachment are presented as percentages and as cells per gram substrate and these values were contrasted with literature values. The rate of attachment observed was calculated and also contrasted with literature. The relative hydrophobicity at pH 1.6 and zeta potential at pH 2 – pH 4 are presented. These were used to provide further insight into the attachment trends observed with cultures of differing growth histories. The results and discussion from the packed column attachment study and metabolic activity tests are presented in Chapter 5. The column experiments were conducted under key parameters and the percentage of cells retained in the column was presented. As the experimental protocol is relatively new, comparison with literature values was restricted to one study and the discussion remained theoretical. A comparison between the results obtained with the two experimental set-ups was presented and the differences observed discussed and analysed with reference to accepted theory. Due to the relatively long residence time of the column, metabolic activity of the cells under experimental conditions was determined to assess if this was a contributing factor in the attachment observed in the

packed column study. Results from oxygen utilisation and ferrous iron oxidation tests were presented and discussed with regard to the effect of metabolic activity on attachment. Chapter 6 contains a discussion of the entire study, focussing on how and if the objectives and hypotheses of the study were met and proven. The gaps in current literature that this study addresses were highlighted and further experiments to fill the remaining gaps in the area of thermophilic attachment with regards to chalcopyrite bioleaching were proposed. Chapter 7 contains a summary of the conclusions drawn and the recommendations made from this study.

# 2. Literature Review

## *2.1. Introduction*

The demand for copper remains great and as the easily extracted high grade (mineral oxides) surface mineral deposits are depleted it is becoming increasingly important to mine low grade ore deposits and waste tailings. The demand for copper is expected to exceed supply by 132000 tons in 2011 and by 386000 tons in 2012 (Logistics Weekly, http://logisticsweek.com/news/2010/08/copper-demand-to-exceed-supply-in-2011/). It is not economically viable to extract copper from the low grade ores via conventional methods. Roasting or smelting of the low grade ores requires a large amount of energy and produces harmful gaseous emissions such as sulphur dioxide making the process both environmentally and economically unfavourable. Bioleaching is a promising alternative technology as it has very low capital costs and energy requirements, does not release the harmful gases associated with conventional extraction techniques and the acid mine drainage potential of the waste tailings is reduced as they are less chemically active and the microbial population that can be supported is diminished (Rawlings *et al.,*2003).

There are still challenges associated with bioleaching, especially with the extraction of copper from chalcopyrite. As chalcopyrite is the most abundant copper containing ore and the extraction of it is not economically viable using conventional methods bioleaching is an important alternative technology. Studies conducted under mesophilic conditions have shown poor copper recoveries from chalcopyrite. This is mainly due slow dissolution kinetics and passivation of the surface which renders the process economically unviable (Yu *et al.,* 2008; Klauber, 2008). Recent research on chalcopyrite leaching has focussed on high temperature bioleaching tests and promising results have been obtained (Rodriguez *et al.,* 2003a; Gautier *et al.,* 2008). The high temperature results in higher dissolution rates as well as inhibiting the formation of the products which lead to passivation of the mineral surface. High temperature bioleaching processes have therefore been identified as having an important role in the economic extraction of copper from the chalcopyrite deposits.

Bioleaching technology has been successfully implemented commercially and an example of such a process is the BIOX® Process. Refractory sulphide gold deposits are treated in the process as the sulphides are broken down, exposing the encapsulated gold for further cyanidation. The process involves a continuous feed on the flotation concentrate slurry to three stirred reactors. The conditions within the reactors are maintained at optimum conditions for sulphide oxidation and bacterial activity

and aeration of the reactors is controlled. There are currently four BIOX® plants in operation in South Africa, Ghana, Brazil and Australia. The most successful of these is Ashanti's Sansu plant in Ghana and processes 960 tons per day. The BIOX® process clearly shows the bioleaching technology has economic benefits when implemented commercially and justifies extensive research into improving this technology to extract copper from chalcopyrite (Gold Fields, http://www.goldfields.co.za).

## *2.2. Bioleaching Process*

### 2.2.1. Chemistry of mineral sulphide oxidation

Bioleaching is the process where metals are extracted from mineral ores due to both chemical and biological processes. The extraction of mineral sulphides is achieved by oxidative processes in the presence of bacteria, which act as catalysts (Crundwell, 2003). In the extraction of copper, ferric ions and hydrogen ions are responsible for the dissolution of the metal.

The role of the bacteria is to regenerate the ferric and sulphuric acid required in the dissolution process above, by the oxidation of ferrous iron and reduce sulphur species (equation 3 and 4). Equations 3 and 4 highlight the necessity for oxygen in the bioleaching process. Oxygen is required for the bacteria to obtain their energy as it acts as an electron acceptor in the oxidation reactions. In this way, the amount of metal that can be dissolved for a specific initial ferric ion concentration is increased.

Different minerals proceed through various intermediates before finally forming sulphuic acid. For acid-insoluble minerals such as pyrite, thiosulphate is the main intermediate and for acid-soluble minerals such as chalcopyrite it is a polysulphide (Rawlings *et al.*, 2003).



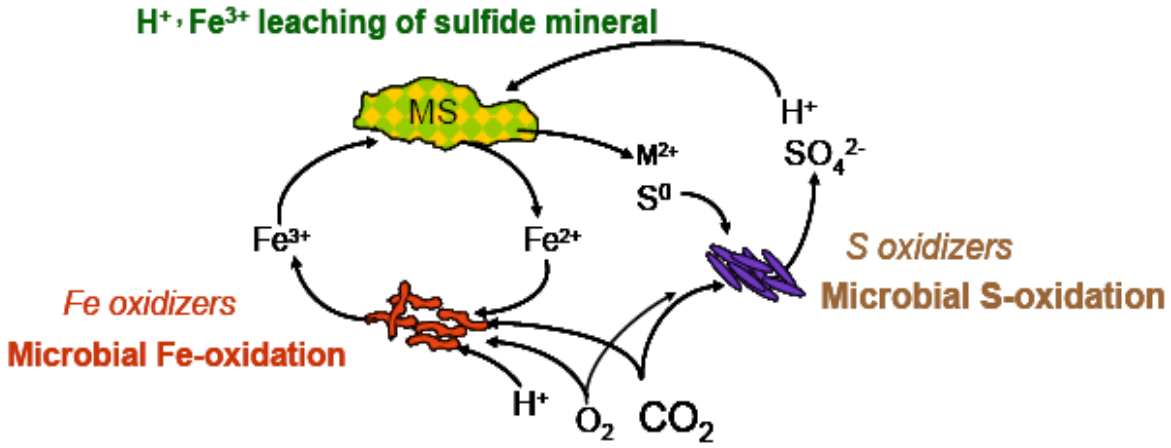



**Figure 2.1:** Role of bacteria in bioleaching process (Ojumu, 2008)

Effective leaching by the above leaching mechanism is only made possible by the abundance of iron and sulphur oxidising bacteria that thrive in the environment that is created. The reactions that take place are exothermic and therefore release energy in the form of heat during the bioleaching process. A microbial consortium that can operate across a wide range of temperatures is necessary in order to ensure that the heap can operate optimally throughout the process.

## *2.3. Bioleaching*

There are two broad categories of bioleaching processes used in industry, namely tank bioleaching and heap bioleaching.

### 2.3.1. Tank Bioleaching

During tank bioleaching a finely milled mineral concentrate is placed in a stirred tank where it is vigorously aerated. The mineral decomposition only takes days in a stirred tank reactor as the concentrate is finely milled which increases the exposed surface area substantially. There are limitations to the loading of the reactor as it high loadings the shear stress is detrimental to microbial growth. The costs of operating a tank bioleaching process are approximately 20% greater than those required to operate a heap bioleaching process. This is due to the high energy cost associated with finely milling the ore. Tank bioleaching processes are therefore limited to use in extracting high value minerals in order to offset this cost (Rawlings *et al.*, 2003).

### 2.3.2. Heap Bioleaching

For heap bioleaching, ore is placed in a heap or dump where it is then irrigated and sometimes aerated. Mineral decomposition can take months or even years in a heap process. However, the slow dissolution rate is offset by the reduced cost of construction and operation. Heap processes are used in the extraction of metal from lower grade ores as the low costs make the process economically feasible. The conditions within a heap are not homogenous, like in the stirred tank reactor, making them far more difficult to control (Rawlings *et al.*, 2003).

In a heap process agglomerated ore is piled on an impermeable base. The heap is constructed with a leach liquor distribution and collection system. Microbes within the heap carry out the oxidation reactions described earlier which allow the leaching of the metals into solution. The microorganisms require oxygen and carbon dioxide for growth and this can be achieved passively, with air being drawn into the heap by the flow of liquid, or actively with air pumped through an engineered aeration system

into the heap. The pregnant leach solution, containing the soluble metal, is then collected and sent for metal recovery (Rawlings *et al.*, 2003; Brierley, 2001). A schematic of the process of heap bioleaching can be seen in Figure 2.2

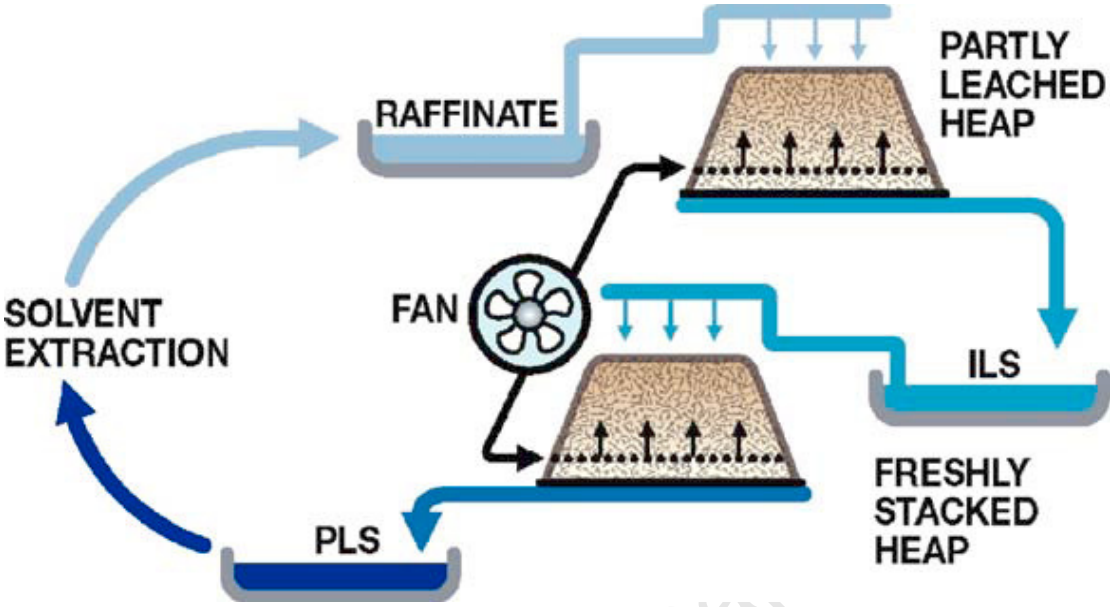



**Figure 2.2:** Schematic of overall bioleaching heap process (Watling, 2006)

Due to the non-homogeneous conditions within the heap, pH gradients exist. This is a result of the ore being a mixture of mineral and gangue. The oxidation of some sulphide minerals (pyrite, chalcopyrite) produces acid while the gangue materials present are generally acid consuming. This makes it difficult to maintain the pH in the desired range 1.8 – 2.2 in order to prevent the ferric iron from precipitating out at a pH of 2.5 or higher (Rawlings *et al.*, 2003).

Inorganic fertiliser can be added to the heaps in order to provide the microorganisms with nutrients such as nitrogen, phosphate, potassium and trace elements (Rawlings *et al.*, 2003). Provision of nutrients is also complicated in heaps as the main nutrient is ammonia, which if added to areas where the pH is too high will cause jarosite formation. Jarosite is an iron containing compound ($MFe_3(SO_4)_2(OH)_6$ where M = $K^+$, $Na^+$ or $NH_4^+$ (Watling, 2006)) which not only removes ferric ions from solution during formation, but also coats the ore surface inhibiting metal dissolution.

Uneven distribution of the microorganisms is initially experienced following inoculation due to inhomogeneity within the heap and preferential flow paths. The mobility of the microorganisms allows for a more even distribution within the heap after a period of time. However this variation in

distribution can be minimised by the careful inoculation of the heap during construction, leading to enhanced rates of dissolution. An option that can be used to ensure even distribution is to add a microbial inoculum to the ore when agglomerating it with acid. A disadvantage of this is that if the acid levels are too high, the microorganisms' viability can be compromised (Rawlings *et al.*, 2003). Inoculation of the heap occurs at temperatures often below ambient and this will have an impact on the microorganisms' metabolic activity and subsequently on the colonisation of the heap under these sub-optimum conditions. This is particularly significant for thermophilic archeae, which perform optimally at temperatures above 60°C. These organisms have shown poor attachment at ambient conditions (Africa, 2009). This poor attachment leads to poor colonisation or even substantial washout of the thermophiles.

The rate of mineral dissolution increases with increasing temperature in the heap. The temperature of a heap is increased due to the mineral oxidation, which involves exothermic reactions, but can be further increased, either by insulation or by optimising the rate of aeration to minimise heat stripping from the heap while still ensuring maximum microbial activity. Insulation however is not a feasible option for large heap process for practical reasons (Rawlings *et al.*, 2003).

## *2.1. Bioleaching of chalcopyrite*

### 2.1.1. Mechanism

The leaching of chalcopyrite proceeds through the two chemical reactions which follow.

$$CuFeS_2 + 4Fe^{3+} \rightarrow 5Fe^{2+} + Cu^{2+} + 2S^0 \quad (1)$$

$$CuFeS_2 + 4H^+ \rightarrow Fe^{2+} + Cu^{2+} + 2H_2S \quad (2)$$

The role of the bacteria is to replenish the ferric and hydrogen ions by the following two reactions.

$$2Fe^{2+} + 2H^+ + 0.5\ O_2 \rightarrow 2Fe^{3+} + H_2O \quad (3)$$

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \quad (4)$$

In the case of chalcopyrite, the process is complicated by passivation of the ore surface. The passivation of chalcopyrite has been the focus of sustained research. While there is consensus on the existence of the layer there is not yet a universally accepted mechanism regarding its formation. A number of theories have been proposed, including the formation of a copper-rich polysulphide layer (Tshilombo and Dixon, 2003), an elemental sulphur layer (Muñoz *et* al., 1979), a jarosite layer (Córdoba *et* al., 2008)

and a combination of chemical and diffusion control (Hackl *et* al., 1995). The rate of mineral dissolution is decreased by this phenomenon and it is therefore necessary to operate at conditions which reduce its impact. Operation at high temperature and low pH has been shown to minimise the effect of passivation (Watling, 2006). Microorganisms with optimum growth conditions at low pH's and high temperatures will therefore play a key role in improving the rate of metal dissolution that can be achieved from chalcopyrite with bioleaching.

The chalcopyrite ore used in this study had a low copper content with only 0.5% chalcopyrite and 4.0% pyrite. The gangue material therefore makes up the majority of the substrate and plays a critical role in the leaching kinetics that take place. The gangue components of the ore will vary with location and extraction efficiency will change from site to site. The ore used in this study contained mainly quartz and muscovite as gangue components. The properties of these minerals are discussed in the next section.

## *2.1. Mineral substrates associated with low grade chalcopyrite ores*

Although the focus of this study is on the copper containing ores the material loaded onto the heap will contain other sulphide minerals and be predominantly gangue. Therefore a chalcopyrite concentrate, pyrite concentrate and a low grade ore were investigated. The main gangue components of the low grade ore were quartz and muscovite. As the mineral surfaces often contain elemental sulphur due to the oxidation of the sulphur containing components, the properties of sulphur are be discussed.

**Table 2.1**: Characterisation of important mineral components of chalcopyrite ore (http://webmineral.com/data.shtml, http://geology.com/minerals.shtml)

| Mineral | Lustre | Diaphaneity | Cleavage | Fracture | Hardness | SG | Crystal |
|---|---|---|---|---|---|---|---|
| Pyrite $FeS_2$ | metallic | opaque | indistinct | irregular, conchoidal | 6 – 6.5 | 4.9 – 5.2 | isometric |
| Chalcopyrite $CuFeS_2$ | metallic | opaque | poor | brittle and conchoidal | 3.5 - 4 | 4.1 – 4.3 | tetragonal |
| Quartz $SiO_2$ | vitreous | transparent - translucent | none | conchoidal | 7 | 2.6 – 2.7 | hexagonal |
| Muscovite $KAl_2(Si_3Al)O_{10}(OH,F)_2$ | vitreous | transparent - translucent | perfect | brittle, sectile | 2.5 - 3 | 2.8 – 2.9 | monoclinic |
| Sulphur $S_8$ | | | | brittle | | 2.07 | rhombic |

Pyrite is a pale brass yellow colour with a greenish black to brownish black streak. It is one of the most ubiquitous minerals of the earth's crust. Pyrite is a polymorph of marcasite with the same chemical composition, but a different structure. Pyrite is currently mined for its sulphur, rather than iron, content. The sulphur in the mineral makes the iron brittle and there are other iron oxides from which it is more economical to extract the iron. In the event of a scarcity of these iron oxide ores, pyrite is a potential source of iron which could be exploited.

Chalcopyrite is also a brass yellow colour with a greenish black streak, but is slightly darker than pyrite. It is the most abundant copper mineral and is found in almost all sulphide deposits. The yield of copper in chalcopyrite is rather low in terms of atoms per molecule (± 25%), compared to 50% - 67% with the other copper containing minerals. However, the large quantities of chalcopyrite make it a leading source of copper.

Quartz can be found in virtually every colour, the common colours are clear, white, gray, purple, yellow, brown, black, pink, green and red. It is the most abundant mineral at the earth's surface and is ubiquitous, easily mined and durable. A few of the uses of quartz are glass making, foundry sand, hydrofrac sand, optical materials, components in electronic products, traction sands, sharpening media, polishing compounds, grinding compounds, fillers and extenders.

Muscovite is colourless, yellow, brown, green or red with a white streak and is the most common mineral of the Mica Group minerals. It is a rock forming mineral, has a high temperature resistance and

is easily recognisable by its perfect cleavage into thin sheets (http://webmineral.com/data.shtml; http://geology.com/minerals.shtml and http://www.mindat.org/min-2815.html).

At ambient conditions sulphur is found as rhombic sulphur, a light yellow brittle powder. At temperatures around 95°C rhombic sulphur becomes the allotrope monoclinic sulphur. It is one of the best thermal insulators, ranking with wood and mica. Sulphur is not easily wetted or dissolved by water and elemental deposits are often found coating the mineral sulphides (http://www.gps.caltech.edu/~vijay/Papers/Chemistry/Meyer-76.pdf).

## *2.2.Microorganisms involved in bioleaching*

The most important microorganisms involved in bioleaching are iron and sulphur oxidising chemolithoautotrophs. These microorganisms obtain their carbon from fixing the carbon dioxide in the atmosphere. However, unlike other autotrophs, chemolithoautotrophs do not obtain their energy from the sun, but rather by using either ferrous iron or reduced sulphur compounds as an electron donor and oxygen as an electron acceptor (Rawlings *et al.*, 2003).

In bioleaching processes that operate around 40°C the most important organisms are a consortium of Gram-negative bacteria which include the iron and sulphur oxidising *Acidithiobacillus ferrooxidans,* the sulphur oxidising *Acidithiobacillus thiooxidans, Acidithiobacillus caldus* and the iron oxidising *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum.* When operated at 70°C, the consortium of microorganisms found within the heap is dominated by archeae rather than bacteria. The most prominent genera found at these conditions are *Acidianus, Sulfolobus* and *Metallosphaera* (Rawlings *et al.*, 2003).

**Table 2.2:** Classification of microorganism by optimal temperature range

| Type of microorganism | Optimum temperature range |
|---|---|
| Mesophile | 25°C - 40°C |
| Moderate thermophile | 40°C – 55°C |
| Extreme thermophile | Above 65°C |

**Table 2.3:** Classification of microorganism by energy source

| Type of microorganism | Carbon or energy source |
|---|---|
| Autotrophic | Carbon from $CO_2$ |
| Heterotrophic | Carbon from organic carbon |
| Lithotrophic | Energy from the oxidation of inorganic compounds |
| Organotrophic | Energy from the oxidation of organic carbons |

**Table 2.4:** Classification of microorganism by structure

| Type of microorganism | Cell wall structure |
|---|---|
| Gram-positive | - Consists primarily of peptidoglycan<br>- Relatively thick<br>- Negatively charged and is partly responsible for the overall negative surface charge of the microorganism |
| Gram-negative | - multilayered<br>- relatively complex |
| Archeae | - Do not contain peptidoglycan<br>- Contain a variety of related and unrelated polysaccharides<br>- Most common type is the paracrystalline surface layer (S-layer) which consists of protein or glycoprotein and is generally of hexagonal symmetry |

Other heterotrophic bacteria are required in conjunction with the chemolithoautotrophs for the bioleaching process. These bacteria utilise the organic compounds produced by the other cells for growth in and ensure that the concentration of these compounds does not get too high and inhibit the iron and sulphur oxidisers.

A list of microorganisms isolated from mineral sulphide ores and commonly used in heap bioleaching processes is shown in Table 2.4 (Watling, 2006). The optimum growth conditions of these bacteria can be used to optimise the bioleaching process.

**Table 2.5:** Iron and Sulphur Oxidising Acidophiles (Watling, 2006)

| Organism | Reported growth substrates | Characeristics |
|---|---|---|
| *Acidianus ambivalens* | S oxidation and reduction | Hyperthermophiles |
| *Acidianus brierleyi* | Sulphides | pH opt 1.5-2.5 |
| *Acidianus infernus* | Poor, if any, Fe oxidation | |
| *'Acidianus tengchangensis'* | | |
| *Acidimicrobium ferrooxidans* | Mixotroph, Fe oxidation and reduction, Sulphides (poor) | Moderate thermophile pH opt 2 |
| *Acidipjilium spp* | Obligate heterotrophs | Mesophiles |
| *Acidiphilium SJH* | S oxidation, Fe(III) reduction | pH opt ~2-3 |
| *Acidiphillium acidophilum* | Facultative autotroph, S oxidation, Fe(III) reduction | Mesophile pH opt ~2-3 |
| *Acidithiobacillus albertensis* | Autotrophs | Mesophiles |
| *Acidithiobacillus ferrooxidans* | S oxidation, sulphides | pH range 2-4 |
| *Acidithiobacillus thiooxidans* | (*Af,* Fe(II) oxidation; Fe(III) reduction as a facultative anaerobe) | |
| *Acidithiobacillus caldus* | Mixotroph, 3S oxidation, Sulphides | Moderate thermophile |
| *Acidolobus aceticus* | Heterotroph, S reduction to $H_2S$ | Hyperthermophile |
| *Alicyclobacillus spp* | S oxidation, sulphides | Mesophiles – moderate thermophiles |
| *'Alicyclobacillus disulfidooxidans'* | (*Ad,* facultative autotroph; | pH 1.5-2.5 |
| *'Alicyclobacillus tolerans'* | *At,* mixotroph, Fe(III) reduction) | |
| *'Ferrimicrobium acidiphilium'* | Heterotroph, Fe(II) oxidation, sulphides, Fe(III) reduction | Mesophile |
| *Ferroglobus placidus* | Fe oxidation | Thermophile, pH neutral |
| *Ferroplasma acidarmanus* | Possibly autotroph | Moderate thermophiles |
| *Ferroplasma cyprexacervatum* | Iron oxidation | pH range <1-2 |
| *Ferroplasma acidophilium* | Pyrite oxidation poor | |
| *Ferroplasma MT17* | | |
| *Hydrogenobaculum acidophilus* | S, H oxidation to produce sulphuric acid | Thermophile, pH opt 3-4 |
| *Leptospirillum ferriphilum* | Fe oxidation | Mesophiles, some thermo tolerant strains |
| *Leptospirillum thermoferrooxidans* | Pyrite | pH range 1.6-1.9 |
| *Leptospirillum ferrooxidans* | Fe oxidation, pyrite | Mesophile, pH opt 1.5-1.7 |
| *Metallosphaera sedula* | S oxidation | Thermophiles |
| *Metallosphaera prunae* | Sulphides | pH 1-4 |
| *'Metallosphaera hakonensis'* | | |
| *Sulfobacillus acidophilus* | Fe(II) oxidation; Fe(III) reduction, sulphides | Moderate thermophiles |
| *Sulfobacillus thermosulfidooxidans* | S oxidation | pH 1-2.5 |
| *Sulfolobus metallicus* | Strict chemoliyhoautotroph | Hyperthermophiles |
| *'Sulfolobus rivotincti'* | S oxidation, sulphides | Various pH in range 1-4.5 |
| *Sulfolobus shibatae* | | |
| *'Sulfolobus tokodail'* | | |
| *Sulfolobus yangmingensis* | | |
| *'Sulfolobus' JP2 and JP3* | | |
| *Sulfolobus acidocaldarius* | Heterotrophs | Hyperthermophiles |
| *Sulfolobus solfataricus* | Not S oxidation | pH 2.-4.5 |
| *Sulfurococcus yellowstonensis* | S and Fe oxidation | Hyperthermophile |
| *Thiobacillus prosperus* | S and Fe oxidation, Sulphides | Mesophile, halophile, pH opt 2 |
| *Thiomonas cuprina* | S oxidation, sulphides | Mesophile, p opt 3-4 |

## *2.3. Extreme thermophiles*

As mentioned previously, extreme thermophiles play a crucial role in facilitating the extraction of copper from chalcopyrite. The most abundant extreme thermophiles found in heap bioleaching systems, *Acidianus, Sulfolobus* and *Metallosphaera,* all belong to the order *Sulfolobales* (Mikkesen *et al.,* 2007). The *Sulfolobales* were first identified in 1989 by Stetter and are thermoacidophilic regular or irregular cocci that favour terrestrial geothermal habitats. The order has one known family, Sulfolobaceae, comprosing of 6 genera, *Sulfolobus, Metallosphaera, Sulfurococfolobus, Acidianus, Sulfurisphaera and Stygiolobus* (Itoh, 2003).

**Table 2.6:** Important bioleaching thermophilic archeae belonging to the order Sulfolobales (Itoh, 2003)

| Genus | Temp. Range (°C) | Opt. Temp. (°C) | Opt. pH | $O_2$ requirement | DNA G+C mol% | Energy | Fe + S oxidiser |
|---|---|---|---|---|---|---|---|
| *Sulfolobus* | 50-95 | 65-80 | 2.0-4.0 | Aerobic | 33-42 | Autotroph | Both |
| *Acidianus* | 45-96 | 70-90 | 1.5-2.5 | Aerobic/ Anaerobic | 31-33 | Autotroph/ heterotroph | Both |
| *Metallosphaera* | 50-80 | 70-75 | 3.0 | Aerobic | 45-46 | Autotroph/ heterotroph | Both |

*Sulfolobus* cultures have shown a unique ability to extensively oxidise chalcopyrite and further research needs to be conducted on these microorganisms in order to further enhance the leaching of copper from chalcopyrite (Clark *et al.,* 1996). This study focuses on one of these microorganisms, namely *Metallosphaera hakonensis*, which was found to dominate thermophilic tank and column leach experiments in the UCT laboratories.

### *2.3.1. Metallosphaera hakonensis*

The microbial population in a set of large simulated column reactors were monitored and during the high temperature operation, were found to be dominated by *Metallosphaera hakonensis* (unpublished data). This microorganism was formerly known as *Sulfolobus hakonensis* before being reclassified as belonging to the genus *Metallosphaera.* It is a facultative chemolithotroph and can obtain its energy either from organic sources or through the oxidation of iron and sulphur (Johnson, 2006).

## *2.4. Attachment*

The attachment of microorganisms to solid surfaces is well established and has been demonstrated in numerous studies (Watling, 2006). van Loosdrecht and co-workers (1990) showed that the highest level of microbial activity was found at theses surfaces. A biofilm is formed around attached cells and provides them with an optimum, homogenous environment. Attached cells are able to function, even when the surrounding conditions are not optimal (Watnick *et al.*, 2000). Attachment of the cells to the ore surface is therefore an important phenomenon as it allows for optimum microbiological activity to take place. Research is therefore required into the process of attachment.

### 2.4.1. How attachment enhances mineral dissolution

Numerous studies have shown that the attachment of microorganisms to the ore surface enhances the rate of leaching. Examples of a few such studies follow in the text below.

Gehrke (1998) performed experiments which looked at the dissolution rates of pyrite using abiotic leaching, inactive bacteria with EPS, active bacteria with no EPS and active bacteria with EPS. *A. ferrooxidans* was used in these experiments. The highest dissolution rate was obtained with active bacteria containing EPS. As EPS assists the attachment of cells, a correlation between cell attachment and mineral dissolution rate exists.

Rodriguez (2003) conducted tests to prove a direct relationship between bioleaching kinetics and attachment during the high dissolution period. The attachment of mesophilic bacteria to pyrite was shown to be faster than that of thermophilic archeae and this coincided with the best kinetic dissolution observed. However with chalcopyrite and sphalerite, attachment was higher with thermophilic archeae and this again coincided with a higher mineral dissolution rate.

Two reasons for the increased rate of mineral dissolution observed have been suggested and research has been conducted into both. One reason is that the attached bacteria are in close contact with their required energy source (Watnick *et al.*, 2000). Therefore in a nutrient poor environment they are able to position themselves near the energy source and continue to function optimally, as the biofilm provides a constant, favourable environment for the microorganisms. An alternative is that the EPS provides a reaction space with the ferric ions and mineral substrate in close proximity to one another. The ferric ions in the EPS are then able to efficiently leach the sulphide mineral via equation 1 (Harneit *et al.*, 2006; Kinzler *et al.*, 2003). Usher *et al.* (2010) have discovered evidence of ferric ions present in the EPS of *Metallosphaera hakonensis*, so the proposed theory is of relevance to this microorganism.

### 2.4.2. Mechanism of attachment

Zobell (1943) proposed an attachment model that assumes that adsorption of the cells follows second order irreversible kinetics with respect to the bacterial concentration and substrate surface area. The model consists of 2 steps, namely an initial stage of reversible adhesion followed by irreversible adhesion. This model was then adapted by van Loosdrecht (1990) to incorporate a 4 step process as outlined below.

#### 2.4.2.1. Transportation

The first step involves transport of the cell to the ore surface. There are three modes of transport that a cell can undergo in order to reach the ore surface, namely:

- **Diffusive Transport**

Diffusive transport is caused by non-negligible Brownian motion (40 μm/h). It involves the random contacts of organisms with interfaces and is responsible for crossing any diffusive layer over which convection cannot take place. This method of transport is slower than both convective transport and active movement.

- **Convective Transport**

Convective transport is a result of liquid flow and is several orders of magnitude faster than diffusive transport. However, situations occur in which the last part of the route is diffusion controlled and this therefore becomes the rate limiting step.

- **Active Movement**

In active movement, the microorganisms can actively move into the vicinity of the interface. Flagella, which are force generating organelles, are used for movement (Watnick *et al.*, 2000). Once the microorganism is in the vicinity, it can either encounter the interface by chance or it can chemotactically respond to a concentration gradient. Chemotaxis is a response to a stimulus which can either be an attractant or a repellent. Bacteria therefore move up or down the concentration gradient depending on the particular stimulus it is exposed to. The process makes use of signal transduction proteins containing two functional domains. Recent research has identified quorum sensing communication, using acyl-homoserine lactones, in bioleaching organisms and suggests this may play a role in attachment to pyrite (Ruiz *et al*., 2008). Additional research is still required in order to determine the mechanisms involved

(Rojas-Chapana *et al.,* 1998). The common bioleaching bacteria, *Leptospirillum ferrooxidans*, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, are all motile (Jerez, 2001).

#### 2.4.2.2. Initial Adhesion

Once at the ore surface, the microorganism initially becomes associated with the surface by a physicochemical process which can be either reversible or irreversible. The microorganisms exhibit Brownian motion and can be readily removed by mild shear or the bacteria's own motile movement during reversible adhesion and the converse during irreversible adhesion. Attachment occurs more readily to positively charged surfaces (Zobell, 1943), but under normal heap conditions the mineral sulphide is generally negatively charged (Poortinga *et al.,* 2002). The extracellular polymeric substances (EPS) of *A. ferrooxidans* or *L. ferrooxidans* incorporate uronic acids and ferric ions rendering the cell surface charge positive (Gehrke *et al.*, 1998). The incorporation of uronic acids and ferric ions has not been shown for thermophilic archeae, but it is hypothesised that they will behave in a similar manner, despite the differences in surface structure between the mesophiles and thermophiles. Therefore, these microorganisms are electrostatically attracted to the mineral surfaces (Harneit *et al.*, 2006). This results in the initial attachment of bioleach organisms being mainly due to hydrophobic or electrostatic forces (van Loosdrecht *et al*., 1990; Devasia *et al.,* 1993; Harneit *et al.,* 2005).

#### 2.4.2.3. Firm Attachment

Special cell surface structures (polymers, fibrils etc) then form strong links between the cell and the ore surface during firm attachment. Polysaccharides are not required during the initial adhesion stage but are necessary at this stage in order to form surface films (van Loosdrecht *et al.*, 1990). Devasia and co-workers (1993) suggested the involvement of a proteinaceous cell surface appendage in adhesion of *A. ferrooxidans* to solid mineral surfaces of sulphur grown cells. According to this model of initial adhesion, followed by firm attachment, a metastable compound must first be formed. This is illustrated in the relationship below, from Rodriguez and co-workers (2003).

$$B + S \leftrightarrow [BS]^{*} \rightarrow BS \qquad (5)$$

where B is the bacterial cell and S represents the surface sites

This process of initial attachment followed by firm attachment can then be modelled as follows:

$$K_a t = \frac{1}{\frac{1}{a}A_o - X_{bo}} Ln\left[\frac{X_{bo}\left(\frac{1}{a}A_o - X_{bs}\right)}{\frac{1}{a}A_o\left(X_{bo} - X_{bs}\right)}\right] \quad (6)$$

Where $K_\alpha$ is the attachment rate constant (ml/cell.h); $A_o$ is the concentration of surface adsorption sites ($cm^2/cm^3$); α is the projected area per bacteria ($cm^2$/cell); $X_{bo}$ is the initial concentration of free bacteria (cell/ml); $X_{bs}$ is the concentration of attached bacteria on the solid surface (cell/ml); and t is the time (h). (Rodríguez *et al.*, 2003)

#### 2.4.2.4. Colonisation

After the microorganisms have firmly attached to the ore surface, they continue growing and newly formed cells remain attached to each other. This can then lead to the formation of a biofilm. Biofilms are complex communities of differentiated sessile microorganisms which are embedded in a polysaccharide matrix. The colonisation and formation of biofilms is highly specific and can be affected by mineral and bacterial type as well as the external environment. Studies have shown that the biofilms formed during bioleaching are monolayered and are usually surrounded by a mucilaginous layer of EPS (Harneit *et al.*, 2006). Close contact with the ore surface appears to be important owing to the fact that the biofilm is always monolayered (Watnick *et al.*, 2000).

Biofilm formation has been shown to be coordinated by diffusible signal transduction mechanisms, such as quorum sensing. Researchers have isolated genes which may encode for a functional type AI-1 quorum sensing system in *At. ferrooxidans*, but there is a large gap in the literature on the formation of biofilms (Farah *et al.,* 2005).

#### 2.4.2.5. Importance of EPS

Lipopolysaccharides or extracellular polymeric substances (EPS) are excreted by the microorganisms and mediate attachment and biofilm formation. The composition and functionality of EPS has been extensively researched, but there are still gaps in our knowledge (Harneit *et al.*, 2006; Kinzler *et al.*, 2003; Savage *et al.*, 1985; Gehrke *et al., 1998*). The EPS of *A. ferrooxidans* is made up of predominantly sugars and lipids as well as some phosphorous, nitrogen and free fatty acids (Harneit *et al.*, 2006; Kinzler *et al.*, 2003; Gehrke *et al.,* 1998).

The different amounts of EPS that were obtained from different strains of *A. ferrooxidans* in a study by Kinzler and co-workers (2003) did not correlate with the known oxidation activities of the strains. However the amount of EPS-bound ferric ions was found to vary with the metabolic activity of the cells. It was thus suggested that the ferric ions complexed within the EPS of the cells play an important role in the attachment of these cells to the ore surface (Kinzler *et al.*, 2003). Harneit (2006) showed that the attachment of cells of *A. ferrooxidans* to pyrite or sulphur and the attachment of *A. thiooxidans* is influenced by their EPS, as attachment was significantly decreased by a factor of 2.5 to 3.4 when the cells were depleted of their EPS.

A study done by Usher *et al.* (2010) showed that EPS production in *M. hakonensis* only occurred when the microorganism was contacted with a solid substrate and even then, there was a relatively small amount of EPS produced when compared with *At. ferrooxidans* cells. The cells had a speckled appearance, when examined by HAADF STEM imaging, when grown in the presence of Fe. STEM EDS analysis showed that the granules contained iron and phosphorous and further analysis with EELS showed that the iron was present as $Fe^{3+}$. The presence of ferric ions in the EPS corresponds to what is known about *At. ferrooxidans* cells and it appears that the EPS of *M. hakonensis* enhances attachment in a similar way to *At. ferrooxidans.*

The mediation of attachment is explained in more detail in Section 2.4.2.2. The role of the EPS is to create a positive cell surface charge or increase the cells hydrophobicity, allowing for electrostatic interactions to take place.

The properties and composition of the EPS are dependent on the growth media of the microorganisms and this is further discussed in Section 2.5.4. In order to enhance the attachment of the bacteria and therefore the mineral dissolution, a growth media which increases the concentration of ferric ions in the EPS of the cells should be used for the inoculum of industrial bioleaching heaps. Further research into different growth media and the resulting EPS composition is therefore important for enhancing the overall process.

#### 2.4.2.6. Langmuir adsorption isotherm

The Langmuir isotherm is developed assuming that the adsorbent has *n* identical binding sites and that binding is the same and unhindered for all molecules. For one molecule we then have:

$$B + S \leftrightarrow BS \quad (7)$$

where S represents the substrate or absorbent, B represents the adsorbate or bacteria and BS is the binding complex. This is very similar to the equation that is used to describe initial adhesion and it follows that this model is also applicable to this process (Liu, 2006).

The Langmuir isotherm was developed to describe the adsorption of gas molecules onto solid surfaces. It has since been modified to describe the adsorption of solutes onto solid surfaces in aqueous systems. The adsorption of microorganisms in a bioleaching heap system can be modelled using this same equation. The main difference between the modified and original models is the introduction of a concentration dependant factor. This allows for the effect that the solute concentration, or in this case the planktonic cell concentration, has on the adsorption and desorption of the molecules to be accounted for. The Langmuir isotherm equation for aqueous systems is then:

$$\frac{X_e}{q} = \frac{X_e}{q_m} + \frac{1}{K_a q_m} \quad (8)$$

Where $X_e$ represents the concentration of adsorbate at equilibrium, q represents the amount of adsorbate absorbed per gram of adsorbent, $q_m$ is the amount of adsorbate required to form a monolayer on the adsorbent (maximum adsorption capacity) and $K_a$ is the Langmuir adsorption equilibrium constant.

Data has been accurately fitted to the modified Langmuir isotherm model indicating that the planktonic cell concentration is a key parameter in modelling the adsorption of cells onto solid substrates (Sohn *et al.,* 2005). There is a substantial amount of work that has been done in other areas of research such as biosorption which have used the Langmuir Isotherm model to adequately describe the adsorption or attachment occurring in the system. Literature which supports the applicability of the Langmuir isotherm to a system such as microbial attachment in bioleaching heaps is thus extensive (Bayrak, 2006; Amini *et al.,* 2009; Del Bubba *et al.,* 2003; Rudzinski *et al.,* 1996; Misak, 1993; Pagnanelli *et al.,* 2003).

## *2.5. Factors affecting attachment*

### 2.5.1. Temperature

There is a large body of literature which shows that bioleaching organisms have an optimum temperature range outside of which they are less metabolically active. Franzmann and co-workers measured the activity, as a function of temperature, of a variety of microorganisms and used the Ratkowsky equation to predict the optimum temperature as well as at what point cell death occurs. An increase in temperature increases the rate of mineral leaching (Franzmann *et al.,* 2005; Breed *et al.,*

1999). This is specifically relevant to chalcopyrite, where the low rate of copper dissolution under mesophilic conditions affects the economically viability of the process. The effect of temperature on microbial attachment is not well understood as the majority of attachment studies have been done at either ambient temperature or the optimal growth conditions of the microorganism. Increasing temperature has been shown to have a significant effect on the activity and leaching efficiency of the microorganisms and leaching efficiency has been linked to microbial attachment and colonisation of the ore. If this relationship is consistent it implies that increasing temperature will lead to enhanced levels of attachment by thermophilic microorganisms.

A study by Zou *et al.* (2006) supports the idea of a 2-step inoculation process based on poor attachment of thermophiles to the mineral sulphides at ambient temperature (Africa *et al.,* 2009). In such a system the heap would be inoculated with a mixed mesophilic culture and only once the maximum temperature attainable by the mesophilic culture (±45°C) was reached would the thermopliles be introduced.

### 2.5.2. Mineralogy

Previous studies have shown the preferential attachment of microorganisms to different ores types (Harneit *et al.*, 2006; Afzal Ghauri *et al.*, 2007; Sampson *et al.*, 2000; Ohmura *et al.*, 1993; Gonzalez *et al.*, 1999; Rodríguez *et al.*, 2003; Africa *et al.,* 2009) and the effect of particle size on attachment (Afzal Ghauri *et al.*, 2007). Rodriguez and co-workers (2003a) showed that mesophiles attach preferentially to pyrite, followed by chalcopyrite and then sphalerite. Thermophiles exhibited no preferential attachment to the substrates investigated. The results for the mesophiles were confirmed by the work of several authors (Harneit *et al.,* 2006; Gonzalez *et al.,* 1999) and the thermophiles by Etzel and associates (2008). The preferential attachment of mesophiles has been further confirmed by Ohmura (1993) who demonstrated selective adhesion of *At. ferrooxidans* to sulphide minerals relative to other minerals in shake flask experiments. A study using mesophiles and moderate thermophiles (Sampson, 2000) showed preferential attachment to sulphide minerals by all species tested, which contradicted the Rodriguez work. However, this could possibly be attributed to structural differences between bacteria and archeae. Africa *et al.* (2009) showed a clear preference of *Acidithiobacillus ferrooxidans* to both chalcopyrite and pyrite (97% and 93% respectively) compared to low grade ore (52%) and quartz (50%). Usher and co-workers (2010) obtained High Angle Annular Dark Field Scanning Transmission Electron Microscopy (HAADF STEM) images showing *M. hakonensis* cells attached to jarosite on a chalcopyrite ore. This supported the reduced selectivity observed with thermophilic archeae.

From the above literature it can be seen that the preferential attachment of mesophilic cultures to sulphide minerals has been clearly proven, but the attachment behaviour of thermophilic cultures is less well understood and there are discrepancies between different studies.

Bioleaching microorganisms have been shown to attach preferentially to surface defects on the ore (Harneit *et al.*, 2006; Gehrke *et al.,* 1998). In the study done by Gehrke (1998), using *A. ferrooxidans,* 76% of all attached cells were attached to mineral imperfections. This could be due to concentration gradients emanating from these sites, marking them as preferential for attachment. This is due to the ready availability of sulphide and ferrous ions in these areas, which provide the cells with a readily available energy source (Harneit, 2006).

The spatial arrangement of the bacteria on the ore resulted in only a small percentage of the surface being covered by the microorganisms. Afzal Ghauri (2007) conducted experiments investigating the attachment of *A. ferrooxidans* on pyrite ore samples with a size fraction of 10 – 60 µm. The results of this study showed that although there was an available ore surface area of 54 $cm^2$, the attached cells only occupied 2.5 $cm^2$ at equilibrium. Both Moon (1996) and Africa *et al.* (2010) showed that microorganisms typically do not completely colonise the available surface area.

Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) or Fluorescence Microscopy (FM) can be used to view the spatial arrangement of the bacteria to the ore surface. With these techniques it is possible to visually confirm the number of attached cells, determine where the cells are congregating and visualise the formation of the biofilm (Edwards *et al.,* 2001; Sampson *et al.,* 2000; Lei *et al.,* 2009; Etzel *et al.,* 2008; Mangold *et al.,* 2008; Muller *et al.,* 2007; Mikkelsen *et al.,* 2007).

### 2.5.3. Surface Chemistry

As discussed in Section 2.4.2.2., the initial adhesion of the cells to the mineral surface is a physicochemical process. Colloid chemical theories have been used to explain the initial adhesion phase, as the bacterial suspension can be viewed as a living colloidal suspension. One of these theories is the Extended Derjaguin, Landau, Verwey, Overbeek (XDLVO) theory. This theory is an extension of the DLVO theory which states that adhesion is governed by long range forces; namely Lifshitz-van der Waals interactions and interactions from overlapping electric double layers which are assumed to be additive. If steric effects do not play a role, the total interaction Gibb's free energy can be determined from the summation of the van der Waals and electrostatic forces. The van der Waals forces are generally attractive and the electrostatic forces usually repulsive as both bacteria and the mineral surfaces are

predominantly negatively charged. The XDLVO theory then includes the short range Lewis acid-base or hydration interactions that occur (van Loosdrecht *et al.*, 1990; Poortinga *et al.*, 2002). Rijnaarts *et al.* contradict the theory that these short ranger hydration interactions exist and claim that initial adhesion is governed by the DLVO interactions and steric interactions only.

The XDLVO theory does not always accurately describe the attachment trends observed in bacterial systems. This is due to the cell surface being highly dynamic with charged groups associating or dissociating depending on changes in pH or ionic strength of the suspending fluid; or the approach of a charged surface. It also does not account for ligand-receptor binding which is typical of bio-systems. As the XDLVO theory is based on non-living particles it does not account for the changes that take place in the system such as the activity, size and shape of *M. hakonensis*, but it does provide a good basic understanding of the forces that are involved in the initial adhesion process and how they interact with one another.

Rijnaarts *et al.* (1993) conducted a study on the effect of ionic strength on the relative contributions of the DLVO and steric interactions on a number of different bacterial species. The study highlighted the difference between different strains, but there were general trends that were observed for all strains tested. The ionic strength of the suspending fluid plays an important role in determining which interactions will dominate in the initial adhesion. At a low ionic strength (<0.001 M), the initial adhesion of the cells is inhibited by DLVO-type electrostatic repulsion and at a high ionic strength (>0.1 M) it is dominated by steric interactions. As in bioleaching, the surfaces of the cells and mineral substrates are usually hydrophobic and the steric interactions typically manifest as bridging between the two surfaces. As the PLS usually has a relatively low ionic strength and electrostatic interactions are only suppressed at an ionic strength of approximately 0.5 M, it is necessary to consider all the interactions in order to properly understand the initial attachment process in the bioleaching system.



#### 2.5.3.1. Microbial Surface Properties

This study focuses on a thermophilic archeaeal species, which has significantly different surface characteristics to the more commonly investigated gram-negative bacteria involved in mesophilic bioleaching processes.

As explained in Section 2.4.2.2., the initial adhesion is mainly due to physicochemical interactions between the mineral substrate and the microorganism. The differences in surface structure between

gram negative bacteria and archeae will have an impact on these so an understanding of the surface characteristics should allow for a better understanding of the initial attachment process.

The cell wall polymers of various archeaeal lineages are chemically unrelated and differ considerably from peptidoglycan found in bacteria. The fact that no common target for antibiotics directed against the cell wall synthesis exists is a consequence of this. Classic antibiotics, such as fosfomycin, vancomycin and β-lactams have little effect effect against archeae (Kandler *et al.*, 1998).

Gram negative bacterial walls are multilayered, relatively complex and contain the molecule peptidoglycan, which forms rigid cell wall saculli in bacterial cells. Unlike bacterial cell walls, archeael cell walls do not contain peptidoglycan, but rather a variety of related and unrelated polysaccharides. The most common type of archeaeal cell wall is the paracrystalline surface layer (S-layer) which consists of protein or glycoprotein and is generally of hexagonal symmetry. Archeae therefore build the same cellular structures as bacteria, but out of different building materials. The main differences between these microorganisms lie in the cell membrane and are:

- Chirality of glycerol
- Ether linkage
- Isoprenoid chains
- Branching of side chains

The basic unit for all cell membranes is the phospholipid, which contains a molecule of glycerol with a phosphate added to one end and two side chains added to the other end. When the cell membrane is constructed, the glycerol and phosphate are on the outside and the long chains are sandwiched in the middle, creating a strong chemical barrier around the cell. The archeael glycerol (L-glycerol) is a sterieoisomer of the bacterial glycerol (D-glycerol). This has an impact on the cell structure, as only specific enzymes with the correct chirality are able to build the correct glycerol stereoisomer for the archeael phosphlipid.

Unlike in bacterial cell membranes, where the side chains are joined by ester linkages, the side chains in archeaeal cell membranes are joined by ether linkages. Archeaeal cell membranes therefore lack the protruding oxygen atom, present in ester linkages. This results in different chemical properties. Side chains in bacterial membranes are made from fatty acids, but in archeae these are made from chains of 20 carbon atoms built from isoprene. Isoprene can be joined together in numerous ways to form a variety of terpene compounds such as beta-carotene, natural and synthetic rubbers, plant essential oils

and steroid hormones. Due to the nature of isoprene, the side chains of archeaeal cell membranes have side chains off the main chain and this creates interesting properties in the archeaeal membrane which are not present in bacterial membranes.

In archeaeal cell membranes, the side chains of a phospholipid can be joined together, or joined to the side chain of another phospholipid. This is unique to archeael cell membranes and is not possible in any other group of organisms. The side branches are also able to form carbon rings by curling around and bonding to a carbon higher up the chain, forming a 5 carbon ring. These carbon rings are thought to provide stability to the microorganism as they are generally found in thermophilic microorganisms (Brock *et al.,* 1994).

### *Electrokinetic properties of microbial surfaces*

In systems with low ionic strength and where hydrophobicity is not dominant, the electrostatic forces between the microbe and the mineral play a dominant role in the initial adhesion of the microbe (Rijnaarts *et al.,* 1993). The electric charge of the cell is measured via the zeta potential and has been reported for numerous different microorganisms under different conditions. Relevant results from a few of these studies are discussed below.

Natarajan *et al.* (2003) showed that when suspended in a 1 mM $KNO_3$ solution, *At. ferrooxidans* cultured on both ferrous iron and elemental sulphur was negatively charged at a pH of 4 and below. Sharma *et al.* (2003) also determined the surface charge of *At. ferrooxidans* cultured on ferrous iron, elemental sulphur and pyrite. The cells were resuspended in a $10^{-3}$ M KCl solution before the measurements were taken. The cells cultured on ferrous iron had an isoelectric point (IEP) at pH 2 whereas the cells cultured on pyrite and elemental sulphur had an IEP at pH 3. The surface charge of the cells cultured on pyrite and elemental sulphur appeared to decrease more rapidly with an increase in pH than the cells cultured on ferrous iron. Huan *et al.* (2008) conducted a study on *Acidianus manzaensis,* a novel thermoacidophilic archeae. The cells were cultured on ferrous iron, elemental sulphur, pyrite and chalcopyrite and exhibited an IEP of pH 2 for the ferrous iron grown culture and an IEP of between 3.4-3.7 for the cultures grown on solid substrate. The maximum negative value was achieved at a pH of 7.5 and was -25 mV, -21 mV, -16 mV and -7 mV for the cells cultured on chalcopyrite, pyrite, elemental sulphur and ferrous iron. The IEP of the cultures is an indication of the functional groups such as carboxyl (-COOH), amino (-NH) and hydroxyl (-OH) which are present as components of the cell surface polymers of the culture. These functional groups determine the surface charge of the cell.

### *Hydrophobicity of microbial surfaces*

The hydrophobicity has been found to be the dominating interaction in initial adhesion when both the microbe and mineral surfaces are highly hydrophobic and was less significant when the cells were hydrophilic (van Loosdrecht *et al.,* 1987). In order to understand the interactions that take place during the initial adhesion, it is critical that both the electrostatic and hydrophobic interactions are considered.

There are various methods for determining the hydrophobicity of the cells and all appear to have an intrinsically large variance in the results that are obtained. Sampson and collaborators (2000) used contact angle measurements to determine the hydrophobicity of moderate thermophiles. Although there was a large variance in the results, they were able to show that cells cultured on chalcopyrite were

generally more hydrophobic than cells cultured on elemental sulphur, which in turn were more hydrophobic than the cells cultured on ferrous iron. This result was confirmed by Huan *et al.* (2008) who used 2-phase partitioning with hexadecane as a measure as hydrophobicity and showed that 17.75% of the cells cultured on chalcopyrite, 16.17% of the cells cultured on pyrite, 6.5% of the cells cultured on elemental sulphur and only 1.2% of the cells cultured on ferrous iron were partitioned into the organic hexadecane phase. Studies were also conducted on *At. ferrooxidans* and 2-phase portioning showed that cells cultured on sulphur were more hydrophobic than cells cultured on ferrous iron (Natarajan *et al.,* 2003). Sharma and co-workers (2003) used Raman spectroscopy and speculated that higher levels of protein are indicative of greater hydrophobicity. *At. ferrooxidans* grown on solid substrates (pyrite and sulphur) exhibited a greater hydrophobicity than the cells that had been cultured on ferrous iron.

#### 2.5.3.2. Mineral Surface Properties

The work of Yee and associates (2000) suggested that electrostatic attraction, van der Waals force, hydrophobicity, surface tension and surface roughness are the main factors in the initial adhesion of microbes to rock surfaces. The attachment of the microorganisms to surface defects has been discussed in Section 2.5.2. and the electrokinetic and hydrophobic properties of the mineral are discussed below.

***Electrokinetic properties of mineral surfaces***

Both the microbial cell surface and natural surfaces, such as the mineral sulphides used in bioleaching, are typically negatively charged (Poortinga *et al.,* 2002). However, the magnitude of the surface charge is dependent on the mineral properties as well as the environment in which it is found. Various studies have been conducted on determining the charge of the different minerals important in bioleaching (Table 2.7).

**Table 2.7:** Iso-electric points of various mineral substrates determined in different studies which will influence the electrostatic interactions with the microorganisms

| Author | Mineral | IEP | Suspending medium | Mineral Concentration |
|---|---|---|---|---|
| Vilinska *et al.* (2008) | Pyrite | 7.5 | $10^{-2}$ M $KNO_3$ | 0.25 g/l |
| | Chalcopyrite | 6.5 | | |
| Devasia *et al.* (1993) | Sulphur | 2.0 | $10^{-3}$ M $KNO_3$ | 0.1 g/l |
| | Pyrite | 2.9 | | |
| | Chalcopyrite | 2.6 | | |
| Natarajan *et al.* (2003) | Sulphur | 2.2 | $10^{-3}$ M $KNO_3$ | - |
| | Pyrite | 2.2 | | |
| | Quartz | <2.0 | | |

The difference in the results obtained is due to the ionic strength of the suspending medium used as well as the concentration of the mineral that was tested. In all studies the IEP of the substrates, other than quartz, was shifted after interaction with the different microorganisms. The IEP of the mineral substrates shifted towards the IEP of the microorganisms that it was contacted with. This indicates that the surface properties of the substrates are altered during interaction with the microorganisms. Quartz is the only substrate that appears to be positively charged under bioleaching conditions (pH 1.8-2.2). Pyrite, chalcopyrite and sulphur are natural surfaces and have been experimentally determined to have a negative surface charge as expected.

### *Hydrophobic properties of mineral surfaces*

It is difficult to precisely determine the hydrophobicity of mineral surfaces, but the contact angle measurement provides information on the relative hydrophobicity of the different substances of interest. The information from a study conducted by Ohmura *et al.* (1993) is summarised in Table 2.8.

**Table 2.8:** Contact angle measurements of various mineral substrates indicating the relevant hydrophobicities which will influence the hydrophobic interactions with the microorganisms

| Author | Mineral | Contact angle | Suspending medium |
|---|---|---|---|
| Ohmura *et al.* (1993) | Pyrite | 68.9 ± 2.1 | pH 2, $H_2SO_4$ |
| | Chalcopyrite | 83.4 ± 4.5 | |
| | Galena | 80.9 ± 2.5 | |
| | Quartz | 28.4 ± 4.3 | |

This study indicates that chalcopyrite and galena are the most hydrophobic, followed by pyrite, with quartz being the least hydrophobic. Given the variance associated with the contact angle measurement, the difference in contact angle between chalcopyrite, galena and pyrite is relatively small and all three substrates can be considered hydrophobic under bioleaching conditions. Quartz, however, is hydrophilic under bioleaching conditions and interacts significantly differently when in the presence of microorganisms.

### 2.5.4. Growth Media

Cells grown on sulphur, pyrite or chalcopyrite have been shown to be more hydrophobic and generate more EPS than cells grown on ferrous ions. This results in increased attachment of these cells to sulphide minerals (Sampson *et al.*, 2000; Harneit *et al.*, 2006; Devasia *et al.*, 1993; Sharma *et al.*, 2003).

A study by Sampson (2000) showed that moderate thermophiles exhibited greater attachment when grown on sulphur or chalcopyrite which was contrary to the results obtained with *A. ferrooxidans*, a mesophile.

The growth media affects the composition of the cells' EPS and this change in composition leads to a change in the relative attachment of cells to the ore. The accumulation of ferric ions in the EPS is dependent on the growth media. High ferric ion accumulation leads to increased cell attachment, as the cell surface is more positively charged and the hydrophobicity is increased (Kinzler *et al.*, 2003; Harneit *et al.*, 2006).

The culture history of the cells also affects the morphology of the cells. *M.hakonensis* cells cultured on ferrous iron have a roughly spherical shape, whereas those cultured on elemental sulphur have irregular geometric shapes and are larger in size (1.9 µm compared to 1.4 µm). HAADF STEM showed that the cells cultured on ferrous iron have a speckled appearance and those cultured on elemental sulphur did not appear to contain these same granules (Usher *et al.,* 2010).

The differences in IEP that have been reported for cells cultured in different media indicate that the growth medium has an effect on the surface properties of the cells. Huan and co-workers (2008) hypothesised that the lower IEP of ferrous iron grown cells (pH 2) is due to the presence of glucuronic acids or other polysaccharides containing negatively charged phosphate and/or carboxyl groups. The slightly higher IEP of cells cultured on a solid substrate (pH 3.4-3.7) is then due to the presence of $NH_3$ groups and suggests that there are more proteins present on the surface of these cells.

### 2.5.5. Solution Chemistry

#### 2.5.5.1. Effect of pH

A decrease in the solution pH increases the hydrophobicity of the cells, allowing them to attach more readily to hydrophobic surfaces such as sulphides (Solari *et al.,* 1992). Heaps are generally operated at a pH range of between 1.8 and 2.2 and under these conditions, the surface properties of the cells will be altered in such a way that their hydrophobicity will be increased allowing for better attachment. The pH of the solution will also affect the extent of dissociation of cell wall components and therefore affect the charge of the microorganism and its ability to attach (Yee *et al.,* 2000).

#### 2.5.5.2. Effect of ferrous:ferric ratio

The ratio of ferrous to ferric ions also impacts the attachment of cells. The accumulation of ferric ions causes the cell surface to become positively charged due to the accumulation of ferric ions in the EPS (Gonzalez *et al.*, 1999). The ore surfaces are usually negatively charged so this is likely to result in an increase in the number of attached cells. The addition of ferrous ions has a negative affect on attachment as it is, in most cases, a more readily available energy source (Ohmura *et al.* 1993).

#### 2.5.5.3. Effect of chloride ions

Studies have shown the concentration of chloride ions in solution to affect the growth kinetics of the microorganisms (Shiers *et al.*, 2005). However, little work has been done on the effect chloride ions have on attachment. Relatively low concentrations of chloride ions (5 g/L) were shown to have an inhibitory affect on the cells. These concentrations can occur in heaps, dependent on the mineralogy, making it is necessary to determine the overall affect they will have on the leaching process.

### 2.5.6. Cell Concentration

Attachment of cells to the ore surface increases with cell concentration until an equilibrium between planktonic and sessile cells is reached. At this equilibrium, the ore surface is only partially covered by cells even though there are still planktonic cells present which could attach. This could be due to preferential attachment of the cells to ore surface defects as mentioned in Section 5.1 or due to the repulsive forces which exist between the cells (Olubambi *et al.*, 2007).

## *2.6. Review of Attachment Literature and Experimental Methodology*

The attachment of microorganisms to sulphide minerals has been the focus of extensive research.

Early research focused on the mechanism of attachment. Numerous studies were conducted investigating the surface chemistry of the cells and ore surface in order to better understand the attachment process. It was concluded that initial attachment is mainly due to hydrophobic and electrostatic forces between the cells and the ore (van Loosdrecht *et al.*, 1990; Zobell, 1943; Devasia *et al.*, 1993). The role of chemotaxis (Rojas-Chapana *et al.*, 1998) and quorum sensing (Farah *et al.*, 2005) in the attachment of cells to the surface has been suggested, but not yet conclusively proven.

Research then shifted to investigating the spatial arrangement of the bacteria attached to the ore. Studies showed that bacteria exhibited different affinities to different minerals and attached predominantly to surface defects (Harneit *et al.*, 2006; Rodríguez *et al.*, 2003; Sampson *et al.*, 2000). However, this work has largely been restricted to pure cultures and not mixed cultures.

The effect of various process variables on the attachment of cells has been investigated. These include the composition of the growth media (Sampson *et al.*, 2000; Harneit *et al.*, 2006; Devasia *et al.*, 1993), the pH of the solution (Solari *et al.*, 1992) and the ferrous and ferric iron concentrations (Gonzalez *et al.,* 1999, Ohmura *et al.,* 1993).

Research has been conducted into the effect that the attachment of the bacteria has on the bioleaching process. Rodriguez (2003) showed a direct correlation between the attachment of bacteria and the rate of mineral dissolution. The close proximity to the energy source (Watnick *et al.*, 2000) and the concentration of ferric ions in the EPS (Kinzler *et al.,* 2003; Harneit, 2006) have been suggested as possible explanations for the increase in mineral dissolution with an increase in cell attachment.

Previous studies have shown that microbial attachment can be modelled using the Langmuir adsorption model (Rodriguez *et al.,* 2003). A basis of this model is that the extent of adsorption is a function of the number of open sites on the adsorbent and the concentration of adsorbing species. In a shake flask there is conservation of material and an equilibrium between the sessile and planktonic cells is reached. However, in a heap the flow of liquid is continuous which prevents a static equilibrium from being reached. This, coupled with a degree of attachment, results in the concentration of planktonic cells decreasing as the liquid flows through the bed, affecting the driving force for microbial attachment. Batch data indicate that the cells attach preferentially to the sulphide minerals, so the use of concentrates results in a much higher cell loading than what is obtained on low grade ores. Shake flask

studies using concentrates are therefore a relatively poor representation of the conditions found within a heap. Experiments conducted in a flow-through system, with a composite ore, may better represent the conditions within the heap.

Studies have investigated the effect of increased temperature on the growth and leaching rate obtained within a heap leaching system. An increase in temperature results in an increase in microbial growth rate, to a point, as well as a decrease in the formation of passivating layers on the ore. This results in an increased leaching rate (Rawlings *et al.,* 2003). However, if the temperature exceeds the tolerance of the microbial species, cell death occurs and leaching is compromised. There is, however, very little literature addressing how a change in temperature affects the attachment of cells to the ore surface.

The attachment of thermophilic bioleaching microorganisms has been investigated at ambient temperatures in previous studies, largely due to the experimental constraints. These include the operation of the analytical equipment as well as the material of construction of equipment (Sampson *et al.,* 2000). New analytical techniques should be developed which can allow for the attachment of these organisms to be tested at their optimum growth conditions.

The majority of the previous experiments were carried out using pure cultures, which is not representative of the mixed community that is found in a heap. Interactions between the different microbial species have therefore not been considered and these interactions could have a significant impact on the results obtained. The use of mixed cultures has been limited due to difficulties in enumerating the different species in a mixed culture. Real-time PCR could be used as a quantification method, but needs to be refined as the high level of organic compounds found within the heap environment affects the accuracy of the results obtained with this method (Escobar *et al.*, 2008; Watling, 2006). Another possible method of quantification is epifluorescence microscopy using multiple probes. Each bacterial strain is targeted with a different probe, to which a distinctly coloured fluorochrome is attached, so that they can be visually distinguished from one another.

Detachment of the microorganisms from the ore surface has been observed in previous studies and is thought to be as a result of solution chemistry or physical forces (hydrodynamic shear) (Ghannoum *et al.*, 2004). In a heap bioleaching environment, solution chemistry is more likely to be the cause, as shear stress is limited. An abundance of ferrous iron in solution could cause cells to detach in order to use the readily available energy source. Conversely, if changes in solution chemistry lead to alteration of the ore surface, such that it no longer provides optimum conditions for the microorganism, they could detach themselves and move to a more suitable area. The validity of this explanation for the detachment has

not been tested and further experimental analysis is required to confirm this. Detachment has, however, not been investigated specifically and very little literature has been published in this area. The ability of one strain of bacteria to cause another strain to detach has not been investigated. This links to mixed culture studies, where the interactions that take place between the different microorganisms are considered.

Gautier and co-workers (2008) conducted a study in which they considered the effect of planktonic cells versus sessile cells in the leaching of copper. Their experimental setup used a filter to separate the cells from the ore in order to assess whether attachment was a necessary step. Their experiment showed that it is in fact essential for at least a small number of cells to attach to the surface in order for optimum growth and leaching rates. There were, however, limitations in the experimental setup, particularly relating to whether or not the two regions separated by the filter were homogenous or whether the filter became blocked during the course of the experiment. Their work represents a novel approach to investigating the effect of attached cells on the rate of leaching and further refinement of the system could provide valuable data. Although extensive research has been conducted into the attachment of microorganisms to the ore surface, there is a need for experiments to be carried out using new techniques in order to provide data that is more relevant to the heap bioleaching process.

This study focuses on two of the key issues that have been raised, namely the effect of temperature on the attachment of thermophilic microorganisms and the evaluation of the applicability of the results obtained in a shake flask and flow-through column configuration to a heap bioleaching system. This study further investigates the effect that the culture history has on the attachment efficiency of a microorganism and the levels of attachment that *M. hakonensis* exhibits when contacted with different sulphide minerals.



## *2.7. Objectives, Key Questions and Hypotheses*

### 2.7.1. Objectives

As outlined in the literature review, there are clear gaps in the knowledge relating to the attachment of microorganisms to lower grade ore, such as that used in bioleach heaps. This study aims to target these gaps by investigating the factors affecting the attachment of an iron and sulphur oxidising thermophilic microorganism, *Metallosphaera hakonensis,* to sulphide and associated gangue minerals. The specific objectives of the study are outlined below:

- To investigate the trends in attachment of *M. hakonensis* to pyrite and chalcopyrite concentrates as well as low grade ores with quartz as a control.
- To investigate the effect of culture history on the surface properties of *M. hakonensis* and relate this to attachment of the cells to mineral concentrates and gangue.
- To investigate the effect of temperature and the possible role of metabolic activity on the attachment of *M. hakonensis* to mineral concentrates and gangue.
- To develop a flow-through experimental system and compare the results obtained using this configuration to those obtained using conventional shake flask, with the aim of assessing relevance to a heap leach environment.

### 2.7.2. Hypotheses

In order to address the areas of interest that have been raised above, the following set of hypotheses have been formulated.

- The surface properties of *M. hakonensis* are altered by their growth history and exhibit different levels of attachment to the mineral concentrates and low grade ore. Microorganisms adapted to the mineral concentrates as well as those cultured on elemental sulphur exhibit greater levels of attachment than the cells cultured on ferrous iron.
- Thermophilic archeae show different attachment behaviour compared to mesophilic bacteria due to the differences in their surface structure. Preferential attachment to mineral sulphides is not pronounced. Attachment to non-sulphide gangue material is significant.
- An increase in temperature results in elevated levels of attachment of thermophilic archeae. Initial attachment is a rapid process so increased attachment is due to physicochemical factors and not as a consequence of increased metabolic activity.
- Attachment is lower in the flow-through system compared to agitated shake flasks as it is not a closed system and conservation of material is not guaranteed.

### 2.7.3. Key questions

The following key questions were then raised when considering the objectives of this study:

- How does *M. hakonensis* attach to mineral surfaces?
- Is the initial adhesion of *M. hakonensis* purely a physicochemical process?

- Does the culture history affect cell surface properties and consequently its ability to attach?
- Are the trends reported in literature in shake flask studies applicable to flow-through systems?
- Do thermophilic archeae show the same preferential attachment to sulphide minerals as has been reported for mesophiles?
- Are archeae able to attach to other components in low grade ore other than the mineral sulphide?
- As the temperature is raised to the optimum temperature of thermophilic archeae does the expected increased metabolic activity of the cells lead to enhanced levels of attachment?
- Are there any other factors that would be affected by this increase in temperature?
- Due to the different surface structure of archeae from bacteria, is it possible to extrapolate general trends across both microorganisms?

# 3. Methodology

In this chapter, the protocols and techniques used to investigate attachment are discussed. The microbial species used and their growth conditions are presented in section 3.1. and a description of the mineralogy and preparation of the mineral substrates in Section 3.2. The packed column configuration follows in Section 3.3. and then a description of the analytical techniques in Section 3.4. The two experimental set-ups for investigating attachment of the cells will be presented in Sections 3.5.1. and 3.5.2. Finally, the experimental protocols for determining surface properties and metabolic activity are discussed in Sections 3.5.3. and 3.5.4.

## *3.1. Microorganisms and cultivation*

An environmental isolate of *Metallosphaera hakonensis,* a thermophilic archeae was used in this study. *Metallosphaera.* Cells with four different growth histories were used in the attachment experiments.

### 3.1.1. Stock cultures

*Metallosphaera hakonensis* was maintianed in 500 ml Erlenmeyer flasks at 65°C, agitated at 180 rpm in an orbital shaking incubator. Cells were cultivated in two different growth media, as recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ): medium 88 with with ferrous iron as the energy source and medium 88 with elemental sulphur as the energy source (Appendix A).

*At. ferrooxidans* was used as a control microorganism as it has been extensively studies. Cells were cultivated in an Erlenmeyer flask at 30°C in 9K medium (Appendix A), agitated at 180 rpm on an orbital shaker.

Sub-culturing took place weekly and 30% of the liquid in the flask was removed and replaced with the appropriate fresh growth medium. This was done in the laminar flow hood and all feed and culture containing vessels were flamed in order to maintain sterile conditions. Cultures were regularly viewed under an Olympus BX-40 microscope to visually assess purity. Due to the high frequency of the sub-cultures, it was assumed that the cells remained in an actively growing state. However, the growth kinetics of the cultures was not monitored.

### 3.1.2. Mineral adapted cultures

*Metallosphaera hakonensis* was adapted to both chalcopyrite and pyrite concentrates. A continuous stirred tank reactor (CSTR) was used to adapt cells to the chalcopyrite concentrate. Cells from the mineral free cultures were added to a CSTR containing Norris medium (Appendix A) with a 2% w/v chalcopyrite concentrate loading. Cells from the mineral free cultures were adapted to a pyrite concentrate in a 500 ml Erlenmeyer flask containing Norris medium with a 2% w/v loading. The flasks were maintained at 65°C and agitated at 180 rpm in a shaking incubator.

Both cultures were sub-cultured weekly and 30% of the liquid was removed and replaced with fresh Norris medium. The culture in the Erlenmeyer flask was supplemented with 0.5 g of mineral concentrate at each sub-culturing to account for any loss that may have occurred.

### 3.1.3. Preparation of cell inocula

Cells were harvested by centrifugation (Beckman centrifuge, JA 20 rotor), initially at 2000xg for 3 min, to remove mineral particles and precipitates, and then at 10000xg for 10 min to recover the cells. The cells were resuspended in 0K media (Appendix A) with a pH of 1.6 and cell concentration was determined by direct counting using a Thoma counting chamber.

## *3.2. Substrates used in Attachment studies*

### 3.2.1. Sulphide minerals

A chalcopyrite (79% chalcopyrite) mineral concentrate (Escondida mine B, courtesy of BHP Billiton), a pyrite (92% pyrite) mineral concentrate (courtesy of BHP Billiton) and a low grade copper sulphide ore (0.69% copper, primarily as chalcopyrite, Escondida mine B, courtesy of BHP Billiton) were used in the experiments. The predominant gangue material present in the low grade ore sample was quartz. A full mineralogical report can be found in Appendix C. The pyrite and low grade ore were wet-milled in a rod mill and then wet sieved and the 38-75 µm size fraction retained. The chalcopyrite concentrate was dry sieved to obtain the 38-75 µm size fraction.

The mineral substrates were conditioned to remove surface oxidation products by washing with 0.1 M HCl for 5 min, with acetone for 10 min, twice with distilled water and finally with acidified distilled water (pH 1.6). The minerals were dried (80°C) prior to use.

### 3.2.2. Mineral coated beads

The packed column attachment studies required uniform mineral surfaces with a quantifiable surface area available for attachment. Glass beads (6 mm diameter, Lasec) were coated in the desired mineral (± 3.6 g) using clear glue (Bostik) and allowed to air dry for a minimum of 24 h. The glue was shown to have a negligible effect on the activity of the microorganisms (Appendix D). An even coverage of the bead surface was maintained to ensure that a uniform mineral surface area was achieved for attachment. It was assumed that each mineral particle has approximately half of its surface area exposed and available for microbial attachment.

### 3.2.3. Control substrates

A portion of the low grade ore sample was floated to generate a low-sulphide tailings. Quartz sand was dry sieved to obtain the 38-75 µm size fraction. The quartz sand was then conditioned in the same way as the mineral sulphides (Section 3.2.1.). The tailings and quartz sand were used for control experiments in the shake flask studies.

## *3.3. Attachment columns*

Glass columns, with a diameter of 2.5 cm and a length of 19 cm, were manufactured by Glasschem (Stellenbosch). The ends were closed with screw-cap lids fitted with a rubber stopper and glass nipple. A peristaltic pump (Masterflex Console Drive, 7521-57) was connected to the glass nipple to allow solution to be pumped in from the bottom of the column, with collection of liquid out the top.

Glass wool and 100 glass beads (4 mm diameter, Lasec) were placed in the bottom of the column in order to promote uniform distribution of liquid throughout the column. The columns were loaded with 300 mineral coated beads. 0K media (pH 1.6) was pumped through the column for approximately 2 hours in order to remove any non-adhered mineral particles. A previous study conducted with these columns, determined the residence time to be between 52 and 56 min (Africa 2009). In the case of the elevated temperature experiments, heating tape (MRC Heating tape, 200 W) was wrapped around the column to provide temperature control. Due to the slow flow rate used, heating the feed was not effective as the heat was lost from the feed before reaching the column. The temperature profile along the length of the column was measured to ensure the liquid inside the column was being heated efficiently.

## *3.4. Analytical methods*

### 3.4.1. pH measurement

The pH measurements were performed using a Metrohm 713 pH meter with a Metrohm 6.0258.000 probe that was calibrated at pH 4 and pH 1 before each use.

### 3.4.2. Redox potential analysis

Redox potential measurements were determined using Metrohm 827 pH lab meter and a Metrohm 6.0451.100 probe. The accuracy of the probe was tested before use with a Crison standard redox solution having a potential of 468 mV at 25°C.

### 3.4.3. Conductivity readings

Conductivity readings were taken using an AZ 86555 pH/mV/Cond./TDS/Temp. meter fitted with a SIN:9665110 probe.

### 3.4.4. Cell concentration

Cell concentration was determined microscopically using a Thoma counting chamber and an Olympus BX-40 epifluorescent microscope. Cells were observed at 1000x magnification using oil immersion and phase contrast. The cell concentration was then determined with the following equation:

$$\text{Volume of small square} = \text{depth} \times \text{area}$$

$$= \frac{0.02 \times (0.05 \times 0.05)}{1000} \qquad (9)$$

$$= 5 \times 10^{-8}\ \text{cm}^3$$

$$\text{Cell conc (cells/ml)} = \text{dilution factor} \times \frac{\text{cell count} \times \frac{N}{n}}{\text{vol one small square} \times \text{total number of small squares}}$$

Where N = total number of big squares (16)

n = number of big squares counted (4) (10)

For the hydrophobicity tests, cell concentration was determined by measuring absorbance (400 nm) with a Unicam Heliosα spectrophotometer.

### 3.4.5. Dissolved oxygen measurement

The dissolved oxygen was measured by placing a Mettler Toledo MT4304 K6/8 DO probe, connected to a Mettler Toledo $O_2$4100 meter, in a Schott bottle with an airtight rubber stopper. The zero point of the probe was calibrated by purging the system with nitrogen to ensure an oxygen free environment. The system was then saturated with compressed air and the probe calibrated to 100% saturation. As temperature and solution chemistry affect the solubility of oxygen, it was important to set the 100% calibration point for each set of parameters tested.

### 3.4.6. Determination of ferrous iron concentration

The ferrous iron concentration was determined using the 1-10 phenanthroline method (Muir and Anderson, 1977). For each sample, 2 ml of ammonium acetate buffer and 2 ml of 1-10 phenanthroline indicator solution were added to a test tube. The final volume was made up to 5 ml by addition of 1 ml of appropriately diluted sample. A blank, containing the reagents and 1 ml deionised water, was prepared to zero the spectrophotometer. The solutions were fully mixed using a vortex mixer and allowed to react for 5 minutes, after which absorbance (510 nm) was measured using a Unican Heliosα spectrophotometer. A five point standard curve (0 – 50 mg/l Fe) was used for calibration.

### 3.4.7. Zeta potential measurement

The zeta potential of the cells was determined using a Malvern Zeta-sizer and readings of each sample were repeated a minimum of 5 times.

## *3.5. Experimental design and data analysis*

### 3.5.1. Shake Flask Attachment Experiments

Previous attachment studies have been conducted predominantly in well agitated batch systems (Ghauri *et al.,* 2007; Rodriguez *et al.,* 2003b; Sampson *et al.*, 2000; Harneit *et al.*, 2006; Ohmura *et al.,* 1993). In order to draw meaningful comparisons in the attachment trends observed, a similar experimental protocol was implemented.

Duplicate experiments were carried out in 250 ml Erlenmeyer flasks with a total volume of 100 ml, comprising 0K medium (pH 1.6), a 2% (w/v) solids loading and a total cell inoculum of $2x10^9$ cells.

The flasks were placed in orbital shaking incubators at 180 rpm and set to the desired temperatures. Samples (1 ml) were taken over a 2 h period at the following intervals: 1, 5, 10, 20, 30, 60 and 120 min

and the cell concentration of the samples determined by direct counting. The following equation was then used to determine the number of cells attached to the mineral:

$$\%\ \text{attached} = \left( \frac{\text{total cell inoculum - } \left(\text{planktonic cell concentration} \times 100\ \text{ml}\right)}{\text{total cell inoculum}} \right) \times 100$$

$$= \left( \frac{2 \times 10^{9} - \left(\text{planktonic cell concentration} \times 100\right)}{2 \times 10^{9}} \right) \times 100 \quad \textbf{(11)}$$

The matrix for the shake flask experiments is detailed in Table 1. The matrix was repeated for 25°C, 45°C and 65°C resulting in 36 unique experimental conditions.

**Table 3.1:** Experimental matrix used to assess the effect of temperature and growth history on attachment.

| | Mineral Concentrate | | |
|---|---|---|---|
| Growth History | Pyrite | Chalcopyrite | Low grade ore |
| Ferrous iron | X | X | X |
| Elemental sulphur | X | X | X |
| Pyrite adapted | X | X | X |
| Chalcopyrite adapted | X | X | X |

Control experiments were conducted using sand (quartz) and the gangue material of the low grade ore. The experimental protocol was the same as for the other shake flask attachment experiments. The full experimental matrix was not implemented and only the attachment of cells cultured on elemental sulphur was investigated as a representative of all the cultures. Attachment to the gangue material was investigated at 45°C and 65°C and attachment to the quartz was investigated at 65°C as at this temperature attachment is assumed to be a maximum.

### 3.5.2. Column Experiments

As explained in Section 2.6., the hydrodynamic conditions in a shake flask set-up are not representative of the conditions within a heap system. In order to account for the fluid flow that occurs within a heap, a column set-up was implemented. This allowed for the comparison of the attachment data generated from the two experimental set-ups and ascertain the validity of the results obtained in shake flask studies to heap bioleaching systems. Due to the small scale of this particular column set-up, it is possible to control the conditions within the system and therefore accurately determine the effect of different parameters.

Before running the experiment, the effect of the Bostik glue, used to coat the beads, on the metabolic functioning of the cells was determined. Erlenmeyer flasks (500 ml) containing 0K medium (Appendix A) were autoclaved and 9 ml of filter sterilised ferrous sulphate solution added (final $Fe^{2+}$ concentration 2.5 g/l). The following was then added to the flasks: nothing (control), 20 glass beads, 20 glass beads coated in glue and 20 chalcopyrite concentrate coated beads. All four flasks were inoculated with *At. ferrooxidans* to achieve a final cell number of $1x10^8$ cells in each flask. This resulted in four flasks which were sampled daily and the following analyses performed: pH, redox potential and ferrous iron concentration. The results indicated that the Bostik glue did not affect the metabolic activity of the cells (Appendix D) and it was therefore assumed that the attachment mechanism of the cells would also not be affected. Once the validity of the experimental set-up had been confirmed, the following attachment experiments were conducted.

Duplicate columns were loaded with 300 beads coated with the appropriate mineral. The inoculum (10 ml of approximately $1x10^8$ cells/ml) was fed into the bottom of the column as a pulse. The inoculum was pumped into the column at a rate of 1 ml/min and thereafter 0K medium (pH 1.6) was pumped in at the same flow rate. Effluent fractions (15 ml) were collected continuously over 15 min periods for three hours, yielding a total of 12 samples. A control column, loaded with uncoated glass beads, was run at 65°C in order to assess potential retention of unattached cells within the bead matrix.

The following analyses were done on each sample: pH, redox potential, conductivity and cell counts. The percentage of cells retained within the column and assumed to be mainly attached to the mineral coated beads was calculated using the following equation:

$$\% \text{ retained} = \left( \frac{\text{total cell inoculum - total cell number washed out of column}}{\text{total cell inoculum}} \right) \times 100$$

$$= \left( \frac{\left( 1\times10^{9} - \sum_{\text{sample 1}}^{\text{sample 12}} (\text{cell concentration of sample} \times 15) \right)}{1\times10^{9}} \right) \times 100$$

**(12)**

The surface area available for attachment was calculated to be $3.4\text{x}10^{-2}$ $m^2$ and the maximum number of cells able to attach to this area was determined to be $1.25\text{x}10^{10}$ cells (Appendix B). This is an order of magnitude higher than the number of cells fed into the column, theoretically allowing for complete attachment of all the cells to occur.

Key parameters determined from the shake flask studies were investigated and the experimental matrix used for the column experiments is detailed in Table 2.

**Table 3.2:** Experimental matrix used to assess the effect of temperature and growth history on attachment to mineral coated glass beads in column experiments.

| Growth History | Chalcopyrite concentrate | | | Low grade ore | | |
|---|---|---|---|---|---|---|
| | 25°C | 45°C | 65°C | 25°C | 45°C | 65°C |
| Ferrous iron | X | X | X | | | X |
| Elemental sulphur | X | X | X | X | X | X |
| Chalcopyrite adapted | X | X | X | | | X |

### 3.5.3. Activity tests

To assess the potential influence of metabolic activity on attachment, activity tests were conducted to determine ferrous iron oxidation and oxygen utilisation by cells under the experimental conditions investigated in the attachment studies.

#### 3.5.3.1. Iron Oxidation Assays

All experiments were performed in duplicate. An un-inoculated control flask, with the same iron concentration, was included to quantify abiotic iron oxidation at the relevant temperatures. The assays were conducted in Erlenmeyer flasks (500 ml), containing 300 ml Norris media, adjusted to a pH of 1.6. These were autoclaved and 9 ml of filter sterilised ferrous iron solution was added as the energy source (final $Fe^{2+}$ concentration 2.5 g/l). The flasks were then inoculated to achieve a total cell number of $1\text{x}10^{8}$

cells. This concentration was chosen as it is just above the detection limit by direct microscopic counting and allowed for several cell replication cycles to occur before the substrate was exhausted. The flasks were covered and placed in an incubator at 25°C, 45°C and 65°C at 180 rpm. The liquid level was monitored and adjusted with distilled water to compensate for evaporation at 65°C.

The flasks were sampled (3 ml) twice daily and the following parameters measured: pH, redox potential and ferrous iron concentration.

A duplicate set of iron oxidation assays were performed using high inoculum concentration ($2x10^7$ cells/ml) to replicate the cell loadings in the shake flask experiments. The greater cell number would accelerate any observed iron oxidation.

#### 3.5.3.2. Oxygen Utilisation Rate

A 250 ml Schott bottle fitted with an airtight rubber stopper, modified to accommodate the DO probe and inlet and outlet ports for nitrogen sparging, was used to determine oxygen utilisation. A magnetic stirrer bar was placed in the bottle to achieve mixing. Heating tape was wrapped around the bottle and set to 50°C in order to achieve a liquid temperature inside the bottle of 45°C.

The bottle was filled with 250 ml Norris medium and 22.5 ml ferrous sulphate solution (final $Fe^{2+}$ concentration 2.5 g/l) and allowed to equilibrate for at least 1 h. Headspace was minimised in order to minimise oxygen diffusion out of solution. The probe was calibrated such that this equilibrium was set to 100% saturation. The microbial inoculum ($5x10^9$ cells) was added to the bottle. The cells were harvested as described above and re-suspended in Norris medium. DO was recorded over a 3 h period or until the percentage saturation reached zero. Experiments were repeated to assess reproducibility. An abiotic control was run in order to quantify oxygen utilisation due to chemical iron oxidation or diffusion out of the system. A second, positive control using *At. ferrooxidans* was run at 30°C to confirm the validity of the experimental design.

### 3.5.4. Cell surface characterisation

The initial attachment of the cells to the solid surface is a physio-chemical process and it is therefore critical to understand the surface properties of the cells which will drive this interaction.

#### 3.5.4.1. Zeta potential

Cells were prepared for zeta potential analysis by harvesting (Section 3.1.3.) and then re-suspending them in 0K media at pH 2, 3 and 4. The pH of the 0K medium was adjusted with a dilute sulphuric acid

solution. The cell solutions were diluted such that the conductivity and attenuation of the samples were approximately the same. A refractive index of 1.33 was selected, based on published values for cells (Liang *et al.,* 2007; Wilson *et al.,* 2001; Stramski, 1999). The absorbency refers to the transparency of the sample and as the samples were dilute, an absorbency of 1 could be used for the analysis. The zeta potential of the cells was determined using a Malvern Zeta-sizer and readings of each sample were repeated a minimum of 5 times per sample in order to assess reproducibility.

#### 3.5.4.2. Hydrophobicity

The harvested cells were diluted until the $OD_{400}$ was between 0.3 and 0.4. Experiments were carried out multiple times in order to assess the reproducibility of the results. The cell suspension (3 ml) and hexadecane (0.8 ml) were placed in a test tube and vortexed for 1 min, before allowing them to stand for 30 min. The $OD_{400}$ of the aqueous solution was then measured and this was used to determine the hydrophobicity of the solution using the following formula (Natarajan *et al* 2003):

$$\text{Hydrophobicity} = \left(1 - \frac{\text{OD}_{\text{final}}}{\text{OD}_{\text{initial}}}\right) \times 100 \quad \textbf{(13)}$$

These results were then verified by repeating the experiment at an $OD_{400}$ of 0.1 and performing cell counts at a 2x dilution.

# 4. Shake Flask Attachment Study

## *4.1. Introduction*

A review of the literature confirms that attachment studies using bioleaching organisms have been carried out predominantly in agitated or shake flask systems. In this chapter, the attachment of the thermophilic archeae, *M. hakonensis*, in a typical agitated system is assessed. Experiments were carried out in Erlenmeyer flasks in an orbital shaking incubator at 180 rpm and a 2% w/v substrate loading (particle size fraction: +38 -75 µm). Direct cell counts of the planktonic cell population allowed the number of cells attached to the substrate to be inferred (equation 10).

A number of different parameters were investigated in this study, namely temperature (25°C, 45°C and 65°C), culture history (ferrous iron, elemental sulphur, chalcopyrite adapted and pyrite adapted) and substrate mineralogy (chalcopyrite concentrate, pyrite concentrate and a low grade ore). The overall results obtained will first be presented and briefly discussed followed by a more in-depth analysis and discussion of the results for each of the parameters that were investigated.

## *4.2. Attachment Results and Discussion*

### 4.2.1. Overall attachment trends observed

The attachment of *M. hakonensis*, cultured on elemental sulphur, to a chalcopyrite concentrate at 65°C is presented in Figure 4.1 (raw data for full experimental matrix – Appendix E). Attachment was rapid during the first 10 minutes, with an equilibrium established within approximately 20 minutes.

This trend of rapid attachment was observed for the attachment of *M. hakonensis* to all mineral types (chalcopyrite, pyrite and low-grade ore mineral systems) and was not affected by the growth history of the culture.

To assess the microbe-mineral affinity during the initial attachment stage quantitatively, the initial rate of attachment to all mineral systems under investigation was calculated. The initial rates of attachment were calculated from the slope of the curve (Figure 4.1) during the first 10 minutes and these are summarized in Table 4.1. The rate constant was then calculated by taking the rate over the initial cell concentration.

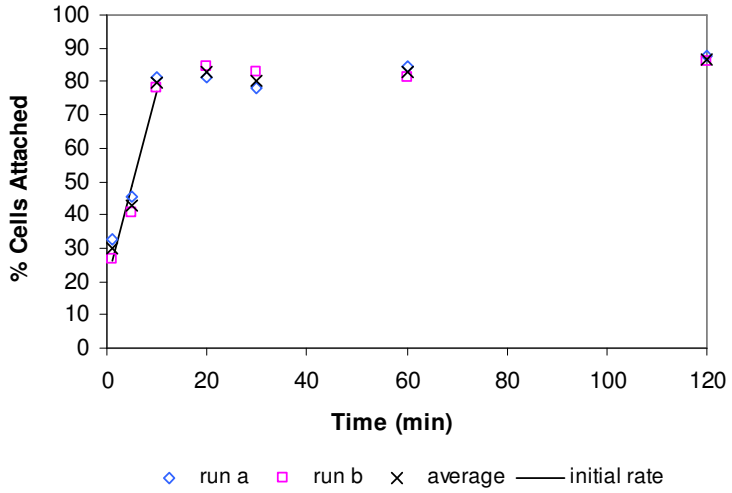



**Figure 4.1:** Attachment of a sulphur grown *M. hakonensis* culture ($2x10^9$ cells total) to a chalcopyrite concentrate (2 % wt $vol^{-1}$) at 65°C (pH 1.6) in an Erlenmeyer shake flask over a 2 hour time period. Results are representative of duplicate experiments represented by (a) and (b) on the graph. Initial rate of attachment was calculated from the straight line indicated in the figure.

**Table 4.1:** Maximum rate of attachment ($x10^8$ cells.$min^{-1}$) and the rate constant (k, $min^{-1}$) for the attachment of *M. hakonensis* to different mineral substrates, in batch flasks, as a function of temperature. Results represent the mean of duplicate experiments.

| | Culture growth history | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mineral Substrate | Sulphur | | Ferrous iron | | Pyrite | | Chalcopyrite | |
| | rate | *k* | rate | *k* | rate | *k* | rate | *k* |
| Chalcopyrite | | | | | | | | |
| 25°C | 0.454 | 0.023 | 0.292 | 0.015 | 0.400 | 0.020 | 0.242 | 0.012 |
| 45°C | 1.142 | 0.057 | 0.668 | 0.033 | 0.766 | 0.038 | 0.516 | 0.026 |
| 65°C | 1.126 | 0.056 | 0.580 | 0.029 | 0.938 | 0.047 | 0.588 | 0.029 |
| Pyrite | | | | | | | | |
| 25°C | 0.334 | 0.017 | 0.188 | 0.009 | 0.586 | 0.029 | 0.340 | 0.017 |
| 45°C | 0.874 | 0.044 | 0.506 | 0.025 | 0.846 | 0.042 | 1.144 | 0.057 |
| 65°C | 1.078 | 0.054 | 0.606 | 0.030 | 1.066 | 0.053 | 1.264 | 0.063 |
| Low grade ore | | | | | | | | |
| 25°C | 0.516 | 0.026 | 0.396 | 0.020 | 0.308 | 0.015 | 0.626 | 0.031 |
| 45°C | 1.320 | 0.066 | 0.432 | 0.022 | 1.084 | 0.054 | 0.886 | 0.044 |
| 65°C | 2.344 | 0.117 | 0.980 | 0.049 | 1.028 | 0.051 | 1.330 | 0.067 |

The maximum level of attachment, determined once the equilibrium had been reached, to three mineral substrates is presented in Figure 4.2.

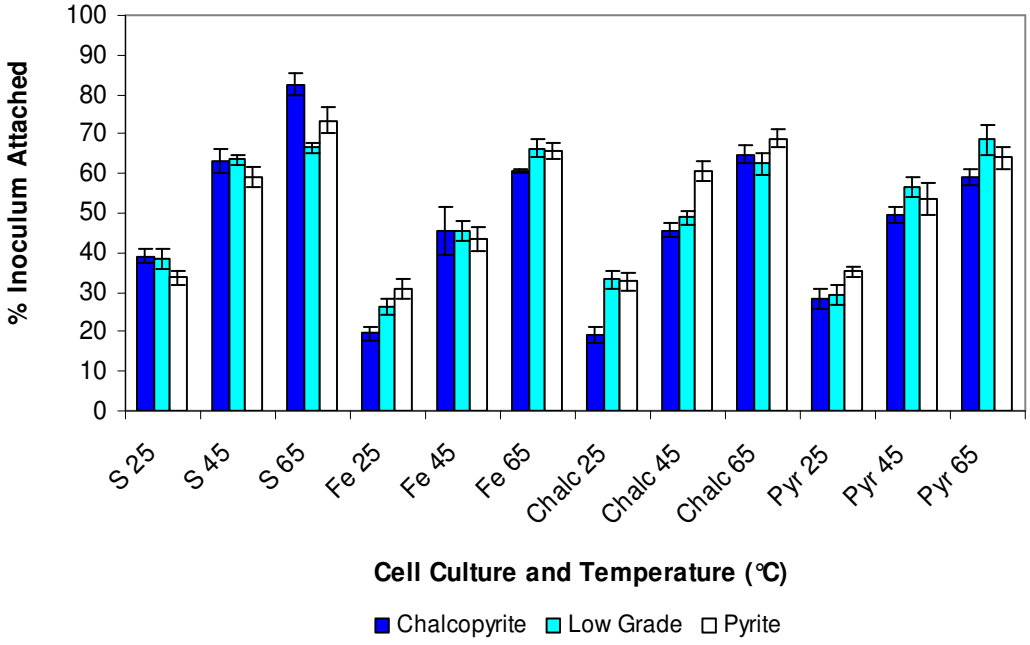



**Figure 4.2:** Attachment of *M. hakonensis*, cultured on four different growth media: ferrous iron (Fe), elemental sulphur (S), pyrite (Pyr) and chalcopyrite (Chalc) to three mineral substrates (chalcopyrite concentrate, pyrite concentrate and low grade ore. Experiments were conducted for two hours at 25°C, 45°C and 65°C.

In all cases, the initial rates of attachment positively correlate well with overall extents of attachment although the magnitude of the relationship differed in certain circumstances. This is consistent with the system reaching equilibrium within 20 minutes.

The cells cultured on elemental sulphur exhibited the greatest levels of attachment to the chalcopyrite concentrate at all three temperatures investigated. An overall attachment of 39%, 63% and 83% was achieved at 25°C, 45°C and 65°C respectively. Slightly lower levels of attachment were observed for cells adapted to the mineral concentrates and ferrous iron. The maximum extent of attachment to chalcopyrite was obtained at 65°C irrespective of culture history. The extent of attachment to chalcopyrite achieved at 65°C was 61%, 65% and 59% with cells cultured on ferrous iron, chalcopyrite concentrate and pyrite concentrate respectively. Similar levels of attachment were observed for all

mineral substrates with sulphur grown cells exhibiting the greatest levels. Overall, the greatest extent of attachment (83%) was achieved with the sulphur adapted cells on chalcopyrite concentrate at 65°C.

A similar study conducted by Rodriguez and co-workers (2003b) showed attachment of a mineral adapted mixed thermophilic culture to be greater than 90% to chalcopyrite and pyrite at 68°C with a Ka value of $1.2x10^{-8}$ and $4.2x10^{-10}$ ml/cells.h respectively. The Ka value is dependent on the cell concentration, whereas the rate constant k is independent of the cell concentration. The Ka values for the attachment of *M. hakonensis* to chalcopyrite and pyrite at 65°C were calculated from the rate constants determined in this study and the average values were determined to be $1.2x10^{-7}$ and $1.5x10^{-7}$ ml/cells.h respectively. This indicates that although there was a slightly lower overall extent of attachment achieved in this study, there was a slightly faster rate of attachment. Attachment of the thermophiles exhibited a greater rate of attachment to chalcopyrite than pyrite in both studies. However, their study used a pulp density of 5% and a 10 ml inoculum of exponential phase (> $10^8$ cells/ml) culture. The total number of cells was similar to this study, but the greater solids loading likely contributed to the increased attachment. Etzel *et al.* (2008) exposed *Metallosphaera sedula* to synthetic pyrite crystals and showed levels of attachment of 60% after 16 days of exposure. The synthetic crystals were significantly larger (± 2 mm) than the material used in the current study. Harneit and co-workers (2006) conducted attachment studies with *At. ferrooxidans* in 50 ml volumes with 10 g of mineral and $2.5x10^{10}$ cells at 21°C and achieved Ka values of $6.0x10^{-9}$ and $1.92x10^{-8}$ with chalcopyrite and pyrite respectively. The values are within the same range as the studies conducted with thermophiles, but the rate of attachment to pyrite was greater than the rate to chalcopyrite, which is not consistent with the other two studies. There is also a significant discrepancy between the extents of attachment of *At. ferrooxidans* to chalcopyrite (±50%) and pyrite (±80%), whereas the other studies show similar levels of attachment to both substrates. This can be attributed to the differences in attachment of mesophiles and thermophiles. The direct comparison of results with other published studies is complicated by differences in inoculum concentration, solids loading, particle size and the expression of results as % attachment rather than cells attached per unit surface area. Despite these differences, the shake flask experimental protocol followed in most studies (Rodriguez *et al*., 2003b; Ohmura *et al*., 1993; Sampson *et al*., 2000) is similar.

The following trends can be seen in the data presented above:

- increase in the rate and extent of attachment with an increase in temperature
- cells cultured on elemental sulphur exhibited the greatest levels of attachment

- preferential attachment to mineral sulphides was not pronounced

These are discussed in more detail in sections below.

### 4.2.2. Effect of temperature

The microorganism used in this study is a thermophilic archeae and it was hypothesised that by raising the temperature of the system to the ideal growth temperature of the microorganism would enhance the attachment of the microorganism. The poor attachment of thermophiles at ambient conditions observed by Africa *et al,* (2009) supports this hypothesis, but attachment of the thermophiles at intermediate temperatures has not been investigated. Attachment at three temperatures was investigated in this study and the trends which were observed are presented and discussed below.

The initial rate of attachment increased with increasing temperature for all mineral substrates (Table 4.1). The trend was consistent for all growth histories. However, the extent to which the rate increased varied with growth culture history, with the highest attachment rates observed for sulphur grown cells. This trend was consistent across all mineral systems, with the initial attachment rate more than doubling when the temperature was increased from 25°C to 45°C and increasing further, by up to 50%, when it was increased from 45°C to 65°C. The relationship between increased temperature and extent of attachment was similar to that between temperature and attachment rate, with the greater impact observed between 25°C and 45°C.

To further analyse the relationship between temperature and attachment of the microorganisms, the rate data were fitted to the Arrhenius equation. The Arrhenius equation relates rates to temperature as follows:

$$k = Ae^{\frac{-Ea}{RT}} \quad \textbf{(14)}$$

where *k* is the rate of microbial attachment, A the 'pre-exponential factor', Ea the activation energy, R the gas constant (8.314 J/K.mol) and T the temperature (K). The linearised equation:

$$\ln k = -\frac{Ea}{RT} + \ln A \quad (\textbf{15})$$

was then plotted for all the rate data and the goodness of fit determined by fitting a straight line (Figure 4.3).

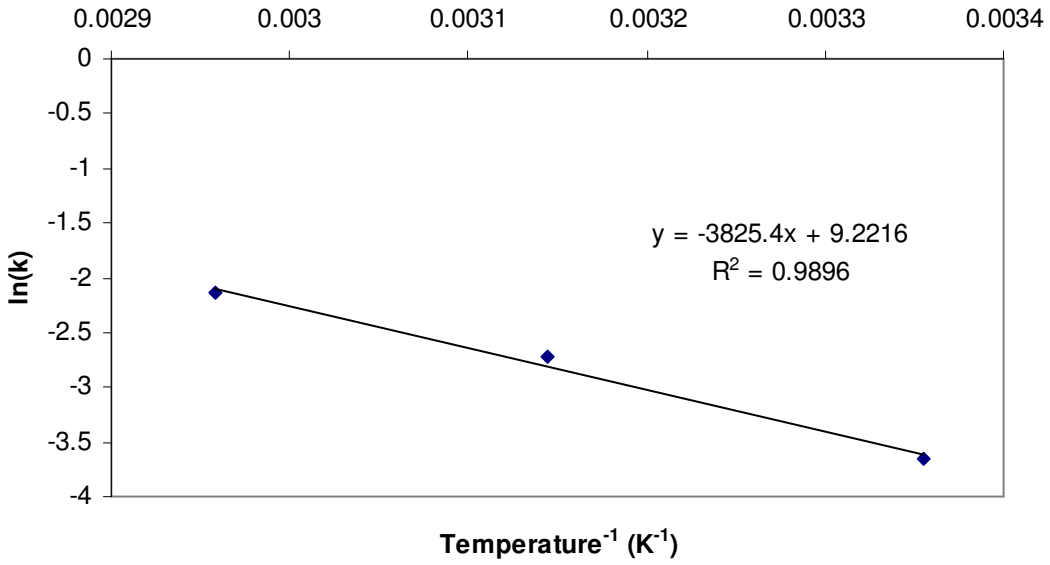



**Figure 4.3**: Example of the Arrhenius equation fit to rate data from the attachment studies of a sulphur grown *M. hakonensis* culture ($2x10^9$ cells total) to a low grade ore (2 % wt $vol^{-1}$) at 65°C (pH 1.6) in an Erlenmeyer shake flask over a 2 hour time period. The constants were determined to be 10113 and 31804 for A and Ea respectively.

All the data were plotted as in Figure 4.3 and the $R^2$ values (Table 4.2) were used in conjunction with the standard deviation between the calculated and experimental values, to determine the applicability of fitting the data using the Arrhenius equation.

**Table 4.2:** $R^2$ values determined from plotting the linearised Arrhenius equation and fitting a straight line to it. The values obtained provide an indication of the goodness of fit of the experimental data to the Arrhenius equation.

| | Culture History | | | |
|---|---|---|---|---|
| Mineral Substrate | Sulphur $R^2$ | Ferrous iron $R^2$ | Pyrite $R^2$ | Chalcopyrite $R^2$ |
| Chalcopyrite | 0.7696 | 0.6361 | 0.9350 | 0.8820 |
| Pyrite | 0.9019 | 0.8864 | 0.9911 | 0.8345 |
| Low grade ore | 0.9896 | 0.7927 | 0.7492 | 0.9934 |

The relationship between temperature and rate provided a good fit to the Arrhenius equation with an average standard deviation and $R^2$ value of 0.0037 and 0.86 respectively and the highest standard deviation observed was 0.0129 (full set of data - Appendix E). This suggests that the increase in the rate

of attachment is due to the intrinsic properties of an increase in temperature as opposed external factors such as the increase in metabolic activity, substantiating the claim that initial attachment is a purely physicochemical process (van Loosdrecht *et al*., 1990).

In order to provide insight into the initial attachment mechanism and how an increase in the temperature of the system impacts it, metabolic activity tests were conducted (Section 5.4).

### 4.2.3. Effect of growth history

Previous studies (Harneit *et al.,* 2006; Yu *et al.,* 2008; Porro *et al.,* 1997; Devasia *et al.,* 1993) have established that the growth medium used to cultivate the cells clearly affects the ability of the cells to attach to the mineral surfaces. The growth media affect the composition of the extracellular polymeric substance (EPS) secreted by the microorganisms. The EPS composition affects the surface properties, which influence the microorganisms' ability to attach to sulphide mineral surfaces.

In this study, the cells cultured on elemental sulphur exhibited the greatest extent of attachment compared with both mineral concentrate adapted and ferrous iron grown cells. This enhanced level of attachment with cells cultured on elemental sulphur is similar to results obtained by Sampson and co-workers (2000) for two species of *Sulfobacillus*.

Kinzler and co-workers (2003) conducted studies to determine the composition of the EPS of *Acidithiobacillus ferrooxidans*. The study concluded that ferric iron complexed with the EPS significantly improved the rate and extent of attachment to pyrite. Harneit *et al.* (2006) observed greater levels of EPS production in *At. ferrooxidans* cells that were cultivated on solid substrates and sulphur. The composition of the EPS of cells grown on ferrous iron and cells grown on pyrite were comparable, but cells grown on elemental sulphur differed significantly as the fatty acid content was higher. Attachment studies were only conducted with ferrous iron grown cells so the impact of EPS composition on attachment was not investigated.

The levels of attachment obtained for all cultures were similar and indicates that the cells either have similar EPS components or that the effect of culture history on attachment was distorted by the removal of the loosely bound EPS during harvesting. Studies that analysed the components in the EPS showed that it was possible to remove the cell's EPS by centrifugation (Kinzler *et al.,* 2003). If all the EPS was removed, the capsular polysaccharide would then form the outer surface layer of all cells and would account for the similarity of the results obtained. Another harvesting technique, such as filtration, should be investigated in order to assess the impact of centrifugation on the surface properties of the

cells and the effect this has on the cells' ability to attach to the mineral substrates. However, studies done with mesophilic cultures harvested in the same manner as the current study showed significant differences in the attachment of cultures with varying growth histories (Africa *et al.,* 2009). This suggests that the harvesting technique may not be responsible for the results observed, but that the differences in surface structure between archeae and mesophilic bacteria could also play a significant role.

Mineral sulphides contain a large amount of sulphur which is oxidised to elemental sulphur over time. The ores were conditioned to remove any oxidised compound on the mineral surface, but if elemental sulphur remained on the surface it would increase the hydrophobicity of the mineral surface. Cells cultured on elemental sulphur contain sulphur in their structure and the oxidised sulphur on the mineral surface could account for enhanced levels of attachment observed with cells cultured on elemental sulphur. In a bioleaching heap the mineral will be exposed to the air and attachment results to these oxidised surfaces therefore offer valuable information for practical application. However, fundamental attachment studies to pristine surfaces are also of fundamental interest as they provide insight into a variable of choice. In order to ensure that there is minimal oxidation of the surface, the minerals should be stored under nitrogen after being conditioned.

The surface properties of the cells were investigated (Section 4.3) to provide further insight into the role that culture history plays in the attachment studies.

### 4.2.4. Effect of substrate mineralogy

The attachment at each set of conditions did not vary greatly with mineral substrate, indicating that *M. hakonensis* did not attach preferentially to the different mineral sulphides. Non preferential attachment has been observed in previous studies with archeae (Rodríguez *et al*, 2003b, Usher *et al.,* 2010; Etzel *et al.,* 2008). However, the relatively high level of attachment to the low grade ore is not consistent with previous studies using mesophilic organisms (Gonzalez *et al.* 1999, 2007; Rodriguez *et al.,* 2003; Harneit *et al.,* 2006) where a clear preference between sulphide minerals and gangue was observed.

Analysing the data as a percentage of the initial inoculum that has attached to the surface may not be an accurate reflection of the results as it does not account for the surface area available for microbial attachment. The surface area of the low grade ore (0.558 $m^3$) was found to be significantly greater than the mineral concentrates (0.1118 $m^3$ and 0.1766 $m^3$ for pyrite and chalcopyrite concentrates respectively) when using the particle size distribution (PSD) of the samples and assuming spherical particles to calculate surface area. When analysing the number of cells attached per total available

surface area, there appeared to be preferential attachment to the mineral concentrates due to the greater area made available by the low grade ore. However, the maximum cell loadings possible on all three substrates were calculated assuming spherical microbial and mineral particles and that the whole mineral surface is available for attachment (Appendix B) to be an order of magnitude greater than the inoculum used in this study. This indicates that the available surface area was not a limiting factor in this study. If we assume that the composition of the minerals remain consistent across the size distribution, the mineral sulphide surface area can be calculated. This was determined to be significantly lower for the low grade ore (0.028 $m^3$), compared to the pyrite and chalcopyrite concentrates (0.103 $m^3$ and 0.140 $m^3$ respectively) which corroborates the non-preferential attachment of *M. hakonensis* observed.

To further investigate the relatively high degree of attachment to the low grade ore, additional experiments were conducted assessing the attachment of sulphur grown *M. hakonensis* to a flotation tailings and quartz sand. The low grade ore was floated and the tailings, consisting of the gangue material, was collected and used in the attachment experiment. Attachment levels to the tailings were slightly lower than those observed with the mineral sulphides. Equilibrium attachment levels of 49% and 61% were achieved for the tailings at 45°C and 65°C respectively, compared to 63% and 66% for low grade ore and 63% and 83% for chalcopyrite concentrate at 45°C and 65°C respectively. However, attachment of the cells to the quartz sand at 65°C over the 2 h period was negligible. In conclusion, *M. hakonensis* does attach better to mineral sulphides but also attaches to the non-quartz components in the gangue material.

The relatively high level of attachment to the low grade ore and specifically to the gangue material of the low-grade ore suggests that the surface properties of *M. hakonensis* differ appreciably from the mesophilic cultures studied. Africa *et al.* observed levels of attachment of *At. ferrooxidans* to low grade ore and quartz of 50% compared with >90% attachment to mineral sulphide concentrates. This difference influences the physicochemical interactions between the microorganism and the mineral, influencing the initial adhesion that is observed (van Loosdrecht *et al.,* 1990, 1987).

Attachment to mineral sulphides ensures that the cell is in close proximity to an energy source and is able to function and leach the metal optimally. Cells that are attached to gangue material and not in the vicinity of the sulphide mineral are compromised in their ability to create the reaction space necessary for leaching to occur. The observed attachment of *M. hakonensis* to components in the gangue will have an impact on the leaching efficiency that is obtained and a better understanding of what gangue components are involved is critical for optimal leaching.

## *4.3. Microbial surface property results and discussion*

As the rate data showed a good fit to the Arrhenius equation, it can be assumed that the initial attachment of the cells is a purely physicochemical process. The cells' surface properties play an important role and understanding these is important in analysing the attachment results. Cell surface properties influence the hydrophobic and electrostatic interactions, which can be complementary or one can dominate the other.

Previous studies have shown that the growth history of microorganisms affects the extracellular polymeric substances (EPS) present (Kinzler *et al.*, 2003; Harneit *et al.*, 2006). The composition of the EPS affects the surface properties of the cells. Uronic acids and the complexing of $Fe^{3+}$ ions in the EPS have been shown to result in a net positive charge in *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* cells, which would aid the attachment to negatively charged substrates (Harneit *et al.*, 2006).

In this study the hydrophobicity and zeta potential of the microbial cultures were determined.

### 4.3.1. Hydrophobicity

The relative hydrophobicity of *M. hakonensis* cultured on elemental sulphur, ferrous iron and chalcopyrite, was determined using 2-phase partitioning with hexadecane. The protocol developed by Natarajan *et al.* (2003) was followed and repeated in triplicate. The results from this can be seen in Table 4.3 below. The results indicated that chalcopyrite-adapted cells were the most hydrophobic, with an average of 14.34% of the cells partitioning into the organic phase, compared to 6.51% and 5.95% for iron- and sulphur-grown cultures respectively.

**Table 4.3:** Relative hydrophobicity of the cultures resuspending in 0K medium (pH 1.6) using the protocol developed by Natarajan *et al.* (2003) and $OD_{400}$ measurements to determine cell concentration.

| | Percentage partitioning into hexadecane | | | |
|---|---|---|---|---|
| Culture history | Test 1 | Test 2 | Test 3 | Average |
| Sulphur | 2.0 | 3.0 | 6.0 | 3.6 ± 2.1 |
| Ferrous iron | 8.5 | 2.7 | 6.5 | 5.9 ± 2.9 |
| Chalcopyrite | 10.9 | 16.3 | 15.9 | 14.3 ± 3.0 |

During the mixing of the two phases, hexadecane droplets formed in the aqueous phase and affected the absorbance of the aqueous phase and thus the final cell concentration which is used to determine relative hydrophobicity. This resulted in the large degree of variance observed. In an attempt to decrease this, direct cell counting was used to determine cell concentration. The same hydrophobicity protocol was implemented, but at an initial $OD_{400}$ reading of 0.1, so that a 2x dilution was required for cell counting. The results were of the same order of magnitude as the initial tests, but the cells cultured on elemental sulphur appeared to be more hydrophobic than the cells cultured on ferrous iron (Table 4.4).

**Table 4.4:** Comparison of $OD_{400}$ and cell counts for cell concentration enumeration during the hydrophobicity tests. The protocol developed by Natarajan *et al.* (2003) was adjusted and an initial $OD_{400}$ measurement of 0.1 as this lower cell concentration ensured the cell counts were accurate. Tests were conducted in duplicate.

| | Percentage partitioning into hexadecane | |
|---|---|---|
| Culture history | $OD_{400}$ | Cell counts |
| Sulphur | 15.1 ± 6.0 | 13.2 ± 2.2 |
| Ferrous iron | 21.6 ± 3.2 | 4.4 ± 0.9 |
| Chalcopyrite | 29.1 ± 4.2 | 18.4 ± 0.0 |

These results are of a similar magnitude to those obtained by Natarajan and Das (2003) for *At. ferrooxidans*, although their study showed sulphur-grown cells to be more hydrophobic, which was only evident in this study when cell concentration was enumerated using direct cell counts. Huan *et al.* (2008) obtained similar results to this study using 2-phase partitioning with hexadecane and a thermophilic archeae, *Acidianus manzaensis.* The chalcopyrite and pyrite adapted cells had the highest hydrophobicity, 17.75% and 16.17% respectively, followed by the cells cultured on elemental sulphur with 6.5% and the most hydrophilic cells were those cultured on ferrous iron with 1.2%. Natarajan and Das (2003) showed that hydrophobicity of both iron- and sulphur-grown cells was substantially increased in the presence of increasing concentrations of potassium phosphate. A number of ionic species have been shown to have a similar effect, attributed to the dehydration of proteins, which increases the surface area of their hydrophobic domains.

The reproducibility of the assays in this study was relatively poor, so while chalcopyrite-adapted cells appeared most hydrophobic, the difference between iron- and sulphur-grown cells was not statistically significant. Using direct cell counting to determine cell concentration decreased the variance, but

contact angle measurements may be a more effective way of assessing cell hydrophobicity. An alternative solution could be to refine the 2-phase partitioning method by placing the test tubes in a shaker for a set period of time. This will ensure that the mixing conditions remain constant between samples and that the results can be compared directly without having to account for mixing effects.

Copper sulphide minerals have been reported to be hydrophobic under the experimental conditions used in this study (Ohmura *et al*., 1993). This suggests that if hydrophobic interactions were dominant in the initial attachment of *M. hakonensis* to the substrates, chalcopyrite- adapted cells should have exhibited the greatest degree of attachment. This was not the case, with sulphur-grown cells showing significantly greater attachment in all cases. The data cannot be assessed based on hydrophobic interactions alone as the surface charge also plays an important role in the initial attachment process and could possibly be the dominant factor under the experimental conditions investigated.

### 4.3.2. Zeta potential

The zeta potential of *M. hakonensis* cultured on elemental sulphur, ferrous iron and chalcopyrite, was determined using a Malvern Zetasizer Nano Series. Cells were re-suspended in 0k medium at pH 2, pH 3 and pH 4 to remain consistent with the pH and solution chemistry of the attachment studies, as well as to mimic the conditions encountered in an industrial bioheap. The measured potentials are presented in Table 4.5.

**Table 4.5:** Zeta potential of the three cultures of *M. hakonensis* (elemental sulphur, ferrous iron and chalcopyrite adapted), at a pH of 2 and 3, measured using a Malvern Zetasizer. The conductivity of all samples tested was kept approximately constant in order to ensure that the results obtained were comparable.

| | Cell growth history | | |
|---|---|---|---|
| | Sulphur | Ferrous iron | Chalcopyrite |
| pH | Zeta (mV) | Zeta (mV) | Zeta (mV) |
| 2 | -2.115 ± 0.200 | -1.077 ± 0.266 | -2.758 ± 0.212 |
| 3 | -2.436 ± 0.069 | -4.826 ± 0.184 | -8.642 ± 0.549 |
| 4 | -3.144 ± 0.366 | -11.740 ± 1.254 | -9.522 ± 1.175 |

The zeta potential of the sulphur-grown cells remained relatively constant and only decreased by 1.029 mV when the pH was increased from 2 to 4, whereas the ferrous iron-grown and chalcopyrite-adapted cultures showed a much greater change in the zeta potential when the pH was increased to 4 (Table 4.5). This suggests that the composition of the cell surface components differed and that the surface

components of the cells cultured on elemental sulphur are less easily deprotonated than the other cultures.

Huan *et al.* (2008) determined the zeta potential over a pH range for *A. manzaensis* cultured under different growth conditions. The results are of the same order of magnitude as the results from this study, with the IEP between pH 2.5 and pH 3.7. The trend observed with an increase in pH differs from this study in that over the pH range of 2 to 4, the cells cultured on ferrous iron and those cultured on elemental sulphur had very similar zeta potentials and only the chalcopyrite adapted cells exhibited a steep decrease in zeta potential.

The addition of acid during the initial stage of heap bioleaching results in the dissolution of some of the acid consuming gangue material. This causes the pH within the heap to increase and pH profiles are created throughout the heap. At the low pH values, it has been determined that the mineral substrates investigated typically exhibit a negative charge (Poortinga *et al.*, 2002). The zeta potential of the cells cultured on elemental sulphur remained relatively constant at the increased pH while the ferrous iron- and chalcopyrite-adapted cells became more negative. This would reduce the repulsive force between the cells and the negatively charged minerals. This explanation is consistent with work on bacteria that concluded that electrostatic interactions play an important role in solutions with low ionic strength and when hydrophobic interactions were not prevalent. Although the initial pH of the system was 1.6, it is assumed that the pH will rise due to dissolution of the mineral. This was not explicitly determined in the shake flask studies but the flow-through column experiments (Section 5) exhibited this initial rise in pH. These studies suggest that the electrostatic forces between the mineral and the cell are the fundamental factors in the initial attachment of *At. ferrooxidans* to mineral substrates and that the attachment of the cells decreases with increasing electrostatic repulsion (Rodriguez *et al.*, 2003b; Devasia *et al.*, 1993).



## *4.4. Conclusions*

Shake flask attachment studies were conducted using similar experimental set-ups to those published in literature. The effect of temperature, culture history and mineralogy were investigated for a thermophilic archeae, *M. hakonensis.* Investigating the attachment of the thermophilic archeae, *M. hakonensis* and over a temperature range provided a novel contribution to the current literature where attachment studies have been predominantly conducted with bacteria and at a single temperature. The surface property studies allowed for a more fundamental analysis of the attachment results.

The rate and extent of attachment increased with an increase in temperature from 25°C to 65°C for all cultures and substrates investigated. The increase in attachment was most pronounced when the temperature was raised from 25°C to 45°C. The Arrhenius equation provided an adequate fit to the rate data, indicating that the increase in attachment observed is most likely to be due to temperature effects and that initial attachment is a physicochemical process. Further studies were conducted on the metabolic activity of the cells to determine evaluate this (Section 5.4).

The cells cultured on elemental sulphur exhibited the greatest levels of attachment under all conditions investigated. Centrifugation of the cells during harvesting may disrupt the loosely bound EPS leaving the capsular polysaccharide to interact with the mineral. The effect of culture history is dependent on differentiation in the EPS composition and alternative harvesting techniques should be investigated in order to eliminate this factor. However, studies conducted with mesophilic cultures exhibited clear differences in attachment of the different cultures (Africa *et al.,* 2009) after harvesting the cells by centrifugation, indicating that the EPS of these cultures were not significantly depleted.

The surface properties of the cells were investigated in hydrophobicity and zeta potential studies. Both the zeta potential and the hydrophobicity of the cultures varied with the different growth histories. This variation between cultures is indicative of a difference between the surface properties, suggesting the EPS composition or cell envelope composition of the cultures differ. The cells adapted to a chalcopyrite concentrate exhibited the greatest hydrophobicity, with the elemental sulphur- and ferrous iron-grown cells exhibiting significantly lower values. Refinement of the experimental protocol is required for an improved quantitative analysis of the relative hydrophobicities. As the mineral substrates are hydrophobic under the experimental conditions and the sulphur-grown cells do not exhibit the greatest hydrophobicity, it is likely that under the conditions of this study the dominating forces involved in initial adhesion are electrostatic interactions. The zeta potential of the cultures decreased with an increase in pH from 2 to 4. For the ferrous iron- and chalcopyrite-grown cells, this decreased more rapidly than the sulphur-grown cells. The mineral substrates will be negatively charged under the experimental conditions and the lower negative charge of the sulphur-grown cells at higher pH's would reduce the repulsive force to the mineral substrate relative to the other cultures. This is particularly relevant during initial adhesion as the pH of the leach solution is elevated due to acid consumption by gangue minerals..

Preferential attachment to mineral sulphides was not pronounced with *M. hakonensis*, which is consistent with other attachment studies using archeae. The surface area available for attachment in the low grade ore sample was greater than in the other substrates, but the available surface area was

orders of magnitude greater than the area required for 100% attachment. While not a factor in this study, relative surface area must be considered when mineral addition is based on mass. Mesophilic cultures however, have shown selective affinity for sulphide minerals. This suggests that either the initial attachment mechanism or the surface properties of the microorganisms differ. Attachment to gangue material was evident and further experimental work should be carried out in order to determine which components in the gangue the microorganisms are attaching to.

The shake flask attachment studies have clearly shown differences between the attachment of archeae and mesophilic bacteria, but the results obtained are in agreement with current literature. The effect of temperature on the attachment of microorganisms provides new insight into the attachment mechanism and is a key finding from the study. The continuous flow-through system of the columns, are more representative of the hydrodynamic conditions within a bioleaching heap. The key parameters from the general trends mentioned above were investigated in this system in order to investigate the effect that a non agitated, flow-through system has on attachment.

# 5. Column Attachment Study

## *5.1. Introduction*

The column experiments were designed to better approximate conditions within a heap, while maintaining a greater degree of control and reproducibility than could be achieved by packing columns with agglomerated ore. The columns were packed with 300 glass beads (6 mm diameter) evenly coated with mineral substrate to ensure a relatively consistent surface area for attachment. Solution was pumped in from the bottom so the columns were fully saturated to minimise channelling. Direct cell counts were performed on the effluent to infer the number of cells retained within the column. The solution chemistry was monitored by measuring pH, redox potential and conductivity to assess the stability of the system and provide a basis for discussion should changes in solution chemistry influence attachment.

The residence time within the column was approximately 60 minutes, so the activity of the cells under the experimental conditions was investigated to assess whether the attachment observed in the columns was an active process, or a physicochemical one, as observed in the shake flask studies. Ferrous iron oxidation rates and oxygen utilisation rates were measured on a culture of sulphur-grown cells under the experimental conditions used in the attachment studies.

The results from the column attachment studies are presented and discussed, followed by a comparison of the experimental set-ups used to analyse microbial attachment. The results from the metabolic activity tests are presented and discussed, before a summary of the key points is presented in the conclusion.



## *5.2. Attachment results and discussion*

Data are presented as % cells retained within the column, rather than % attached, as the planktonic cells have a certain residence time within the column. However, once the retention had stabilised (Figure 5.2), the remaining cells were assumed to be attached to the mineral. A control experiment, using uncoated glass beads, was conducted at 65°C with cells cultured on elemental sulphur. This confirmed that the cells were not retained in the column in hydraulic “dead zones” or attached to the column walls or glass beads. The combination of factors (culture history and temperature) chosen showed the greatest levels of attachment in the shake flask studies, so it was assumed that other combinations would show lower levels of attachment. The data from the control experiments is presented in Figure

5.1 and indicates a final retention of less than 10% in the control column for all runs. The results are consistent with the negligible attachment to quartz in the shake flask experiments.

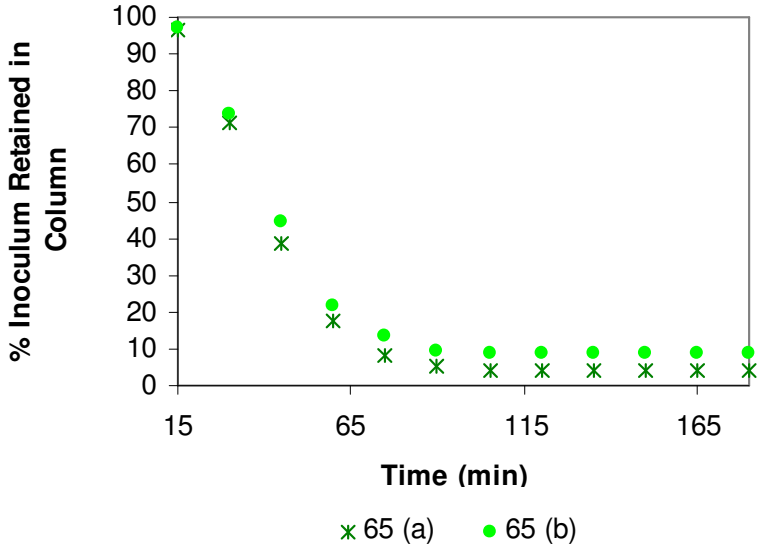



**Figure 5.1:** Retention of sulphur grown *M. hakonesis* ($1x10^9$ cells total) in the packed-column reactor with uncoated glass beads, operated at 65°C.

A typical profile obtained in the column experiments is presented in Figure 5.2. The final value for the cells retained in the column was determined as the mean value from the time the retention line stabilised (75 min in Figure 5.2). The results obtained across the experimental matrix are summarised in Figure 5.3.

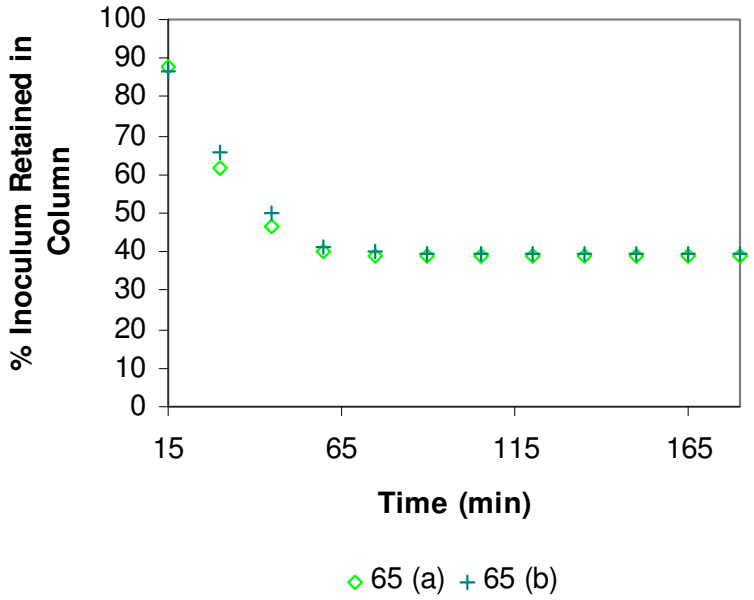



**Figure 5.2:** Retention of sulphur grown *M. hakonesis* ($1x10^9$ cells total) in the packed-column reactor with chalcopyrite concentrate coated beads operated at 65°C. The results are representative of duplicate experiments, indicated by (a) and (b) on the graph.

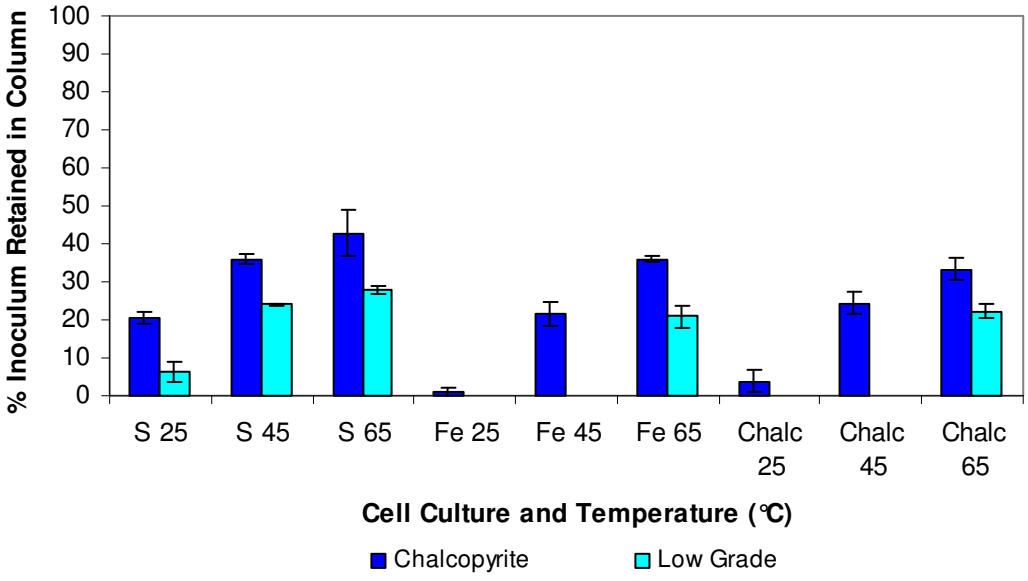



**Figure 5.3:** Final percentage of *M. hakonensis* retained in columns loaded with chalcopyrite and low-grade ore coated beads as a function of temperature and culture history. Experiments with low-grade ore coated beads at 25°C and 45°C were only conducted with sulphur grown cells.

The trends observed in the column experiments were similar to the shake flasks, with the retention of cells in the column increasing at higher temperatures. Similarly, the sulphur grown cells were retained to a greater degree. However, the level of retention (Figure 5.3) was significantly lower than the attachment observed in shake flasks (Figure 4.2). The mineral loadings should provide a comparable surface area for attachment as approximately double the mass was used in the columns with only half the surface area available for attachment, the other half facing the beads. The shake flask experiments had an initial cell concentration of 2 x $10^7$ cells/ml (2 x $10^9$ cells total) and the columns were inoculated with a 10 ml pulse of ~1 x $10^8$ cells/ml (1 x $10^9$cells total).

The attachment to the low-grade ore followed the same trend as to the chalcopyrite concentrate, but the levels of attachment were lower. This is different to the trend observed with the shake flask studies where there was no substantial difference between attachment to the different substrates. The difference in the trends observed between the shake flask and column data may be a result of the differences in the experimental systems, but additional research is required to provide conclusive evidence.

The experimental set-up used in this study was developed by Africa and colleagues (2009) and is unique, making it difficult to draw comparisons with existing literature. The previous study (Africa *et al*., 2009) investigated attachment of mesophilic and thermophilic cultures at ambient conditions in the same column experiment. Although the experimental conditions differ, the effect of the experimental set-up on the attachment results and the levels of attachment achieved can be compared. Complete washout or very low levels of attachment of the thermophile, *S. metallicus*, were observed. This is consistent with the current study, where *M. hakonensis* exhibited very little or no attachment to the substrates at ambient temperature. The highest degree of attachment, using *At. ferrooxidans*, was shown to be 63% to a chalcopyrite concentrate and 25% to a low grade ore (Africa *et al.,* 2009). The attachment of *M. hakonensis* to the low grade ore at the optimum growth conditions (65°C) was similar, with a maximum attachment of 28% achieved using cells adapted to elemental sulphur. The attachment to the chalcopyrite concentrate was lower for *M. hakonensis*, with maximum attachment of 43% achieved using cells cultured on elemental sulphur.

While there appeared to be some preferential attachment, the difference between the extent of attachment to the low grade ore and chalcopyrite concentrate observed with *M. hakonensis* was lower than the difference observed with the mesophilic cultures using the same experimental set-up (Africa *et*

*al.,* 2009). Under optimal culture growth conditions, the difference for *M. hakonensis* was approximately 14% compared to 42% for *At. ferrooxidans* (Africa *et al.,* 2009).

## *5.3. Comparison between the experimental set-ups*

Both experimental set-ups showed the same trend of increased attachment with increasing temperature. However, the levels of attachment were substantially lower in the flow-through column studies and there was some evidence of preferential attachment. The packed column provides a better representation of the hydrodynamic conditions within the heap as there is a constant flow through the column, whereas the shake flasks represent a batch system, with conservation of all material.

Previous studies have shown that microbial attachment can be modelled using the Langmuir adsorption model (Liu, 2006; Sohn *et al.,* 2005). A basis of this model is that the extent of adsorption is a function of the number of open sites on the adsorbent and the concentration of adsorbing species. The equation that is generally used to describe the Langmuir model is:

$$\frac{X_e}{q} = \frac{X_e}{q_m} + \frac{1}{K_a q_m} \quad \textbf{(16)}$$

where $X_e$ represents the concentration of adsorbate at equilibrium, q represents the amount of adsorbate absorbed per gram of adsorbent, $q_m$ is the amount of adsorbate required to form a monolayer on the adsorbent (maximum adsorption capacity)and $K_a$ is the Langmuir adsorption equilibrium constant.

Rodriguez and co-workers (2003) modelled the adsorption of cells onto solid surfaces using a similar model, which was also dependent on the number of available adsorption sites and the concentration of planktonic cells. The equation used to describe this model is:

$$K_a t = \frac{1}{\frac{1}{a}A_o - X_{bo}} Ln\left[\frac{X_{bo}\left(\frac{1}{a}A_o - X_{bs}\right)}{\frac{1}{a}A_o\left(X_{bo} - X_{bs}\right)}\right] \quad \textbf{(17)}$$

where $K_\alpha$ is the attachment rate constant (ml/cell.h); $A_o$ is the concentration of surface adsorption sites ($cm^2/cm^3$); α is the projected area per bacteria ($cm^2$/cell); $X_{bo}$ is the initial concentration of free bacteria (cell/ml); $X_{bs}$ is the concentration of attached bacteria on the solid surface (cell/ml); and t is the time (h).

Both equations have been successfully used to model attachment data in simulated bioleaching systems. The Langmuir model is based on three main assumptions – monolayer coverage, adsorption site equivalence and independence (Sohn *et al.,* 2005). The heterogeneity of solid substrates as well as the forces that are exerted between the microorganisms will therefore impact these assumptions and result in deviations from theoretical values, but experimental data has shown a good fit to Langmuir type models (Rodriguez *et al.,* 2003).

A clear dependence of attachment on both the adsorption sites available and the concentration of planktonic cells is highlighted in both models. The theoretical maximum cell loading in the columns used in this study were determined to be $1.25 \times 10^{10}$ cells, which is an order of magnitude greater than the cell inoculum of approximately $1 \times 10^{9}$ cells. It can therefore be assumed that the surface area available for attachment was not a limiting factor in the attachment observed in the columns. Another factor is the microbe-mineral contact time, but it was clearly shown in the shake flask studies that attachment occurred rapidly, predominantly within the first 10 min, and the residence time in the column (± 60 min, Africa *et al.* 2009) exceeded this. The key factor impacting the rate and extent of attachment according to the above, generally accepted, equations is the planktonic cell concentration.

In the shake flasks there is conservation of cells within the system, while in the columns the planktonic cells move through the column and unattached cells are eventually washed out. As the planktonic cell concentration is an important driving force in the attachment of the microorganisms, according to the Langmuir theory, this could account for the different extents of attachment observed. The planktonic cell concentration decreased with flow through the column, as some cells interacted with the mineral, which resulted in a reduction in the driving force for attachment. This could account for the reduced attachment relative to the shake flasks, where the cells were conserved within the system. This is an important finding and validates the development of the experimental system.

The initial cell concentration in the column experiments was approximately $1 \times 10^{8}$ cells/ml (10 ml) compared to $2 \times 10^{7}$ cells/ml (100 ml) in the shake flask experiments. As it has been clearly defined that planktonic cell concentration drives cell attachment, this will clearly impact the attachment that occurs regardless of the fact that the total cell number is comparable between the experiments. The flow regimes in the systems also differ as turbulent flow is experienced in the shake flasks and laminar flow in the column experiments. The turbulent flow will result in better mixing of the system, resulting in better microbe-mineral contacting which will facilitate attachment. Conversely, if too much force is exerted

with the turbulent mixing the cell surface could be damaged which would effect the cells ability to attach.

Bioleaching heaps are flow through systems and the attachment will more likely follow a similar model to that seen in the columns, rather than the shake flasks. The columns that were used in these experiments were only 20 cm in length, compared to the average heap height of approximately 6 m (Watling *et al.,* 2006) and a greater theoretical level of attachment can therefore be expected as bed height increases. This could be assessed by running a number of columns in series and determining the change in the rate of attachment as the planktonic cell concentration decreases.

The other main difference between the two systems is the preferential attachment to sulphide minerals. The shake flask results showed attachment to different solid substrates did not differ significantly (within one standard deviation), indicating non preferential attachment to mineral sulphides. However, in the column experiments, the level of attachment to the chalcopyrite concentrate was clearly greater than the levels observed for the low grade ore. A possible explanation is the flow through nature of the system and the decreasing planktonic cell concentration which leads to a lower attachment driving force. The lower driving force exhibited along the length of the column, would provide increasingly poor conditions for attachment. Shake flasks provide a constant attachment environment which is equivalent to the initial and best attachment conditions in the column. Further research is required to substantiate this theory.

The differences in the results obtained indicate that while shake flask experiments are sufficient in identifying attachment trends, they tend to overestimate the level of attachment likely to occur in packed beds (heaps). Although the results from a single column (20 cm bed height) experiment will likely underestimate attachment in a heap, this should not be a gross overestimation given the retention time (60 minutes) and the rapid nature of initial attachment. The data from a set of columns in series would be able to more accurately predict the attachment of microorganisms within a bioleaching heap. Models require accurate data in order to correctly adjust parameters to optimise the process and obtain efficient copper extraction. The data from the columns is significantly different from the values obtained in shake flask studies and the column data would be better suited and provide advantages in developing models of the bioleaching process.

## *5.4. Metabolic activity results and discussion*

The level of attachment of *M. hakonensis* was higher with an increase in temperature, peaking at the temperature at which the cells would show optimal metabolic activity. In view of this correlation, activity tests were conducted to investigate whether initial attachment could be influenced by metabolic activity. This was of particular interest in the packed column studies where the residence time of 60 min is significantly longer than the 10 minutes that were required for equilibrium to be reached in the shake flask studies. Ferrous iron oxidation and oxygen utilisation tests were selected to assess metabolic activity.

The results from these tests and the role that metabolic activity played in the attachment studies are presented and discussed in the following sections.

### 5.4.1. Ferrous iron oxidation

For the iron oxidation tests the length of the lag phase and subsequent iron oxidation rate were used to assess activity. The lag phase was defined as the time required before rapid, consistent iron oxidation was observed. It was shortest for cells that had been adapted to ferrous iron (24.5 h) and longest for cells adapted to grow on sulphur (79.3 h), with chalcopyrite adapted cells showing an intermediate lag (46.3 h). These results are consistent with the growth history, but indicated that negligible iron oxidation would have occurred over the three hour duration of the column experiments.

The rate of ferrous iron oxidation increased with an increase in temperature, but was negligible over the attachment study time period of 3 hours (Appendix G).

In order to ensure that the negligible metabolic activity was not due to the cell concentration, a ferrous iron oxidation test was carried out with the same cell concentration as the shake flask attachment studies (2 x $10^8$ cells/ml). The experiment was conducted at 65°C using the cells cultured on elemental sulphur, as the greatest extent of attachment was achieved under these conditions. The ferrous iron concentration and redox potential remained relatively constant over the 3 h period (Appendix G), confirming negligible iron oxidation activity even at the high cell concentration.

### 5.4.2. Oxygen utilisation rate

The oxygen utilisation rate (OUR) for sulphur grown cells at 45°C was shown to follow the curve of the abiotic control over a 3 h period (Figure 5.4). This confirmed the negligible metabolic activity observed over a 3 h period in the ferrous iron oxidation tests. The OUR was determined at 45°C and not 65°C due

to the constraint of the temperature limit of the dissolved oxygen (DO) probe. *At. ferrooxidans* was used in the positive control, confirming the validity of the assay. The *At. ferrooxidans* culture exhibited rapid oxygen utilisation at 30°C and complete dissolved oxygen utilisation was achieved after only 2 h (Figure 5.4).

In order to ensure that the negligible metabolic activity is not due to adaptation period, cells were harvested and resuspended in Norris medium, with ferrous iron, at 45°C for 2 days before conducting the oxygen utilisation test. The tests run with this culture followed the same curve as the other tests as well as the abiotic control (Figure 5.4).

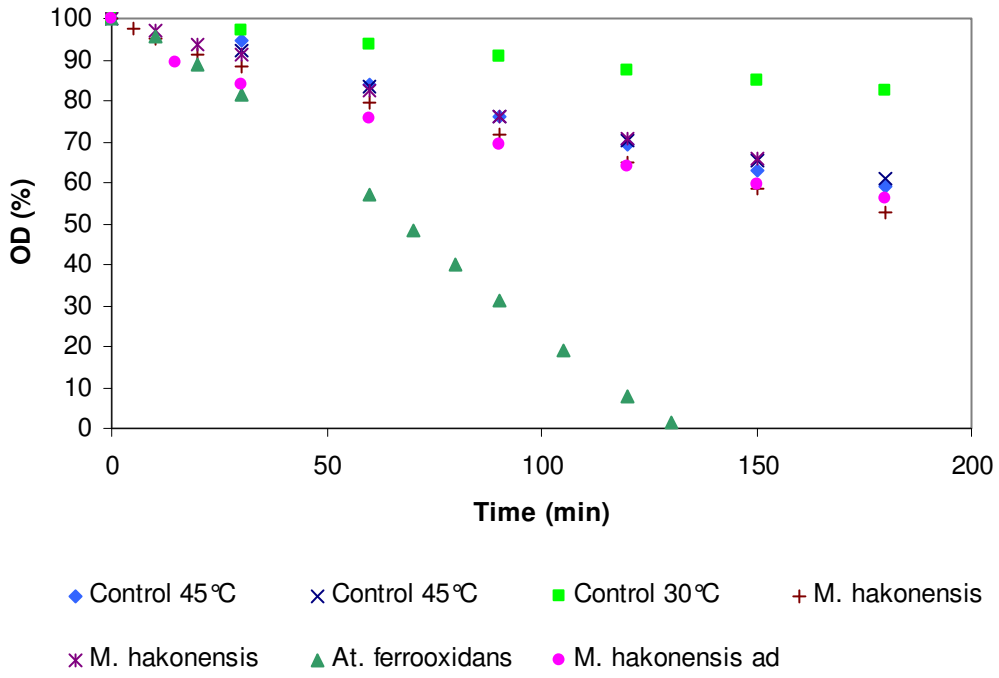



**Figure 5.4:** % of dissolved oxygen measured for *M. hakonensis* at 45°C (stock culture (duplicated) and culture adapted to 45°C and ferrous iron (ad)) and *At. ferrooxidans* at 30°C used as a measure of metabolic activity.

### 5.4.3. Role of metabolic activity in attachment

The very low metabolic activity of the cells observed over the experimental period suggests that the initial attachment process is independent of the metabolic activity of the cells and is a purely physicochemical process. It is possible that *M. hakonensis* behaves in the same way as *Leptospirillum* and requires an elevated redox potential before iron oxidation can take place, resulting in the extended lag period observed. The Brownian motion of the cells will increase with an increase in temperature and

this could increase the number of collisions between the cells and mineral substrate. The effect of temperature on solution chemistry and surface properties of the cells and minerals needs to be investigated further to determine the role that they could play in the observed increase in attachment.

## *5.5. Conclusions*

Attachment studies were conducted in packed, flow-through columns in order to better simulate the hydrodynamic conditions experienced in a bioleaching heap system. Key parameters were determined in the shake flask studies and attachment of *M. hakonensis* in the packed columns was investigated for these key conditions.

The same trend of increasing levels of attachment with increasing temperature was observed in the column studies. However, the levels of attachment were significantly lower in the packed column studies and preferential attachment of the cells to the chalcopyrite concentrate was observed.

The differences in the attachment of the microorganisms between the two experimental set-ups was not due to microbe-mineral contact time as the residence time of the packed column exceeded the time necessary to achieve maximum attachment in the shake flask studies. The Langmuir adsorption model has been used to accurately model microbial attachment in previous studies and states that attachment is a function of the planktonic cell concentration. As the cell concentration decreases as the fluid moves through the column, the driving force of attachment will decrease resulting in lower extents of overall attachment in the columns. The data suggest that packed column attachment studies more accurately account for the hydrodynamic conditions within a bioleaching heap and the data generated will be better suited to validate attachment models.

The residence time of the columns is approximately 60 minutes, which is significantly greater than the time required for maximum attachment in the shake flasks. Metabolic activity tests were conducted to assess the potential for an active component to the trends observed. Ferrous iron oxidation tests showed a minimum lag period of 24.5 h, which exceeded the experimental period of 3 h. The lag period was determined as the time from initial cell inoculation of the system until ferrous iron oxidation was observed. Negligible ferrous iron oxidation over a 3 h period was confirmed with tests conducted at high cell concentration. Oxygen utilisation tests showed negligible metabolic activity over the 3 h period, as the oxygen utilisation followed the abiotic control curve. A positive control conducted with *At. ferrooxidans* showed rapid oxygen utilisation and confirmed that the negligible metabolic activity was not due to the experimental set-up. Experiments were also conducted with cells that were adapted to

ferrous iron as a growth substrate and a temperature of 45°C. These tests also illustrated negligible metabolic activity of the cells over the 3 h period. The evidence suggests the attachment observed in both the shake flasks and packed columns is a physicochemical process and the important factors to consider in the initial adhesion of cells are therefore the electrostatic and hydrophobic interactions. The differences observed in the attachment results between the two experimental systems suggests that the contacting mechanism also plays a significant role in the initial adhesion of cells.

# 6. Integrated Discussion

The objectives of this study were achieved by conducting attachment experiments in both shake flask and packed, flow-through column configurations, using microbial cultures with four different growth histories and three different mineral substrates over an appropriate temperature range. Further analyses were conducted to assess the metabolic activity and surface properties of the cells in order to provide insight into the trends that were observed.

The study was able to show distinct differences in the surface properties of the microbial cultures used. Cells adapted to chalcopyrite concentrate were significantly more hydrophobic than the cell cultured on ferrous iron and elemental sulphur. All cultures were shown to have a negative charge under the experimental conditions, but this was lowest for the cells cultured on elemental sulphur. The surface charge was most stable for these cells, decreasing less rapidly with an increase in pH. During the inoculation phase on heap operation the solution pH is typically elevated above the normal operating pH due to acid consumption by alkaline gangue minerals. The relationship between surface charge and pH may impact the efficiency of heap inoculation. The differences observed suggest culture history affects the composition of organic molecules (proteins, lipids) at the cell surface. More focused research, using Raman or IR spectroscopy could provide additional information regarding the relationship between composition and cell surface properties. Cells cultured on elemental sulphur exhibited the greatest levels of attachment across all experimental conditions and with both experimental set-ups. This result is consistent with the electrostatic charge of the cell and it appears the hydrophobic interactions do not play as significant a role under the conditions investigated.

It is possible, although not proven, that the loosely bound EPS was lost during the harvesting process, resulting in the capsular polysaccharide being exposed. Alternative harvesting techniques such as microfiltration could be used to ensure that the EPS layer of the cell remains intact so the affect of culture history can be accurately determined. The data generated in this study support the first hypothesis, that culture history affects surface properties and ultimately attachment efficiency, but further studies, with more gentle harvesting techniques, will provide information on the extent that the culture history affects attachment.

There are distinct differences in the surface properties of mesophilic bacteria and thermophilic archeae and the attachment trends were shown to differ significantly. The hypothesis that preferential attachment to sulphide minerals would not be evident with thermophilic organisms could not be

conclusively disproved or supported. In the shake flask studies the values were all within one standard deviation and appear to substantiate the hypothesis. However, the column studies showed preferential attachment to the mineral sulphides. There was substantial attachment to gangue material (flotation tails) in the shake flasks and the column studies showed that attachment to the low grade ore was still significant. This suggests that, unlike the mesophilic cultures where negligible attachment to the low grade ores is observed, *M. hakonensis* is capable of significant attachment to gangue material. The gangue component is complex and the exact nature of the interaction could not be determined in this study. Africa and co-workers (2009) developed a biofilm reactor, where the special distribution of the organisms on the mineral surface can be visualised. In order to quantify the distribution of cells attaching to mineral sulphides and gangue material respectively, this experimental protocol should be utilised.

The affect of temperature on the attachment of thermophilic microorganisms was identified as a gap in the existing literature and was addressed for *M. hakonensis* in this study. The dominant trend observed across all experimental conditions and both experimental set-ups was the increase in the extent of attachment with an increase in temperature. The hypothesis that initial attachment is a physicochemical process, rather than being related to increased metabolic activity of the cells, was supported in this study. The metabolic activity was shown to be insignificant under the experimental conditions using both ferrous iron oxidation and oxygen utilisation tests. In order to conclusively prove that there is not an active component to the initial attachment observed, attachment tests with dead or metabolically inactive (sodium azide treated) cells should be conducted. This could confirm that the microbial surface properties are responsible for the observed attachment and that the increased levels of attachment with temperature are due to the increased convective motion or lowering of activation energy.

Increasing the system temperature enhances the levels of attachment of *M. hakonensis* and although not substantiated, it can be postulated that this will hold true for all thermophilic archeae. This is a reasonable assumption due to the similarity in archeael surface properties and the significant role that they play in the initial attachment process. This observation supports the proposal of a two-step inoculation process (Zou *et al.,* 2006), but further research in this area will be required before it can be conclusively shown to enhance the overall leaching efficiency. Column studies which can assess the rate of leaching achieved as well as studies conducted with mixed cultures should be the next step in assessing the benefit of this proposed inoculation process thoroughly.

The final hypothesis, that shake flask data does not accurately describe the attachment in flow-through systems such as a bioleaching heap, was addressed by comparing data from the two systems. The shake flasks data showed significantly higher levels of attachment than in the flow-through columns, despite the systems being designed to use similar cell concentrations and available mineral surface area. The broad trends, such as the increase in attachment with an increase in temperature and the greater levels of attachment achieved with cells cultured on elemental sulphur, were consistent across both systems implying that the attachment mechanism did not change. The column studies were designed to provide a more accurate representation of the hydrodynamic conditions within a bioleaching heap. While this assertion is intuitively sound a focused study comparing the solution flow in the columns to heap simulations could substantiate this and conclusively confirm that the data from the column experiments more accurately represent the real world situation. The column has a much shorter bed height than a stacked heap and due to the changing conditions within the heap, one column is not sufficient in providing to model the entire heap. Experiments with columns run in series should be conducted. A sample should be taken between reactors, before the solution is fed into the next column and this will provide a number of data points for short lengths (19 cm) which can be used to extrapolate the complete set of data points through the heap. The limitations of the experimental design are acknowledged, however this study has made progress toward developing a system which more accurately represents attachment within a heap. The data strongly suggest that shake flask data overestimate the attachment efficiency and may introduce inaccuracies when incorporated into integrated bioleaching models. The value of the column data could be increased by performing attachment isotherm studies and using the derived Langmuir constants to develop a model to describe the laboratory scale columns.

This study addressed a number of gaps in the existing literature, as outlined above. However, all studies were conducted with a pure culture of *M. hakonensis*. The interactions that occur between microorganisms within a mixed culture were not addressed in this study and it was assumed that attachment of the microorganisms occurs independently of each other which is most likely not the case. In addition, the data suggest that a two-stage inoculation system may have value, in which case the thermophiles would be exposed to pre-colonised material and not carefully prepared substrates (as used in this study) The first step toward addressing these issues would be to initially saturate a column with a mixed mesophilic culture and once these microorganisms are established on the ore surface, increase the temperature of the heap to 45°C and feed in the thermophilc culture. This will further assess the validity of introducing a two-step inoculation process as well as providing an analysis of the

interactions between two species. The analysis of such a study could be done using FISH or another discriminatory molecular technique. The biofilm reactor (Africa *et al.,* 2009) could also be used to investigate the special orientation of the mesophilic bacteria relative to the thermophilic archeae, providing further insight into the interactions that occur between the microorganisms. Column studies should also be conducted to assess the impact that a two-step inoculation system would have on the rate of mineral leaching.

Another area that this study did not address was the detachment of the microorganisms due to changes in the solution chemistry. This could be assessed by loading a column with microorganisms and then altering the composition of feed solution into the column. The number of originally attached cells could be determined, as in this study, and then the number of cells which detach due to the change could be determined by counting the cells leaving the column. The percentage of cells which detach could then be determined and used to assess the detachment likely to occur in a heap bioleaching system. Conditions which are likely to change within a heap bioleaching system should be investigated in this way. These include the salt concentration, the redox potential, the pH and the temperature.

This study addressed a number of gaps in the existing literature, including the affect of temperature on attachment, the affect of culture history on attachment, attachment to low grade ores. It focussed on a thermophilic archeael species which is prevalent in bioleaching systems, but has not been extensively studied to date. As mentioned above, the research area is vast and there are still a number of gaps that need to be addressed in order to obtain a comprehensive understanding of the microbial attachment that occurs within a heap bioleaching process.

# 7. Conclusions and Recommendations

Bioleaching is a relatively environmentally benign and economically attractive alternative to conventional processes for the extraction of metals from low grade ores. In industry, both stirred tank and heap bioleaching systems are implemented. The focus of this study is on heap bioleaching, which is being used to extract copper from low grade sulphide ores, such as chalcopyrite. Additional research is required to improve the efficiency of the process and make it more robust.

The extraction of copper in a heap bioleaching process occurs via the attack of ferric and hydrogen ions, resulting in mineral dissolution. The microorganisms act as catalysts and regenerate the ferric and hydrogen ions required. Attachment of the microorganisms to the mineral substrate has been shown to enhance the rate of metal extraction. Two explanations for the increased rates are that the cells are in close proximity to their energy source when attached and that the EPS produced by the cells creates a reaction space for mineral dissolution to occur (Harneit *et al.*, 2006; Kinzler *et al*., 2003 and Watnick *et al.*, 2000). In a heap system, attachment of the microorganisms also ensures that the microbial population is retained within the heap, and thereby more prolific throughout the heap.

The initial attachment of the microorganisms to the mineral substrate is a physicochemical process and the surface properties of the cells and the mineral are the main factors affecting this process (van Loosdrecht *et al*., 1990; Devasia *et al.,* 1993; Harneit *et al.,* 2005). The culture history of the microorganism affects the EPS composition (Harneit *et al.,* 2006; Kinzler *et al.,* 2003) and therefore the surface properties.

In this study, the affect of different growth media on the surface properties of the microorganisms and the effect this had on the cells ability to attach was investigated as previous studies have shown that culture history has a significant impact on microbial attachment. The low grade ores which are commonly used in bioleaching heaps contain large amounts of gangue material and microbial attachment to concentrates as well as low grade ores was therefore investigated in this study. The extraction of copper from chalcopyrite requires high temperatures to overcome passivation. The heap is able to achieve high temperatures due to the exothermic oxidation of reduced sulphur species. In this study the attachment of a thermophilic archeae, *M. hakonensis,* was investigated at 25°C, 45°C and 65°C. These temperatures cover ambient conditions, optimum microbial growth conditions as well as an intermediate temperature which represents the transition between mesophilic and thermophilic temperatures. Bioleaching heaps have a temperature profile from ambient to thermophilic

temperatures as they do not utilise external heating and the effect that this has on the microbial attachment is important.

The shake flask attachment studies were conducted in order to position this study within the current literature as well as to provide a direct comparison between the agitated batch and flow-through column configurations. Levels of attachment consistent current literature values (60-90% attachment) were observed in the shake flask studies. Direct comparisons were difficult due to slight deviations in experimental protocols. Results were represented as percentage attachment and cells attached per unit area in order to provide a better comparison with other studies where different mineral loadings were used.

The flow-through column studies were conducted using a novel experimental set-up making comparison with existing literature difficult. The same experimental set-up was used by Africa *et al.* (2009) who showed that limited attachment of the thermophile, *S. metallicus,* occurred under ambient conditions.

Both the shake flask and column attachment studies showed a clear trend of increased levels of attachment as the experimental temperature was increased from 25°C to 65°C. The rate data from the shake flask attachment study showed a good fit to the Arrhenius equation, indicating that the increased levels of attachment were most likely a function of temperature and not a consequence of cellular physiology. Both ferrous iron oxidation and oxygen utilisation tests were conducted with adapted and unadapted cells. The metabolic activity of the cells was shown to be limited under the experimental conditions and time period, confirming that the initial attachment was a physicochemical phenomenon. The third hypothesis was therefore confirmed as increasing levels of attachment with increasing temperature as well as the lack of an active component in the attachment process was shown.

The sulphur grown cells exhibited the greatest levels of attachment to all mineral substrates. The hydrophobicity of the cells was determined using 2-phase partitioning with hexadecane and the cells adapted to chalcopyrite were shown to be the most hydrophobic. The zeta potential of the cells was determined at pH 2, 3 and 4. The zeta potential of the cells cultivated on elemental sulphur decreased less rapidly than the other two cultures. The lower negative charge resulted in a smaller repulsive force between the microbe and the mineral and would facilitate attachment. This suggests that the surface charge played a more important role in attachment than hydrophobic interactions. The enhanced levels of attachment observed with cells cultured on elemental sulphur support the first hypothesis.

In contrast to previous work with mesophiles, *M. hakonensis*s did not show pronounced affinity for sulphide minerals and significant attachment to the non-quartz fraction of the gangue was observed.

The surface area of the low grade ore (per mass) was greater than the concentrates, but on further analysis, the maximum cell loading capacity for all substrates were at least an order of magnitude greater than the cell inoculums, so surface area was therefore not a limiting factor.

The cell wall and membrane composition of archeae differ significantly from bacteria. These differences most likely contribute to the differences observed. The packed column data showed slight preferential attachment, but significant attachment to the low grade ore was observed. This finding supports the second hypothesis, but further research is required in order to completely understand the role of mineralogy and gangue composition on the attachment of thermophilic archeae.

The extent of attachment was lower in the column experiments due to the flow through nature of the experiments. The Langmuir adsorption isotherm has been used to describe microbial attachment and effectively model experimental data in numerous studies. Attachment is a function of planktonic cell concentration according to this model. This relationship provides an explanation for the difference in the levels of attachment observed as the planktonic cell concentration decreases as the inoculums pulse moves through the column. This validated the development of the experimental set-up, as the flow-through column provided attachment data is a system more representative of a bioleaching heap. This is consistent with the fourth hypothesis.

The study confirms that attachment of thermophilic archeae is suppressed at mesophilic temperatures. The data suggest that a secondary inoculation of thermophiles once the heap has reached 40 to 45°C may enhance their retention and improve subsequent colonisation. Studies should be conducted with mixed cultures in order to determine the interactions that occur between the microorganisms and how the attached mesophilic cultures affect the subsequent attachment of the thermophiles. To better model the attachment of *M. hakonensis*, attachment isotherms should be conducted in order to fit constants to the Langmuir model and infer the 'activation energy' required for attachment of the microorganism.

# References

Africa, C.-J., 2009. Microbial attachment to sulphide minerals in a bioleach environment. MSc thesis, University of Cape Town, Cape Town, pp 171

Akcil, A., Ciftci, H., Deveci, H., 2007. Role and contribution of pure and mixed cultures of mesophiles in bioleaching of a pyritic chalcopyrite concentrate. Minerals Engineering 20, 310-318.

Amini, M., Younesi, H., Bahramifar, N., 2009. Biosorption of nickel(II) from aqueous solution by *Aspergillus niger*: Respponse surface methodology and isotherm study. Chemosphere 75(11), 1483-1491.

Anonymous (a), n.d. Retrieved January 2011, from http://logisticsweek.com/news/2010/08/copper-demand-to-exceed-supply-in-2011/

Anonymous (b), n.d. Retrieved January 2011, from http://www.goldfields.co.za/com_technology.php

Anonymous (c), n.d. Retrieved December 2010, from http://webmineral.com/data.shtml

Anonymous (d), n.d. Retrieved December 2010, from http://geology.com/minerals.shtml

Anonymous (e), n.d. Retrieved December 2010, from http://www.mindat.org/min-2815.html

Anonymous (g), n.d. Retrieved December 2010, from http://www.gps.caltech.edu/~vijay/Papers/Chemistry/Meyer-76.pdf

Anonymous (i), n.d. Retrieved December 2010, from http://www.back-to-basics.net/agrifacts/pdf/b2b29b.pdf

Bayrak, Y., 2006. Application of Langmuir isotherm to saturated fatty acid adsorption. Microporous and Mesoporous Materials 87(3), 203-206.

Breed, A.W., Dempers, C.J.N., Searby, G.E., Gardner, M.N., Rawlings, D.E., Hansford, G.S., 1999. The effect of temperature on the continuous ferrous-iron oxidation kinetics of a predominantly Leptospirillum ferrooxidans culture. Biotechnology and Bioengineering 65(1), 44-53.

Brock, T.D., Madigan, M.T., Martinko, J.M., Parker, J. 1994. *Biology of Microorganisms,* 7th Edition, Prentice Hall, New Jersey.

Clark, D.A., Norris, P.R., 1996. Oxidation of mineral sulphides by thermophilic microorganisms. Minerals Engineering 9(11), 1119-1125.

Córdoba, E.M., Munõz, J.A., Blázquez, M.L., González, F., Ballester, A., 2008. Leaching of chalcopyrite with ferric iron. Part I: general aspects. Hydrometallurgy 93, 81–87.

Del Bubba, M., Arias, C.A., Brix, H., 2003. Phosphorous adsorption maximum of sands for use as media in subsurface flow constructed reed beds as measured by the Langmuir isotherm. Water Research 37(14), 3390-3400.

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

Edwards, K.J., Rutenberg, A.D., 2001. Microbial response to surface microtopography: the role of metabolism in localized mineral dissolution. Chemical Geology 180, 19-32.

Escobar, B., Bustos, K., Morales, G., Salazar, O., 2008. Rapid and specific detection of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* by PCR. Hydrometallurgy 92(3-4), 102-106.

Etzel, K., Klingl, A., Huber, H., Rachel, R., Schmalz, G., Thomm, M., Depmeier, W., 2008. Etching of {111} and {210} synthetic pyrite surfaces by two archael strains, *Metallosphaera sedula* and *Sulfolobus metallicus*. Hydrometallurgy 94, 116-120.

Farah, C., Morin, D., Haras, D., Jerez, C.A. and Guiliani, N., 2005. Evidence for a functional quoram-sensing type *Al-1* system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*. Applied and Environmental Microbiology 71, 7033-7040.

Fowler, T.A., Crundwell, F.K., 1999. Leaching of zinc sulphide by *Thiobacillus ferrooxidans*: Bacterial oxidation of the sulfur product layer increases the rate of zinc sulfide dissolution at high concentrations of ferrous ions. Applied and Environmental Microbiology 65, 5285-5292.

Franzmann, P.D., Haddad, C.M., Hawkes, R.B., Robertson, W.J., Plumb, J.J., 2005. Effects of Temperature on the rates of iron and sulfur oxidation by selected bioleaching Bacteria and Archae: Application of the Ratkowsky equation. Minerals Engineering 18, 1304-1314.

Gautier, V., Escobar, B., Vargas, T., 2008. Cooperative action of attached and planktonic cells during bioleaching of chalcopyrite with *Sulfolobus metallicus* at 70°C. Hydrometallurgy 94(1-4), 121-126.

Gehrke, T., Telegdi, J., Thierry, D., Sand, W., 1998. Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. Applied and Environmental Microbiology 64, 2743-2747.

Ghannoum, M., O'Toole, G.A., 2004. Microbial Biofilms. *American Society for Microbiology Press*. Washington DC.

Ghauri, A.M., Okibe, N., Johnson, B.D., 2007. Attachment of acidophilic bacteria to solid surfaces: The significance of species and strain variations. Hydrometallurgy 85(2-4), 72-80.

Gonzalez, R., Gentina, J.C., Acevedo, F., 1999. Attachment behaviour of *Thiobacillus ferrooxidans* cells to refractory gold particles. Biotechnology Letters 21, 715-718.

Hackl, R.P., Dreisinger, D.B., Peters, E., King, J.A., 1995. Passivation of chalcopyrite during oxidative leaching in sulfate media. Hydrometallurgy 39, 25–48.

Harneit, K., Goksel, A., Kock, D., Klock, J.H., Gehrke, T., Sand, W., 2006. Adhesion to metal sulfide surfaces by cells of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. Hydrometallurgy 83, 245-254.

He, H., Yang, Y., Xia, J., Ding, J., Zhao, X., Nie, Z., 2008. Growth and surface properties of new thermoacidophilic Archae strain *Acidianus manzaensis* YN-25 grown on different substrates. Transactions of Nonferrous Metals Society of China 18, 1374-1378.

Itoh, T., 2003. Review – Taxonomy of Nonmethanogenic Hyperthermophilic and Related Thermophilic Archaea. Journal of Bioscience and Bioengineering 96(3), 203-212.

Jerez, C.A., 2001. Chemotactic transduction in biomining organisms. Hydrometallurgy 59, 347-356.

Johnson, B., 2006. Biohydrometallurgy and the environment: Intimate an important interplay. Hydrometallurgy 83, 153-166.

Kandler, O., Konig, H., 1998. Cell wall polymers in Archae (Archaebacteria). CMLS 54, 305-308.

Kinzler, K., Gehrke, T., Telegdi, J., Sand, W., 2003. Bioleaching - a result of interfacial processes caused by extracellular polymeric substances (EPS). Hydrometallurgy 71(1-2), 83-88.

Klauber, C., 2008. A critical review of the surface chemistry of acidic ferric sulphate dissolution of chalcopyrite with regards to hindered dissolution. International Journal of Mineral Processing 86, 1-17.

Lei, J., Huaiyang, Z., Xiaotong, P., Zhonghao, D., 2009. The use of microscopy techniques to analyze microbial biofilm at the bio-oxidized chalcopyrite surface. Minerals Engineering 22, 3-42.

Liang, X.J., Liu, A.Q., Limb, C.S., Ayi, T.C., Yap, P.H., 2007. Determining refractive index of single living cell using an integrated microchip. Sensors and Actuators A 133, 349–354.

Liu, Y., 2006. Some consideration on the Langmuir isotherm equation. Colloids and Surfaces 274, 34-36.

Mangold, S., Harneit, K., Rohwerder, T., Claus, G., Sand, W., 2008. Novel Combination of Atomic Force Microscopy and Epifluorescence Microscopy for Visualization of leaching bacteria on Pyrite. Applied and Environmental Microbiology 74(2), 410-415.

Mikkelsen, D., Kappler, U., Webb, R. I., Rasch, R., McEwan, A.G., Sly, L.I., 2007. Validation of pyrite leaching by selected thermophilic archae: Nature of microorganism-ore interactions during bioleaching. Hydrometallurgy 88, 143-153.

Misak, N.Z., 1993. Langmuir isotherm and its application in ion-exchange reactions. Reactive Polymers 21(1-2), 56-64.

Moon

Muir, M.K., Anderson, T., 1977. Determination of ferrous iron in copper process metallurgical solutions by o. phenanthroline colorimetric methods. Metallurgical and Materials Transactions B 8(2), 517–518.

Muller, R., Groger, G., Hiller, K.A., Schmalz, G., Ruhl, S., 2007. Fluorescence-Based Bacterial Overlay Method for Simultaneous In Situ Quantification of Surface-Attached Bacteria. Appled and Environmental Microbiology 73(8), 2653-2660.

Muñoz, P.B., Miller, J.D., Wadsworth, M.E., 1979. Reaction mechanisms for the acid ferric sulfate leaching of chalcopyrite. Metallurgical Transactions B 10B, 149–158.

Natarajan, K.A., Das, A., 2003. Surface chemical studies on '*Acidithiobacillus'* group of bacteria with reference to mineral flocculation. International Journal of Mineral Processing 72, 189-198.

Ohmura, N., Kitamura, K., Saiki, H., 1993. Selective adhesion of *Thiobacillus ferrooxidans* to pyrite. Applied and Environmental Microbiology 59, 4044-4050.

Ojumu T.V., 2008. The Effects of Solution Conditions on the Kinetics of Microbial Ferrous-Iron Oxidation by *Leptospirillum Ferriphilum* in Continuous Culture. PhD Thesis, University of Cape Town, South Africa.

Olubambi, P.A., Ndlovu, S., Potgieter, J.H., Borode, O.J., 2007. Effects of ore mineralogy on the microbial leaching of low grade complex sulphide ores. Hydrometallurgy 86(1-2), 96-104.

Pagnanelli, F.,Beolchini, F., Di Biase, A., Vegkio, F., 2003. Effect of equilibrium models in the simulation of heavy metal biosorption in single and two-stage UF/MF membrane reactor systems. Biochemical Engineering Journal 15(1), 27-35.

Poortinga, A.T., Bos,R., Norde, W., Busscher, H.J., 2002. Electric double layer interactions in bacterial adhesion to surfaces. Surface Science Reports 47, 1-32.

Porro, S., Ramfrez, S., Reche, C., Curutchet, G., Alonso-Romanowski, S., Donati, E., 1997. Bacterial attachment: its role in bioleaching processes. Process Biochemistry 32(7), 573-578.

Pradhan, N., Nathsarma, K.C., Srinivasa Rao, K., Sukla, L.B., Mishra, B.K., 2008. Heap bioleaching of chalcopyrite: A review. Minerals Engineering 21(5), 355-365.

Rijnaarts, H.H., Norde, W., Bouwer, E.J., Lyklema, J., Zehnder, A.J., 1993. Bacterial Adhesion under Static and Dynamic Conditions. Applied and Environmental Microbiology 59, 3255-3265.

Rodriguez, Y., Ballester, A., Blazquez, M.L., Gonzalez, F., Munoz, J.A., 2003(a). New information on the chalcopyrite bioleaching mechanism at high and low temperature. Hydrometallurgy 71, 47-56.

Rodríguez, Y., Ballester, A., Blázquez, M.L., Gonzalez, F., Munoz, J.A., 2003(b). Study of bacterial attachment during the bioleaching of pyrite, chalcopyrite, and sphalerite. Geomicrobiology Journal 20(2), 131-141.

Rojas-Chapana, J.A., Bartels, C.C., Pohlmann, L. and Tributsch, H., 1998. Co-operative leaching and chemotaxis of *Thiobacilli* studies with spherical sulphur/sulphide substrates. Process Biochemistry 33, 239-248.

Rudzinski, W., Dominko, A., Wojciechowski, B.W., 1996. Mixed-gas adsorption on real solid surfaces: Lack of correlations between adsorption energies of various components related to the wide applicability of the generalized Langmuir-Freundlich isotherm equation. The Chemical Engineering Journal and the Biochemical Engineering Journal 64(1), 85-95.

Sampson, M.I., Phillips, C.V., Blake, R.C., 2000. Influence of the attachment of acidophilic bacteria during the oxidation of mineral sulfides. Minerals Engineering 13(4), 373-389.

Savage, D.C., Fletcher, M., 1985. Bacterial adhesion, Plenum Press, New York, 349-351.

Sharma, P.K., Das, A., Hanumantha Rao, K., Forssberg, K.S., 2003. Surface characterization of *Acidithiobacillus ferrooxidans* cells grown under different conditions. Hydrometallurgy 71, 285-292.

Shiers, D.W., Blight, K.R., Ralph, D.E., 2005. Sodium sulphate and sodium chloride effects on batch culture of iron oxidising bacteria. Hydrometallurgy 80(1-2), 75-82.

Silverman, M.P., Lundgren, D.G., 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. An improved medium and a harvesting procedure for securing high cell yields. Journal of Bacteriology 77(5), 642–647.

Sohn, S., Kim, D., 2005. Modification of Langmuir isotherm in solution systems – definition and utilization of concentration depenent factor. Chemosphere 58, 115-123.

Solari, J.A., Huerta, G., Escobar, B., Vargas, T., Badilla-Ohlbaum, R., Rubio, J., 1992. Interfacial phenomena affecting the adhesion of *Thiobacillus ferrooxidans* to sulphide mineral surfaces. Colloids and Surfaces 69, 159-166.

Stramski, D., 1999. Refractive index of planktonic cells as a measure of cellular carbon and chlorophyll a content. Deap-Sea Research I 46, 335-351.

Tshilombo, A.F., Dixon, D.G., 2003. Kinetic study of chalcopyrite passivation during electrochemical and chemical leaching. In: Doyle, F.M., Kelsall, G.H., Woods, R. (Eds.), Proceedings of the Sixth International Symposium on Electrochemistry in Mineral and Metal Processing, The Electrochemical Society, pp. 108–119.

Usher, K.M., Shaw, J.A., Kaksonen, A.H., Sauders, M., 2010. Elemental analysis of extracellular polymeric substances and granules in chalcopyrite bioleaching microbes. Hydrometallurgy, doi: 10.1016/j.hydromet.2010.02.028

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Zehnder, A.J.B., 1990. Influence of interfaces on microbial activity. Microbiological Reviews 54(1), 75-87.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G., Zehnder, A.J.B., 1987. The role of bacterial cell wall hydrophobicity in adhesion. Applied and Environmental Microbiology 53(8), 1893-1897.

Vilinska, A., Nanumantha Rao, K., 2008. *Leptospirillum ferrooxidans* – sulphide mineral interactions with reference to bioflotation and bioflocculation. Trsnsactions of Nonferrous Metals Society of China 18, 1403-1409.

Watling, H.R., 2006. The bioleaching of sulphide minerals with emphasis on copper sulphides – A review. Hydrometallurgy 84, 81-108.

Wilson, W.W., Wade, M.M., Holman, S.C., Champlin, F.R., 2001. Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements. Journal of Microbiological Methods 43, 153-164.

Yee, N., Fein, J.B., Daughney, C.J., 2000. Experimental study of the pH, ionic strength, and reversibility behaviour of bacterial-mineral adsorption. Geochimica et Cosmochimica Acta 64, 609-617.

Yu, R., Tan, J., Yang, P., Sun, J., Ouyang, X., Dai, Y., 2008. EPS-contact-leaching mechanism of chalcopyrite concentrates by *A. ferrooxidans*. Transactions of Nonferrous Metals Society of China 18(6), 1427-1432.

Zobell, C., 1943. Bacterial adhesion model. Journal of Bacteriolog. 46, 39-52.

Zou, P., Zhang, W.B., Lei, T., Wang, J.K. 2006. Study on bioleaching of primary chalcopyrite ore with thermoacidiphilic archae. Acta Metallurgica Sinica 19(5), 341-346.

# Appendix A: Media and buffer composition

**Medium 88**

| Chemical | Mass (g) |
|---|---|
| $(NH_4)_2SO_4$ | 1.3 |
| $KH_2PO_4$ | 0.28 |
| $MgSO_4.7H_2O$ | 0.25 |
| $CaCl_2.2H_2O$ | 0.07 |
| **Trace element solution** | |
| $MnCl_2.2H_2O$ | 0.062 |
| $ZnCl_2$ | 0.068 |
| $CoCl_2.6H_2O$ | 0.064 |
| $H_3BO_3$ | 0.031 |
| $Na_2MoO_4$ | 0.01 |
| $CuCl_2.2H_2O$ | 0.067 |

**Ferrous based medium:**

The main salts and 0.2 g of yeast extract were added to 1 l of distilled water and the pH adjusted to 1.6 with $H_2SO_4$ and then autoclaved. The trace element solution was dissolved in 1 l of distilled water and the pH adjusted to 1.6. The following was added to the autoclaved solution: 1 ml of the trace element solution, 13.9 g $FeSO_4.7H_2O$ (50 mM) and 10 ml of Tetrathionate (50 mM) stock. The entire solution was readjusted to a pH of 1.6 with $H_2SO_4$.

**Elemental sulphur based medium:**

The main salts and 1.0 g of yeast extract were added to 1 l of distilled water and the pH adjusted to 2.0 with $H_2SO_4$ and then autoclaved. The trace element solution was dissolved in 1 l of distilled water and the pH adjusted to 1.6. The following was added to the autoclaved solution: 0.05 g of elemental sulphur and 1 ml of the trace element solution.

## Norris Medium

| Chemical | Mass (g) |
|---|---|
| $MgSO_4.7H_2O$ | 0.5 |
| $(NH_4)_2SO_4$ | 0.4 |
| $KH_2PO_4$ | 0.2 |
| KCl | 0.1 |

The salts were dissolved in 1 l of distilled water and the pH adjusted to 1.6 with $H_2SO_4$ and then autoclaved.

## 0K Medium

| Chemical | Mass (g) |
|---|---|
| $(NH_4)_2SO_4$ | 3 |
| KCl | 0.1 |
| $K_2HPO_4$ | 0.5 |
| $MgSO_4.7H_2O$ | 0.5 |
| $(CaNO_3)_2.4H_2O$ | 1.45 |

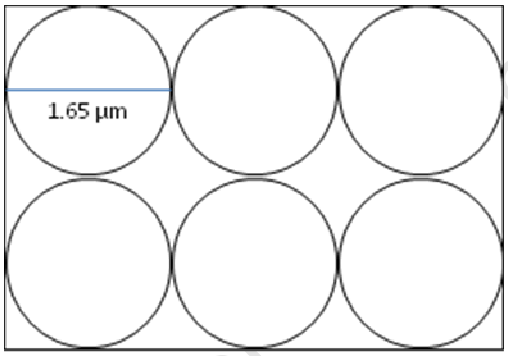
The salts were dissolved in 1 l of distilled water and the pH adjusted to 1.6 with $H_2SO_4$ and then autoclaved.

# Appendix B: Quantification of saturation cell number

In order to ensure that there was a large enough surface area available for all the cells to attach to the substrate in the columns, the maximum number of cells that could be accommodated was calculated.

The following assumptions were made in order to determine the maximum attachment:

- Repulsive forces between cells and minerals were ignored
- Attachment was monolayered with the greatest cell surface area parallel to the substrate
- Cells were taken as spherical in shape
- A diameter of 1.65 µm was used to determine surface area of the cell (average of 1.4 and 1.9 µm, Usher *et al.,* 2010)
- The available surface area of the substrate was taken as the surface area of the glass beads




**Surface area occupied by microbial cell:**

$$Surface\ area = l * l$$

$$= 1.65 * 1.65$$

$$= 2.7225\ \mu m^2$$

**Surface area available for attachment in shake flasks:**

| Mineral substrate | PSD surface area ($m^3/g$) | Total surface area |
|---|---|---|
| Pyrite | 0.0559 | 0.1118 |
| Chalcopyrite | 0.0883 | 0.1766 |
| Low grade ore | 0.279 | 0.558 |

**Saturation cell number in shake flasks:**

$$Maximum\ number\ of\ cells = \frac{Total\ surface\ area\ available\ for\ attachment}{Surface\ area\ of\ microbial\ cell}$$

| Mineral substrate | Saturation cell number (x $10^{10}$) |
|---|---|
| Pyrite | 4.11 |
| Chalcopyrite | 6.49 |
| Low grade ore | 20.5 |

**Surface area available for attachment in column:**

$$Surface\ area = 300 * (4 * \pi * r^2)$$

$$= 300 * (4 * \pi * 0.003^2)$$

$$= 0.034\ m^2$$

**Saturation cell number in columns:**

$$Maximum\ number\ of\ cells = \frac{Total\ surface\ area\ available\ for\ attachment}{Surface\ area\ of\ microbial\ cell}$$

$$= \frac{0.034\ m^2}{2.7225 * 10^{-12}\ m^2}$$

$$= 1.25 * 10^{10} cells$$

# Appendix C: Mineralogy of substrates used

**Table C.1:** Composition of low grade ore with the main gangue and mineral components highlighted

| Minerals | Composition of low grade ore |
|---|---|
| Chalcocite | 0.2 |
| Chalcopyrite | 0.5 |
| Covellite | 0.3 |
| Bornite | 0.1 |
| Atacamite | 0.0 |
| Brochantite | 0.0 |
| Chrysocolla | 0.0 |
| Cuprite | 0.0 |
| Native_Copper | 0.0 |
| Tenorite | 0.0 |
| Turquoise | 0.0 |
| Enargite | 0.1 |
| Pyrite | 4.0 |
| Sphalerite | 0.0 |
| Molybdenite | 0.0 |
| Galena | 0.0 |
| Ilmenite | 0.0 |
| Monazite | 0.0 |
| Rutile | 0.2 |
| Thorite | 0.0 |
| Zircon | 0.0 |
| Muscovite | 28.6 |
| Biotite | 0.5 |
| Chlorite | 0.7 |
| Kaolinite | 7.4 |
| Calcite | 0.0 |
| Goethite | 0.1 |
| Hematite | 0.2 |
| Magnetite | 0.0 |
| Albite | 5.7 |
| Na_orthoclase | 2.9 |
| Plagioclase | 1.7 |
| Alunite | 0.5 |
| Jarosite | 0.2 |
| Gypsum | 0.0 |
| Barite | 0.0 |
| Svanbergite | 0.6 |
| Apatite | 0.1 |
| Hornblende | 0.2 |
| Iron | 0.0 |
| Cromite | 0.0 |
| Corundum | 0.0 |
| Aluminium_Phosphate | 0.0 |
| Quartz | 44.8 |

# Appendix D: Effect of Bostik glue on metabolic activity of microorganisms

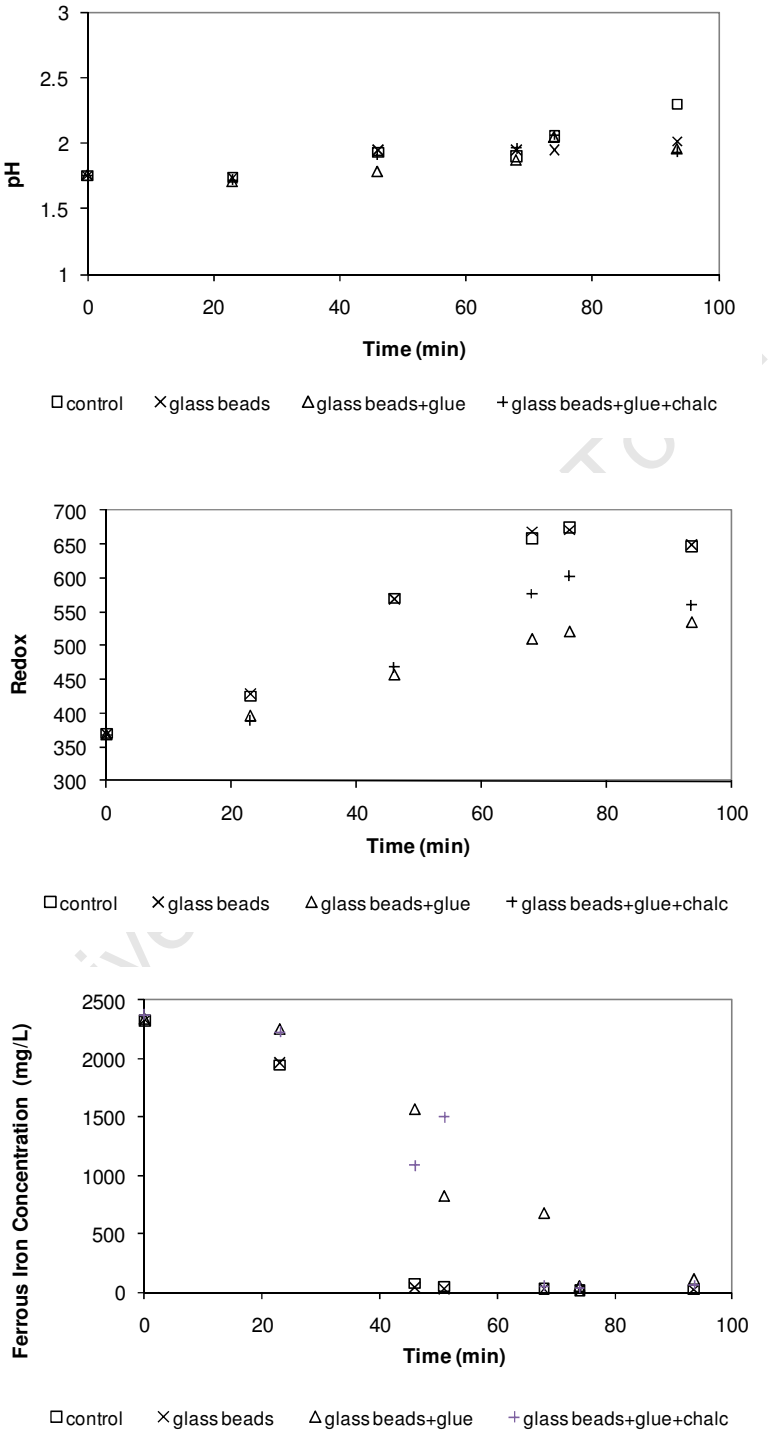



**Figure D.1:** pH, redox potential and ferrous iron concentration assessing the affect of using Bostik glue to coat the beads.

# Appendix E: Shake flask attachment study raw data

The following tables show and example of the raw data generate. The complete data set is available on the CD included.

| Mineral system: Chalcopyrite concentrate | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature: 25°C | | | | | | | | | | | | |
| Culture history: elemental sulphur | | | | | | | | | | | | |
| Time (min) | Planktonic cell count | | Planktonic cell number | | Cell number attached | | Number of cells attached per $m^3$ substrate | | Percentage attached | | Average percentage attached | Std dev (%) |
| | Flask 1 | Flask 2 | Flask 1 | Flask 2 | Flask 1 | Flask 2 | Flask 1 | Flask 2 | Flask 1 | Flask 2 | | |
| 1 | 54 | 50 | 1.69E+09 | 1.56E+09 | 3.13E+08 | 4.38E+08 | 1.77E+09 | 2.48E+09 | 16% | 22% | 19% | 4% |
| 5 | 47 | 48 | 1.47E+09 | 1.50E+09 | 5.31E+08 | 5.00E+08 | 3.01E+09 | 2.83E+09 | 27% | 25% | 26% | 1% |
| 10 | 38 | 40 | 1.19E+09 | 1.25E+09 | 8.13E+08 | 7.50E+08 | 4.60E+09 | 4.25E+09 | 41% | 38% | 39% | 2% |
| 20 | 40 | 41 | 1.25E+09 | 1.28E+09 | 7.50E+08 | 7.19E+08 | 4.25E+09 | 4.07E+09 | 38% | 36% | 37% | 1% |
| 30 | 38 | 37 | 1.19E+09 | 1.16E+09 | 8.13E+08 | 8.44E+08 | 4.60E+09 | 4.78E+09 | 41% | 42% | 41% | 1% |
| 60 | 40 | 37 | 1.25E+09 | 1.16E+09 | 7.50E+08 | 8.44E+08 | 4.25E+09 | 4.78E+09 | 38% | 42% | 40% | 3% |
| 120 | 40 | 39 | 1.25E+09 | 1.22E+09 | 7.50E+08 | 7.81E+08 | 4.25E+09 | 4.42E+09 | 38% | 39% | 38% | 1% |

| Mineral system: Chalcopyrite concentrate | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature: 45°C | | | | | | | | | | | | |
| Culture history: elemental sulphur | | | | | | | | | | | | |
| Time (min) | Planktonic cell count | | Planktonic cell number | | Cell number attached | | Number of cells attached per $m^3$ substrate | | Percentage attached | | Average percentage attached | Std dev (%) |
| | Flask 1 | Flask 2 | Flask 1 | Flask 2 | Flask 1 | Flask 2 | Flask 1 | Flask 2 | Flask 1 | Flask 2 | | |
| 1 | 57 | 58 | 1.78E+09 | 1.81E+09 | 2.19E+08 | 1.88E+08 | 1.24E+09 | 1.06E+09 | 11% | 9% | 10% | 1% |
| 5 | 64 | 60 | 2.00E+09 | 1.88E+09 | 0.00E+00 | 1.25E+08 | 0.00E+00 | 7.08E+08 | 0% | 6% | 3% | 4% |
| 10 | 27 | 25 | 8.44E+08 | 7.81E+08 | 1.16E+09 | 1.22E+09 | 6.55E+09 | 6.90E+09 | 58% | 61% | 59% | 2% |
| 20 | 24 | 25 | 7.50E+08 | 7.81E+08 | 1.25E+09 | 1.22E+09 | 7.08E+09 | 6.90E+09 | 63% | 61% | 62% | 1% |
| 30 | 24 | 24 | 7.50E+08 | 7.50E+08 | 1.25E+09 | 1.25E+09 | 7.08E+09 | 7.08E+09 | 63% | 63% | 63% | 0% |
| 60 | 23 | 22 | 7.19E+08 | 6.88E+08 | 1.28E+09 | 1.31E+09 | 7.26E+09 | 7.43E+09 | 64% | 66% | 65% | 1% |
| 120 | 22 | 20 | 6.88E+08 | 6.25E+08 | 1.31E+09 | 1.38E+09 | 7.43E+09 | 7.79E+09 | 66% | 69% | 67% | 2% |

| | Culture growth history | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sulphur | | | | Ferrous iron | | | | Pyrite | | | | Chalcopyrite | | | |
| Mineral Substrate | K (exp) $min^{-1}$ | K (model) $min^{-1}$ | A | $E_a$ | K (exp) $min^{-1}$ | K (model) $min^{-1}$ | A | $E_a$ | K (exp) $min^{-1}$ | K (model) $min^{-1}$ | A | $E_a$ | K (exp) $min^{-1}$ | K (model) $min^{-1}$ | A | $E_a$ |
| Chalcopyrite | | | 66 | 19402 | | | 7 | 14773 | | | 31 | 18016 | | | 27 | 18836 |
| 25°C | 0.023 | 0.026 | | | 0.015 | 0.017 | | | 0.020 | 0.021 | | | 0.012 | 0.013 | | |
| 45°C | 0.057 | 0.043 | | | 0.033 | 0.025 | | | 0.038 | 0.034 | | | 0.026 | 0.021 | | |
| 65°C | 0.056 | 0.066 | | | 0.029 | 0.034 | | | 0.047 | 0.050 | | | 0.029 | 0.033 | | |
| Pyrite | | | 418 | 24828 | | | 237 | 24826 | | | 5 | 12570 | | | 1579 | 27942 |
| 25°C | 0.017 | 0.019 | | | 0.009 | 0.011 | | | 0.029 | 0.030 | | | 0.017 | 0.020 | | |
| 45°C | 0.044 | 0.043 | | | 0.025 | 0.020 | | | 0.042 | 0.041 | | | 0.057 | 0.041 | | |
| 65°C | 0.054 | 0.066 | | | 0.030 | 0.035 | | | 0.053 | 0.054 | | | 0.063 | 0.06 | | |
| Low grade ore | | | 10113 | 31804 | | | 32 | 18624 | | | 616 | 25774 | | | 18 | 15730 |
| 25°C | 0.026 | 0.027 | | | 0.020 | 0.018 | | | 0.015 | 0.019 | | | 0.031 | 0.031 | | |
| 45°C | 0.066 | 0.060 | | | 0.022 | 0.028 | | | 0.054 | 0.036 | | | 0.044 | 0.046 | | |
| 65°C | 0.117 | 0.12 | | | 0.049 | 0.043 | | | 0.051 | 0.064 | | | 0.067 | 0.065 | | |

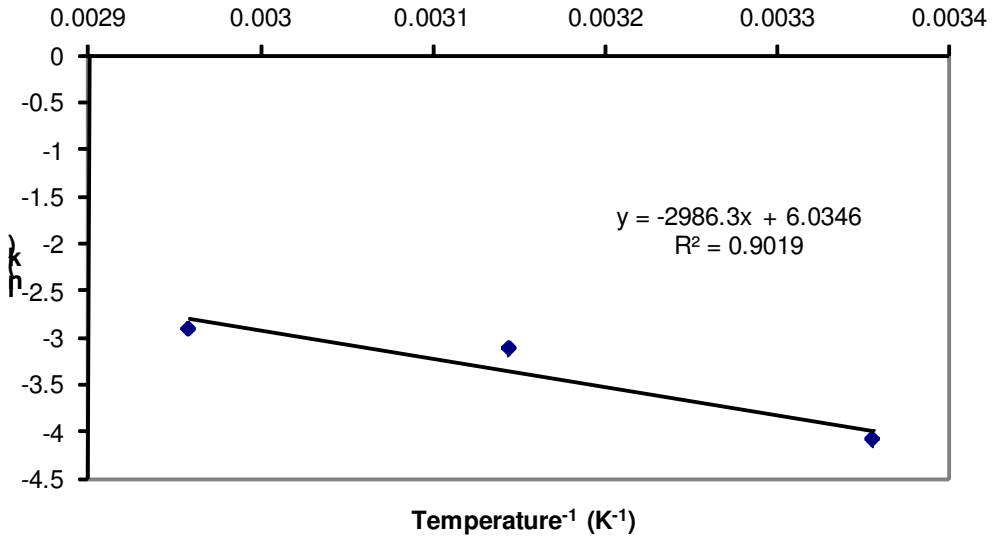



**Figure E.1: Example of the Arrhenius equation fit to rate data from the attachment studies of a sulphur grown *M. hakonensis* culture ($2x10^9$ cells total) to a pyrite concentrate (2 % wt vol-1) at pH 1.6 in an Erlenmeyer shake flask over a 2 hour time period.**

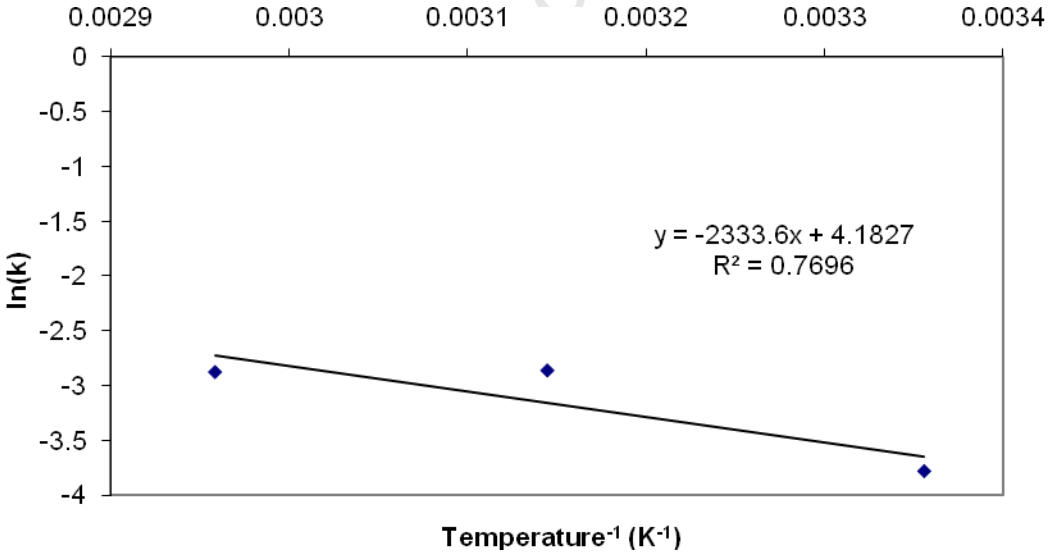



**Figure E.2: Example of the Arrhenius equation fit to rate data from the attachment studies of a sulphur grown *M. hakonensis* culture ($2x10^9$ cells total) to a chalcopyrite concentrate (2 % wt vol-1) at pH 1.6 in an Erlenmeyer shake flask over a 2 hour time period.**

# Appendix F: Column attachment study raw data

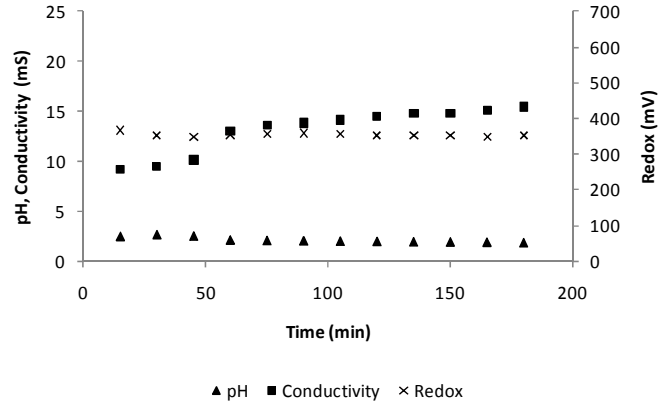
The following tables show and example of the raw data generate. The complete data set is available on the CD included.

| Mineral system: Chalcopyrite concentrate | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature: 25°C | | | | | | | | | | | | | | | | | |
| Culture history: elemental sulphur | | | | | | | | | | | | | | | | | |
| Inoc cell no. | | | Time (min) | Cell count on effluent | | | Cumulative number cells eluted | | | Cumulative number cells retained | | | Percentage cells retained | | | Average (%) | Std dev (%) |
| Col 1 | Col 2 | Col 3 | | Col 1 | Col 2 | Col 3 | Col 1 | Col 2 | Col 3 | Col 1 | Col 2 | Col 3 | Col 1 | Col 2 | Col 3 | | |
| 1.56E+09 | 1.14E+09 | 1.09E+09 | 15 | 0 | 36 | 44 | 0.00E+00 | 1.69E+08 | 2.06E+08 | 1.13E+09 | 9.67E+08 | 8.82E+08 | 100 | 85 | 81 | 89 | 10 |
| | | | 30 | 9 | 66 | 36 | 4.22E+07 | 4.78E+08 | 3.75E+08 | 1.14E+09 | 6.57E+08 | 7.14E+08 | 97 | 58 | 66 | 74 | 21 |
| | | | 45 | 59 | 52 | 37 | 3.19E+08 | 7.22E+08 | 5.48E+08 | 1.22E+09 | 4.14E+08 | 5.40E+08 | 80 | 36 | 50 | 55 | 22 |
| | | | 60 | 77 | 27 | 44 | 6.80E+08 | 8.48E+08 | 7.55E+08 | 1.32E+09 | 2.87E+08 | 3.34E+08 | 56 | 25 | 31 | 37 | 17 |
| | | | 75 | 67 | 11 | 14 | 9.94E+08 | 9.00E+08 | 8.20E+08 | 1.40E+09 | 2.35E+08 | 2.68E+08 | 36 | 21 | 25 | 27 | 8 |
| | | | 90 | 37 | 3 | 4 | 1.17E+09 | 9.14E+08 | 8.39E+08 | 1.45E+09 | 2.21E+08 | 2.49E+08 | 25 | 19 | 23 | 23 | 3 |
| | | | 105 | 14 | 0 | 1 | 1.23E+09 | 9.14E+08 | 8.44E+08 | 1.47E+09 | 2.21E+08 | 2.45E+08 | 21 | 19 | 22 | 21 | 1 |
| | | | 120 | 4 | 0 | 0 | 1.25E+09 | 9.14E+08 | 8.44E+08 | 1.47E+09 | 2.21E+08 | 2.45E+08 | 20 | 19 | 22 | 21 | 2 |
| | | | 135 | 0 | 0 | 0 | 1.25E+09 | 9.14E+08 | 8.44E+08 | 1.47E+09 | 2.21E+08 | 2.45E+08 | 20 | 19 | 22 | 21 | 2 |
| | | | 150 | 0 | 0 | 0 | 1.25E+09 | 9.14E+08 | 8.44E+08 | 1.47E+09 | 2.21E+08 | 2.45E+08 | 20 | 19 | 22 | 21 | 2 |
| | | | 165 | 0 | 0 | 0 | 1.25E+09 | 9.14E+08 | 8.44E+08 | 1.47E+09 | 2.21E+08 | 2.45E+08 | 20 | 19 | 22 | 21 | 2 |
| | | | 180 | 0 | 0 | 0 | 1.25E+09 | 9.14E+08 | 8.44E+08 | 1.47E+09 | 2.21E+08 | 2.45E+08 | 20 | 19 | 22 | 21 | 2 |

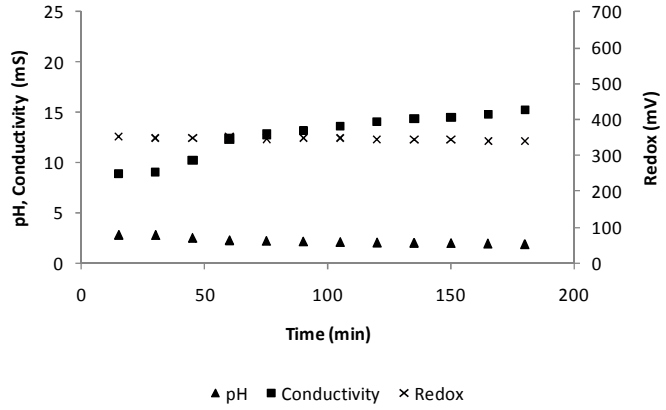
| Mineral system: Chalcopyrite concentrate | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature: 45°C | | | | | | | | | | | | | | | | | |
| Culture history: elemental sulphur | | | | | | | | | | | | | | | | | |
| Inoc cell no. | | | Time (min) | Cell count on effluent | | | Cumulative number cells eluted | | | Cumulative number cells retained | | | Percentage cells retained | | | Average (%) | Std dev (%) |
| Col 1 | Col 2 | Col 3 | | Col 1 | Col 2 | Col 3 | Col 1 | Col 2 | Col 3 | Col 1 | Col 2 | Col 3 | Col 1 | Col 2 | Col 3 | | |
| 1.56E+09 | 1.14E+09 | 1.09E+09 | 15 | 1 | 18 | 25 | 4.69E+06 | 8.44E+07 | 1.17E+08 | 1.56E+09 | 1.05E+09 | 9.71E+08 | 100 | 93 | 89 | 94 | 5 |
| | | | 30 | 9 | 50 | 29 | 4.69E+07 | 3.19E+08 | 2.53E+08 | 1.51E+09 | 8.17E+08 | 8.35E+08 | 97 | 72 | 77 | 82 | 13 |
| | | | 45 | 50 | 49 | 42 | 2.81E+08 | 5.48E+08 | 4.50E+08 | 1.28E+09 | 5.87E+08 | 6.39E+08 | 82 | 52 | 59 | 64 | 16 |
| | | | 60 | 71 | 27 | 46 | 6.14E+08 | 6.75E+08 | 6.66E+08 | 9.46E+08 | 4.60E+08 | 4.23E+08 | 61 | 41 | 39 | 47 | 12 |
| | | | 75 | 44 | 7 | 8 | 8.20E+08 | 7.08E+08 | 7.03E+08 | 7.40E+08 | 4.28E+08 | 3.85E+08 | 47 | 38 | 35 | 40 | 6 |
| | | | 90 | 35 | 1 | 2 | 9.84E+08 | 7.13E+08 | 7.13E+08 | 5.76E+08 | 4.23E+08 | 3.76E+08 | 37 | 37 | 35 | 36 | 1 |
| | | | 105 | 2 | 0 | 0 | 9.94E+08 | 7.13E+08 | 7.13E+08 | 5.66E+08 | 4.23E+08 | 3.76E+08 | 36 | 37 | 35 | 36 | 1 |
| | | | 120 | 0 | 0 | 0 | 9.94E+08 | 7.13E+08 | 7.13E+08 | 5.66E+08 | 4.23E+08 | 3.76E+08 | 36 | 37 | 35 | 36 | 1 |
| | | | 135 | 0 | 0 | 0 | 9.94E+08 | 7.13E+08 | 7.13E+08 | 5.66E+08 | 4.23E+08 | 3.76E+08 | 36 | 37 | 35 | 36 | 1 |
| | | | 150 | 0 | 0 | 0 | 9.94E+08 | 7.13E+08 | 7.13E+08 | 5.66E+08 | 4.23E+08 | 3.76E+08 | 36 | 37 | 35 | 36 | 1 |
| | | | 165 | 0 | 0 | 0 | 9.94E+08 | 7.13E+08 | 7.13E+08 | 5.66E+08 | 4.23E+08 | 3.76E+08 | 36 | 37 | 35 | 36 | 1 |
| | | | 180 | 0 | 0 | 0 | 9.94E+08 | 7.13E+08 | 7.13E+08 | 5.66E+08 | 4.23E+08 | 3.76E+08 | 36 | 37 | 35 | 36 | 1 |

Example of graphs illustrating pH, conductivity and redox potential of collected fractions

a)




b)




c)

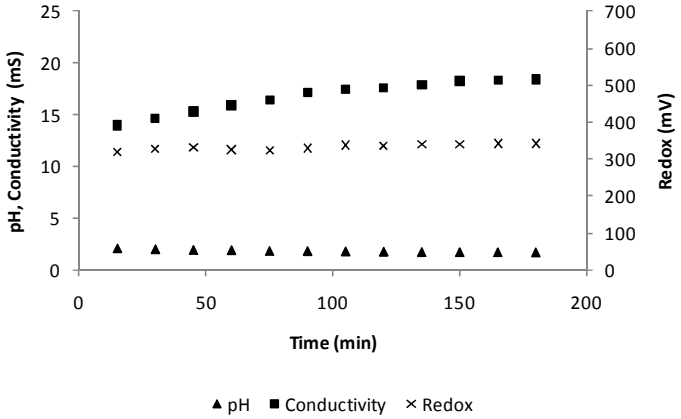



**Figure F.1:** Solution chemistry of cells cultured on elemental sulphur attaching to a chalcopyrite concentrate at a) 25°C, b) 45°C and c) 65°C

Example of temperature profiles through the columns

**Table F.1:** Temperature profile along column with heating tape set to 65°C

| Length from bottom (cm) | Initial temperature (°C) | Temperature at equilibrium (°C) |
|---|---|---|
| 0 | 32.7 | 28.7 |
| 2 | 42.7 | 39.5 |
| 4 | 62.0 | 50.5 |
| 7 | 66.6 | 67.0 |
| 10 | 72.7 | 71.4 |
| 15 | 69.8 | 67.7 |

**Table F.2:** Calculation of average mass of mineral used to coat the beads in the column experiments

| | Mass of 20 beads coated in glue (g) | Mass of 20 beads coated in chalcopyrite concentrate (g) | Mass of 20 beads coated in low grade ore (g) |
|---|---|---|---|
| | 5.8115 | 6.0539 | 6.0497 |
| | 5.7877 | 6.1382 | 6.0522 |
| | 5.8284 | 6.0063 | 6.0669 |
| | 5.8124 | 6.0557 | 6.0470 |
| | 5.7948 | 5.9882 | 5.9990 |
| Average | 5.8070 | 6.0485 | 6.0430 |
| Standard deviation | 0.0160 | 0.0582 | 0.0258 |

# Appendix G: Metabolic activity tests raw data

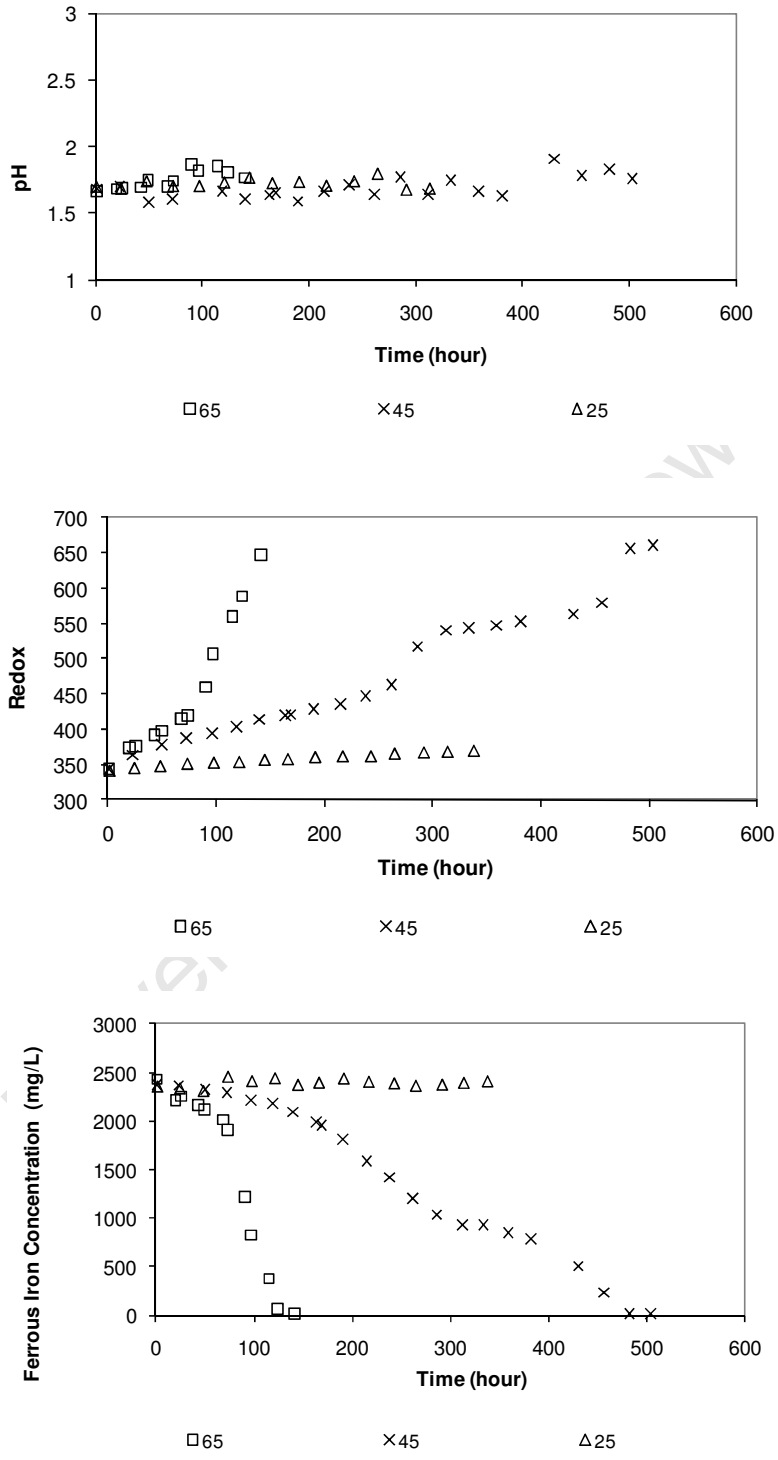



**Figure G.1:** Ferrous iron activity tests with cells cultured on elemental sulphur ($3.33x10^5$ cells/ml initial cell concentration). All experiments run in duplicate.

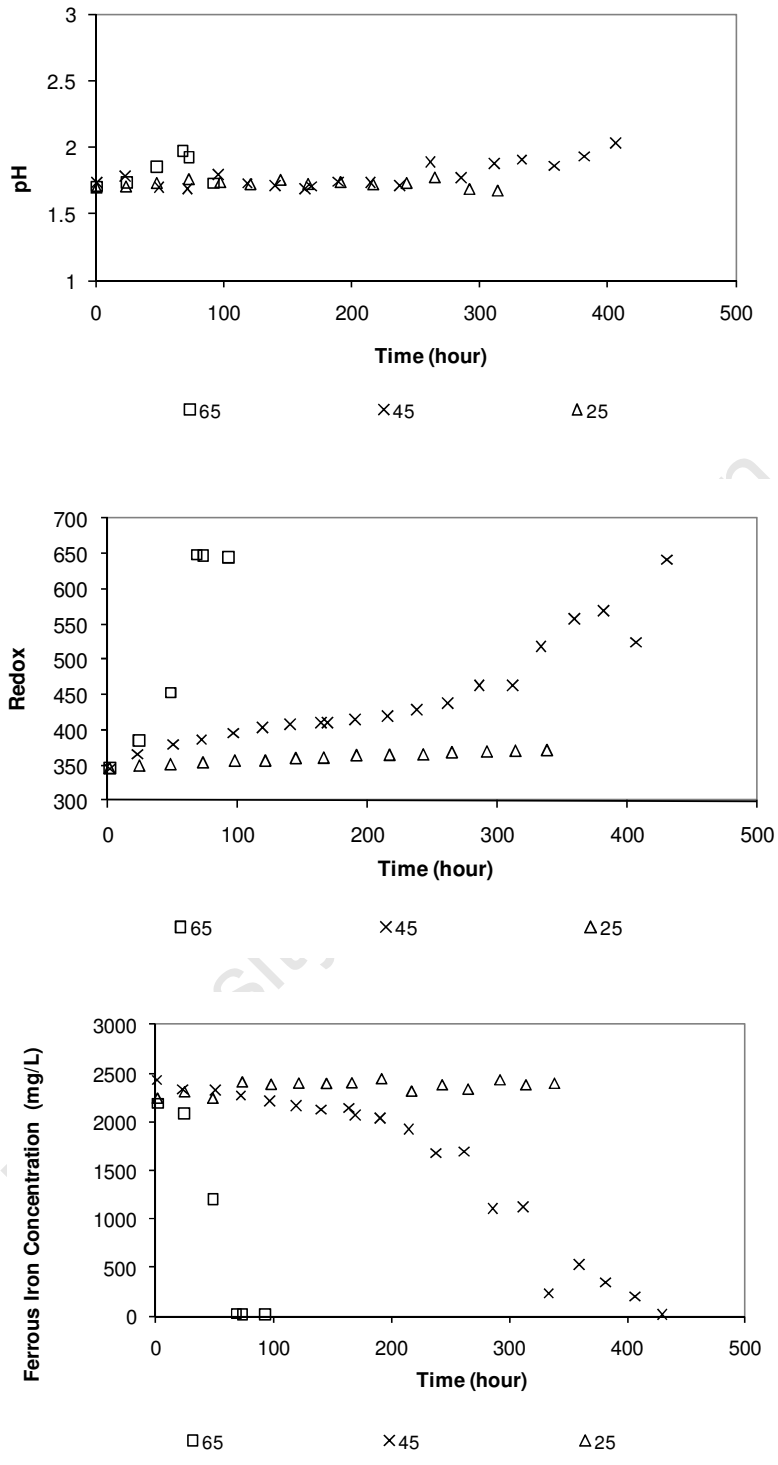



**Figure G.2:** Ferrous iron activity tests with cells cultured on ferrous iron iron ($3.33x10^5$ cells/ml initial cell concentration). All experiments run in duplicate.

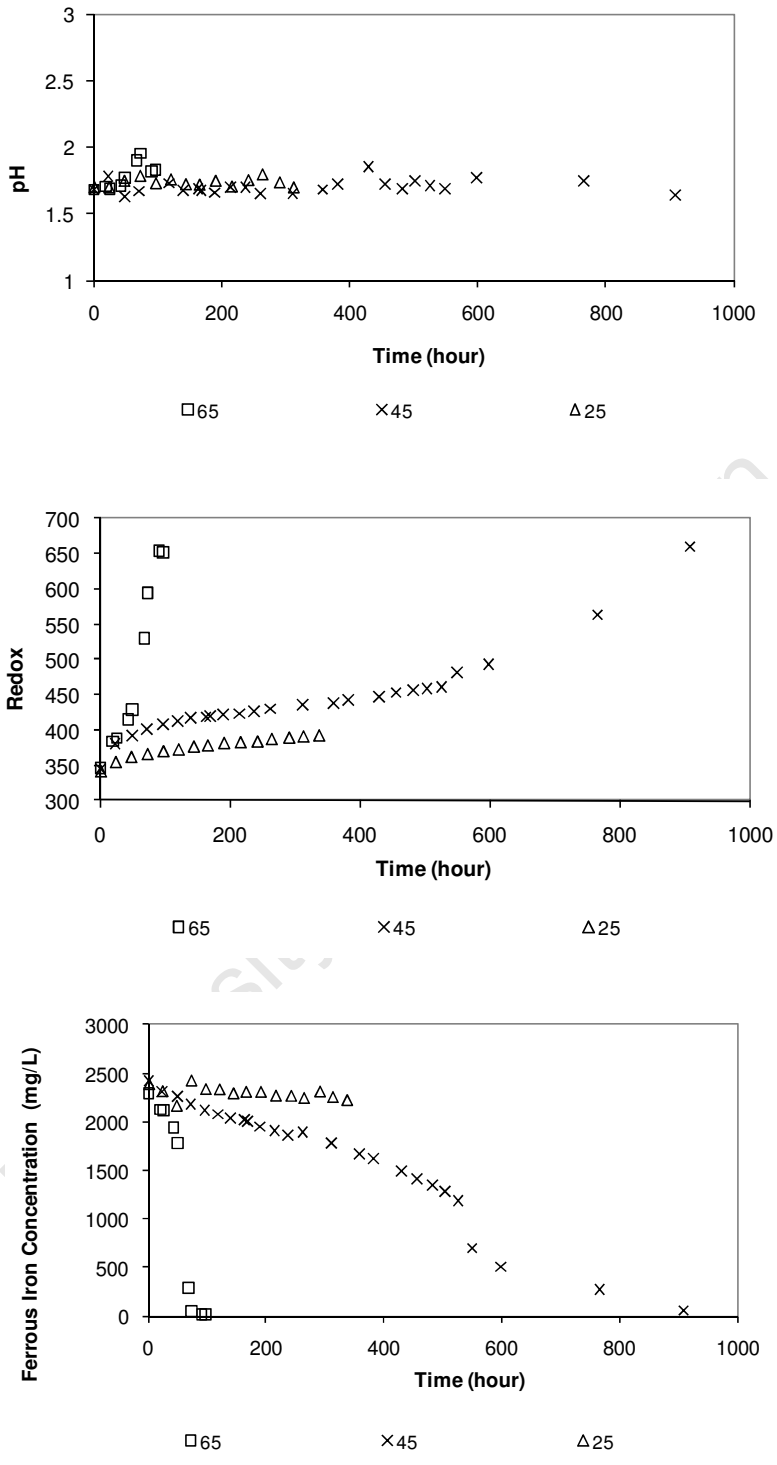



**Figure G.3:** Ferrous iron activity tests with cells adapted to chalcopyrite concentrate ($3.33x10^5$ cells/ml initial cell concentration). All experiments run in duplicate.

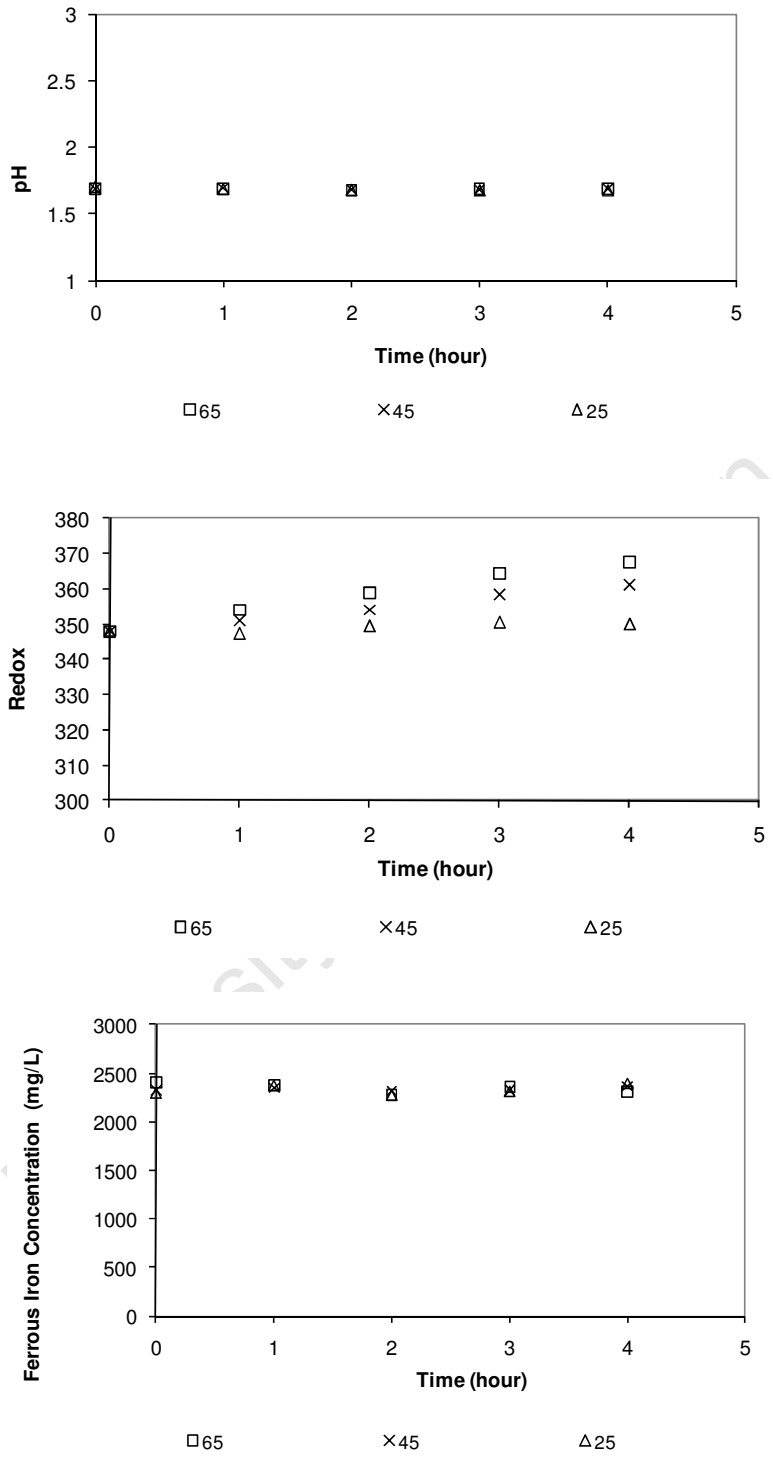



**Figure G.4:** Ferrous iron activity tests with cells cultured on elemental sulphur ($2x10^7$ cells/ml initial cell concentration). All experiments run in duplicate.

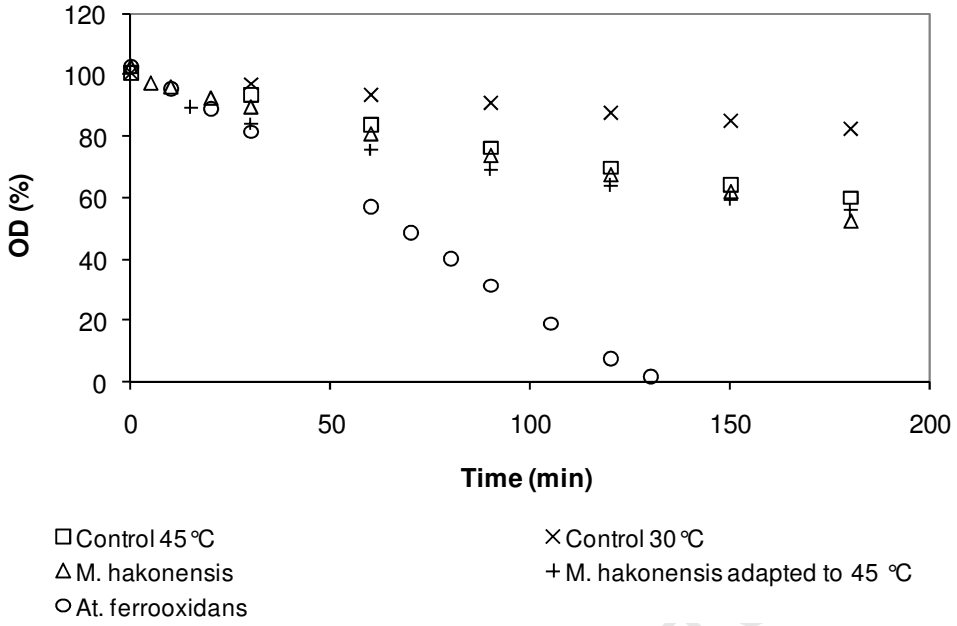



**Figure G.5:** Oxygen utilisation tests with *M. hakonensis* cells cultured on elemental sulphur at 45 and *At. ferrooxidans* cells cultured in standard medium ($5x10^9$ cells in 250 ml).

# Appendix H: Cell surface property tests raw data

**Table H.1:** First hydrophobicity test using two-phase partitioning into hexadecane with an initial $OD_{400}$ between 0.3 and 0.4.

| Growth history | $OD_{400, initial}$ | $OD_{400, final}$ a | $OD_{400, final}$ b | Index a | Index b | Average partitioning into hexadecane |
|---|---|---|---|---|---|---|
| Elemental sulphur | 0.405 | 0.397 | 0.393 | 1.98 | 2.96 | 2.47 |
| Ferrous iron | 0.354 | 0.324 | - | 8.47 | - | 8.47 |
| Chalcopyrite | 0.313 | 0.279 | 0.262 | 10.86 | 16.29 | 13.58 |

**Table H.2:** Second hydrophobicity test using two-phase partitioning into hexadecane with an initial $OD_{400}$ between 0.3 and 0.4.

| Growth history | $OD_{400, initial}$ | $OD_{400, final}$ a | $OD_{400, final}$ b | Index a | Index b | Average partitioning into hexadecane |
|---|---|---|---|---|---|---|
| Ferrous iron | 0.297 | 0.289 | - | 2.69 | - | 2.69 |

**Table H.3:** Third hydrophobicity test using two-phase partitioning into hexadecane with both cell counts and $OD_{400}$ measurements used to determine cell concentration.

| Growth history | $OD_{400, initial}$ | $OD_{400, final}$ | Partitioning into hexadecane | Initial cell count | Final cell count | Partitioning into hexadecane |
|---|---|---|---|---|---|---|
| Elemental sulphur | 0.21 | 0.196 | 6.67 | 480 | 230 | 52.08 |
| | 0.21 | 0.199 | 5.24 | 480 | 440 | 8.33 |
| Ferrous iron | 0.384 | 0.359 | 6.51 | 650 | 580 | 10.77 |
| Chalcopyrite | 0.353 | 0.297 | 15.86 | 700 | 690 | 1.43 |

**Table H.4:** Fourth hydrophobicity test using two-phase partitioning into hexadecane with both cell counts and $OD_{400}$ measurements used to determine cell concentration. A lower cell concentration was used to improve the accuracy of cell counts.

| Growth history | $OD_{400, initial}$ | $OD_{400, final}$ | Partitioning into hexadecane | Initial cell count | Final cell count | Partitioning into hexadecane |
|---|---|---|---|---|---|---|
| Elemental sulphur | 0.129 | 0.104 | 19.38 | 190 | 162 | 14.74 |
| | 0.129 | 0.115 | 10.85 | 190 | 168 | 11.58 |
| Ferrous iron | 0.111 | 0.087 | 21.62 | 158 | 152 | 3.80 |
| Chalcopyrite | 0.134 | 0.091 | 32.09 | 261 | 213 | 18.39 |
| | 0.134 | 0.099 | 26.12 | 261 | 213 | 18.39 |

**Table H.5:** Zeta potential of cells in their respective growth medium.

| | Elemental sulphur | | Ferrous iron | | Chalcopyrite concentrate | |
|---|---|---|---|---|---|---|
| Replications | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) |
| 1 | -1.83 | 11.5 | -0.792 | 33.2 | -1.62 | 16.9 |
| 2 | -1.98 | 11.5 | -0.72 | 33.8 | -1.78 | 18.2 |
| 3 | -1.87 | 11.5 | -0.682 | 33.9 | -2.73 | 18.4 |
| 4 | -1.95 | 11.5 | -0.564 | 33.8 | -2.38 | 18.5 |
| 5 | -1.94 | 11.4 | -0.885 | 33.6 | -2.34 | 18.6 |
| 6 | | | | | -1.55 | 18.3 |
| **Average** | **-1.914** | **11.48** | **-0.729** | **33.66** | **-2.067** | **18.15** |

**Table H.6:** Zeta potential of cells in 0K medium at pH 1.6.

| | Elemental sulphur | | Ferrous iron | | Chalcopyrite concentrate | |
|---|---|---|---|---|---|---|
| Replications | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) |
| 1 | -1.85 | 21.1 | -2.01 | 20.6 | -1.58 | 20.7 |
| 2 | -2.36 | 21.1 | -1.84 | 21.0 | -1.86 | 21.2 |
| 3 | -2.12 | 22.7 | -2.07 | 21.6 | -2.44 | 21.9 |
| 4 | -1.97 | 22.7 | -2.29 | 22.9 | -2.17 | 21.8 |
| 5 | -2.16 | 22.4 | -2.15 | 22.7 | -2.15 | 21.6 |
| 6 | | | | | -2.28 | 21.3 |
| **Average** | **-2.092** | **22.0** | **-2.072** | **21.8** | **-2.080** | **21.4** |

**Table H.7:** Zeta potential of cells in 0K medium at pH 2.

| | Elemental sulphur | | Ferrous iron | | Chalcopyrite concentrate | |
|---|---|---|---|---|---|---|
| Replications | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) |
| 1 | -2.12 | 11.7 | -1.03 | 13.1 | -2.84 | 12.6 |
| 2 | -1.81 | 12.5 | -0.989 | 13.8 | -2.84 | 12.9 |
| 3 | -1.95 | 12.5 | -1.45 | 13.8 | -2.98 | 12.9 |
| 4 | -2.22 | 12.5 | -0.728 | 13.7 | -2.80 | 12.4 |
| 5 | -2.34 | 12.5 | -1.19 | 13.6 | -2.73 | 12.5 |
| 6 | -2.25 | 12.2 | | | -2.36 | 12.4 |
| **Average** | **-2.10** | **12.3** | **-1.077** | **13.6** | **-2.758** | **12.6** |

**Table H.8:** Zeta potential of cells in 0K medium at pH 3.

| | Elemental sulphur | | Ferrous iron | | Chalcopyrite concentrate | |
|---|---|---|---|---|---|---|
| Replications | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) |
| 1 | -2.38 | 8.71 | -4.99 | 8.67 | -7.68 | 8.47 |
| 2 | -2.37 | 9.38 | -4.67 | 9.01 | -8.75 | 8.64 |
| 3 | -2.54 | 9.41 | -4.98 | 9.12 | -8.8 | 8.82 |
| 4 | -2.46 | 9.43 | -4.90 | 9.18 | -8.96 | 8.9 |
| 5 | -2.43 | 9.2 | -4.59 | 9.23 | -9.02 | 9.06 |
| **Average** | **-2.436** | **9.2** | **-4.826** | **9.0** | **-8.642** | **8.8** |

**Table H.9:** Zeta potential of cells in 0K medium at pH 4.

| | Elemental sulphur | | Ferrous iron | | Chalcopyrite concentrate | |
|---|---|---|---|---|---|---|
| Replications | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) | Zeta (mV) | Conductivity (mS/cm) |
| 1 | -3.19 | 8.48 | -11.6 | 8.19 | -8.64 | 8.09 |
| 2 | -3.08 | 8.71 | -10.1 | 8.49 | -10.5 | 8.33 |
| 3 | -3.07 | 9.05 | -13.3 | 8.62 | -9.42 | 8.51 |
| 4 | -3.70 | 9.12 | -11.1 | 8.71 | -10.9 | 8.71 |
| 5 | -2.68 | 8.97 | -12.6 | 8.79 | -8.15 | 8.78 |
| **Average** | **-3.144** | **8.9** | **-11.740** | **8.6** | **-9.522** | **8.5** |