Quantification of Biomass in a Biooxidation System

By

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SYNOPSIS

The aim of this study was to investigate and compare various methods to enumerate the number of bacteria in a minerals biooxidation system. In this system most of the bacteria are attached to fine particles of ore and therefore cannot be enumerated by direct cell counting. This has hindered attempts to understand the mechanism by which the bacteria assist in the leaching process.

The methods reported in the literature to enumerate both the free and attached bacteria in a biooxidation system can be divided into 2 categories: direct methods and indirect methods. The direct methods involve the quantification of the bacteria by direct observation. It is difficult to enumerate attached bacteria by direct observation but attempts have been made to desorb or dislodge these bacteria. Such experiments have had limited success in achieving dislodgement of all the attached bacteria. However, the results have shown that desorption of the bacteria from the mineral surface is possible. Indirect methods involve the monitoring of a cell component such as protein, nitrogen and carbon. Biomass concentrations have been estimated using its metabolic activity by means of a maximum specific oxygen utilisation rate.

The purpose of this study was to compare the various methods and test their suitability to the quantification of biomass in a biooxidation system. In particular the biooxidation system investigated treated an arsenopyrite-pyrite concentrate from Fairview Gold Mine, Barberton, South Africa. The elemental analysis of the concentrate is 5.84% arsenic, 21.71% sulphur, 24.01% iron and 1.41% carbon. The dominant bacteria present in the biooxidation system were *Leptospirillum ferrooxidans* and *Thiobacillus thiooxidans* as shown by 16S rDNA analysis.

The methods investigated are microscopic counting, gravimetric dry weight determination, desorption, determination of chemical oxygen demand, ashing, protein analysis, nitrogen analysis, total organic carbon analysis and measurement of oxygen utilisation rate. The oxygen utilisation rate method differs from the other methods as it uses the metabolic activity of the bacteria to measure the bacterial concentrations.

The presence of attached and free bacterial populations was confirmed by scanning electron microscopy and light microscopy respectively.

The desorption data was fitted to a Langmuir isotherm to calculate the maximum adsorption capacity of the mineral ore. A maximum adsorption capacity of between 6×10^{10} and 7.8×10^{11} cells/g ore was obtained.

The chemical oxygen demand of a washed cell suspension was linearly related to the dry weight of the sample. Thus, chemical oxygen demand was suitable for enumerating the free bacteria in a biooxidation system.

Ashing of the leached arsenopyrite-pyrite concentrate was unsatisfactory for enumerating the number of attached bacteria as the contribution of the ore to the change in mass on ashing could not be determined.

The Lowry protein assay was suitable for determining the cell concentration of a washed cell suspension and thus could be used to enumerate the free bacteria. However, the Lowry protein assay was unsuitable for the estimating the attached bacterial population. This was a result of interference experienced by the inorganic ions released from the mineral ore during digestion of the ore. On the other hand, the Coomassie Blue protein assay showed potential use for the enumeration of both the free and attached bacterial concentrations.

The nitrogen analysis could be used to enumerate the attached bacterial concentrations provided that the inorganic nitrogen present in the leached ore or the interference from the arsenopyrite-pyrite concentrate could be measured. Assuming that the difference in the nitrogen content of the leached and unleached ore was due to bacterial nitrogen, an attached bacterial concentration of 8.3×10^{9} cells/g ore was obtained.

The total organic carbon analysis was suitable for determining the cell concentration of the free bacteria. However, carbonate and inorganic ions released from the mineral ore during digestion interfered with the analysis. Thus, the total organic carbon analysis was unsuitable for enumerating the attached bacterial concentration with this ore.

The oxygen utilisation rate of a sample is a function of the cell concentration of the sample and the specific oxygen utilisation rate of the bacteria. The oxygen utilisation rate of a washed suspension was dependent on the ferrous iron concentration below a critical value. At non-limiting ferrous iron concentrations above 10 g/ℓ, the oxygen utilisation rate was constant. This enabled the calculation of the maximum specific oxygen utilisation rate. A maximum specific oxygen utilisation rate at 12 g Fe^2/ℓ of 0.57 mol O_2/mol C/hr was obtained.

It was hypothesised that the maximum specific oxygen utilisation rate of the attached bacteria was the same as that of the free bacteria. This enabled the calculation of the number of attached bacteria from measured oxygen utilisation rates of an ore sample at 12 g Fe^{2+}/ℓ. An attached bacterial concentration of $5.7\times10^{10} \pm 1.6\times10^{10}$ cells/g ore for a 15 day residence time and of $2.8\times10^{10} \pm 0.8\times10^{10}$ cells/g ore for a 6 day residence time were determined for the bacteria-ore suspension obtained from the primary tank of the continuous miniplant. Although the hypothesis requires further validation, close agreement with the maximum adsorption capacities reported in literature suggested that this method could be used to enumerate the number of attached bacteria.

The percentage of attached bacteria of the total bacterial concentration was calculated as 88% for a 6 day residence time from the oxygen utilisation rate measurements. This implies that the majority of the active bacteria in the biooxidation system are attached to the mineral ore.

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NOMENCLATURE

AAS	Atomic absorption spectroscopy
BCA	Bicinchoninic acid
BOM	Biological oxygen monitor
BSA	Bovine serum albumin
COD	Chemical oxygen demand
CTC	Copper-tartrate-carbonate
DAPI	4'-6'-Dimindino-2-phenylindole
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetate
FA	Fluorescent antibody staining
FAS	Ferrous ammonium sulphate
MPN	Most probable number
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TOC	Total organic carbon
UCT	University of Cape Town
VVS	Volatile suspended solids

a	Surface area occupied per bacterium (m^2/cell)
A_t	Total surface area
c_x	Bacterial concentration (cells/ml or cells/g ore)
$c_x(o)$	Bacterial concentration at time t=0 (cells/ml or cells/g ore)
k_1, k_{-1}	Rate constants in the Langmuir isotherm
K_A	k_1/k_{-1}
K_i	Inhibition constant of ferric iron
K_S	Saturation constant for ferrous iron
q_{O2}	Specific oxygen utilisation rate (mol O_2/mol C/hr)
q_{O2}^{max}	Maximum specific oxygen utilisation rate (mol O_2/mol C/hr)
$-r_{CO2}$	Carbon dioxide consumption rate (mol CO_2/ℓ/s)

$-r_{O2}$	Oxygen consumption rate (mol $O_2/\ell/s$)
r_x	Rate of bacterial growth (mol $C/\ell/s$)
X_A	Concentration of attached bacteria (cells/m^2 or cell/g)
X_{AM}	Maximum adsorption capacity (cells/g ore)
X_L	Concentration of bacteria in solution (cells/ml)
θ	Fraction of the solid surface covered by bacteria

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The recovery of gold from refractory ore is difficult as the leaching agent (usually cyanide) cannot reach the gold particles encapsulated within the pyritic matrix (Paponetti *et al.*, 1991). To increase the gold recovery from the cyanidation process, the refractory ores must first be pretreated. There are three options of pretreatment: roasting, pressure oxidation or biooxidation. Roasting involves high temperatures to convert the sulphides to oxides. Pressure oxidation involves treating the ore in a pressurized vessel containing a large partial pressure of oxygen, while biooxidation involves the solubilization of mineral sulphides in the presence of bacteria to release the ions (Fe^{2+}, As^{3+}). By releasing these, the gold in the mineral becomes accessible to the cyanide and thereby recovery is increased. The advantages of using biooxidation over roasting or pressure oxidation include lower capital and operating costs. The waste products from biooxidation are also easier to dispose than those from the alternative pretreatments. Roasting can lead to toxic elements such as arsenic, lead and sulphur oxide being present in the off-gas, whereas in biooxidation the arsenic can be recovered as a precipitant and handled appropriately. The major disadvantage of biooxidation is the high residence time required to achieve sufficient oxidation. This results in large leaching vessels. Other disadvantages include the low solids content of the slurry, the need to supply oxygen and nutrients to the bacteria (Paponetti *et al.*, 1991) and the susceptibility of the bacteria to process perturbation (Free, 1992).

The bacteria, in the biooxidation system, may be attached or in suspension. The state of knowledge does not indicate conclusively whether the free bacteria, attached bacteria or both are involved in the solubilization of the mineral sulphide. The understanding of the role of bacteria and the effect of operating conditions and perturbations on the process in limited by the inability to enumerate the attached bacterial population.

The bacteria that are free in solution can be enumerated by conventional methods which include direct cell counts, protein and nitrogen analysis (McGoran *et al.*, 1969; Le Roux *et al.*, 1973 and DiSpirito *et al.*, 1983). However, difficulty is expected with these methods when quantifying the bacteria which are directly associated with the mineral surface. The major reasons for this are the difficulty in observing the attached bacteria directly, the low cell densities found in the bioleaching systems and the interference caused by the mineral ore

during analyses (Yeh *et al.*, 1987).

The knowledge gained from the enumeration of the free and attached bacteria would assist in the understanding of the role of the bacteria in the bioleaching process. It would allow the determination of the location of most of the bacteria. It would also assist in the study of the effect of hydrodynamic forces on both the location of the bacteria and the metabolism of the bacteria.

The objectives of this study are:

- To review the different methods reported in the literature for the enumeration of the bacteria in a biooxidation system
- To investigate these and other possible methods for enumerating the free bacterial population and the attached bacterial population associated with a complex sulphide ore, thereby quantifying the entire bacterial population in a biooxidation system
- To provide experimental comparison of the various enumeration methods
- To calculate the percentage of attached bacteria in the biooxidation system
- To draw conclusions with respect to the different methods that can be used to determine the number of bacteria in a biooxidation system

The literature review presented in Chapter 2 contains an overview of a biooxidation system with particular reference to the type of bacteria found in the system. A brief discussion of the mechanism of leaching and the mechanism of attachment is given with the stoichiometry of pyrite and arsenopyrite leaching. The methods used to enumerate the number of bacteria reported in the literature are also discussed. These methods are then drawn together for a particular mineral ore and compared. Finally, the maximum coverage of the mineral surface is presented.

Chapter 3 sets out the experimental procedures and assays used in the enumeration of the number of bacteria in a minerals biooxidation system.

Chapter 4 presents the results and discussion of the investigation into the different methods used for the enumeration. The results of both the free and attached bacterial populations are presented for each method investigated. The results are discussed as to the applicability of the method in enumerating the bacteria in a minerals biooxidation system. The characterisation of the cell in terms of the cell mass and elemental composition is also presented. A calculation of the maximum possible number of attached bacteria is then given. Finally, the relative size of the attached bacterial population obtained by the various methods is compared with each other and with that reported in literature.

Chapter 5 contains a summary of conclusions drawn from this thesis.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. THE BIOOXIDATION PROCESS

Biooxidation involves the solubilisation of the mineral sulphides to release the metal ions into solution. The bacteria present in the biooxidation system assist in the solubilisation of the mineral ore and in many cases the rate of oxidation of the mineral ore has been enhanced by the presence of the bacteria (Corrans *et al.*, 1972).

2.2. THE BACTERIA

The dominant bacteria involved in the biooxidation process are *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. These bacteria are gram-negative, mesophilic, acidophilic chemolithoautotrophs. They grow at moderate temperatures in an acidic environment. Their carbon source is obtained by fixing atmospheric carbon dioxide or carbonate while their energy is derived from the oxidation of inorganic ions. These bacteria are strictly aerobic as oxygen acts as the electron acceptor in the oxidation reactions.

Thiobacillus ferrooxidans are rod-shaped organisms (0.5×1.0 μm) which usually occur in single units or in pairs. They are motile by a single polar flagellum. They oxidize and grow on ferrous iron (Fe^{2+}), pyrite (FeS_2), numerous sulphide minerals, sulphur (S^0), thiosulphate ($S_2O_3^{2-}$) or tetrathionate ($S_4O_6^{2-}$). Their optimum temperature for growth is between 30 and 35°C and the optimum pH is approximately 2.5 (Staley *et al.*, 1989).

Thiobacillus thiooxidans are rod-shaped bacteria (0.5×1.0-2.0 μm) which appears either singularly, in pairs or as short chains. They are motile by means of a polar flagellum. They grow on elemental sulphur, thiosulphate or tetrathionate. *Thiobacillus thiooxidans* cannot oxidize ferrous iron or pyrite. Their optimum temperature for growth is between 28 and 30°C while the optimum pH is between 2.0 and 3.0 (Staley *et al.*, 1989).

Leptospirillum ferrooxidans are vibroid-shaped cells usually forming chains of 3-12 cells. The cell size varies between 0.9 and 1.1 μm in length and between 0.2 and 0.4 μm in diameter

(Rossi, 1990). They grow on iron but not on sulphur (Staley *et al.*, 1989). *Leptospirillum ferrooxidans* are able to grow at low pH values (Tuovinen *et al.*, 1991).

The relative amount of each type of bacteria found in the leaching system depends on the environmental conditions, namely the pH, temperature and ferric to ferrous ratio. At low pH values (pH 1.5) *L. ferrooxidans*, grown on ferrous iron, has been shown to dominate over *T. ferrooxidans* while at pH 2.3 the reverse was found to be true (Helle and Onken, 1988). They found the oxidation activity of *T. ferrooxidans* is more sensitive to extremely acidic conditions than the activity of *Leptospirillum*-like bacteria, in that *T. ferrooxidans* experienced a decrease in respiratory activity to 15% when the pH was decreased from 2.0 to 1.0. The respiratory activity of *Leptospirillum*-like bacteria was, however, found to be at least 64% at pH 1.0 compared to that at pH 2.0.

2.3. MECHANISM OF LEACHING

There are two proposed mechanisms by which the bacteria assist in the dissolution of the mineral sulphides (Figure 2.1). Both mechanisms occur only under aerobic conditions where oxygen acts as the electron acceptor. In the first mechanism, often referred to as the direct mechanism, it is assumed that the bacteria directly attack the mineral surface, *i.e.* the bacteria act as the primary oxidant. This mechanism requires direct contact between the bacteria and the mineral surface (Silverman, 1967). The direct mechanism is not clearly understood but is thought to be an enzymatic conversion. The products of this mechanism are ferric sulphate and sulphuric acid (Le Roux *et al.*, 1973).

The second mechanism, often referred to as the indirect or two-stage mechanism, involves the oxidation of the mineral surface by ferric iron (Fe^{3+}). Ferrous iron is released from this reaction and the role of the bacteria is to reoxidize the ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). The bacteria thereby regenerate the ferric iron (a strong oxidant) which then acts as the primary oxidant (Silverman, 1967).

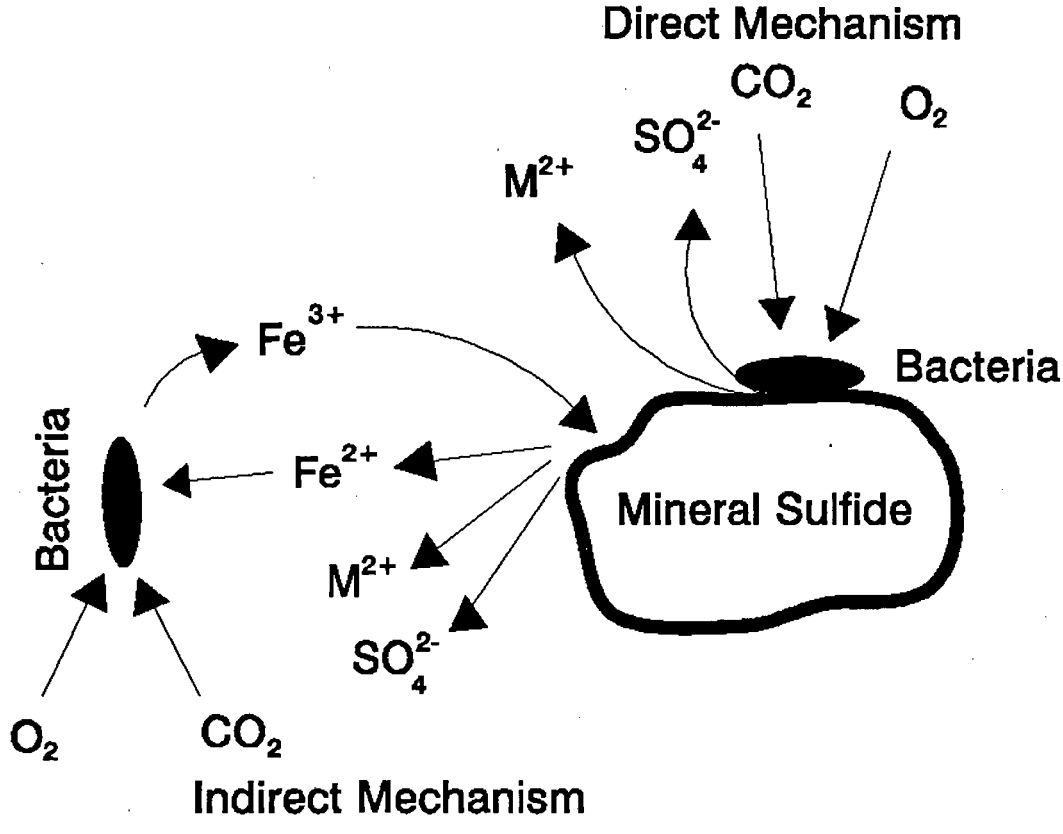


Figure 2.1. The mechanisms by which the bacteria assist in the leaching process.

The mechanism, which is occurring or the predominant mechanism, has been a subject of debate for many years. An understanding of the degree of attachment and relative activity of attached and free populations will aid in determining this mechanism.

2.4. STOICHIOMETRY OF THE SYSTEM

For the oxidation of ferrous iron, the following chemical reaction occurs (Le Roux *et al.*, 1973):

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

For the oxidation of elemental sulphur (Le Roux *et al.*, 1973):

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$

For the oxidation of pyrite by the indirect mechanism (Le Roux *et al.*, 1973):

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

$$4Fe^{2+} + O_2 + 4H^+ \xrightarrow{\text{bacteria}} 4Fe^{3+} + 2H_2O$$

For the oxidation of pyrite by the direct mechanism the overall reaction is (Boon and Heijnen, 1993):

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

The exact stoichiometry for arsenopyrite oxidation is unclear and there have been many postulations. A possible stoichiometry is given by Monroy-Fernandez (1995b):

$$4FeAsS + 3O_2 + 2H_2O \rightarrow 4Fe^{2+} + 4HAsO_2 + 4S^0_{surface}$$

2.5. ATTACHMENT

2.5.1. Mechanism of Attachment

The bacteria in the biooxidation system are located in two areas: attached to the mineral surface (as shown by scanning electron microscopy) and free in solution (as shown by light microscopy). The attachment of bacteria to mineral surfaces as well as the selectivity of the attached bacteria for sulphidic regions has been observed with the scanning electron microscope (SEM) (Bagdigian and Myerson, 1986; Murthy and Natarajan, 1992). In addition selectivity based on surface topography is also evident. The bacteria have an affinity for dislocations, grain boundaries and other nonuniformities in the pyrite crystal surface (Bagdigian and Myerson, 1986). This seems to indicate that the bacteria have some chemotactic sense which allows them to locate a favourable site for attachment (Rodriguez-Leiva and Tributsch, 1988).

The mechanism of bacterial attachment is not fully understood. Rapid initial adsorption rates have been reported (DiSpirito *et al.*, 1983 and Myerson and Kline, 1983). Myerson and Kline (1983) found that up to 90% of the bacteria were adsorbed within the first 5 minutes. Bagdigian and Myerson (1986) found that up to 100% of the total bacterial population adsorbed within 2 minutes of inoculation provided sufficient coal was present. This behaviour is shown in Figure 2.2, for the attachment of *Thiobacillus ferrooxidans* to coal, where the bacterial concentration in solution decreases with time.

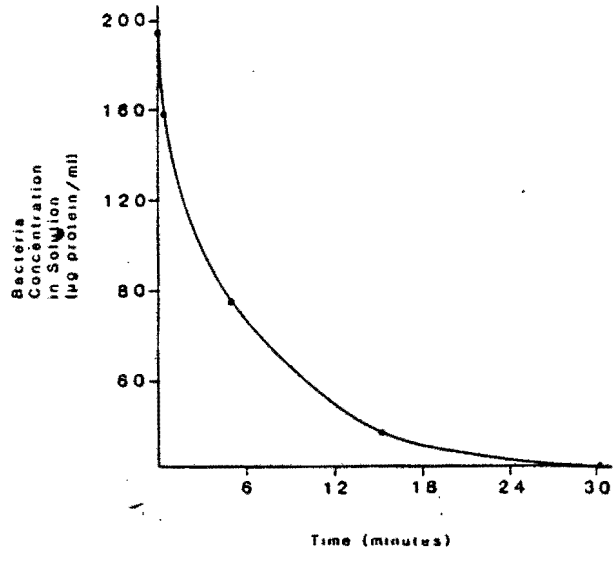


Figure 2.2. The adsorption of *Thiobacillus ferrooxidans* onto coal (Myerson and Kline, 1983).

The rapid initial adsorption rates are explained by DiSpirito *et al.* (1983) in terms of electrostatic interactions such as London van der Waal's forces and electrical double-layer forces. However, they could not exclude factors such as wettability characteristics since the hydrogen ion concentration did not influence cell sorption.

Murthy and Natarajan (1992) found that bacterial attachment to the pyrite surface was not instantaneous. They observed a minimum lag period of 3 days for the -75+63 μm and the -1000+600 μm size fraction and 10 days for the -4750+4000 μm size fraction, for the onset of bacterial attachment. The time delay could be due to the involvement of certain bacterially-synthesized adhesive polymers in the attachment as well as changes in the quality

and quantity of the mineral surface (Murthy and Natarajan, 1992). Bagdigian and Myerson (1986) suggested that time-independent irreversible adhesion involves the production of extracellular polymers necessary to bridge the electrostatic barrier holding the cell at a finite distance from the substratum surface. These polymers are typically glycoprotein or lipopolysaccharide in nature and assume the form of capsular holdfasts, thin pili or extracellular secretions. Schaeffer and Umbreit (1963) were able to identify a major component of the wetting agent produced by *Thiobacillus thiooxidans*, grown on sulphur, as phosphotidylinositol (also known as inositol phosphotide or phosphoinositide).

Zobell's bacterial adhesion model (as explained by Bagdigian and Myerson, 1986) consists of 2 stages: an initial stage of reversible adhesion followed by time independent irreversible adhesion:

$$B + S \rightleftharpoons [BS]^* \rightarrow BS$$

According to this model, an initial interaction between a bacterial cell and a surface site involves the formation of a metastable complex $[BS]^*$, in which the cell is held at a finite distance from the surface by a balance of repulsion and attraction forces. Once formed, the complex may dissociate reversibly thereby regenerating a free bacterium and surface site. Alternatively, the bacterium may expend metabolic energy for the production of biopolymers necessary to establish a permanent bacterium-surface site BS.

Bagdigian and Myerson (1986) studied whether the adsorption of *Thiobacillus ferrooxidans* on coal changes from reversible to irreversible. They found that only a small fraction was reversibly adsorbed (as determined by rinsing the filtered coal with 9K media) and that up to 100% of the total population was adsorbed within 2 minutes provided sufficient coal was present. The production of biopolymers for this very rapid adsorption is unlikely as this requires both metabolic energy and an induction period. Duddrige *et al.* (1982) suggested that irreversible adsorption may in some cases be due to the compatibility of the substratum and the molecular structure of microbial surface. Takakuwa and co-workers (1957), as cited by Bagdigian and Myerson (1986), came to the same conclusion by showing that instantaneous adhesion of *Thiobacillus thiooxidans* to sulphur particles involved thiol groups located in the cell membrane.

Bagdigian and Myerson (1986) showed that the reversibly adsorbed population increased as the coal concentration decreased. Since bacterial cells generally have charged surfaces, electrostatic repulsion forces are expected to increase as bacterial surface density increases. Hence they suggested that electrostatic repulsion forces may be responsible for the fraction of bacteria reversibly adsorbed. Devasia *et al.* (1993) found that the isoelectric point of *Thiobacillus ferrooxidans* grown on pyrite is at a pH of approximately 3.8, hence the bacteria are positively charged below pH 3.8. This finding supports the electrostatic repulsion of positively charged bacteria when the bacterial surface density was increased.

Blake *et al.* (1994) found that ferric irons are adsorbed onto *Thiobacillus ferrooxidans*, neutralizing the net charge on its surface. This reduced or removed any double-layer repulsive barriers to the cell's interaction with charged particles. Blake *et al.* (1994) found *Thiobacillus ferrooxidans* to regulate the net charge on its surface in order to minimize double-layer repulsive forces between the cells and the charged substrate.

2.5.2. The Role of Attached Bacteria

The mechanism by which the attached bacteria assist the leaching process is unknown. Scanning electron microscopic observations by Rodriguez-Leiva and Tributsch (1988) of the surface of bacterially leached synthetic pyrite shows an organic film around the bacteria. They proposed that these organic substances may either be part of a "pseudo capsule" which shields the contact area or a sulphur absorbing and transporting organic film. The degradation of the sulphide occurs in the contact area below the bacteria leading to a corrosion pit (Figure 2.3) which the bacteria abandon after it has reached a depth of bacterial dimensions. They thus concluded that the bacterial leaching mechanism of metal sulphides by *Thiobacillus ferrooxidans* is a heterogeneous process, in which the bacterial cell attaches itself to the sulphide and corrosion occurs in a thin film between the bacterial outer membrane and the sulphide interface. The chemical degradation of the sulphide is determined by the composition of the film.

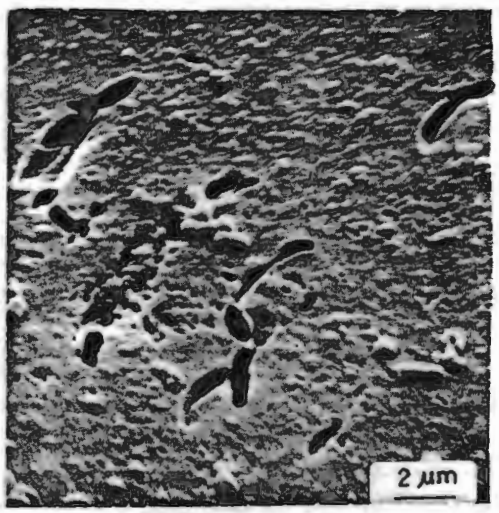


Figure 2.3. Bacterial-shaped corrosion pits on a pyrite surface (Vargas *et al.*, 1993).

2.6. METHODS OF ENUMERATING THE NUMBER OF BACTERIA IN A BIOOXIDATION SYSTEM

The bacterial population can be enumerated by direct observation, indirect determination of a cell component or by measuring metabolic activity. Enumeration of the bacteria in a bioleaching system is difficult due to the attachment of the bacteria to the mineral surface and inorganic precipitants and by the presence of interfering compounds released by the ore.

Counting by direct means (*i.e.* by light microscopy using a counting chamber or by using a Coulter Counter) is a means of enumerating the free bacteria in solution alone. In order to count the number of attached bacteria directly, these bacteria would first have to be desorbed from the mineral surface. This has been attempted in literature with limited success (Le Roux *et al.*, 1973; Myerson and Kline, 1983; Murthy and Natarajan, 1992). Alternatively, epifluorescent techniques (Yeh *et al.*, 1987, Huber *et al.*, 1985 and Olson and Kelly, 1986) and fluorescent antibody (FA) staining techniques (Apel *et al.*, 1976; Gates and Pham, 1979) have been developed in order to enumerate attached bacteria directly.

The enumeration of attached bacteria has been reported mainly by indirect methods. Indirect

methods reported in literature for quantifying the number and activity of the bacteria include the monitoring of metabolic activity through ferrous iron oxidation rate, titratable acidity, rate of oxygen uptake, metal dissolution rate and analysis of cell components such as protein and cellular nitrogen (Brierley, 1978). The problems associated with indirect methods include the interference from the mineral ore and the low cell densities in bioleaching systems (Yeh *et al.*, 1987). Hence the indirect method should include a blank which measures the contribution to the assay from the mineral ore. The method should be relatively sensitive to measure low cell densities.

This section presents the methods reported in the literature to enumerate the bacteria as well as the results obtained. The methods are divided into two groups: direct and indirect methods. Finally a summary and comparison of the methods and results for the different mineral ores used, are given.

2.6.1. Direct Methods of Enumerating the Number of Bacteria in a Biooxidation System

Direct methods include those methods by which the bacteria are enumerated by direct observation.

2.6.1.1. Counting

The free bacteria can be counted under the light microscope using a counting chamber. This is an established method of enumerating the free bacteria (Le Roux *et al.*, 1973; Konishi *et al.*, 1992; Mustin *et al.*, 1992; Ohmura *et al.*, 1993). Alternatively the free bacteria can be counted using a Coulter Counter (Shuler and Tsuchiya, 1975). However, before the Coulter Counter can be used the precipitates must be removed or minimized. For growth of *Thiobacillus ferrooxidans* on ferrous sulphate, the ferric iron precipitates can be dissolved with a mixture of NaCl and EDTA (ethylenediamine tetraacetate). This method, however, cannot be used for cultures of *Thiobacillus ferrooxidans* grown on finely ground ore as the EDTA does not dissolve the mineral ore (Shuler and Tsuchiya, 1975). One advantage of using a Coulter Counter is that it can also determine the size of the bacteria. This would enable *Leptospirillum ferrooxidans* to be distinguished from the *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. However, distinguishing between *Thiobacillus ferrooxidans*

(0.5×1.0 μm) and *Thiobacillus thiooxidans* (0.5×1.0-2.0 μm) is not possible because of their similar sizes. Direct microscopic observation has also been used to distinguish between *Leptospirillum ferrooxidans* and the *Thiobacilli* (Helle and Onken, 1988) but a high magnification is necessary.

Counting the free bacteria has also been used to determine the number of adsorbed bacteria through adsorption experiments. The number of adsorbed bacteria is calculated from the difference in the free bacteria before and after the addition of the mineral ore (Konishi *et al.*, 1992; Asai *et al.*, 1992; Ohmura *et al.*, 1993 and Konishi *et al.*, 1994). These authors also determined the saturation adsorption capacity of the mineral ore by increasing the cell density added to the ore. The saturation adsorption capacities are given in Table 2.1.

Table 2.1. The saturation adsorption capacity of mineral ore by *Thiobacillus ferrooxidans*.

Author	Mineral ore	Particle size	Saturation adsorption capacity
Konishi *et al.* (1992)	Zinc Sulphide		1.65×10^{10} cells/g
Asai *et al.* (1992)	Pyrite	-44+25 μm	6.61×10^{10} cells/g
		-63+53 μm	2.50×10^{10} cells/g
		-88+63 μm	1.41×10^{10} cells/g
		-177+149 μm	0.912×10^{10} cells/g
Ohmura *et al.* (1993)	pyrite		1.76×10^{10} cells/g
Konishi *et al.* (1994)	Sulphur		4.88×10^{10} cells/g

2.6.1.2. Desorption/dislodgement of attached bacteria

If the bacteria can be desorbed or dislodged from the mineral ore then the bacteria can be enumerated by direct methods. Many authors have investigated the possibility of desorption occurring and many attempts have been made to dislodge the entire biomass from the mineral surface (Le Roux *et al.*, 1973; Myerson and Kline, 1983 and Murthy and Natarajan, 1992). Experiments designed to induce dislodgement of the entire attached bacterial population include agitation, centrifugation and sonication as well as the addition of chemical agents known to release bacteria from flocs. The results indicate that only a fraction of all the attached bacteria were dislodged. However, they do show that the desorption of bacteria from the mineral surface is possible.

Myerson and Kline (1983) detected no desorption of attached bacteria even with changes in pH (up to 10) and agitation. An attempt to dislodge the bacteria by agitation was also tried by Le Roux *et al.* (1973) with limited success. Le Roux *et al.* (1973) attempted to dislodge the bacteria by hand shaking, mechanical shaking and mechanical shaking together with the addition of Lubrol and sodium pyrophosphate (agents which have been used for releasing bacteria from flocs). The number of bacteria in the supernatant was measured by oxygen uptake rates and cell counts. Le Roux *et al.* (1973) made various inferences from their results: (i) the bacteria are very firmly attached, (ii) attached bacteria are little affected by vigorous agitation, (iii) Lubrol and sodium pyrophosphate, although assisting dislodgement of the bacteria, inhibited bacterial activity, and (iv) total number dislodged (1.7×10^8 bacteria per ml) was only a fraction of the number of bacteria adhering to the pyrite. Le Roux *et al.* (1973), using the data obtained above, estimated that between 2.5×10^9 and 1×10^{10} bacteria were attached per ml of pyrite suspension.

Murthy and Natarajan (1992) looked at the effect of the centrifugal forces and ultrasonication on the dislodgement of bacteria. The supernatant was analysed for protein. The results however, were unsuccessful as after one hour of such treatments only a maximum of 0.04 to 0.05 mg protein per gram of ore, was reported. Conversely, the digestion in NaOH dislodged the entire biomass (0.11 to 0.12 mg protein per gram ore) as confirmed by repeated alkaline digestion for prolonged periods. Using the protein content of 2×10^{-8} μg protein/cell (Myerson and Kline, 1983) the bacterial density associated with the ore was 5.5×10^9 - 6.0×10^9 cells/g pyrite (4% pulp density).

Konishi *et al.* (1994) and Monroy Fernandez *et al.* (1995a) used chemical means of dislodging the bacteria. Konishi *et al.* (1994) examined the adsorption of *Thiobacillus ferrooxidans* onto sulphur. In addition to counting the free bacteria after the addition of the sulphur, the adsorbed bacteria were estimated by dissolving the sulphur with CS_2 (carbon disulphide) and counting the adsorbed cells released into solution. The two methods were found to agree within a few per cent.

Monroy Fernandez *et al.* (1995a) leached arsenopyrite with *Thiobacillus ferrooxidans*. The bacteria in the free solution were counted using a counting chamber. A Tween method was used to remove the adhered bacteria into solution. This method involves washing the mineral sample several times with the rinse water, which was collected for cell counts. The mineral sample was then treated with a 1% v/v Tween 80 solution and direct microscopic counts were performed on the resulting solution to enumerate the strongly adhered bacteria. After 4 days growth in a batch culture the free bacterial concentration was 10^7 cells/ml and the attached bacterial concentration was 2.5×10^9 bacteria/g mineral. Thus, 83% of the total bacteria were attached to the mineral surface.

Evidence of naturally occurring desorption is found when the free population is removed. The attached bacteria seem to detach from the solid to repopulate the free solution. This was seen by Bailey and Hansford (1993) when they washed out the free population in a fluidized bed reactor. They showed that the free cell recovery occurred in 2 stages. In the first stage the rate of free cell repopulation is greater than the growth rate in the control experiment. In the second stage the rate of cell recovery is similar to the growth rate in the control flask. This indicated that the bacteria detach from the mineral surface until equilibrium is achieved between the free and attached population.

Other evidence of desorption occurring includes the size of the corrosion pits observed in the leached mineral surface by scanning electron microscope. The pits' depth is observed to be of bacterial dimensions. This indicates that the bacteria detach from the mineral surface probably when some aspect becomes limiting, eg. the limitation of CO_2 and O_2 by diffusion (Rodriguez-Leiva and Tributsch, 1988).

Murthy and Natarajan (1992) found that, under agitated conditions, there was a continuous

variation in bacterial growth. This could be due to the constant attachment and detachment of the bacteria from the mineral substrate. From this observation, together with that of Rodriguez-Leiva and Tributsch (1988), Murthy and Natarajan (1992) concluded that there must be a mechanism that is causing the bacteria to leave unfavourable sites. Either the bacteria are first released into the liquid phase and then reattach or they move on the mineral surface itself to locate favourable sites.

2.6.1.3. Epifluorescence of attached bacteria

Another method of enumerating the attached bacteria directly is to make the bacteria visible for microscopic examination. This can be achieved by staining the cells with fluorescent dyes, such as DAPI (4'-6'- Diamidino-2-phenylindole) and acridine orange. These dyes cause the bacteria to fluoresce when viewed under a fluorescent microscope. This would enable the number of attached bacteria to be enumerated by counting the fluorescing cells attached to a small area of ore particle. This is, however, a crude method of quantifying the number of attached bacteria as it involves working with a two-dimensional projection of a three-dimensional surface. The cell counts only give estimates of the actual adsorbed population.

DAPI becomes fluorescent in the inciting light after binding to a cell component such as DNA. Huber *et al.* (1985) reported a modified DAPI staining method in which the cells are made permeable by low amounts of detergent allowing the stain to form a fluorescing complex with the DNA. The bacteria then fluoresce a bright blue when viewed under a fluorescent microscope.

Yeh *et al.* (1987) tried to correlate the colour of fluorescence of the cell stained with acridine orange, to the activity of the cells. Cells stained with acridine orange exhibit either green or red fluorescence depending on the nature of the nucleic acid with which the acridine orange molecules interact. Acridine orange can interact in two ways: it either forms a strong complex at low dye to nucleotide ratios in which the dye molecules intercalate between 2 adjacent base pairs (green fluorescence) or each dye molecule is held by an ionic bond between the nuclear nitrogen atom and a phosphate residue of DNA at high dye to nucleotide ratios or for single stranded nucleic acids (red fluorescence). RNA fluoresces red when bonded to acridine orange. When both the DNA and the RNA fluoresce, the cell appears either yellow or orange depending on the relative amounts of acridine orange bonded to DNA

and RNA (Yeh *et al.*, 1987). Thus, when the bacteria are growing actively, the cells will fluoresce red-orange due to the predominance of m-RNA while inactive bacteria are low in m-RNA and therefore fluoresce green (Hobbie *et al.*, 1977).

Yeh *et al.* (1987) related the colour of the cell fluorescence to the activity of the cell. They changed the conditions of an actively growing *Thiobacillus ferrooxidans* culture on ferrous iron from aerobic to anaerobic or heat treated actively growing cells. The results showed that the cell fluorescence for both cases changed from green-yellow to orange-red. They also noticed that a culture which fluoresced green or green-yellow has a shorter lag phase on subculturing than cells which predominately fluoresced red.

Yeh *et al.* (1987) then observed the fluorescent nature of the cells free in solution and those attached to the mineral surface when the bacteria were grown on pyrite. Initially the free cells fluoresced green and near the end of the leach the cells fluoresced yellow-orange. The attached cells fluoresced mostly green during the middle of the leaching period and a mixture of yellow and orange at the beginning and end. Yeh *et al.* (1987) therefore concluded that there is a relationship between cell fluorescence colour and biological leaching activity. Therefore, counting the relative number of green and red cells will give an indication of the number of actively growing bacteria in the sample.

2.6.1.4. Dry weight determination and turbidity

Dry weight determination and turbidity is not often used as a measure of growth for bioleaching as most of the growth substrates are particulates or the growth results in the formation of inorganic precipitants (Brierley, 1978). These particulates add to the dry weight and affect the turbidity of the solution giving unreliable and misleading results. The specific gravity of the particulates is between 2.3 and 5 while that of the bacterial suspension is approximately 1. Thus, only a small amount of precipitant in the sample can interfere with the results.

2.6.2. Indirect Methods of Enumerating the Number of Bacteria in a Biooxidation System

Indirect methods involve the quantification of the number of bacteria by monitoring a cell component, for example, the protein, nitrogen and carbon. Indirect methods also include determination of the bacterial population by monitoring the metabolic activity of the bacteria, for example, the oxygen uptake rate of the cells. Such methods are unable to distinguish between types of bacteria.

2.6.2.1. Protein

The most commonly used method for enumerating the bacteria in a bioleaching system is the estimation of the protein content of the sample. The protein content of a cell accounts for approximately 48-66% of the dry weight (Jones and Kelly, 1983). Myerson and Kline (1983) assumed a protein content per cell of 2×10^{-8} μg protein. This assumed protein content corresponds to 17% of the dry weight of a cell and is therefore not the true protein content of the cell.

Protein assays require that the protein be in solution. The method most frequently used to release the protein content of a cell into solution involves the digestion of the sample with an alkaline solution, typically a 0.1 N NaOH solution (Chang and Myerson, 1982; Barros, 1983; DiSpirito *et al.*, 1983; Murthy and Natarajan, 1992 and Yelloji Roa *et al.*, 1992). Other methods used to lyse the cells include digestion with 0.2 NaOH solution (Basaran and Tuovinen, 1987) and treatment with sodium dodecyl sulphate (SDS) (Nagpal *et al.*, 1993).

The protein assays used in literature include the Lowry, Peterson, Coomassie Blue and Bicinchoninic acid (BCA) protein assays. All these methods determine the protein content colorimetrically with bovine albumin (BSA) as a standard. The basic principle of each of the protein methods is given in Table 2.2.

Table 2.2. The principles of the different protein assays.

Protein assay	Principle of the protein assay	Reference
Lowry	There are two steps involved in the colour formation. The first step is the reaction of the copper with the protein in an alkaline solution. The second step is the reduction of the Folin reagent (a phosphomolybdic-phosphotungstic reagent) by the copper-protein complex, thereby producing reduced species of the reagent which have a characteristic blue colour.	Lowry *et al.* (1951)
Peterson - a modified Lowry assay	Same as Lowry with the addition of sodium dodecyl sulphate (SDS) to alleviate possible interference from nonionic and cationic detergents and lipids.	Peterson (1977)
Coomassie Blue	The assay is based on the conversion of Coomassie Blue G-250 from the red form to the blue form upon binding of the dye to protein. This causes a shift in the adsorption maximum of the dye from 465 to 595 nm. The absorbance at 595 nm is monitored.	Bradford (1976)
Bicinchoninic acid (BCA)	In the treatment of the protein with Cu^{2+} in an alkaline solution as done in the Lowry assay, it has been theorized (Smith *et al.*, 1985) that a reduction of the Cu^{2+} to Cu^{1+} occurs at the complexation sites within the copper-protein molecule (Biuret reaction). The BCA reagent, which is highly specific for Cu^{1+}, forms an intense purple complex with Cu^{1+} in an alkaline environment.	Smith *et al.* (1985)

Following the digestion of the protein-mineral ore suspension, a blank is usually employed to allow for the contribution of the ore to the protein content of the sample. The blank used is a protein-free mineral ore (Chang and Myerson, 1982; Myerson and Kline, 1983 and Murthy and Natarajan, 1992). Myerson and Kline (1983) found, however, that the protein

content of the adsorbed bacteria still could not be determined by the digestion of the ore as material, which leached out of the coal during digestion caused interference with the Lowry assay. On the other hand, DiSpirito *et al.* (1983), Basaran and Tuovinen (1987) and Norris *et al.* (1988) digested pyrite and recorded no interference with the Lowry assay. DiSpirito *et al.* (1983) found that the sum of the protein content of the free and attached cells agreed to within $\pm 12\%$ with the protein content of the total sample.

Basaran and Tuovinen (1987) found a protein concentration of 2200 μg protein for the total attached bacteria and a protein concentration of 550 μg protein for the total free bacteria for *Thiobacillus ferrooxidans* grown on pyrite when incubated under static conditions. Under shaken incubation an attached population of 1550 μg protein and a free population of 7250 μg protein was observed.

Norris *et al.* (1988) calculated the adsorbed protein concentration by measuring the protein concentration of the total sample and the supernatant (Table 2.3). The adsorbed protein content was determined from the difference of the total and the supernatant protein concentrations. Norris *et al.* (1988) found that this avoided inaccuracy from over-estimation of attached cells owing to the occlusion of free cells in the solids fraction or from under-estimation if attempts to eliminate free cells by washing dislodged some associated with the pyrite. They found that for *Thiobacillus ferrooxidans* 7% of the population was associated with the pyrite and for *Leptospirillum ferrooxidans* 87% of the bacterial population was associated with the pyrite. They attributed the low percentage of attached *Thiobacillus ferrooxidans* to the cultivation of the bacteria on ferrous iron for many years.

Table 2.3. Analysis of biomass by protein content of supernatant and complete culture (Norris *et al.*, 1988).

Micro-organism	Protein content	
	Supernatant $mg.\ell^{-1}$	Total sample $mg.\ell^{-1}$
T. ferrooxidans	30.9	35.6
L. ferrooxidans	3.13	22.5

Barros (1983) examined the effect of inorganic ions on three different protein assays by enumerating *Thiobacillus ferrooxidans* grown on liquid media. The liquid media contained the inorganic ions potassium, magnesium, ferrous and ferric iron, nitrate, sulphate and chloride. The three different protein assays that Barros (1983) examined include the determination of amino acids with the Ninhydrin reaction after alkaline hydrolysis of the protein, estimation of bacterial protein with the Folin-Ciocalteu reagent and binding of Coomassie Blue to the protein. Barros (1983) found that both the Ninhydrin reaction and the Folin-Ciocalteu reagent experienced interference from the inorganic ions present in the culture media. The inorganic ions which caused the interference were not identified. Washing with acidified distilled water did reduce this interference but the washes were time consuming and there was the potential loss of bacteria during the washing cycle. An additional problem was the insensitivity of the assays to small amounts of protein. On the other hand, Barros (1983) found that Coomassie Blue protein-binding assay was free from any interference from the inorganic ions. A correlation coefficient of 0.9993 was obtained when comparing the protein measured from a sample of oxidised supernatant fluid and BSA with that of water and BSA. Also, the Coomassie Blue assay was found to be able to determine lower cell numbers of 3.6×10^5 cells/ml reproducibly, than the Lowry protein assay (1.0×10^7 cells/ml) (Yeh *et al*, 1987).

Karavaiko *et al.* (1986) and Murthy and Natarajan (1992) used the Peterson protein assay. On the digestion of the ore (arsenopyrite and pyrite, respectively), no interference was recorded. Karavaiko *et al.* (1986) found for a continuously leached arsenopyrite concentrate a maximum biomass concentration of 5 mg biomass/g solid. The total number of cells adsorbed varied between 95% in the first pachuca tank to 70% in the 6th pachuca tank.

Murthy and Natarajan (1992) found a total of 0.11 to 0.12 mg protein/g pyrite to be associated with the pyrite ore after 15 days growth. Murthy and Natarajan (1992) also found that particle size affected the bacterial attachment (Table 2.4).

Table 2.4. The bacterial concentrations attached and free in solution at various particle sizes after 25 days of growth (Murthy and Natarajan, 1992).

	-75+63 μm	-1000+600 μm	-4750+4000 μm
Liquid concentration mg protein	0.19	0.42	0.09
Attached population mg protein	0.29	0.28	0.03

In contrast to Karavaiko *et al.* (1986) and Murthy and Natarajan (1992), Le Roux *et al.* (1973) reported that the biuret reaction for protein determination was only suitable for determination of *Thiobacillus ferrooxidans* cell numbers in a clean supernatant. Le Roux *et al.* (1973) found that the mineral ore interfered with the biuret colour development. Also the Pierce® BCA protein-binding assay has only been used for protein determination of the supernatant (Nagpal *et al.*, 1991, 1993).

The protein assay method has also been used to measure the adsorbed bacterial population in adsorption experiments. The adsorbed bacterial population is determined by the difference in protein concentration of the supernatant before and after the addition of the mineral ore (DiSpirito *et al.*, 1983; Myerson and Kline, 1983 and Bagdigian and Myerson, 1986). These results are given in Table 2.5.

Table 2.5. The adsorption of *Thiobacillus ferrooxidans* onto mineral ore as determined by protein assays.

Author	Mineral ore	Free bacterial concentration μg protein/ml	Attached bacterial concentration μg protein/g	% adsorbed bacteria
DiSpirito *et al.* (1983)	Pyrite - % solids = 0.2	27.8	11950	46 %
	- 0.5	20	5160	56 %
	- 1.0	7.8	5520	88 %
	- 5.0	55	1000	48 %
Myerson and Kline (1983)	coal	360.8	863	32 %
Bagdigian and Myerson (1986)	coal - 25 g/l solids			63 %
	- 50 g/l solids			98 %
	- 100 g/l solids			91 %

2.6.2.2. Nitrogen

The amount of nitrogen in a sample has been used to calculate the number of bacteria in solution and attached to the mineral surface (Gormely and Duncan, 1974; McGoran *et al.*, 1969 and Paponetti *et al.*, 1991). Gormely and Duncan (1974) determined the bacterial nitrogen content per cell of 1.57×10^{-11} mg.

McGoran *et al.* (1969) estimated the bacterial nitrogen content of the unattached bacteria by micro-Kjeldahl and the total bacterial nitrogen by macro-Kjeldahl analysis. The results obtained for the growth of *Thiobacillus ferrooxidans* on ferrous iron, sulphur and

chalcopyrite are given in Table 2.6.

Table 2.6. The free and attached population determined by nitrogen estimation by McGoran *et al.* (1969).

	Nitrogen content of entire sample mg/l	Nitrogen content of filtrate mg/l	% attached bacteria	% bacteria in the filtrate
Ferrous iron	65.6	2.2	96.6	3.4
Sulphur	74.5	17.3	76.7	23.3
Chalcopyrite at 20 g/l solids	127.4	3.3	97.4	2.6
Chalcopyrite at 53.3 g/l solids	341	3.6	98.9	1.1

In contrast to McGoran *et al.* (1969), Gormely and Duncan (1974) found that the total nitrogen in the sample was not a true reflection of the bacterial nitrogen in the sample. Gormely and Duncan (1974) found that nitrogen in the sample can be precipitated in the form of ammoniojarosite ($NH_4Fe_3(SO_4)_2(OH)_6$). Thus, estimating the entire biomass by Kjeldahl does not give a true reflection of the bacterial nitrogen present. Gormely and Duncan (1974) estimated the bacterial nitrogen as non-distillable nitrogen. The non-distillable nitrogen was calculated as the difference between total nitrogen concentration after Kjeldahl digestion and the distillable nitrogen. The latter is determined by steam-distilling the sample with NaOH. It is a measure of the inorganic nitrogen in solution and that which precipitated in the jarosite. Gormely and Duncan (1974) found that the nitrogen present as soluble organic metabolites was less than 1% and therefore this nitrogen contribution was ignored. In order to validate that the bacterial nitrogen does not report as distillable nitrogen and that all the nitrogen in the jarosite reports as distillable nitrogen, they determined the total and distillable

nitrogen content of a washed suspension of cells and of bacteria-free jarosite. They found that less than 5% of the cells reported as distillable nitrogen while the entire nitrogen content of the jarosite reported as distillable nitrogen. This verified that the nitrogen in the jarosite will report as distillable nitrogen while the bacterial nitrogen does not. The results that Gormely and Duncan (1974) obtained for growth of *Thiobacillus ferrooxidans* on a zinc sulphide concentrate and chalcopyrite are given in Table 2.7.

Table 2.7. The nitrogen content of a zinc sulphide concentrate and chalcopyrite as determined by Gormely and Duncan (1974).

Ore	Location	Total Nitrogen mg/l	Distillable Nitrogen mg/l	Non-distillable Nitrogen mg/l	Non-distillable Nitrogen distribution %
Zinc sulphide concentrate	Slurry	646	347	299	100
	Solids	222	26	196	65
	Centrifugate	414	306	108	35
Chalcopyrite	Solids	-	-	128.5	95.6
	Centrifugate	-	-	5.9	4.4

The distillable content of the slurry and the centrifugate is high (zinc sulphide concentrate). Approximately 53% of the slurry total nitrogen, 74% of the centrifugate total nitrogen and 11.7% of the solids total nitrogen reported as distillable nitrogen. The error on the non-distillable nitrogen measurements is 1.7% as determined by the non-distillable content of the slurry and the sum of the non-distillable content of the solid and centrifuge supernatant fractions.

The total nitrogen, however, has still been used as a measure of bacterial nitrogen. Paponetti *et al.* (1991) estimated biomass growth by detecting an increase in the organic nitrogen by

the Kjeldahl technique in both the liquid and solid phase (the Kjeldahl technique gives total nitrogen and not organic nitrogen as assumed by Paponetti *et al.* (1991)). Bacteria were grown on an arsenopyrite concentrate in batch tests. At maximum biomass concentration (after approximately 4 days of leaching), the total biomass concentration was estimated as 2.6 g/l with the attached and free biomass concentration of 2.0 g/l and 0.6 g/l, respectively. Thus, approximately 80% of the bacteria were attached. With increasing leaching time, the attached biomass concentration decreased, the free biomass concentration increased and the total biomass concentration remained relatively constant.

Atkins *et al.* (1986) quantified growth of *Thiobacillus ferrooxidans* by a modified micro-Kjeldahl technique. The total biomass in the leach system and the unassociated cells were measured as cellular nitrogen. The results obtained are given in Table 2.8.

Table 2.8. The free and total nitrogen of *Thiobacillus ferrooxidans* as determined by Atkins *et al.* (1986).

Substrate	Free Nitrogen μg Nitrogen per ml	Total Nitrogen μg Nitrogen per ml	% bacteria associated with solids
Tharsis pyrite	5-10	55-60	87
Chalcopyrite/pyrite	21	40	48
Tynagh sphalerite	33	47	30
Ferrous sulphate	9-11	9-11	0

Le Roux *et al.* (1973) used micro-Kjeldahl digestion to release the ammonia. The amount of ammonia was determined colorimetrically by the phenol hypochlorite method. They found that using this method the pyrite gave an appreciable colour reaction which they assumed was due to nitrogenous compounds present in the pyrite. They found that reproducible results could be obtained when corrections for these reactions were made.

2.6.2.3. Carbon

The total organic carbon (TOC) content of a sample gives information of the biomass content. Gormely and Duncan (1974) measured the total organic carbon content of a washed cell suspension of known bacterial concentration, determined by cell counts. They found that there was 7.67×10^{-11} mg organic carbon per cell.

Blancarte-Zurita *et al.* (1988) measured the total organic carbon of a slurry sample with a TOC analyser. They found that after 900 hours of growth on chalcopyrite the total organic carbon content of the sample was 347 ppm (4.5×10^{9} cells/ml slurry). Liu *et al.* (1988) also measured the inorganic and organic carbon contents using a total carbon analyser.

The carbon dioxide consumption rate has been used as a measure of the biomass concentration. Nagpal *et al.* (1993) found that the rate of protein generation (protein concentration× dilution rate) is proportional to carbon dioxide uptake rate. Therefore, they concluded that carbon dioxide uptake rate is a valid indicator for bacterial growth rate.

The rate of carbon dioxide uptake ($-r_{CO2}$) is equal to the rate of bacterial growth (r_x) (Boon *et al.*, 1995b):

$$-r_{CO_2} = r_x \qquad [2.1]$$

The bacterial concentration can be calculated from the rate of carbon dioxide consumption as follows (Boon *et al.*, 1995b):

$$c_x = c_x(0) - \int_0^t r_{CO_2}.dt \qquad [2.2]$$

Thus, if the carbon dioxide concentration of the off-gas and the inlet gas are measured, the increase in total cell concentration can be calculated from Equation 2.2.

Boon *et al.* (1994a) validated the above equation by measuring the carbon dioxide consumption rates as well as the concentration of the free bacteria by total organic carbon, for growth on ferrous iron. The two methods gave similar results. Therefore, carbon dioxide consumption rate measurements are a valid method of measuring biomass concentrations

provided the initial cell concentration is known.

Boon *et al.* (1994a) compared the biomass concentration as calculated from the carbon dioxide consumption rates with that obtained from total organic carbon analysis of the free supernatant for growth on pyrite. They found that there was no significant difference between the two methods and therefore most of the biomass population was unassociated with the pyrite particles. A possible reason for the low attached bacterial population is the low pyrite concentration (20 g/l) used and a high total iron concentration (12 g/l) in solution.

2.6.2.4. Oxygen utilisation rate

The oxygen utilisation rate or the oxygen consumption rate has been used to determine the activity of the bacteria. Norris *et al.* (1988) used oxygen measurements to determine the iron affinity of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*. The selective inhibition of oxygen uptake by the bacteria by copper and nitrate ions was then used to determine the relative activity of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* in a mixed cell suspension. They mixed two pure cultures and found that after 200 hours of continuous operation with pyrite approximately 92% of the bacterial population was *Leptospirillum ferrooxidans* and 8% *Thiobacillus ferrooxidans*. By microscopic counts, they confirmed that about 95% of the bacterial population was *Leptospirillum ferrooxidans*.

The bacterial activity, measured through oxygen utilisation rate, has been related to cell number by Le Roux *et al.* (1973) and Boon *et al.* (1995d). Boon *et al.* (1995d) related the number of cells, c_x, to the oxygen utilisation rate, r_{O2}, of a sample and the specific oxygen utilisation rate of the bacteria, q_{O2}.

$$-r_{O_2} = q_{O_2} \cdot c_x \qquad [2.3]$$

The specific oxygen utilisation rate, q_{O2}, can be written in terms of a Michaelis-Menton type equation (Boon *et al.*, 1995d) with competitive inhibition of ferric iron:

$$q_{O_2} = \frac{q_{O_2}^{max}}{1 + \frac{K_s}{[Fe^{2+}]} + \frac{K_s[Fe^{3+}]}{K_i[Fe^{2+}]}} \quad [2.4]$$

where q_{o2}^{max} is the maximum specific utilisation rate
K_s is the saturation constant for ferrous iron
K_i is the inhibition constant of ferric iron

If the ferrous iron concentration tends to infinity then q_{O2} tends to q_{O2}^{max}. Boon *et al.* (1995d) found that the q_{O2}^{max} is dependent on the ferrous to ferric ratio, tending to the maximum specific oxygen utilisation at a ferric to ferrous ratio above 1.0 and on the type of bacteria in the system. They determined a q_{O2}^{max} value of 0.65 mol O_2/mol C/hr for a culture of *Leptospirillum*-like bacteria and 0.9 mol O_2/mol C/hr for a pure culture of *Thiobacillus ferrooxidans*. Norris *et al.* (1988) determined oxygen uptake at various ferrous concentrations for the pure cultures of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Figure 2.4). From the data presented in Figure 2.4, a maximum specific oxygen utilisation rate of 0.99 mol O_2/mol C/hr for *Thiobacillus ferrooxidans* and 0.36 mol O_2/mol C/hr for *Leptospirillum ferrooxidans* was calculated at 12 g Fe^{2+}/ℓ. Once the maximum specific oxygen utilisation rate of the bacterial culture has been established, the bacterial concentrations can be calculated from the measured oxygen utilisation rate of the sample and Equation 2.3.

Le Roux *et al.* (1973) determined bacterial activity by measuring oxygen uptake by manometric techniques. They used a Gilson differential respirometer to measure the oxygen uptake due to biological and chemical reactions. The oxygen uptake is measured by a micrometer which records the inward movement of a rod required to keep the system at constant pressure. Le Roux *et al.* (1973) measured the oxygen uptake of the suspension, the supernatant and the settled pyrite which had been resuspended in bacteria-free supernatant (Table 2.9). They found that the oxygen uptake by the resuspended pyrite was 3 to 4 times that of the supernatant. Thus they concluded that there was 3 to 4 times as many bacteria associated with the pyrite than free in the supernatant provided that the activity of the free and attached bacteria are the same and that there are no inactive bacteria in the supernatant.

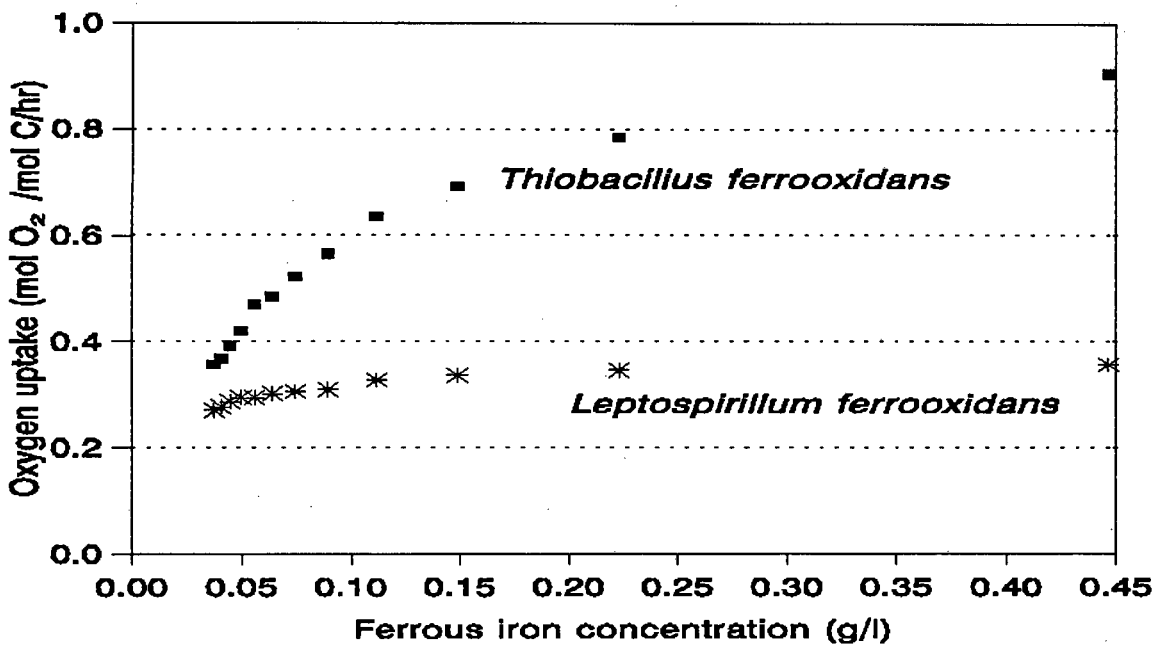


Figure 2.4. The oxygen uptake versus ferrous ion concentration by Norris *et al.* (1988).

An interesting result which Le Roux *et al.* (1973) do not explain is the difference between oxygen uptake of the suspension and the sum of the oxygen uptake of the supernatant and the resuspended pyrite.

Table 2.9. The oxygen uptake of fractions as measured by Le Roux *et al.* (1973).

	Suspension	Resuspended pyrite	Supernatant
Oxygen uptake after 300 min (μl)	367	217	62

Le Roux *et al.* (1973) did, however, stop the biological activity to determine the oxygen uptake due to chemical reactions only. They found that the chemical reactions accounted for less than 3% of the biological activity.

The assumption that the activity of the free and attached bacteria is the same was validated by Le Roux *et al.* (1973) using micro-Kjeldahl digestion. They measured the nitrogen content of the supernatant and the settled pyrite and the oxygen uptake rate of these fractions simultaneously. They found, by micro-Kjeldahl digestions, that there was approximately 6 times more bacteria associated with the settled pyrite than in the supernatant. Their oxygen uptake results gave a similar ratio. They therefore concluded that the oxygen uptake activity of the bacteria associated with the pyrite was equivalent to that of by the free bacteria.

2.6.2.5. Other methods

The most probable number (MPN) method has been used to analyse the viable cell number (Helle and Onken, 1988; Beyer *et al.*, 1986 and Southam and Beveridge, 1992). The most probable number method involves a series of dilutions into media containing ferrous iron when looking at iron-oxidizing bacteria or sulphur for sulphur-oxidizing bacteria. The problem with dilutions of a suspension of solid particles is in obtaining a representative sample of the mineral particles with bacteria (Brierley, 1978).

Atkins *et al* (1986) also estimated culture growth in terms of chemical oxygen demand (COD). The chemical oxygen demand of a sample is a measure of the oxygen equivalent to the portion of the organic matter that is susceptible to oxidation by a strong oxidant in the presence of a catalyst. Atkins *et al.* (1986) determined the chemical oxygen demand of the total organics in solution and of a bacteria-free extract. This enabled a measure of the free organic cell content to be determined together with an indication of the organic products of metabolism. The COD can, however, only be used as a measure of the number of the free bacteria.

Determination of the number of bacteria able to form colonies by plating in a two-layer gel at pH of 2.5 has also been used for enumeration purposes (Espejo and Ruiz, 1987). Espejo and Ruiz (1987) attempted to estimate the quantity of bacteria adhered to the mineral by plating washed ore. Many other authors have, however, reported difficulty in growing *Thiobacillus ferrooxidans* on solid media (Tuovinen and Kelly, 1973 and Barros, 1983). Tuovinen and Kelly (1973) found that colony development of *Thiobacillus ferrooxidans* on solid ferrous sulphate agar was retarded by the agar. A similar result was obtained by Barros (1983).

Espejo and Ruiz (1987) found, from plating the ore (copper containing ore), that the number of colonies did not correspond to the actual number of bacteria because the number of bacteria associated with a single ore particle rendered only one colony. They, however, found that these colonies formed rapidly, indicating that the colony formed from a large number of bacteria attached to the ore particles.

Espejo and Ruiz (1987) then tried to estimate the number of attached bacteria by monitoring the oxidation of ferrous iron on the plates. From their results they determined that the total activity in association with the ore was less than 10% of that present in solution. Espejo and Ruiz (1987) explained that this could be an underestimation if the bacteria utilized sulphur or sulphides as an energy source and if an appreciable amount of time was required for adaptation to ferrous iron utilisation.

Radioactive uptake of $^{14}CO_2$ (Brierley, 1978) and ^{32}P (Espejo and Romero, 1987) has been used to quantify the number of bacteria. Espejo and Romero (1987) determined the growth of *Thiobacillus ferrooxidans* on sulphur by incorporating radioactive ^{32}P into the medium. The number of bacteria free in solution and attached to the sulphur particles were measured in a Packard scintillation counter. The amount of radioactivity was then corrected for the natural decay of ^{32}P. They verified that the ^{32}P detected in the prills were due to bacterial growth. They found the 1.6×10^{7} bacteria per ml were attached to the prills with approximately 12 prills per ml.

2.6.3. Summary of Enumeration Methods and Results

When enumerating the number of bacteria in a bioleaching system, the actual value obtained depends on the methodology of enumeration, the mineral ore to which the bacteria adhere, the bacterial strains used, the conditions of the leaching environment and the history of the bacteria. Methods which have been used for the enumeration include the oxidation of ferrous iron, oxygen uptake rate, carbon dioxide uptake rate, rate of production of acid as well as analysis of intracellular products such as cellular nitrogen and protein. More recently direct methods of enumeration have been used, for example epifluorescence microscopy and antibody fluorescent staining. This section summaries the enumeration techniques and results

obtained for various mineral ores. Some discussion of the results for each mineral ore is also given.

In some cases it has been necessary to convert the reported attached and free bacterial concentrations into cell numbers. The conversions used are 2.0×10^{-8} μg protein/cell (Myerson and Kline, 1983), 1.57×10^{-11} μg nitrogen/cell (Gormely and Duncan, 1974) and 1.12×10^{-7} μg dry weight/cell (Gormely and Duncan, 1974). The protein content per cell corresponds to 17% of the dry cell mass compared to reported values of 41-48% (Jones and Kelly, 1983). Although this value of protein is low, it correlates to the protein extracted per cell over a digestion time of 15 minutes in which only a fraction of the soluble protein has been released. As the 15 minute digestion time was used by these authors, the protein content per cell obtained at this digestion time was used.

2.6.3.1. Pyrite

The various methods which have been used to enumerate the number of bacteria in a pyrite system include cell counts of the free cells, protein, nitrogen and oxygen uptake. The results of these methods are summarized in Table 2.10.

Table 2.10. The enumeration of bacteria on pyrite.

Authors	Method	Attached bacteria	Free bacteria	% Adsorbed	Particle size
Ohmura *et al.* (1993)	Cell counts of free bacteria	1.92×10^{10} cells/g *saturation constant* = *1.76×10^{10} cells/g* = *8.9×10^{11} cells/m^2*	7.7×10^{8} cells/ml	24	-100+40 μm Total surface area = 2.58×10^{9} μm^2

Authors	Methods	Attached bacteria	Free bacteria	% Adsorbed	Particle size
Asai *et al.* (1992)	Cell counts	*saturation constants* = 6.61×10^{10} 2.5×10^{10} 1.41×10^{10} 0.91×10^{10} *cells/g*			-44+25 μm -63+53 μm -88+63 μm -177+149 μm
Le Roux *et al.* (1973)	Oxygen uptake	between 2.5×10^{9} and 1.0×10^{10} cells/ml suspension	10^{9} cells/ml	71-91%	90% finer than 53 μm
Murthy and Natarajan (1992)	Protein	After 15 days: 5.5×10^{9} - 6.0×10^{9} cells/g			
		After 25 days:			
		4.0×10^{9}	1.05×10^{8}	60.4 %	-75+63
		3.9×10^{9}	2.33×10^{8}	40 %	-1000+600
		4.1×10^{8} cells/g	5×10^{7} cells/ml	25 %	-4750 + 4000 μm
DiSpirito *et al.* (1983)	protein				-297+53 μm
	- 0.2 % solids	5.9×10^{11}	1.39×10^{9}	46 %	
	- 0.5 %	2.58×10^{11}	1.0×10^{9}	56%	
	- 1.0 %	2.76×10^{11}	3.9×10^{8}	88%	
	- 5.0 %	5×10^{10} cells/g	2.75×10^{9} cell/ml	48%	

Authors	Methods	Attached bacteria	Free bacteria	% Attached	Particle size
Basaran and Tuovinen (1987)	protein - static - shaken	After 35 days: 1.1×10^{11} 7.75×10^{10} cells/g	 2.75×10^{8} 3.6×10^{9} cells/ml	 80 % 17.6 %	-74 + 44 μm
Norris *et al.* (1988)	protein	*Thiobacillus ferrooxidans* 4.7×10^{10} *Leptospirillum ferrooxidans* 1.94×10^{11} cells/g	 1.5×10^{9} 1.56×10^{8} cells/ml	 7 % 87%	< 75 μm
Atkins *et al.* (1986)	Nitrogen	3.1×10^{9} cell/ml	between 3.18×10^{8} and 6.36×10^{8} cell/ml	87 %	< 45 μm

Asai *et al.* (1992) calculated the saturation concentration of adsorbed bacteria for *Thiobacillus ferrooxidans* attached to pyrite particles of varying particle sizes. They obtained saturation adsorption bacterial densities between 6.61×10^{10} - 0.91×10^{10} cells/g for particle sizes between 25 and 177 μm. Ohmura *et al.* (1993) and Norris *et al.* (1988) measured attached bacterial concentrations which fall into the range of those measured by Asai *et al.* (1992). Conversely, the results of DiSpirito *et al.* (1983) and Basaran and Tuovinen (1987) do not agree with those of Asai *et al.* (1992). Both DiSpirito *et al.* (1983) and Basaran and Tuovinen (1987) measure higher values of attached bacterial concentration. It is important to note that the method of bacterial attachment measurements are different. Asai *et al.* (1992) and Ohmura *et al.* (1993) use cell counts of free bacteria while DiSpirito *et al.* (1983) and Basaran and Tuovinen (1987) measure bacterial protein by the Lowry assay.

The results presented by Murthy and Natarajan (1992) are lower than the results obtained by Asai *et al.* (1992), DiSpirito *et al.* (1983) and Basaran and Tuovinen (1987). However, Murthy and Natarajan (1992) use larger particle sizes. The decrease in attached cell population per unit mass of ore shown by Murthy and Natarajan (1992) agrees with the results of Asai *et al.* (1992) generated using a smaller particle size range. Murthy and Natarajan (1992) used the Peterson protein assay whereas DiSpirito *et al.* (1983) and Basaran and Tuovinen (1987) used the Lowry assay. These results seem to indicate that the methodology of enumeration has an important role in the results obtained.

A wide range of values for the percentage attachment is found in the literature. The values vary from 7 to 94 %. Norris *et al.* (1988) who measured a 7% bacterial attachment for *Thiobacillus ferrooxidans*, states that this low percentage may be due to the cultivation of the *Thiobacillus ferrooxidans* in ferrous media for a few years before being used in this experiment.

2.6.3.2. Chalcopyrite

Only two methods have been used to enumerate the bacteria attached to chalcopyrite, namely cell counts and nitrogen analysis (Table 2.11).

Table 2.11. Enumeration of bacteria on chalcopyrite.

Author	Method	Attached	Free	% Attached	Particle size
Ohmura *et al.* (1993)	Cell counts	9.33×10^{9} cells/g	8.6×10^{8} cells/ml	14 %	-100 + 40 μm
McGoran *et al.* (1969)	Nitrogen	20 g/l solids: 3.95×10^{11} 53.3 g/l solids: 4.03×10^{11} cells/g	2.1×10^{8} 2.2×10^{8} cells/ml	97 % 99%	< 37 μm
Gormely and Duncan (1974)	Nitrogen	7.67×10^{10} cells/g	3.75×10^{8} cells/ml	96%	< 37 μm
Atkins *et al.* (1986)	Nitrogen	1.21×10^{9} cells/ml	1.33×10^{9} cells/ml	48 %	< 45 μm

Little agreement is seen in the attached bacterial concentrations presented for growth on chalcopyrite. The attached bacterial concentration (approximately 4×10^{11} cells/g) measured by McGoran *et al.* (1969) is higher than that measured by Gormely and Duncan (1974) (approximately 7.6×10^{10} cells/g). This is due to Gormely and Duncan (1974) taking into account the nitrogen in the system which is present as inorganic nitrogen or precipitated in the form of ammoniojarosite. The work of Gormely and Duncan (1974) with a zinc sulphide concentrate showed that 53% of the slurry and 74% of the supernatant's total nitrogen were inorganic nitrogen. Thus, the contribution of inorganic nitrogen to the bacterial nitrogen can be substantial.

Ohmura *et al.* (1993) used cell counting of the free population to measure the attached population. They report a lower attached cell population than those obtained by nitrogen analysis. It is noted, however, that Ohmura *et al.* (1993) used a larger particle size.

2.6.3.3. Arsenopyrite

Karavaiko *et al.* (1986), Paponetti *et al.* (1991) and Monroy Fernandez *et al.* (1995a) investigated the biomass population in an arsenopyrite bioleach system. Each author used a different method for enumerating the number of bacteria as can be seen in Table 2.12.

Table 2.12. Enumeration of bacteria on arsenopyrite.

Author	Method	Attached	Free	% Attached	Particle size
Monroy Fernandez *et al.* (1995a)	Cell counts (Tween method)	2.5×10^{9} cells/g	10^{7} cells/ml	83	- 80 + 50 μm
Karavaiko *et al.* (1986)	Protein	maximum concentration = 4.4×10^{10} cells/g		95 - 70 %	93 % less than 44 μm
Paponetti *et al.* (1991)	Nitrogen	1.8×10^{13} cells/l	5.4×10^{12} cells/l	80 %	

The attached bacterial concentration of 2.5×10^9 cells/g (Monroy Fernandez *et al.*, 1995a) is lower than the attached bacterial concentration obtained for pyrite (approximately 10^{10} cells/g). The percentage attached bacteria is high, between 70 and 95%.

2.6.3.4. Zinc sulphide concentrate

A saturation adsorption bacterial concentration of 1.65×10^{10} cells/g was found by Konishi *et al.* (1992). This agrees closely with the results obtained by Asai *et al.* (1992) for the attachment to pyrite. Both these results were obtained from cell counting the free bacteria.

Table 2.13. Enumeration of bacteria on a zinc sulphide concentrate.

Author	Method	Attached	Free	% Attached	Particle size
Konishi *et al.* (1992)	Cell counts	Saturation constant = 1.65×10^{10} cells/g 3.57×10^{11} cells/m^2			-53 + 37 μm
Gormely and Duncan (1974)	Nitrogen	1.24×10^{10} cells/ml	6.87×10^9 cells/ml	65 %	

2.6.3.5. Coal

The only method used to enumerate the number of bacteria attached to coal is the protein analysis. This method has been used by Myerson and Kline (1983) and Bagdigian and Myerson (1986) and the results are presented in Table 2.14.

Table 2.14. Enumeration of bacteria on coal.

Authors	Method	Attached	Free	% Attached	Particle Size
Myerson and Kline (1983)	Protein	4.3×10^{10} cells/g	1.8×10^{10} cells/ml	32 %	< 63 μm
Bagdigian and Myerson (1986)	Protein			solids conc 25 g/l = 71 % Solids conc 25 g/l = 63 % Solids conc 50 g/l = 98 % Solids conc 100 g/l = 91 %	

The percentage attachment varied from 32 to 98%. An increase in percentage bacterial attachment seems to increase with increasing solids concentration. This is expected as an increase in solids concentration increases the area available for attachment.

2.6.3.6. Sulphur

A lower attached bacterial concentration was obtained with the cell counts (Konishi *et al.*, 1994) than the nitrogen analysis (McGoran *et al.*, 1969). A similar trend was noted with chalcopyrite where Ohmura *et al.* (1993) obtained a lower attached bacterial concentration than McGoran *et al.* (1969) and Gormely and Duncan (1974) by nitrogen analysis. It should be noted that McGoran *et al.* (1969) overestimates the number of attached bacteria by not accounting for the inorganic nitrogen obtained by the total nitrogen analysis.

Table 2.15. Enumeration of bacteria on sulphur.

Authors	Method	Attached	Free	% Attached	Particle size
Konishi *et al.* (1994)	cell counts CS_2	saturation constants = 4.88×10^{10} cells/g			
McGoran *et al.* (1969)	Nitrogen	1.36×10^{11} cells/g	1.1×10^{9} cells/ml	77 %	
Espejo and Romero (1987)	^{32}P uptake	1.6×10^{7} bacteria/prill			

2.6.3.7. Copper-containing ore

A copper-containing ore has been examined by Espejo and Ruiz (1987). Their results are given in Table 2.16.

Table 2.16. Enumeration of bacteria on a copper-containing ore.

Authors	Method	Attached	Free	% Attached	Particle Size
Espejo and Ruiz (1987)	plating washed ore and following the ferrous iron oxidation			10 %	

A low percentage attached bacteria was reported by Espejo and Ruiz (1987). This result was obtained by plating washed ore, allowing the bacteria to grow on solid media and monitoring the ferrous iron oxidation. Espejo and Ruiz (1987) explained that this could be an underestimate if the bacteria utilize sulphur or sulphides as an energy source. Many authors have had difficulties in growing *Thiobacillus ferrooxidans* on solid media (Tuovinen and

Kelly, 1973 and Barros, 1983). Tuovinen and Kelly (1973) found that colony development of *Thiobacillus ferrooxidans* on solid ferrous sulphate agar was retarded by the agar. A similar result was obtained by Barros (1983). If the growth of the bacteria is affected by the agar, then the ferrous iron oxidation measured by Espejo and Ruiz (1987) is not a true reflection on the number of bacteria present.

2.7. MAXIMUM COVERAGE OF THE MINERAL SURFACE

For a particular surface area, the maximum covering that can occur is determined by the ratio of the surface area to the area occupied per cell. Deviation from the value could be due to the repulsive forces between the like-charged bacteria or to the fact that not all the mineral surface is suitable for attachment. The latter reason occurs particularly in the case of low-grade waste ores. Berry and Murr (1978) observed selective attachment of *Thiobacillus ferrooxidans* on low-grade copper ores to the energy yielding sulphide phase regions ($CuFeS_2$ and FeS_2).

From literature, the maximum adsorption capacity is obtained from kinetic models of the adsorption or from adsorption equilibrium isotherms. The adsorption isotherm has been modelled by Chang and Myerson (1982), Konishi *et al.* (1992) and Asai *et al.* (1992). In particular, they found that the adsorption isotherm (X_A vs X_L) is characterised by a curve which approaches monotonically a limiting value, as shown in Figure 2.4. This indicates that the adsorption isotherm can be modelled by the Langmuir isotherm:

$$\underset{\text{rate of adsorption}}{k_1(1-\theta)X_L} = \underset{\text{rate of desorption}}{k_{-1}\theta} \qquad [2.5]$$

where k_1, k_{-1} are the rate constants,
X_L is the concentration of bacteria in solution,
θ is the fraction of solid surface covered by bacteria.

$$\theta = \frac{X_A a A_t}{A_t} = X_A a \qquad [2.6]$$

where a is the surface area occupied per bacterium (m^2/cell)

X_A is the concentration of attached bacteria per unit surface area

A_t is the total surface area

Rearranging the isotherm equation gives,

$$X_A = \frac{K_A X_L}{a(1+K_A X_L)} \qquad [2.7]$$

where $K_A = k_1/k_{-1}$.

Equation 2.7 shows that the concentration of bacteria attached to the solid surface is a first order function of the bacterial concentration in solution at low concentration. This changes to zero order at high bacterial concentrations. This is the behaviour obtained in the adsorption experiments as given in literature (Figure 2.5).

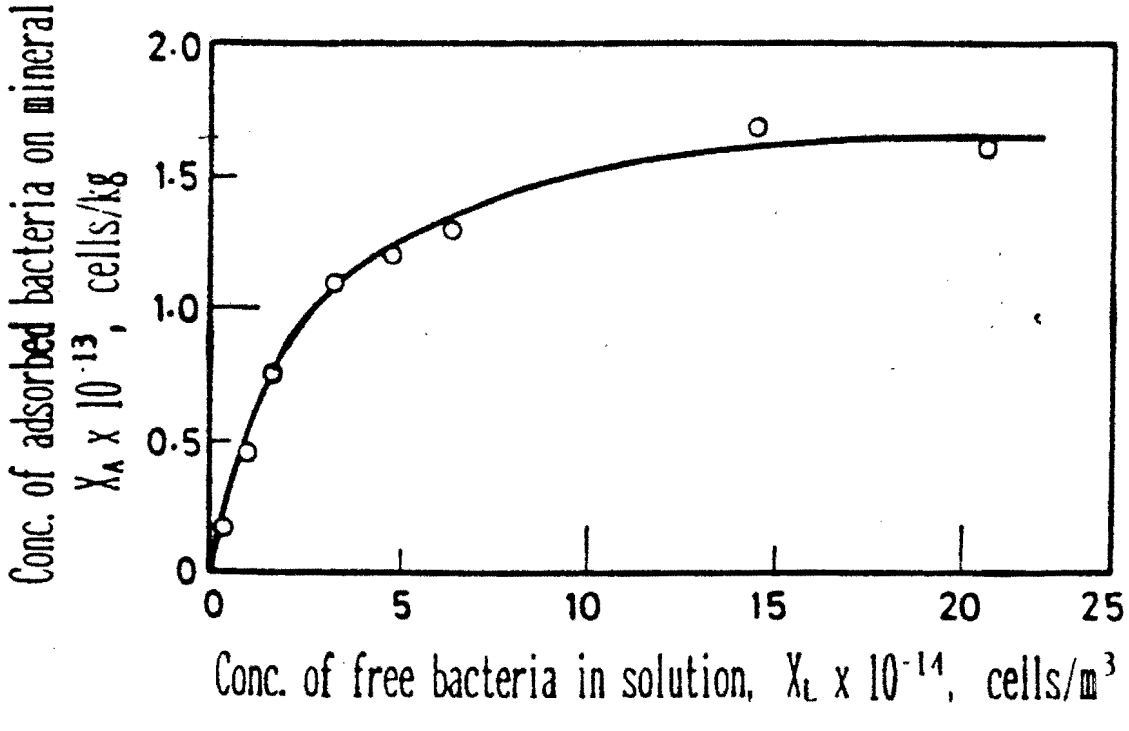


Figure 2.5. Adsorption isotherm for *Thiobacillus ferrooxidans* on ZnS (Konishi *et al.*, 1992).

There are some assumptions which have been made in the development of the Langmuir isotherm (Smith, 1981):

1. All the surface has the same activity for adsorption; *i.e.* it is energetically uniform. The concept of nonuniform surface with active centres can be employed if it is assumed that all the active centres have the same activity for adsorption and that the rest of the surface has none, or that an average activity can be used.
2. There is no interaction between adsorbed molecules. This means that the amount adsorbed has no effect on the rate of adsorption per site.
3. All the adsorption occurs by the same mechanism, and each adsorbed complex has the same structure.
4. The extent of adsorption is less than one complete monolayer on the surface.

It has already been noted that not all the mineral surface is suitable for attachment. In order to satisfy assumption 1, it has to be assumed that an average activity can be used for the entire surface or that the areas on the mineral surface which are suitable for adsorption have the same activity for adsorption and the rest of the surface has none. Assumptions 2 and 3 are valid. Scanning electron micrographs of *Thiobacillus ferrooxidans* indicating that the bacteria adsorb in a monolayer are shown in Figure 2.6 (Berry and Murr, 1978). DiSpirito *et al.* (1983) observed that there was no multiple cell aggregates for adsorption onto pyrite, glass, sulphur and quartz. However, scanning electron microscopic observations by Wakao *et al.* (1984) revealed that *Thiobacillus ferrooxidans* were adsorbed aggregatively on restricted areas of the pyrite particles.

Assuming that the Langmuir isotherm relationship is valid, the surface area per bacterium, a, can be calculated. The maximum adsorbed bacteria per unit area is then $1/a$.

Figure 2.6. Monolayer adsorption of *Thiobacillus ferrooxidans* to pyrite (Berry and Murr, 1978).

Chang and Myerson (1982) modelled the adsorption isotherm for pyrite leached by *Thiobacillus ferrooxidans* in a continuous stirred tank reactor. From the linear regression of data fitted to the Equation 2.8,

$$\frac{X_L}{X_A} = aX_L + \frac{a}{K_A} \qquad [2.8]$$

the calculated value of a is $(4.3\pm0.45)\times10^{-3}$ $m^2/\mu g$ protein. They assumed that the cell contains approximately 2×10^{-8} μg protein and calculated an area occupied per cell of 86 μm^2/cell. *Thiobacillus ferrooxidans* is a rod shape bacterium approximately 1 × 0.5 μm. Therefore, the actual area occupied per cell is 0.5 μm^2. They attributed the difference between the calculated area and the actual area to the unsuitability of the entire surface of the pyrite for attachment. Chang and Myerson (1982) thus calculated a maximum cell concentration adsorbed on the pyrite per unit area of 1.2×10^{10} cells/m^2.

Konishi *et al.* (1992) looked at the attachment of *Thiobacillus ferrooxidans* to ZnS (35-53 μm) in a batch air-sparged agitated vessel. The maximum adsorption capacity was evaluated by the Langmuir isotherm to be 3.5×10^{11} cells/m^2 (1.65×10^{10} cells/g mineral). Good agreement was obtained between the experimental points and the prediction by the Langmuir isotherm shown in Figure 2.5.

Asai *et al.* (1992) modelled the adsorption isotherm of *Thiobacillus ferrooxidans* on pyrite particles of different size fractions in a batch air-sparged agitated vessel. The size fractions used were: -44+25; -63+53; -88+63 and -177+149 μm. They found that as the particle size decreased, the number of adsorbed bacteria per unit mass of ore increased. Also, each size fraction had its own adsorption isotherm. The maximum adsorption capacity per unit weight of pyrite is given in Table 2.17. The decrease in the maximum adsorption capacity per unit weight of pyrite was explained by a decrease in the specific surface area, reflected by the increase in the particle size.

Table 2.17. Maximum adsorption capacity of *Thiobacillus ferrooxidans* for different size fractions of pyrite.

Particle size μm	Maximum adsorption capacity per unit weight of pyrite
-44+25	6.61×10^{10} cells/g-FeS_2
-63+53	2.50×10^{10} cells/g-FeS_2
-88+63	1.41×10^{10} cells/g-FeS_2
-177+149	0.912×10^{10} cells/g-FeS_2

Deviation from Langmuir isotherm behaviour has been shown by Ohmura *et al.* (1993) and Myerson and Kline (1983).

It is important to note that the Langmuir isotherm has been developed for reversible processes, in which desorption is possible. This can be clearly seen in Equation 2.5 where the rate of adsorption is equated to the rate of desorption at equilibrium.

Myerson and Kline (1983) investigated *Thiobacillus ferrooxidans* growth in shake flasks with coal or controlled pore glass. They varied the initial bacterial concentration and observed the bacterial concentration in solution at steady state (Table 2.18 and 2.19). They observed that

at low initial cell concentrations all the bacteria were adsorbed onto the solid surface. Myerson and Kline (1983) thus concluded that the adsorption was irreversible. This was supported by their desorption experiments in which no bacteria were detected in suspension even with variations of pH and agitation (section 2.6.1.2). As a consequence they did not use the Langmuir isotherm to model their data. Instead the maximum cell concentration on the solid surface was obtained from Tables 2.18 and 2.19, where the adsorbed bacterial concentration is seen to approach a limiting value. The results of the maximum cell concentration are shown in Table 2.20.

Table 2.18. Adsorption of *Thiobacillus ferrooxidans* onto coal.

Initial bacterial concentration (μg protein/ml)	Bacterial concentration at equilibrium (μg protein/ml)	Bacteria adsorbed on coal (μg protein/g coal)
54.4	0	267
123	0	615
191.5	30.2	807
250.3	93.4	783
533.6	360.8	863

Table 2.19. Adsorption of *Thiobacillus ferrooxidans* onto controlled pore glass.

Initial bacterial concentration (μg protein/ml)	Bacterial concentration at equilibrium (μg protein/ml)	Bacteria adsorbed on controlled pore glass (μg protein/g)
186	0	2325
377	33	4300
461	118	4288
650	300	4375
1283	922	4513

Table 2.20. The maximum adsorption capacity of *Thiobacillus ferrooxidans* to coal and controlled pore glass.

	Coal	Controlled pore glass
Number of cells adsorbed	4.09×10^{10} cell/g	2.18×10^{11} cell/g
Surface area	5 m^2/g	187.1 m^2/g
Surface area occupied per cell	122 μm^2/ cell	858 μm^2/ cell
Maximum cell concentration	8.2×10^9 cells/m^2	1.2×10^9 cells/m^2

With this concept of irreversible attachment, Myerson and Kline (1983) state that when there is a finite concentration of bacteria in solution with a contact time between the bacteria and the particle greater than 30 minutes, the maximum possible concentration of bacteria will be attached to the solid particles. The maximum concentration can be limited by the number of free bacteria or the surface area available for attachment.

The concept of irreversible attachment is supported by Myerson and Kline (1983) and

Bagdigian and Myerson (1986). Myerson and Kline (1983) came to this conclusion by noting that when the initial cell concentration was low all the bacteria adsorbed onto the mineral surface. Bagdigian and Myerson (1986) found that up to 100% of the bacteria were adsorbed provided that sufficient coal was present. The concept of reversible attachment has also been observed in literature. Bailey and Hansford (1993) found that the free bacterial population was restored after free cell washout in a fluidized bed reactor. They concluded from their results that the bacteria detached from the mineral surface to repopulate the solution. Reversible attachment and specifically an equilibrium between the attached and free cell populations has also been noted by Chang and Myerson (1982), Asai *et al.* (1992) and Konishi *et al.* (1992).

Thus, from the literature it seems that the maximum covering depends on the particle type and particle size used. The maximum extent of adsorption is summarized for various particle types and particle sizes in Table 2.21.

Table 2.21. The maximum extent of adsorption.

Author	Type of bacteria	Particle type	Particle size μm	Maximum adsorption capacity	
				cells/m^2	cells/g ore
Chang and Myerson (1982)	*Thiobacillus ferrooxidans*	Pyrite	-	1.2×10^{11}	
Konishi *et al* (1992)	*Thiobacillus ferrooxidans*	ZnS	-53+35	3.5×10^{11}	1.65×10^{10}
Asai *et al* (1992)	*Thiobacillus ferrooxidans*	Pyrite	-44+25		6.61×10^{10}
			-63+53		2.50×10^{10}
			-88+63		1.41×10^{10}
			-177+149		0.91×10^{10}
Myerson and Kline (1983)	*Thiobacillus ferrooxidans*	Coal	< 63	8.2×10^{9}	
		pore glass	177	1.2×10^{9}	

2.8. CONCLUSIONS

Enumeration of the number of bacteria in a biooxidation system is hindered by the mineral ore and inorganic precipitants found in the system. Methods of enumeration which have been reported in literature include cell counting, desorption/dislodgement of attached bacteria, fluorescent staining of attached bacteria, dry weight determination, protein analysis, nitrogen analysis, carbon analysis, monitoring the oxygen utilisation rate, uptake of radioactively labelled compounds ($^{14}CO_2$ and ^{32}P), and most probable number (MPN). Some of these methods are affected by the fine ore particles and inorganic precipitants present in the system and require lengthy washing steps be performed prior to analysis.

Although the authors have reported various methods of enumerating the bacteria, a study of the different methods applied to the same system has not been reported. An aim of this study is to investigate and compare different methods for enumerating the bacterial population in a biooxidation system. One of the techniques used involves measuring the metabolic activity of the bacteria by means of oxygen utilisation rate measurements. The measurement of oxygen utilisation rate has been used, in various studies, to give an indication of the physiological conditions of bacteria and fungi (Torma *et al.*, 1991). In this study the use of oxygen utilisation rate has been extended to enumerate bacterial population in a biooxidation system. Le Roux *et al.* (1973) and Norris *et al.* (1988) have studied the use of oxygen utilisation rate in a biooxidation system by they concentrated mainly on the free bacteria. This study has furthered the use of oxygen utilisation rate to quantify both free and attached bacteria populations.

The literature has applied the enumeration methods to systems with essentially pure mineral ores such as pyrite and chalcopyrite with a *Thiobacillus ferrooxidans* culture. The ore that is used in this study is a complex arsenopyrite-pyrite concentrate containing carbonate with a mixed bacterial culture. This ore system provides a challenging system in which to compare the enumeration methods.

CHAPTER 3

EXPERIMENTAL METHODS

3. EXPERIMENTAL METHODS

Quantification of the total bacterial population (free and attached) as well as determination of the percentage attachment in a minerals biooxidation system was investigated. The different methods used to enumerate the number of bacteria in the biooxidation system include direct observation of the mineral ore and indirect measurements of a component of the cell or of the metabolic activity of the bacteria. Direct methods used include scanning electron microscopy and the desorption of the bacteria from the mineral ore. The cellular components of the cell which were monitored include protein, nitrogen and carbon and the metabolic activity of the bacteria was measured by monitoring the oxygen utilisation rate of a sample.

3.1. SOURCE OF BACTERIA

A two stage, continuous biooxidation miniplant provided actively growing bacteria. The miniplant consists of two 23 ℓ, baffled, agitated, aerated bioreactors in series operating under controlled conditions of temperature, pH, solids concentration and nutrient supply. Typically the bioreactors operate at a temperature of 40°C, a pH of 1.65 and a solids concentration of 20%. The bioreactors are fed from a 10ℓ, baffled agitated conditioning vessel to which the nutrient solution and dry solids concentrate are added.

The biooxidation system treated an arsenopyrite-pyrite concentrate from Fairview Gold Mine, Barberton, South Africa. Of the feed material, 86.35% was finer than 75 μm. The elemental composition of the mineral ore is given in Table 3.1. The mineral analysis as calculated from the elemental analysis is given in Table 3.2.

Table 3.1. The elemental analysis of the Fairview flotation concentrate.

	Mass percent (%)
As	5.84
S	21.71
Fe	24.01
C	1.41

Table 3.2. The mineral analysis of Fairview flotation concentrate.

	Mass percent (%)
Arsenopyrite	12.7
Pyrite	37.2

The bacteria present in the biooxidation system are *Leptospirillum ferrooxidans*, *Thiobacillus thiooxidans* with the absence of *Thiobacillus ferrooxidans*. The bacterial composition in the bioleaching tanks was identified by Rawlings (1995). The technique used by Rawlings involved the restriction enzyme analysis of restriction enzyme fractions of PCR (polymerase chain reaction) amplified 16S rDNA prepared from the chromosomal DNA of the mixed culture and the pure cultures of *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. Throughout this thesis the mixed culture will be referred to as thiobacilli.

3.2. PREPARATION OF BACTERIAL SAMPLES

Before the number of bacteria in the biooxidation suspension could be enumerated, the bacteria were harvested from the miniplant. The free cells were washed to remove any precipitants and inorganic ions which could interfere with the assaying methods. For the protein and total organic carbon analyses, it was necessary to digest the samples to release the cellular components into solution before analyses could be performed.

3.2.1. Harvesting Procedure

A volume (1 ℓ) of slurry was removed from the miniplant, either from the primary tank or from the overflow of the secondary tank. The slurry was allowed to settle for 1 hour to facilitate easier separation of the solid ore and liquid. The supernatant was decanted and centrifuged (Beckman TJ-6) at 3000 rpm for 10 minutes to remove any remaining ore particles. The solid-free supernatant was then washed before analyses were performed.

The ore was collected from the concentrated slurry after the supernatant had been decanted. The concentrated slurry was either centrifuged at 3000 rpm for 10 minutes to recover the solids or filtered through a Millipore 0.45 μm filter. The solids were either used directly *eg.* for oxygen utilisation rate measurements and nitrogen analysis or digested for protein and total organic carbon analyses.

3.2.2. Washing procedure

The free cells were washed to remove inorganic precipitants and ions associated with the cells. The cells were centrifuged from the supernatant at 15000 rpm (17600g) in a Beckman JA-20 centrifuge. The bacterial pellet was then resuspended in 100 ml acidified (with concentrated sulphuric acid) distilled water at pH 1.8. The resulting suspension was left to stand in a refrigerator for 4 hours to allow the precipitants to settle. The bacterial suspension was decanted from the settled precipitants and re-centrifuged at 15000 rpm (17600g). The washing procedure was repeated at least 3 times. The final bacterial pellet was either

resuspended in distilled water or acidified distilled water.

3.2.3. Digestion Procedure

The bacteria and ore were digested in 0.1 N NaOH solution. The samples (volume of 5 ml for washed cell suspension or between approximately 0.1 and 1 g of wet ore) were boiled in 40 ml NaOH solution for 45 minutes to lyse the cells. The solution was then cooled and filtered (Millipore filtered, 0.45 μm) to remove the solid ore particles and the cell debris. The resultant supernatant was analysed for either total soluble protein or total organic carbon.

3.3. QUANTIFICATION OF BACTERIAL POPULATION

The experimental methods, which were used to measure the number of bacteria free in solution and attached to the mineral ore, include total microscopic counts, gravimetric determination of dry mass, desorption, chemical oxygen demand (COD), ashing, protein, nitrogen, total organic carbon and oxygen utilisation measurements.

3.3.1. Cell Counts

Total cell counts were performed using a Neubauer Improved cell counting chamber and a phase contrast Nikon microscope. An average of 100 to 150 bacteria was counted per sample at a magnification of 400x and each sample was counted twice. Samples were diluted either 1 in 100 or 1 in 20 or 1 in 10 with acidified (acidified with concentrated sulphuric acid) distilled water of pH 1.8. A maximum error of 10% was obtained for the cell counts.

3.3.2. Gravimetric Determination of Dry Cell Weight

The dry weight of a washed cell suspension was determined by centrifuging the bacterial suspension in a micro laboratory centrifuge at 10000g. The microfuge tube was first dried in an oven at 100°C for 24 hours and then allowed to cool in a desiccator prior to weighing to 4 decimal places. After centrifuging the supernatant was decanted and the microfuge tube with the bacterial pellet was placed in an oven at 100°C and left for 24 hours. The microfuge tube and the dried bacterial pellet were then allowed to cool in a desiccator prior to weighing. A maximum error of 5% was obtained for dry cell weight measurements.

3.3.3. Desorption

A volume of suspension (100 ml or 25 ml) was taken from the miniplant and centrifuged at 3000 rpm for 10 minutes to remove the ore. The ore was resuspended in fresh media (9K, Appendix A) with or without ferrous iron. For the media without ferrous iron, acidified distilled water was added to the basal salts media instead of the ferrous iron solution. The resuspended ore was shaken for 5 minutes to keep the ore in suspension. Next the ore was allowed to settle for 1 hour in an ice bath. The ore was then separated from the desorbed cells by centrifuging at 3000 rpm for 10 minutes. This method was repeated five to seven times. The number of cells desorbed was obtained by counting the free cells in the centrifugate. The dry weight of the ore was obtained gravimetrically by placing the ore in an oven (100 °C) overnight at the end of the experiment.

3.3.4. Chemical Oxygen Demand (COD)

The chemical oxygen demand (COD) of a sample of washed cell suspension was performed as follows (American Public Health Association, 1975):

A. A small amount of $HgSO_4$ (spatula tip) was placed in each test tube to avoid chlorine interference.

B. 2 ml sample was added to each test tube.

C. 3 ml Ag_2SO_4 and H_2SO_4 solution and 1 ml 0.25 N $K_2Cr_2O_7$ solution were then

added to each test tube (Appendix B).

D. The test tubes were sealed and shaken.

E. The test tubes were refluxed for 2 hours, before cooling.

F. The resulting solution was titrated with 0.1 N ferrous ammonium sulphate solution (FAS) using 3 drops of 1,10-phenanthroline-ferrous complex solution as the indicator.

G. The COD of the sample was calculated from the following formula:

$$\text{COD (mg/l)} = \frac{(a-b)N \times 8000}{\text{ml sample}} \qquad [3.1]$$

where a is the volume (ml) of FAS used for the blank
b is the volume (ml) of FAS used for the sample
N is the normality of the FAS solution (0.1 N)

As the samples were prepared in acidified water, a blank of acidified distilled water was used. The solution preparations are given in Appendix B.

3.3.5. Ashing

A suspension of washed cells (2 ml) or wet ore (between 0.1 and 1 g) was placed in a dry preweighed petri dish. The petri dish was placed in an oven at 100°C overnight. The petri dish with its contents was cooled in a desiccator before being weighed. The petri dish was placed in an oven at 600°C for 4 hours. The petri dish was then cooled in a desiccator and weighed when cold. The change in weight of the petri dish between 100°C and 600°C is the ashable content of the sample. The cell number in the cell suspension was counted microscopically to calculate the ashable content of a cell.

3.3.6. Protein

The protein analyses used, include the Lowry, Peterson and Coomassie Blue methods. The detailed methodology of these techniques is given in Appendix C. The Lowry and Peterson methods use a phosphomolybdic-phosphotungstic reagent (Folin-Ciocalteu reagent) which is reduced by the copper-treated protein to produce reduced species which have a characteristic blue colour. Coomassie Blue protein assay is a protein dye-binding assay. The dye changes colour from a red to a blue when binding to a protein molecule. All the protein assays used bovine serum albumin as the standard. The spectrophotometer used to measure the absorbance was a Varian Cary 1E UV-Vis spectrophotometer. Maximum errors in the Lowry assay of 10%, in the Peterson assay of 10% and in the Coomassie Blue assay of 5%, were obtained.

The protein analyses require that the protein be in solution. To release the total soluble protein from the bacteria, digestion of the cells in 0.1 N NaOH solution was used. This digestion procedure is described in Section 3.2.3.

3.3.7. Nitrogen

Nitrogen analysis was performed on leached and unleached arsenopyrite-pyrite concentrate using the Kjeldahl technique. This was done by A L ABBOTT and ASSOCIATES (PTY) LTD, Cape Town. This technique was only performed once on the leached and unleached ore and hence the accuracy for the method could not be determined. The Kjeldahl procedure involves the digestion of the sample with concentrated sulphuric acid to convert the nitrogen to ammonium sulphate in solution. By adding an alkaline (sodium hydroxide), ammonia can be steam-distilled off and dissolved in a known concentration of hydrochloric acid (0.1 N). The hydrochloric solution is then titrated with 0.1 N sodium hydroxide to measure the amount of ammonia dissolved in the hydrochloric acid. The amount of ammonia released is equated to the amount of nitrogen in the sample.

3.3.8. Total Organic Carbon (TOC)

Samples were analysed for total organic carbon (TOC) using an ANATOC ™ total organic carbon analyser. The total organic carbon analyser operates by oxidising the organic compounds in the aqueous medium photocatalytically to produce carbon dioxide. This oxidation is achieved by illuminating the sample with near-ultraviolet radiation (300-400 nm) in the presence of a titanium dioxide catalyst. The carbon dioxide is then dissolved in deionised water and quantified by measuring the conductivity of the water. The operating range of the analyser is between 10 and 80 μg C per sample. A maximum error of 3% was obtained using the total organic carbon analyser.

Sample preparation involved the alkaline digestion of the bacteria either free in suspension or associated with the ore to which they are attached (Section 3.2.3). The TOC analyser required that the samples be at a pH of approximately 3.5 $\pm$ 0.2. The pH of the sample was adjusted using 0.1 M perchloric acid. A volume of 1 ml of digested sample was injected into the analyser and the total organic carbon content of the sample read.

3.3.9. Oxygen Utilisation Rate

Oxygen utilisation rate measurements were carried out in a biological oxygen monitor (BOM). The biological oxygen monitor (BOM) consists of a 125 ml conical glass flask with a custom made side port for the dissolved oxygen probe and a rubber stopper with two ports for a thermometer and a vent tube (Figure 3.1). The sample was saturated with oxygen and the rate at which the oxygen was utilised by the bacteria was monitored. A Yellow Springs model number 5739 dissolved oxygen probe and a Hitech Micro Systems Dissolved Oxygen / Utilisation Rate Meter was used. The Hitech Micro Systems Dissolved Oxygen / Utilisation Rate Meter was designed and manufactured in the Department of Chemical Engineering at the University of Cape Town.

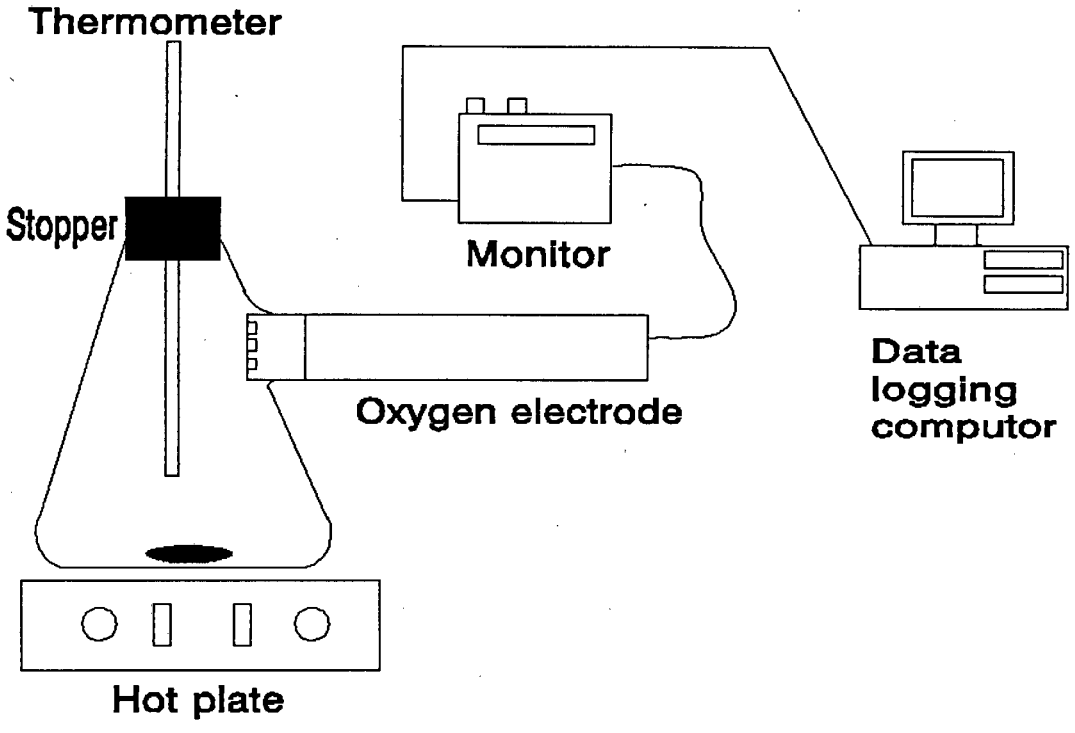


Figure 3.1. Biological oxygen monitor.

The oxygen probe was calibrated by first zeroing it with a solution of sodium sulphite. The probe was left in the sodium sulphite solution until the dissolved oxygen concentration became constant. The zero was then adjusted until the monitor displayed a dissolved oxygen concentration of zero. A flask of distilled water was then agitated for 1 hour to become saturated with oxygen. The temperature of the solution was monitored and the gain adjusted to the saturation oxygen concentration for that temperature.

To measure the maximum specific oxygen utilisation rate it is essential that the energy source, Fe^{2+}, is not limiting. To determine the required concentration, 5 ml of a washed cell suspension was pipetted into each of several flasks containing varying ferrous iron concentration (between 0 and 14 g Fe^{2+}/ℓ). The solution was stirred with air bubbling through the sample to allow the sample to become saturated with oxygen. The flask was then plugged with a stopper and the dissolved oxygen concentration monitored with time. The oxygen utilisation rate was calculated from the slope of the curve of the dissolved oxygen against time. The temperature of the solution at which the oxygen utilisation measurements were made was 30°C. Errors of between 2 and 13% were obtained for the oxygen utilisation

rate measurements.

In the ensuring studies, the maximum oxygen utilisation rate of an ore sample or a free bacterial suspension was measured by placing the sample (between 0.7 and 1.7 g ore or 5 ml free bacterial suspensions) in a 12 g Fe^{2+}/ℓ solution and measuring the dissolved oxygen concentration with time.

3.4. EXPERIMENTAL ANALYSES

Various experimental analyses were used throughout the study. These experimental analyses include scanning electron microscopy (SEM), microanalysis, size analysis and atomic absorption spectroscopy (AAS).

3.4.1. Scanning Electron Microscopy (SEM)

To visualise bacteria attached to leached ore by scanning electron microscopy, the bacteria on the ore were fixed using the glutaraldehyde fixation technique. The sample was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 48 hours. The phosphate buffer preparation is given in Appendix A. The glutaraldehyde was decanted and traces removed by washing. The sample was placed in a 0.1 M phosphate buffer for 10 minutes. The phosphate buffer was decanted and the washing step repeated. To remove water from the sample, dehydration was achieved by allowing the sample to stand in each of the following ethanol solutions for 10 minutes successively: 30% ethanol, 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol and twice in 100% ethanol for 15 minutes. Critical point drying was then performed in which the water and ethanol were replaced with liquid CO_2, which was then vapourised without causing heat or pressure damage. The samples were mounted on aluminium stubs covered with a mixture of water-based glue and colloidal carbon and then covered with a thin layer of Au/Pd.

A Cambridge S200 scanning electron microscope was used to take micrographs of the bacteria attached to leached ore. The SEM operating parameters are given in Table 3.3.

Table 3.3. The SEM operating parameters.

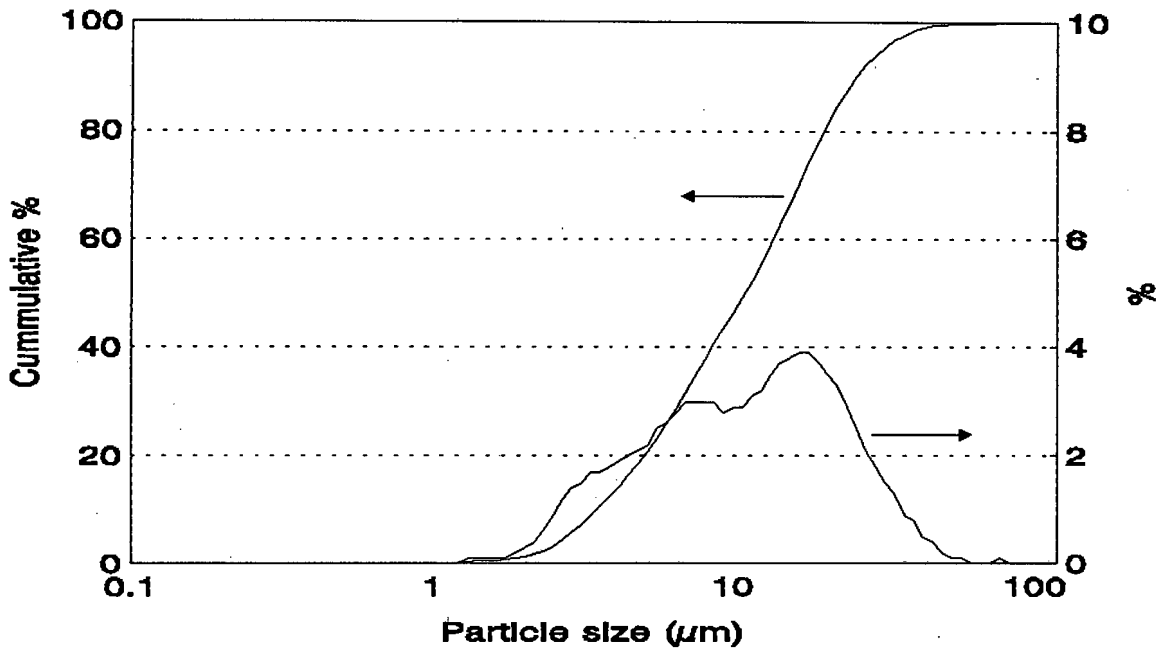
Accelerating voltage	15 keV
Aperture	30
Tilt angle	30°
Resolution	9
Working distance	10-12 mm

3.4.2. Microanalysis

The free bacteria grown on 9K media (Silverman and Lundgren, 1959) were analysed for carbon, nitrogen and hydrogen using a Fisons EA 1108 Elemental Analyser. The sample is combusted in pure oxygen in the presence of copper. The combustion products, CO_2, H_2O and nitrates, are then separated and identified in a gas chromatograph.

3.4.3. Size Analysis

The particle size analysis was determined using the Malvern Laser Master-Sizer Diffraction method. This method utilises the fact that particles suspended in water will diffract the laser beam on passage through the cell and the angle of diffraction will correspond to the diameter of the particles. A dried sample of ore was placed in a stirred ultrasonic bath until the obscuration level was at an acceptable level. The particle size distribution could then be read. A size distribution of leached arsenopyrite-pyrite concentrate from the secondary tank is given in Figure 3.2.



High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	Span 2.02
118	100	53.3	99.8	24.0	87.7	10.8	49.2	4.84	18.5	2.18	1.7	D[3,2] 13.16 μm
110	100	49.5	99.7	22.3	84.8	10.0	46.3	4.50	16.4	2.03	1.3	
102	100	46.1	99.5	20.7	81.5	9.31	43.4	4.19	14.4	1.88	1.0	D[3,2] 7.45 μm
95.2	100	42.8	99.1	19.3	78.0	8.66	40.6	3.89	12.5	1.75	0.8	
88.6	100	39.8	98.6	17.9	74.3	8.05	37.6	3.62	10.7	1.63	0.7	
82.4	100	37.0	97.8	16.7	70.4	7.49	34.6	3.37	9.0	1.51	0.6	D[v,0.9] 25.62 μm
76.6	100	34.4	96.9	15.5	66.5	6.97	31.6	3.13	7.3	1.41	0.5	
71.2	99.9	32.0	95.6	14.4	62.7	6.48	28.6	2.91	5.8	1.31	0.4	
66.2	99.9	29.8	94.1	13.4	59.0	6.02	25.8	2.71	4.4	1.22	0.3	D[v,0.1] 3.51 μm
61.6	99.9	27.7	92.3	12.5	55.5	5.60	23.2	2.52	3.2			
57.3	99.9	25.8	90.2	11.6	52.3	5.21	20.7	2.34	2.3			
Source = : Sample Focal length = 63 mm Presentation = pil				Beam length = 10.0 mm Log. Diff. = 2.737 Obscuration = 0.2683 Volume distribution				Model indp Volume Conc. = 0.0078% Sp. S.A. 0.8053 m²/cc				D[v,0.5] 10.97 μm Shape OFF

Figure 3.2. A size distribution of leached arsenopyrite-pyrite concentrate from the secondary tank with the miniplant operating at a residence time of 15 days.

3.4.4. Atomic Absorption Spectroscopy (AAS)

Total iron analysis of the supernatant of digested ore samples was carried out on a Varian SpectrAA-30 spectrometer attached to a DS-15 data station. The samples were digested in 0.1 N sodium hydroxide as described in Section 3.2.3. An error of 5% was obtained with the atomic absorption spectroscopy.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Various techniques used in the literature to enumerate the number of bacteria in an arsenopyritic biooxidation system have been studied to quantify the free and attached bacterial population. These include direct visualisation of the micro-organisms or indirect quantification of their presence through measurement of cell components or of the metabolic activity of the population. The cell components measured include protein, nitrogen, organic carbon or a mixture of these through chemical oxygen demand. The metabolic activity was measured by means of the maximum specific oxygen utilisation rate.

In this section, the results obtained for the various methods are presented and discussed. The results presented in this thesis are averaged or typical results obtained for that method. Each method was performed at least three time to evaluate the reproducibility and accuracy of the experiment. The only exception to this was the nitrogen analysis, which was only performed once. With methods such as protein, total organic carbon and ashing blanks were used to assess if the results obtained equalled that, which was expected. The accuracy of the different experimental methods is outlined in Chapter 3.

This section also presents the calculation of the maximum possible number of bacteria that can attach to the mineral surface. Finally, a comparison of the results obtained for the different enumeration techniques is given.

4.1. CHARACTERISATION OF THIOBACILLI

The bacteria were characterised by their dry mass per cell and elemental composition.

4.1.1. Dry Cell Mass

The dry mass of a cell was calculated from ashing experiments. The cell concentration of the bacterial suspension was obtained by cell counts. A dry cell mass of $1.18\times10^{-10} \pm 0.2\times10^{-10}$ mg per cell was measured. This agrees with that reported in literature by Gormely and

Duncan (1974), Silverman and Lundgren (1959) and Tuovinen and Kelly (1973) who found dry cell weights for *Thiobacillus ferrooxidans* of 1.12×10^{-10}, 1.36×10^{-10} and 1.55×10^{-10} mg/cell, respectively.

4.1.2. Microanalysis

An elemental analysis of the free bacteria grown in a batch culture on ferrous sulphate, was performed using microanalysis. The results are given in Table 4.1.

Table 4.1. Elemental analysis of the mixed thiobacilli culture grown on ferrous sulphate.

Element	mass % sample 1	mass % sample 2	mass % sample 3	mass % sample 4	Average mass %	Jones and Kelly (1983) continuous culture (mass %)	Jones and Kelly (1983) batch culture (mass %)
Carbon	46.50	47.29	46.26	46.40	46.61±0.46	48.0	74.58
Hydrogen	7.11	7.06	6.98	6.88	7.00±0.10	7.13	10.05
Nitrogen	10.96	10.96	11.04	10.94	10.98±0.04	9.57	7.32

A cell composition of 46.61 mass % carbon, 7.00 mass % hydrogen and 10.98 mass % nitrogen was obtained. These results agree closely with the elemental composition obtained by Jones and Kelly (1983) for *Thiobacillus ferrooxidans* grown on ferrous sulphate (Table 4.1). The differences obtained may result from the different bacterial cultures or culture conditions. Jones and Kelly (1983) used a pure culture of *T. ferrooxidans* while in this study a mixed culture of thiobacilli was used.

The microanalysis results gave a carbon to nitrogen ratio of 4.24 as compared with 4.89 and 3.19 obtained by Gormely and Duncan (1974) for *Thiobacillus ferrooxidans* and Tuovinen

and Kelly (1973) for *T. neapolitanus*, respectively. Although *T. neapolitanus* was not used in this study it was noted that different thiobacilli species have different carbon to nitrogen ratios. Therefore, the carbon to nitrogen ratio of 4.24 obtained for the mixed culture was different from that of a pure culture of *Thiobacillus ferrooxidans*.

The microanalysis results can be used to calculate an empirical formula for the bacterial culture. Assuming the bacterial culture has an elemental composition of carbon, nitrogen, hydrogen and oxygen, an empirical formula $CH_{1.8}O_{0.57}N_{0.2}$ was calculated. This agreed closely with the standard average empirical formula of $CH_{1.8}O_{0.5}N_{0.2}$ for bacteria (Atkinson and Mavituna, 1991).

4.2. SCANNING ELECTRON MICROSCOPY (SEM)

To observe the attached bacteria directly, scanning electron micrographs of leached arsenopyrite-pyrite concentrate were studied. The micrographs in Figure 4.1 show that bacteria are associated with the ore. These bacteria are rod-shaped and S-shaped of size $0.5 \times 1.0\text{-}2.0$ μm. From the size and shape of the bacteria, it was evident that a mixed population, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans*, may exist attached to the mineral surface.

The mineral surface was sparsely populated by the bacteria. It was possible to estimate a cell density per unit area from the micrographs. The cell densities measured from Figure 4.1 (a), (b), (c) and (d) are 3.7×10^{10} cells/m^2, 2.0×10^{11} cells/m^2, 1.2×10^{10} cells/m^2 and 2.0×10^{11} cells/m^2 respectively. The surface area of the ore was not measured and therefore the results could not be reported as cells/g ore. Because Figure 4.1 (b) and (d) are at a high magnification, the small surface area considered did not give a representative picture of the number of attached bacteria. Therefore, an attached bacterial density of between 1.2×10^{10} and 3.7×10^{10} cells/m^2 was estimated.

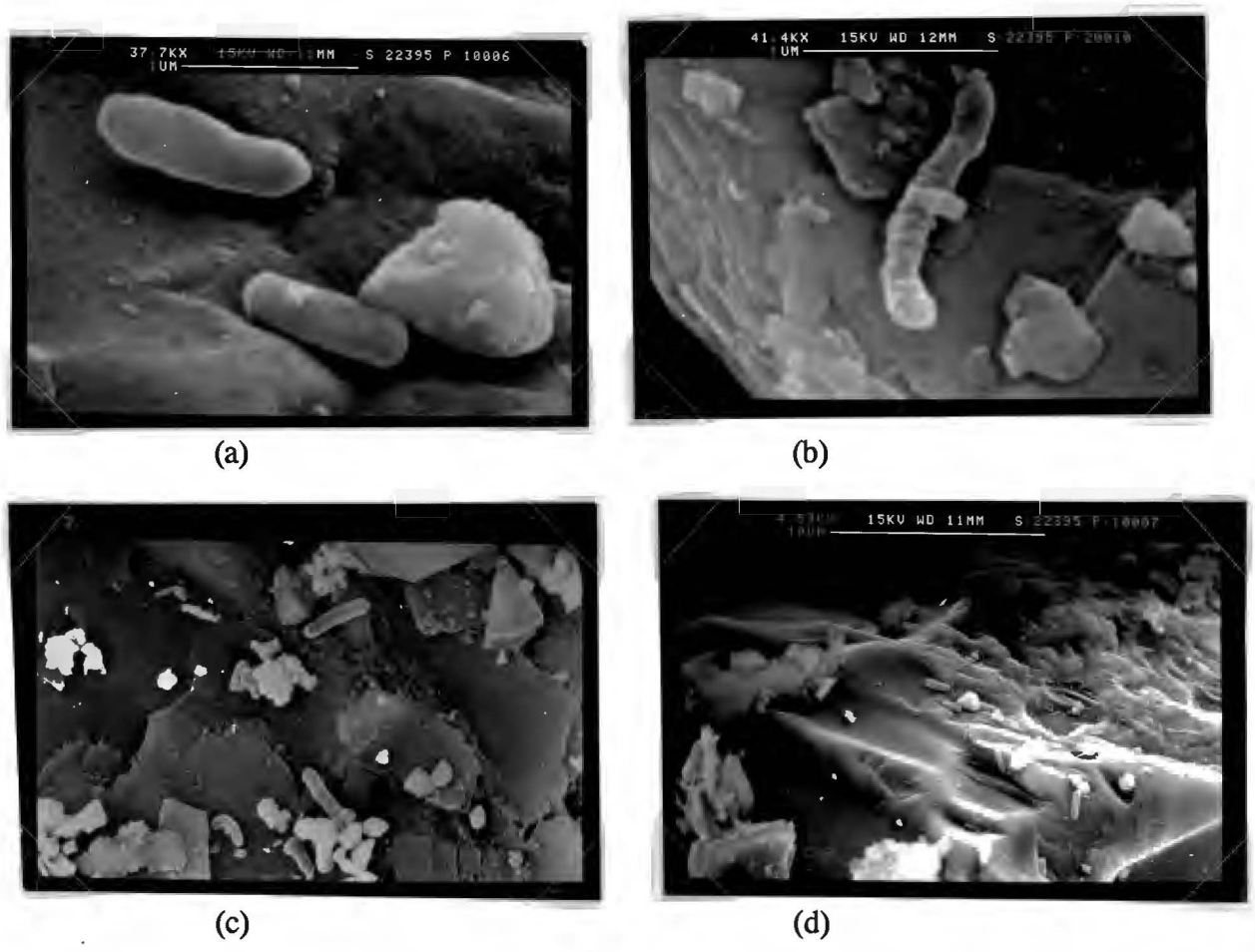


Figure 4.1. Scanning electron micrographs of leached arsenopyrite-pyrite concentrate showing a mixed attached population. The size bar of (a) is 1 μm, (b) is 1 μm, (c) is 2 μm and (d) is 10 μm.

In calculating the number of attached bacteria per unit area, it was important to note that the scanning electron micrographs are a two-dimensional projection of a three-dimensional surface. This means that it was difficult to quantify the surface area to which the bacteria are attached and the surface area was underestimated. Also, only a small sample was viewed. To obtain a representative picture a large area should be viewed.

Berry and Murr (1978) observed the attachment of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* to pyrite (Figure 4.2). Figure 4.2 shows rod-shaped bacteria of sizes between 0.07 and 0.3 μm. The bacteria are notably smaller than the reported sizes for *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* (0.5×1.0-2.0 μm). However, this could result

from a parallax error (a distortion as the sample is not at a right angle to the electron beam) or the incorrect size bar was given. Consequently, cell density per unit area was not calculated.

In Figure 4.2 (b), it is seen that *Thiobacillus ferrooxidans* can populate the pyrite surface as a dense monolayer. *Thiobacillus thiooxidans* do not appear to populate the mineral surface as densely as *Thiobacillus ferrooxidans* (Figure 4.2 (a)).

It is possible that the difference in the bacterial covering the mineral ore in Figures 4.1 and 4.2 could result from the sample preparation. Bacteria could have been lost from the mineral surface during the multiple steps required for the fixation with glutaraldehyde before the samples were mounted (Figure 4.1). Berry and Murr (1978) did not fix the bacteria before they mounted the samples. The absence of washing steps may have led to unattached organisms appearing associated with the ore.

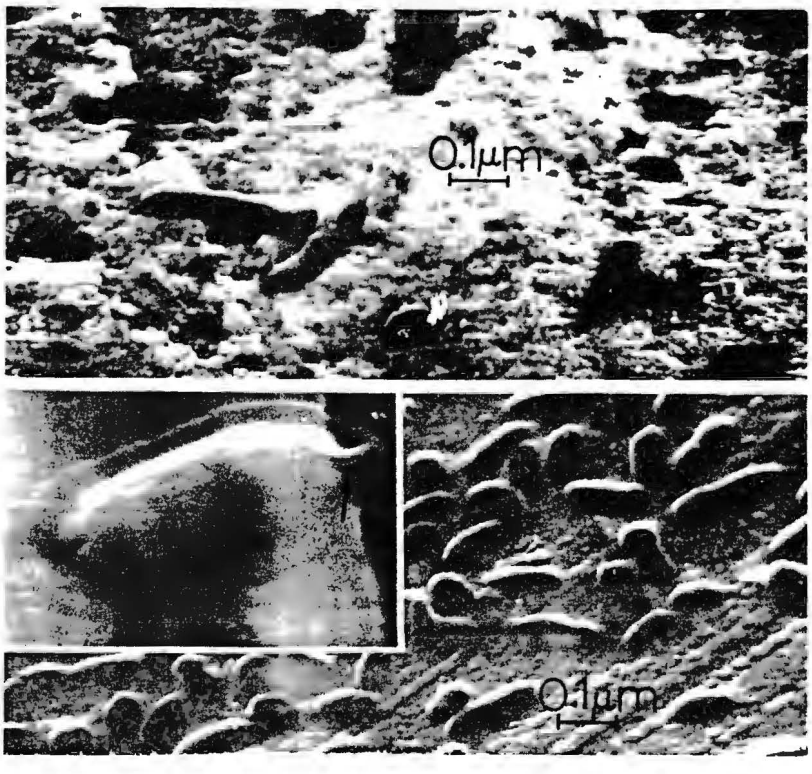


Figure 4.2. Scanning electron micrographs of the attachment of *Thiobacillus thiooxidans* (a) and *Thiobacillus ferrooxidans* (b) to pyrite (Berry and Murr, 1978).

4.3. DESORPTION

Desorption experiments were performed to see if all the attached bacteria could be desorbed from the arsenopyrite-pyrite concentrate and counted directly under a microscope. In this study, desorption of the bacteria from the ore was observed but there have been controversial views reported in the literature whether desorption occurs (Section 2.6.1.2 and 2.7). Desorption of attached bacteria over 8 cycles of washing is shown in Figure 4.3.

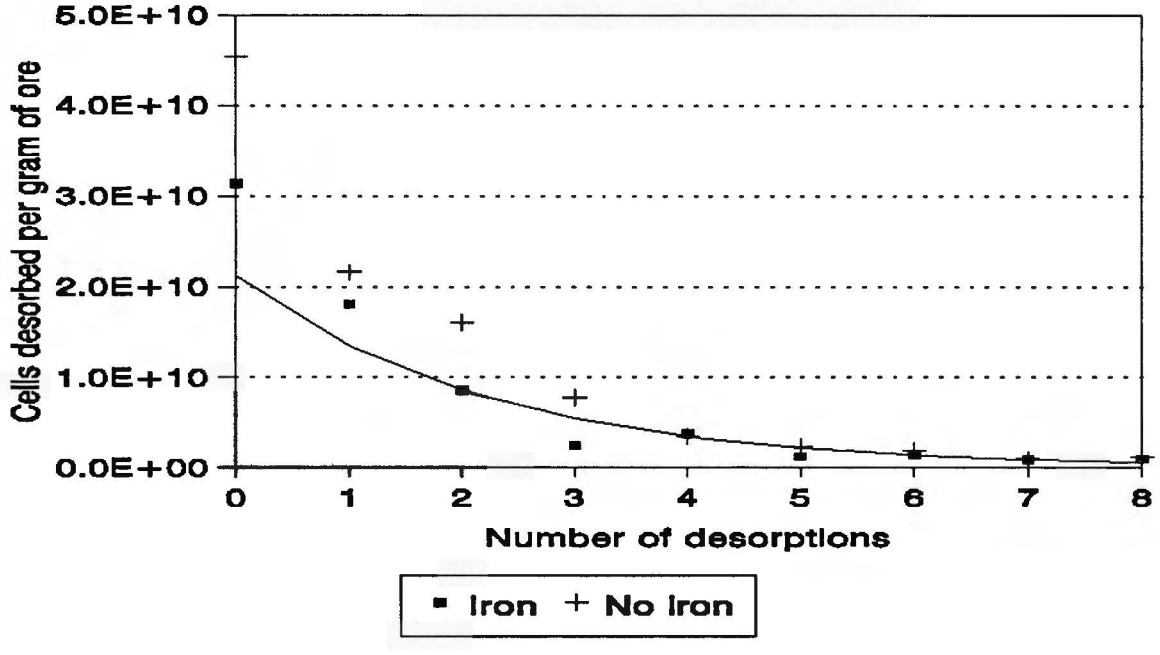


Figure 4.3. Desorption of attached bacteria into media with or without ferrous iron.

Figure 4.3 shows that the number of bacteria desorbed per cycle decreased as the number of desorption cycles increased. This was expected as the number of attached bacteria decreased with each desorption stage and therefore the number of bacteria available for desorption decreased. The number of bacteria first desorbed was 47% of the initial free bacteria. Most of the bacteria had desorbed after 4 desorption steps. The number of bacteria desorbed decreased slightly with each further desorption step.

The desorption experiments were performed with and without ferrous iron in an attempt to encourage desorption by satisfying the affinity of the bacteria to ferrous iron. The addition of ferrous iron to the desorption media was expected to favour the desorption of

Leptospirillum ferrooxidans over *Thiobacillus thiooxidans* as *L. ferrooxidans* can utilise ferrous iron while *T. thiooxidans* cannot. However, the addition of ferrous iron into the media did not enhance desorption as can be seen in Figure 4.3.

The number of attached bacteria can be estimated from the total number of bacteria desorbed. This can be determined by extending the number of desorption cycles by extrapolation. The curve in Figure 4.3 represents an exponential fit of the type Ae^{-Bx}. Although the exponential fit did not seem to correlate well with the first four desorptions, it does correlate well with the last desorption points. Therefore, an exponential fit was used to predict desorption with infinite desorption cycles to enable the calculation of the number of attached bacteria.

The number of bacteria desorbed, with increasing desorption cycles, was summed until a constant value was obtained, which represented the number of attached bacteria before desorption. The number of attached bacteria at the different stages of desorption was calculated from the difference between the number of cells desorbed in that interval and the attached bacteria count at the previous desorption stage. The number of attached bacteria (per gram of ore) was then plotted against the number of free bacteria (per ml) in Figure 4.4. The data in Figure 4.4 approaches a limiting value monotonically, thus confirming that the relationship between the attached and free bacterial populations can be represented by a Langmuir isotherm (shown as curve):

$$X_A = \frac{K_A X_{AM} X_L}{(1 + K X_L)} \qquad [4.1]$$

where X_A is the attached bacterial concentration per unit mass
X_L is the free bacterial concentration per ml
K_A is the adsorption equilibrium constant
X_{AM} is the maximum adsorption capacity per unit mass

The validity of the Langmuir isotherm to a biooxidation system was discussed in Section 2.7. The constants obtained from the Langmuir isotherm fit are given in Table 4.2. The maximum adsorption capacity (X_{AM}) showed that a maximum of between 6×10^{10} and 7.8×10^{11} cells/g ore can adsorb onto the surface of the arsenopyrite-pyrite concentrate. A possible reason for the spread in results could be due to the numerous interruptions in the continuous operation of the miniplant. This could have resulted in more jarosite being associated with the ore and therefore a greater attached population as the bacteria readily adhere to the jarosite.

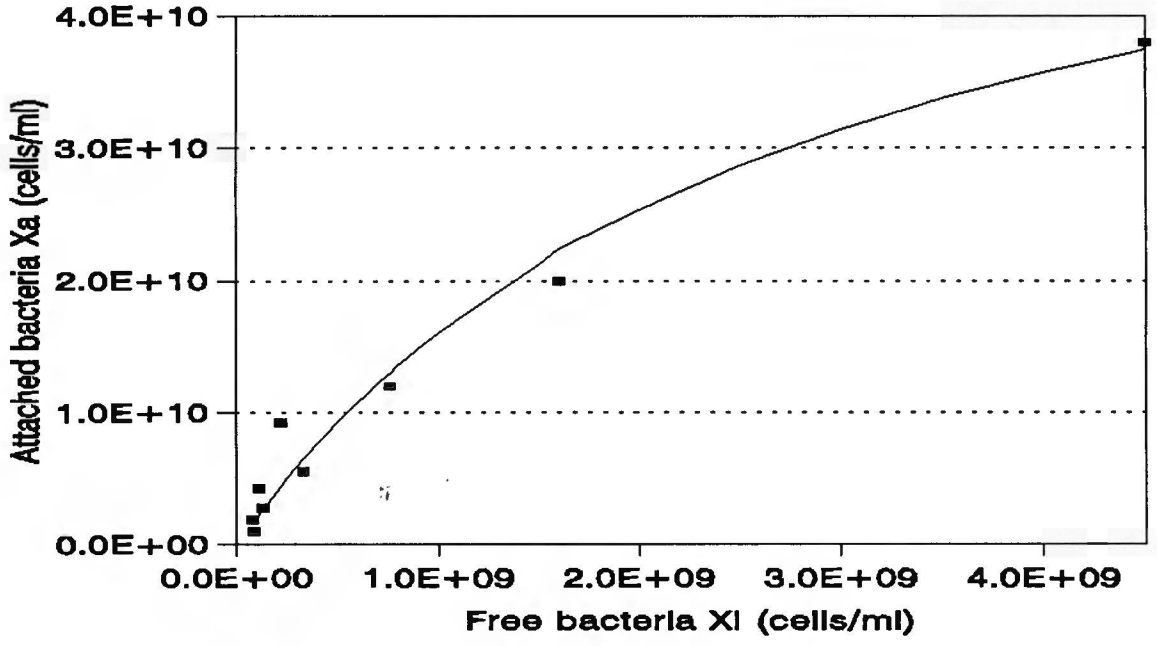


Figure 4.4. Equilibrium relationship between attached and free bacterial populations and the applicability of the Langmuir isotherm to bacterial desorption.

Table 4.2. The Langmuir isotherm constants for a mixed culture of thiobacilli grown on arsenopyrite-pyrite concentrate at a residence time > 15 days for 4 identical desorption experiments.

K_A (ml/cells)	X_{AM} (cells/g)
3.62×10^{-10}	6×10^{10}
4.5×10^{-9}	7.8×10^{11}
5.7×10^{-9}	5.8×10^{11}
5.5×10^{-9}	5.6×10^{11}

Asai *et al.* (1992) suggested that since the rate of bacterial adsorption is higher than the rate of bacterial growth and dissolution, the adsorption equilibrium was achieved during the dissolution process. Therefore, the number of attached bacteria was maintained in equilibrium with the number of free bacteria during leaching. Konishi *et al.* (1992), Asai *et al.* (1992) and Konishi *et al.* (1994) modelled the adsorption by the Langmuir isotherm. The Langmuir isotherm constants obtained in their studies are summarized in Table 4.3.

Table 4.3. The Langmuir isotherm constants as reported in the literature for the adsorption of thiobacilli onto sulphide minerals.

Author	Bacteria	Mineral ore	Particle size	K_A	X_{AM} (cells/g) (ml/cells)
Chang and Myerson (1982)	*Thiobacillus ferrooxidans*	Pyrite		5.8×10^{-10} - 1.62×10^{-9}	
Asai *et al.* (1992)	*Thiobacillus ferrooxidans*	Pyrite	-44+25 μm	4.68×10^{-9}	6.61×10^{10}
			-63+53 μm	3.58×10^{-9}	2.5×10^{10}
			-88+63 μm	4.03×10^{-9}	1.41×10^{10}
			-177+149 μm	5.29×10^{-9}	0.912×10^{10}
Konishi *et al.* (1992)		ZnS	-53+37 μm	5.90×10^{-9}	1.65×10^{10}
Konishi *et al.* (1994)	*Thiobacillus ferrooxidans*	Sulphur	-63+25 μm	2.15×10^{-9}	4.88×10^{10}

The maximum adsorption capacity for the arsenopyrite-pyrite concentrate found by the Langmuir isotherm (6×10^{10} - 7.8×10^{11} cells/g ore) is greater than that predicted from literature for pyrite, zinc sulphide and sulphur. This difference could be due to the type and

size of ore used and a mixed culture of thiobacilli used. As expected, Table 4.3 indicated that the maximum adsorption capacity of the ore was not only a function of the ore but also the particle size. The particle size of the leached arsenopyrite-pyrite concentrate used in the experimental studies reported here can be characterised by 90% smaller than 25.62 μm with 50% smaller than 10.97 μm. From the results of Asia *et al.* (1992), a maximum adsorption capacity in excess of 6×10^{10} cells/g ore is expected for this particle size distribution of the ore.

The desorption experiments cannot give a value for the actual number of bacteria attached to the mineral surface as the point of complete desorption was difficult to define. It did, however, through the Langmuir isotherm show the maximum adsorption capacity of the mineral ore. If the number of attached and free bacteria, are in equilibrium the number of free bacteria can be used to estimate the attached bacterial population. However, should this equilibrium be disturbed by a perturbation to this system, this estimate will be invalid.

4.4. CHEMICAL OXYGEN DEMAND (COD)

The chemical oxygen demand (COD) is a measure of the oxygen equivalent of that portion of the organic matter in a sample that is susceptible to oxidation by a strong chemical oxidant (American Public Health Association, 1975). The chemical oxygen demand has been reported in literature (Section 2.6.2.5) for free cell determination. In this section, the chemical oxygen demand was investigated for use in enumerating the bacterial population in the biooxidation system.

4.4.1. Chemical Oxygen Demand of the Free Bacteria

The chemical oxygen demand (COD) was used to measure the number of bacteria in a washed cell suspension and related to the dry cell mass of the free cell suspension (Figure 4.5). Figure 4.5 shows that the COD of a washed cell suspension is linearly related to the dry weight of the cell suspension. This implies that the COD of a washed cell suspension can be used to enumerate the number of free bacteria. The cells must be washed to remove any

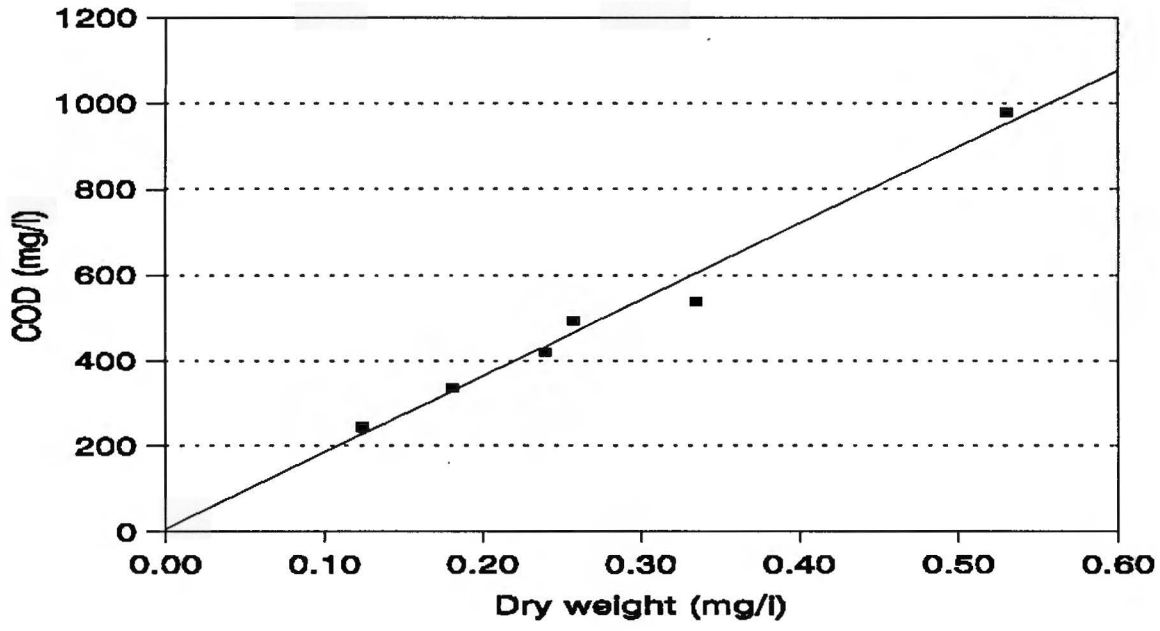


Figure 4.5. The chemical oxygen demand of a washed cell suspension.

interference from inorganic ions and precipitants as they can cause interference with the COD method by consuming additional potassium dichromate.

Atkins *et al.* (1986) used the COD as a measure of the free organic cell content with the organic products of metabolism. For a leach of a Tynagh sphalerite concentrate using *Thiobacillus ferrooxidans*, Atkins *et al.* (1986) found that the bacterial COD increased from 46 mg/ℓ at time zero to a peak of 321 mg/ℓ at time 335 hours. The bacterial COD then declined to a value of 200 mg/ℓ at 500 hours. This bacterial COD curve corresponded to the free nitrogen content measured simultaneously by Atkins *et al.* (1986) to estimate biomass. This implied that the variation in the bacterial COD measured was due to the growth of the bacteria.

In this study, the COD content of the supernatant from the secondary tank was calculated as 142.6 $\pm$ 4.3 mg/ℓ. This was in the range measured by Atkins *et al.* (1986). A COD content of 142 mg/ml corresponds to a dry cell mass of 76 μg/ml (from the linear correlation shown in Figure 4.5) and a cell concentration of 6.4×10^8 cells/ml (the dry mass per cell is given in Section 4.1.1). This free cell concentration was between 4.18×10^7 and 1.5×10^9 cells/ml, which was obtained from cell counts.

4.4.2. Chemical Oxygen Demand of the Attached Bacteria

The COD analysis of an ore sample (leached and unleached) resulted in the potassium dichromate being consumed so that it could not be back titrated. In the COD analysis, the potassium dichromate is used to oxidise the organic matter and the excess potassium dichromate is quantified by titration with ferrous ammonium sulphate. The amount of oxidisable organic matter in the sample is proportional to the potassium dichromate consumed. If the excess potassium dichromate is consumed by the ore, it then not possible to calculate the COD of the attached bacteria.

4.4.3. Conclusions

The chemical oxygen demand of the free cells was linearly related to the dry weight of the bacterial suspension. Thus, the COD analysis can be used to calculate free bacterial concentrations. The COD analysis cannot be used for measuring the attached bacterial concentration.

4.5. ASHING (VOLATILE SUSPENDED SOLIDS VVS)

Volatile suspended solids, measured through ashing, have been successfully applied to a system with a mixed population in waste water treatment (American Public Health Association, 1975). The ashing experiments were performed to measure the number of attached bacteria. It was postulated that the ashable contents of the attached bacteria would volatilize at 600°C while the ore remained uncombusted. Thus, the difference in mass with a knowledge of the ash contents of a cell would enable the calculation of the number of bacteria attached to the mineral ore.

4.5.1. Ashing of the Free Bacteria

On ashing of a bacterial suspension of known cell concentration (a dry cell mass of 0.0069g), a change of mass of 0.0060g was found. Thus, an ash content of the bacteria of 13% by mass was obtained. This agrees with that reported in literature. Lundgren *et al.* (1964), as cited by Rossi (1990), measured the ash content of a *Thiobacillus ferrooxidans* cell of 10%.

4.5.2. Ashing of the Attached Bacteria

Ashing of the arsenopyrite-pyrite concentrate was evaluated in terms of the fractional change in mass of leached and unleached ore as a function of the mass of ore. Figure 4.6 shows no correlation between the mass of the ore and the change in the mass of the ore after ashing, for both unleached and leached concentrates. A maximum change in mass of 0.26 g/g and a minimum change of mass of 0.12 g/g was measured for the unleached concentrate while a maximum and minimum change of mass of 0.11 g/g and 0.07 g/g, respectively, was measured for the leached concentrate. Hence, the "volatile" component of the leached ore is lower than that of the unleached ore.

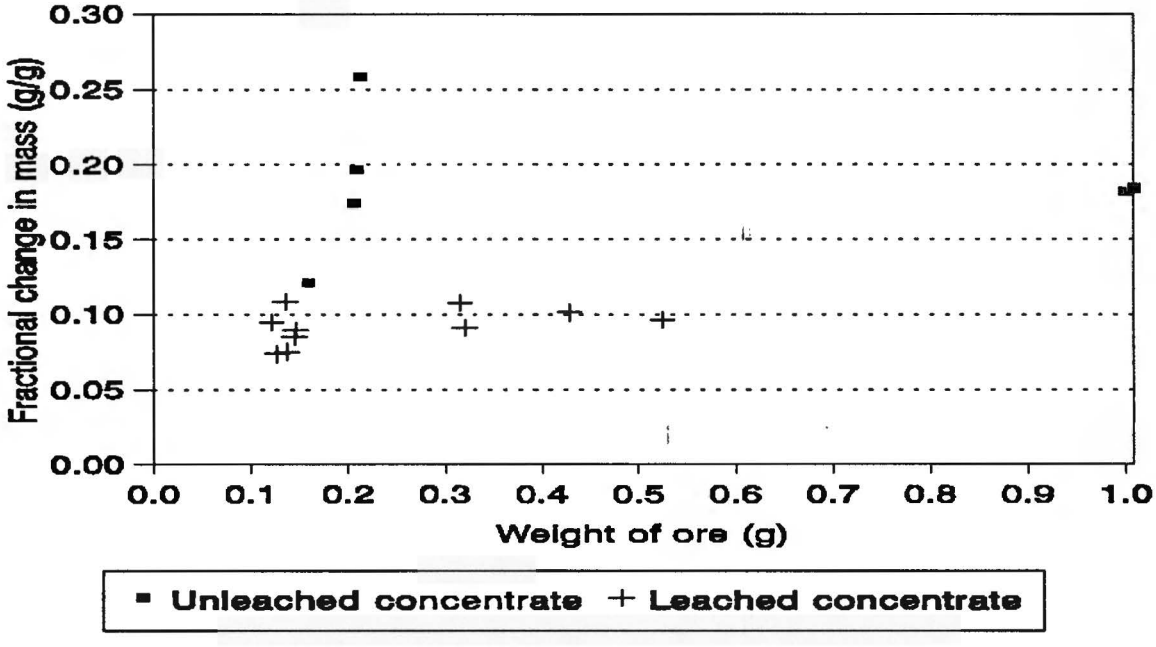


Figure 4.6 The change in mass on ashing of unleached and leached arsenopyrite-pyrite concentrate as a function of dry weight.

The use of ashing to quantify the attached bacterial population was evaluated further using a bacteria-ore suspension made by adding a known bacterial population into a leached or unleached ore suspension. The mass change on ashing was compared with the sum of the mass change of the component parts. The results for unleached and leached arsenopyrite-pyrite concentrates are presented in Figures 4.7 (a) and (b) respectively.

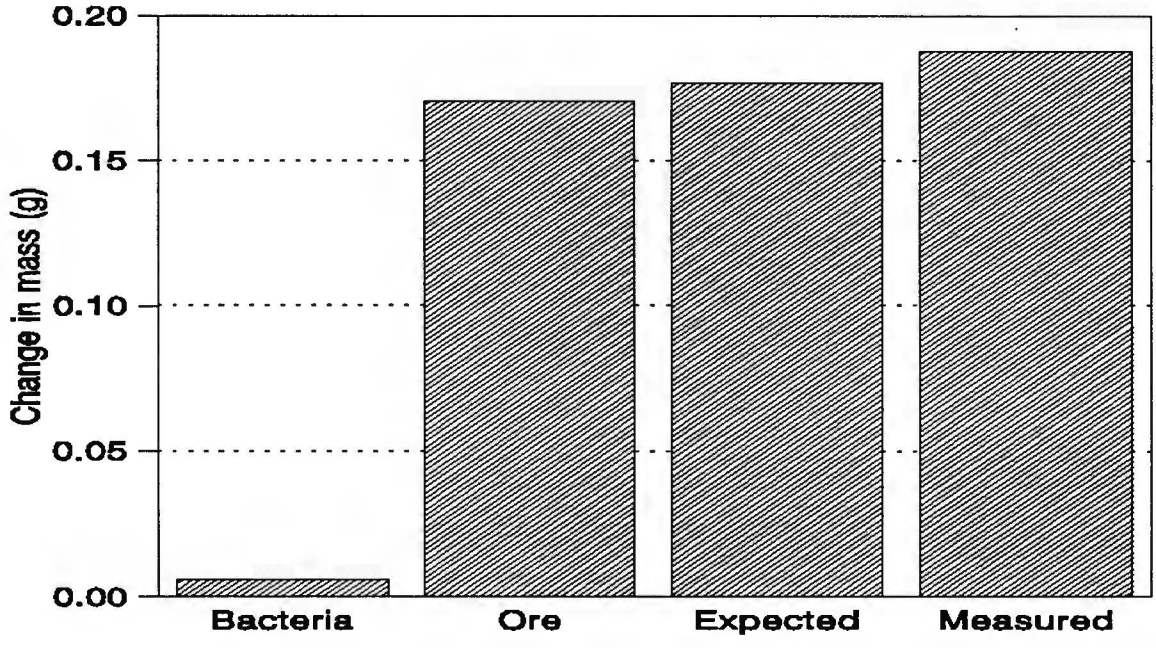


Figure 4.7 (a). The ashing of an ore-bacteria suspension containing unleached concentrate and cells.

In both Figure 4.7 (a) and (b) it can be seen that the measured change in mass does not equal the expected change in mass, calculated from the sum of the change of mass of the component parts. For the unleached arsenopyrite-pyrite concentrate, errors between the measured and expected change in mass of between 7 and 9 % were obtained. For the leached concentrate, errors of between 36 and 50% were obtained. In both cases, the error is approximately equal to the change in mass of the bacteria.

The above results showed that a "volatile" component of the ore was released during ashing. Hence, the hypothesis that only the bacterial fraction is "volatile" or that the ashable component of the ore remains constant on ashing did not hold. Also, the lower contribution of the leached ore to the change in mass than that of the unleached ore, suggested that a "volatile" component was removed during leaching. Furthermore, the change in mass on

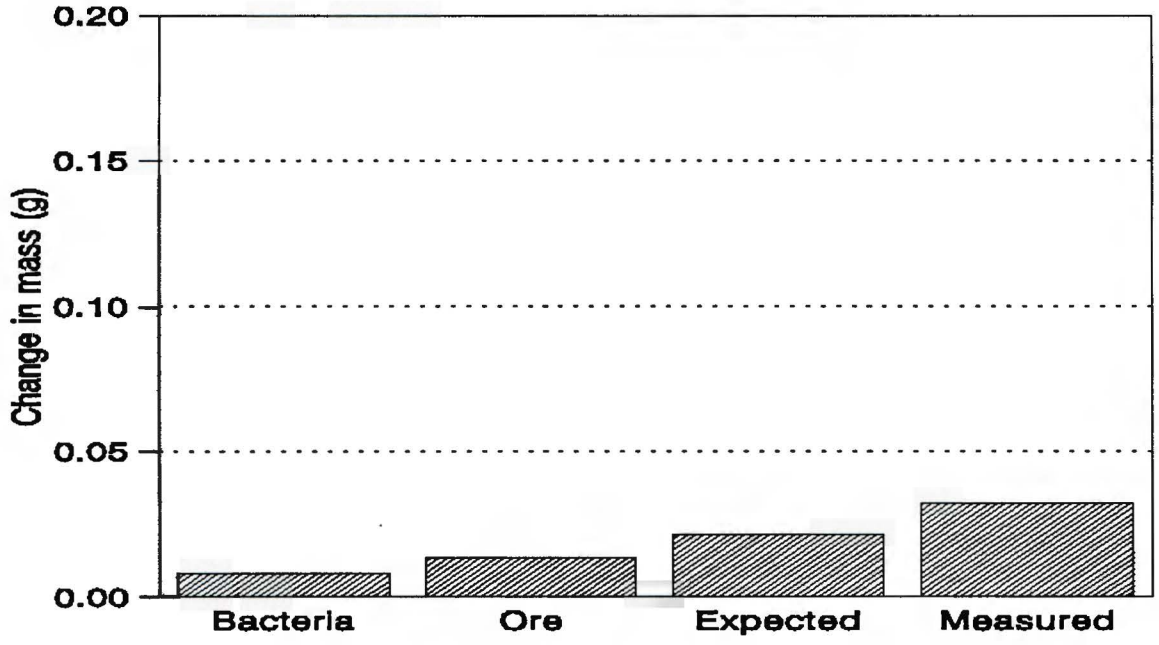


Figure 4.7 (b). The ashing of an ore-bacteria suspension containing leached concentrate and bacteria.

ashing of the bacteria is smaller than the change in mass of the ore. As the former is of interest, errors in the latter may mask the change in mass found for the cells.

The ashing of microbial biomass in the presence of leached ore to calculate the attached bacterial population has not been recorded in the literature. Therefore, a comparison of the results obtained with that of the literature cannot be made.

4.5.3. Conclusions

In investigating the hypothesis that microbial biomass will ash at 600°C without changing the ore, it was found that the ore volatilized to a certain extent. Furthermore, a fixed relationship between the amount of ore ashed and the amount present was not found for either unleached and leached arsenopyrite. The measured change of mass was lower for the leached arsenopyrite with the attached cells than the unleached arsenopyrite, which indicated that a "volatile" component of the ore was removed in the leaching process. As the ashable component of the ore cannot be quantified, this method cannot be used to calculate the

number of attached bacteria.

4.6. PROTEIN

Protein analyses have been used to enumerate bacterial concentrations as discussed in Section 2.6.2.1. The Lowry, Peterson and Coomassie Blue protein assays were investigated for their applicability to an arsenopyrite-pyrite concentrate system. The principle of each protein method is summarized in Section 2.6.2.1. The Peterson protein assay uses the same principle as the Lowry assay.

4.6.1. Protein Release by Alkaline Digestion

The protein must be released from the cell before an assay can be performed. This was done by digesting the cells in 0.1N NaOH as described in Section 3.2.3. To ensure that the digestion time was sufficient for maximum protein release, this parameter was investigated for both a washed cell suspension and leached ore. The results are given in Table 4.4.

Table 4.4. The protein concentration as measured by the Lowry protein assay of a washed cell suspension and leached arsenopyrite-pyrite concentrate given at different digestion times.

	Digestion time (minutes)	Lowry protein results	Peterson protein results
Washed cell suspension	45	15.8×10^{-6} g/ml	-
	30	7.65×10^{-6} g/ml	-
	15	3.53×10^{-6} g/ml	-
Leached arsenopyrite	50	1.24×10^{-3} g/g ore	6.53×10^{-4} g/g ore
	45	1.33×10^{-3} g/g ore	1.46×10^{-3} g/g ore
	30	3.58×10^{-4} g/g ore	3.25×10^{-4} g/g ore
	15	2.02×10^{-4} g/g ore	1.71×10^{-4} g/g ore

Table 4.4 shows that the maximum amount of protein was recorded at a 45 minute digestion time for both the Lowry and Peterson assay. Because the maximum amount of protein was released at a digestion time of 45 minutes, this digestion time was used throughout the study, in contrast to the 15 minute digestion time used by Chang and Myerson (1982), DiSpirito *et al.* (1983) and Myerson and Kline (1983). Murthy and Natarajan (1992) found that a digestion time of 30 minutes was sufficient to release the protein content of a cell into solution.

4.6.2. Protein Analysis of Free Bacteria

The protein content of a cell was determined from the protein concentration of a washed cell suspension measured using the Lowry assay and dry weight measurements. A value of $5.19\times10^{-8} \pm 0.51\times10^{-8}$ μg protein per cell was found. Using a dry cell weight of 1.18×10^{-10} mg per cell (Section 4.2.3), a relative protein content of the cell between 41 and 48% was found. Jones and Kelly (1983) reported a relative protein concentration of between 48-66% for *T. ferrooxidans*. Lundgren *et al.* (1964), as cited by Rossi (1990), found the protein concentration to be 44% of the dry weight for *T. ferrooxidans*.

Myerson and Kline (1983) reported a protein content of 2×10^{-11} mg/cell based on protein analysis following a 15 minute digestion time. This corresponds to a cell protein content of only 17%. This low protein content could be a result of only a fraction of the soluble protein been released during the 15 minute digestion time. Throughout this study, a protein content of 2×10^{-11} mg/cell was used for the conversion of protein results reported in literature using a 15 minute digestion time.

4.6.3. Protein Analysis of the Attached Bacterial Population

In order to evaluate the Lowry and Coomassie Blue protein assays for the determination of biomass concentration in the presence of ore, mixtures of bacteria and leached arsenopyrite-pyrite concentrate were subjected to digestion and protein analysis. The Lowry and Coomassie Blue assays were performed on the same samples. Figure 4.8 shows that the

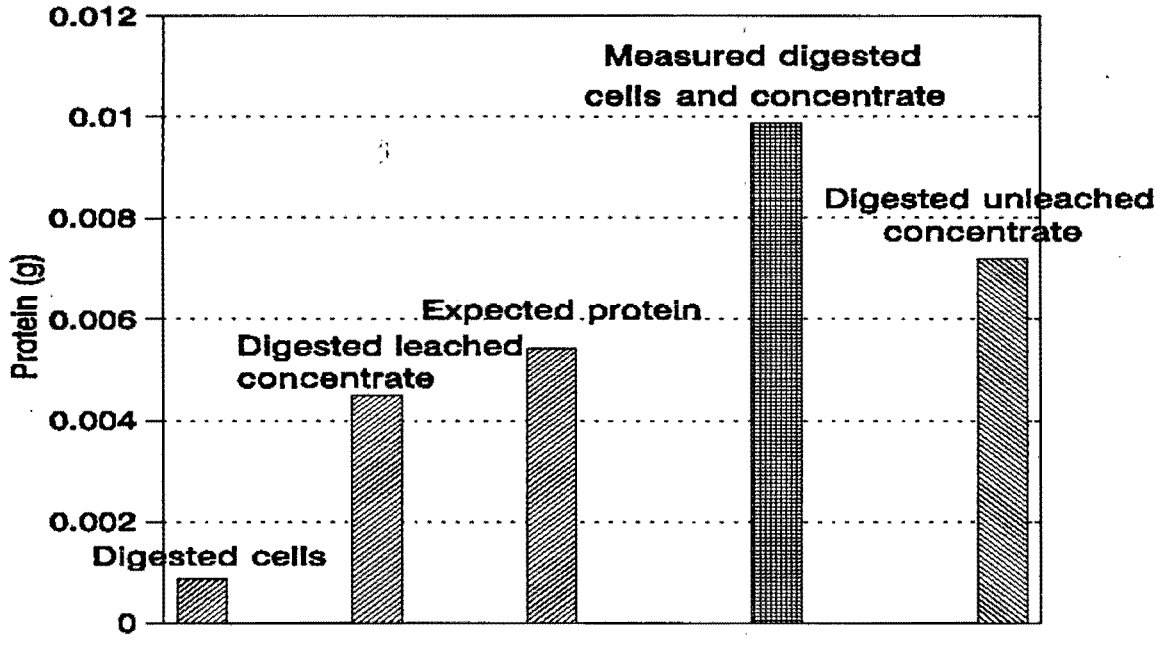


Figure 4.8. The Lowry protein content of a bacteria-ore mixture containing leached arsenopyrite-pyrite concentrate.

soluble protein determined by the Lowry assay resulting from the digested cells in the presence of arsenopyrite-pyrite concentrate was not equal the predicated protein release determined from the sum of the individually digested ore and digested cells. Furthermore, the unleached arsenopyrite-pyrite concentrate gave a positive protein reading.

The results for the Coomassie Blue protein assay (Figure 4.9) shows that the measured protein concentration agreed with that of the expected protein concentration. It was also found that the protein content of the unleached concentrate was negligible.

In comparing the Lowry and Coomassie Blue protein results, it was observed that the protein content of the leached concentrate was a factor of 10 greater for the Lowry assay than for the Coomassie Blue assay. Also the Lowry assay gave a significant protein content of the unleached concentrate.

The above results suggested the presence of a compound in the digested samples which interfered with the Lowry protein assay. This was not observed with the Coomassie Blue protein assay. Inorganic ions which are present and could interfere with the assay include

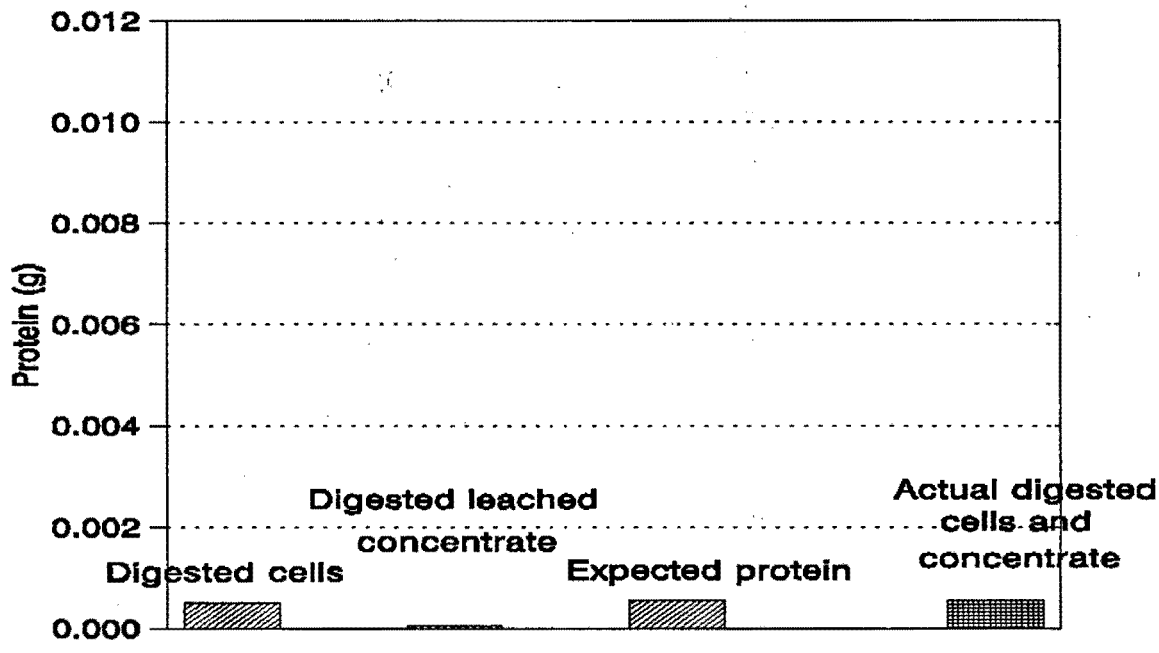


Figure 4.9. The Coomassie Blue protein content of a bacteria-ore mixture containing leached arsenopyrite-pyrite concentrate.

arsenic, sulphur, ferrous and ferric iron.

While the Lowry assay is most frequently used, the literature shows conflicting results with respect to interference. Barros (1983) found that the inorganic ions present in a liquid media of ferrous sulphate interfered with the Lowry protein assay while no such interference was recorded with Coomassie Blue protein assay. Myerson and Kline (1983) found that material leached out of coal during digestion interfered with the Lowry assay. However, DiSpirito *et al.* (1983), Basaran and Tuovinen (1987) and Norris *et al.* (1988) recorded no interference with the Lowry protein assay on digested pyrite. The reason for this may be due to the difference in the ore being used. They used pure pyrite while in this study a complex arsenopyrite-pyrite concentrate was used.

4.6.4. Interference and the Lowry Assay

The effect of ferrous and ferric iron on the Lowry protein assay was investigated to determine if these ions caused the interference. A ferrous iron concentration of between 0.26

and 2.83 ppm and a ferric iron concentration of between 0.16 and 3.04 ppm, was investigated. These concentrations were studied as the total iron concentration, found in the digested supernatant of leached ore by AAS, was in this range. The ferrous and ferric ions were added as $FeSO_4.7H_2O$ and $FeCl_3.6H_2O$. Figure 4.10 shows that the standard curve with the ferrous and ferric iron did not affect the Lowry protein assays at the concentrations tested. It is possible that the arsenic and sulphur present in the ore could cause interference as these compounds can be oxidised further. The effect of these ions on the Lowry assays needs to be investigated.

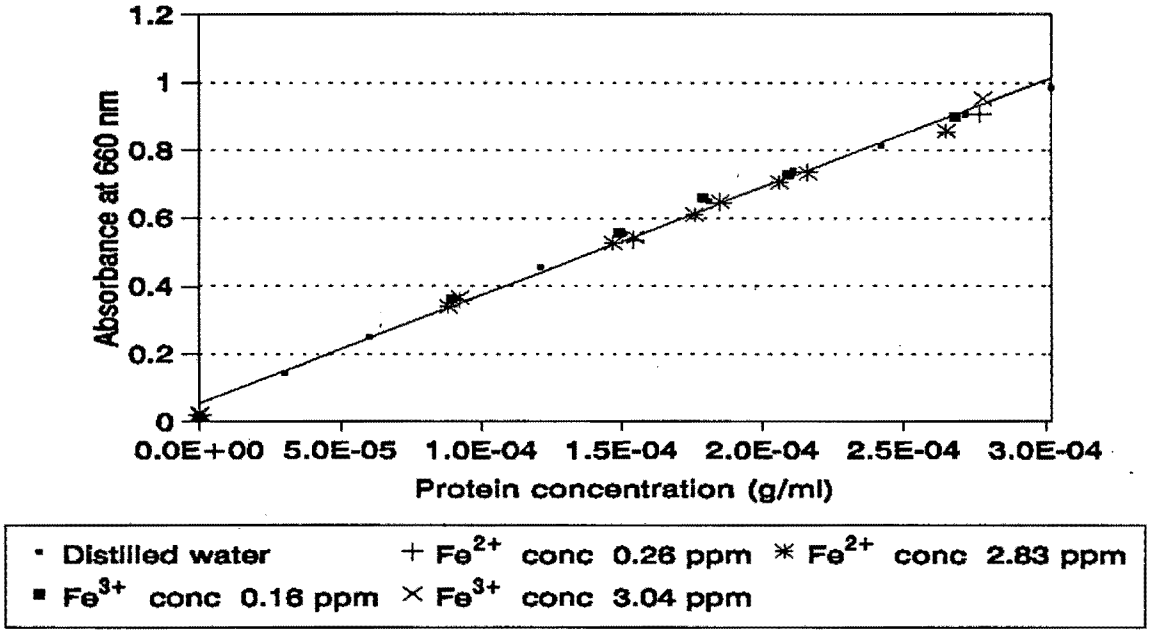


Figure 4.10. The effect of ferrous and ferric iron on the protein standard curve of the Lowry protein assay.

4.6.5. Conclusions

The Lowry protein assay experiences interference from the inorganic ions present in the supernatant of the digested arsenopyrite-pyrite concentrate. Ferrous sulphate and ferric chloride did not to interfere with the Lowry protein assay. No interference with the Coomassie Blue protein assay was found. Thus, the Coomassie Blue protein assay should be used rather than the Lowry protein assay in the bioleaching system.

4.7. NITROGEN

Nitrogen analysis of the bacterial populations in a biooxidation system reported in literature has been discussed in Section 2.6.2.2. This section presents the results of an investigation of the applicability of using nitrogen analysis with the arsenopyrite-pyrite concentrate.

4.7.1. Nitrogen analysis of Attached Bacteria

Nitrogen analysis by total Kjeldahl was done on leached and unleached arsenopyrite-pyrite concentrate. The results are given in Table 4.5.

Table 4.5. The nitrogen content of leached and unleached arsenopyrite-pyrite concentrate measured by Kjeldahl.

Sample	Nitrogen content (mg/kg ore)
Unleached arsenopyrite-pyrite concentrate	141.5
Leached arsenopyrite-pyrite concentrate	250

Table 4.5 shows that the unleached arsenopyrite-pyrite ore has a positive nitrogen content of 141.5 mg nitrogen/kg ore while the leached concentrate had a higher nitrogen content of 250 mg nitrogen/kg ore.

These results indicated that either the unleached arsenopyrite-pyrite concentrate contained nitrogen compounds or that the concentrate interferes with the nitrogen analysis. It was assumed that the nitrogen content of the unleached and leached ore was the same or that the interference from the ores to the nitrogen analysis was the same, for the calculation of the attached bacterial population. Thus, from the difference in the nitrogen content of the unleached and leached ore (108.5 mg nitrogen/kg ore), an attached bacterial concentration of 8.3×10^9 cells/g ore was obtained. In this above calculation, a dry cell mass of 1.18×10^{-10} mg/cells (Section 4.1.1) and a nitrogen content of 10.98 mass % (Section 4.1.2) was used.

The attached bacterial concentration of 8.3×10^9 cells/g was lower than that reported in literature using nitrogen analysis. McGoran *et al.* (1969) found an attached bacterial concentration of 3.95×10^{11} cells/g and 4.03×10^{11} cells/g chalcopyrite at 20 g/ℓ and 53.3 g/ℓ, respectively. Gormely and Duncan (1974) found an attached bacterial concentration of 7.67×10^{10} cells/g chalcopyrite. The difference between the results obtained in this study and those reported in literature can be due to the different ore used and the possible interference of the arsenopyrite-pyrite concentrate with the total Kjeldahl analysis. To further assess the interference of the arsenopyrite-pyrite concentrate with the Kjeldahl analysis, the effect of arsenopyrite in a caustic digest needs to be investigated.

4.7.2. Conclusions

An attached bacterial concentration of 8.3×10^9 cells/g was obtained. This calculation assumed either that the nitrogen-containing compounds in the leached and unleached ore were the same or that the interference of both ores with the nitrogen analysis were the same.

4.8. TOTAL ORGANIC CARBON (TOC)

Total organic carbon (TOC) analysis has been reported in literature (Section 2.6.2.3) for the enumeration of bacteria in a biooxidation system. The TOC analysis was investigated for the biooxidation system using an arsenopyrite-pyrite concentrate.

4.8.1. Total Organic Carbon Analysis of the Free Bacteria

The results of a total organic carbon (TOC) analysis of a washed cell suspension, resuspended in distilled water and digested using the method described in Section 3.2.3, are given in Table 4.6. Table 4.6 shows that a measured TOC content of 115.6 μg/ml of the washed cell suspension of a known cell concentration of 257.6 μg dry weight/ml was obtained. A carbon content of 120.1 μg/ml was calculated from the 46.61 mass % of dry weight (Section 4.1.2). An error of 3.9 % between the measured TOC content and the

calculated TOC content was found.

Table 4.6. The total organic carbon of a washed cell suspension.

	Measured TOC μg C/ml	Dry weight of washed cell suspension μg dry weight/ml	Carbon concentration calculated from dry weight as 46.61 % of the dry cell mass μg C/ml
Washed cell suspension	115.6 ± 3.5	257.6 ± 20.0	120.1 ± 9.3

Using the data presented in Table 4.6, it is possible to calculate a carbon content relative to dry weight of 44.8% (microanalysis obtained 46.61%, Section 4.1.2) and the total organic carbon content of a cell of $5.3\times10^{-11} \pm 0.44\times10^{-11}$ mg/cell (dry cell mass of 1.18×10^{-10} mg/cell, Section 4.1.1). Gormely and Duncan (1974) obtained a total organic carbon content per cell of 7.67×10^{-11} mg/cell for *Thiobacillus ferrooxidans*. The results for the total organic carbon content of a cell reported in this study and by Gormely and Duncan (1974) are of the same order of magnitude. The difference could be due to the mixed culture used in this study compared to a pure culture used by Gormely and Duncan (1974).

4.8.2. Total Organic Carbon Analysis of the Attached Bacteria

To evaluate total organic carbon (TOC) analysis for the determination of biomass concentration, mixtures of bacteria and ore were subjected to digestion (Section 3.2.3) and TOC analysis. Figure 4.11 indicated that the measured TOC content of the digested cells and leached concentrate was not equal to the sum of the individual TOC content of the digested leached concentrate and digested cells. Figure 4.11 also shows that the unleached ore has a positive TOC content and that the TOC content of the unleached ore is higher than that of the leached ore.

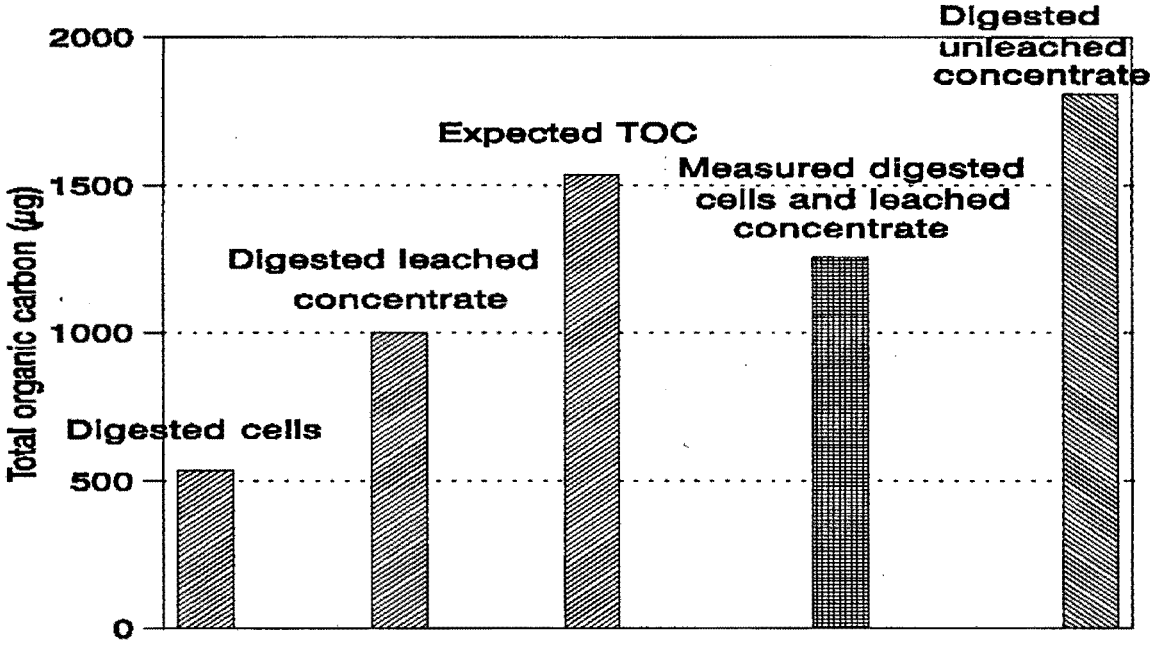


Figure 4.11. The TOC content of an ore-bacteria suspension of leached arsenopyrite-pyrite concentrate and bacteria.

The above results suggested that either carbonate or inorganic ions released during digestion of the ore interfered with the TOC analysis. The results also suggest that the interfering compounds were removed during leaching.

Total organic carbon measurements have been used in literature as a measure of the cell number (Blancarte-Zurita *et al.*, 1988; Lui *et al.*, 1988 and Boon *et al.*, 1995b). Blancarte-Zurita *et al.* (1988) measured the TOC in samples of pulp. However, a detailed methodology was not given. The number of bacteria present was reported as the total population and not divided into attached and free bacterial concentrations.

Lui *et al.* (1988) measured the total organic carbon content of the bacteria grown on ferrous sulphate and thus only the free population was measured. Boon *et al.* (1995b) measured the free bacterial population with a ferrous sulphate and a pyrite leach. Thus, no quantification of the attached bacterial populations by total organic carbon is available with which to compare the results obtained in this study.

4.8.3. Conclusions

TOC analysis was successfully used to determine free cell concentrations. The TOC measurements could not be used to measure the attached bacterial population for this mineral concentrate as either carbonate or inorganic ions released during digested interfered with the TOC analysis.

4.9. OXYGEN UTILISATION RATE

The oxygen utilisation rate, which is a measure of the metabolic activity of the bacteria, has been used to quantify bacterial concentrations using the approach of Boon *et al.* (1995d) presented in Section 2.6.2.4. The oxygen utilisation rate of a sample, at high ferrous concentrations, is related to the cell number and the maximum specific oxygen utilisation rate of the bacteria. To calculate this maximum, the oxygen utilisation rate of a cell suspension of known cell concentration was measured at various ferrous concentrations between 0 and 14 g Fe^{2+}/ℓ. Figure 4.12 shows that the specific oxygen utilisation rate reaches a maximum, independent of ferrous iron concentrations above 10 g Fe^{2+}/ℓ. A maximum specific oxygen utilisation rate obtained was 0.57 mol O_2/mol C/hr at 12 g Fe^{2+}/ℓ. The calculation of q_{O2}^{max} is detailed in Appendix D. The q_{O2}^{max} obtained agreed closely with the results of Boon *et al.* (1995d) of 0.65 mol O_2/mol C/hr for a culture of *Leptospirillum*-like bacteria and 0.9 mol O_2/mol C/hr for a pure culture of *Thiobacillus ferrooxidans*. From the data presented by Norris *et al.* (1988), it was possible to determine a q_{O2}^{max} of 0.36 mol O_2/mol C/hr for a pure culture of *Leptospirillum ferrooxidans* and 0.99 mol O_2/mol C/hr for a pure culture of *Thiobacillus ferrooxidans*.

The q_{O2}^{max} obtained in this study, suggested that most of the bacteria are *Leptospirillum ferrooxidans*. This is supported by Rawlings (1995) who found, by using the polymerase chain reaction and restriction enzyme analysis of 16S rDNA, that most of the bacteria in the continuous bioleaching tanks (at miniplant and full plant scale) were *Leptospirillum ferrooxidans*. This is further supported by Hansford (1995) who found that the bacterial activity of *Leptospirillum ferrooxidans* was higher than for *Thiobacillus ferrooxidans* at the high redox potentials that are found in the continuous bioleaching tanks.

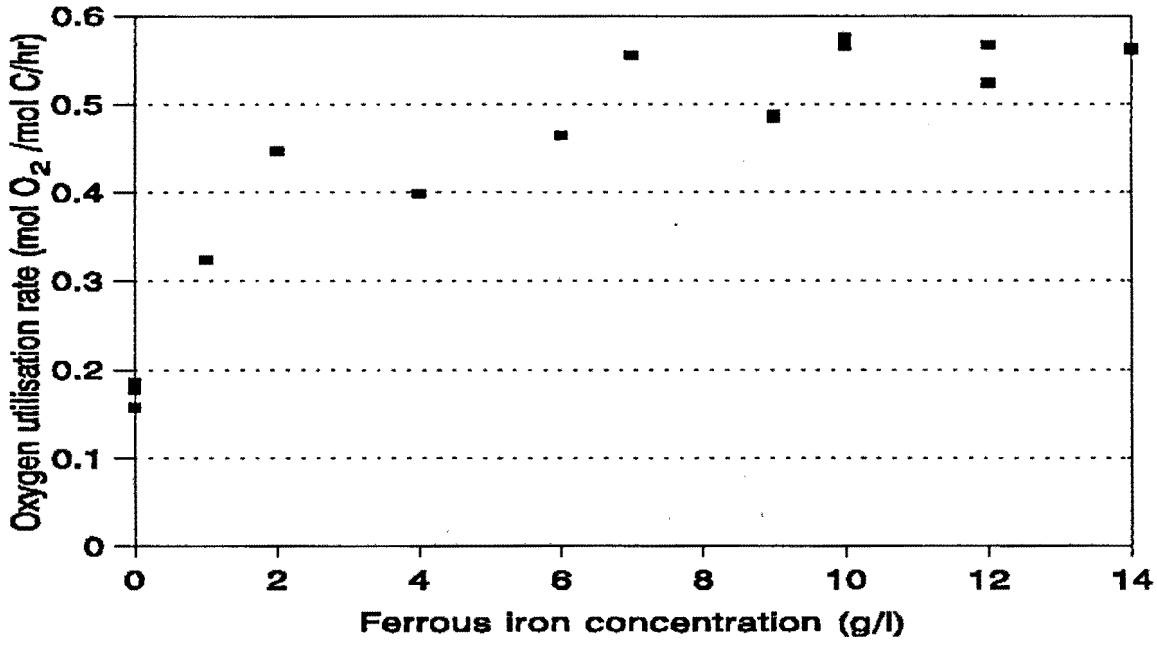


Figure 4.12. The oxygen utilisation rate of a washed cell suspension at various ferrous iron concentrations.

4.9.1. Oxygen Utilisation of Attached Bacteria

It was hypothesised that the attached bacteria have the same maximum specific oxygen utilisation rate as the free bacteria, hence the ferrous iron oxidation kinetics of the attached and free bacteria are the same. If this hypothesis is true then the attached bacterial population can be calculated by measuring the oxygen utilisation rate of the ore and using the following equation:

$$c_x \text{ (attached)} = \frac{r_{O_2} \text{ (ore)}}{q_{O_2}^{max}} \qquad [4.2]$$

where r_{O2} is the measured oxygen utilisation rate of the bacterial-ore concentrate sample at 12 g Fe^{2+}/ℓ

q_{O2}^{max} is the maximum specific oxygen utilisation rate obtained from measuring the free bacteria (0.57 mol O_2/mol C/hr)

The attached bacterial populations calculated from the measured oxygen utilisation rate using the above hypothesis are presented in Table 4.7. An attached population of $5.7\times10^{10} \pm 1.6\times10^{10}$ cells/g ore and $2.8\times10^{10} \pm 0.8\times10^{10}$ cells/g ore was found in the primary tank of the miniplant operated, as described by Breed *et al.* (1995), at a residence time of 15 and 6 days respectively.

Table 4.7. The attached bacterial population calculated from the measured oxygen utilisation rate for ore from the primary tank of a continuous arsenopyrite-pyrite concentrate leach.

Attached bacterial population cells/g ore	Average attached bacterial concentrations cells/g ore	Particle size μm	Residence time
5.3×10^{10}	$5.7\times10^{10}\pm1.6\times10^{10}$	< 76.6 (50% less than 10.97 μm)	15 days
4.9×10^{10}			
7.7×10^{10}			
5.5×10^{10}			
3.6×10^{10}			
7.4×10^{10}			
3.1×10^{10}	$2.8\times10^{10}\pm0.8\times10^{10}$	< 15.5	6 days
1.6×10^{10}		< 14.4	
3.1×10^{10}		< 14.4	
3.2×10^{10}		< 14.4	

The hypothesis that the maximum specific oxygen utilisation rate of both the attached and the free bacterial populations is the same requires validation. It has been shown that the maximum specific oxygen utilisation rate is dependent on the bacterial species present (Norris *et al.*, 1988 and Boon *et al.*, 1995d). Therefore, the composition of the free and attached bacterial population should be the same to obtain the same q_{O2}^{max} values or the maximum specific oxygen utilisation rate should be measured using a free bacterial population with the same composition as the attached bacterial population. A method of determining if the q_{O2}^{max} is the same for the free and attached bacteria is to desorb the bacteria from the mineral surface and measure the q_{O2}^{max} of the desorbed bacteria.

Based on the above determination of the attached population, the attached bacteria can be determined as a percentage of the total number of bacteria in the system. An attached bacterial population of 88% was determined from a free population of 7.6×10^{8} cells/ml and an attached population of 2.8×10^{10} cells/g ore for a 6 day residence time at a 20% solids concentration. This agrees well with the percentage attachment reported in literature for arsenopyrite. Karavaiko *et al.* (1986) found that the percentage of attached bacteria varied from 70 to 95% depending on the tank number in a continuous leach using protein analysis. Paponetti *et al.* (1991), using nitrogen analysis, and Monroy Fernandez *et al.* (1995a), using cell counts, obtained a percentage of attached bacteria of 80 and 83% respectively.

4.9.2. Conclusions

The hypothesis that the maximum specific oxygen utilisation rate of both the attached and the free bacterial populations are the same requires further validation and comparison with literature (see Section 4.11).

4.10. MAXIMUM NUMBER OF ATTACHED BACTERIA

It is possible from the size of the bacteria to calculate the maximum number of bacteria that can attach to a particle of a particular size. This calculation is dependent on the orientation of the bacteria, *i.e.* if the bacteria lie with their major axis parallel or perpendicular to the

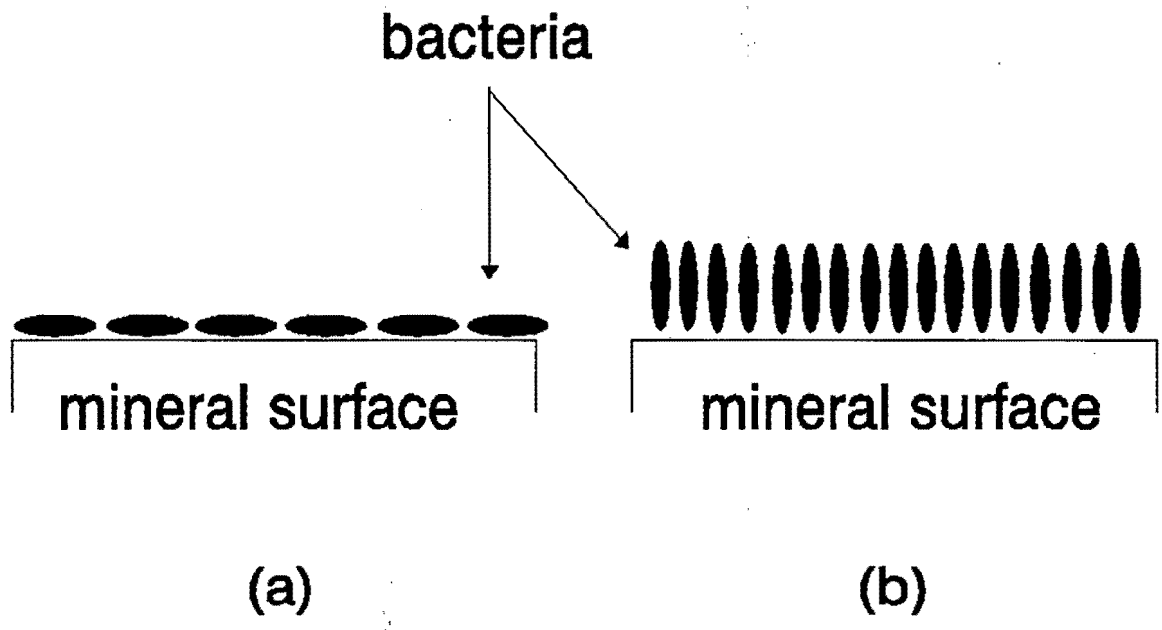


Figure 4.13. The orientation of the bacteria on the surface of the particle for the determination of the maximum possible number of attached bacteria.

surface of the particle (Figure 4.13).

Based on a particle diameter of 13.63 μm, it can be calculated that 2.17×10^{11} cells/g ore can attach if in the orientation portrayed in Figure 4.13 (a) while in the orientation shown in Figure 4.13 (b), 6.5×10^{11} cells/g ore can attach. Detailed calculations are given in Appendix E. It is important to note that these bacterial numbers do not allow for the repulsive forces between the like-charged bacteria and the suitability of the surface of the mineral for attachment. This variable attachment potential to the surface has been documented in the literature (Chang and Myerson, 1982 and Bagdigian and Myerson, 1986). Chang and Myerson (1982) correlated the attachment of a single *Thiobacillus ferrooxidans* cell (rod-shaped cell of 1×0.5 μm) to an area of 86 μm^2 of the pyrite surface. This could be due to the availability of suitable surface area. Bagdigian and Myerson (1986) observed by scanning electron microscopy that the attached bacteria selectively adhere to sulphidic regions and along dislocations, grain boundaries and other nonuniformities in the pyrite crystal surface. The maximum possible number of attached bacteria calculated here is the maximum limit and no enumeration technique should estimate higher numbers if the particle sizes are similar.

4.11. COMPARISON OF THE ENUMERATION TECHNIQUES

As shown in the above chapter and in the literature review (Chapter 2), the enumeration of the attached bacterial population involves many difficulties. These difficulties include the interference of inorganic ions and precipitants in the analyses. This section summarizes and compares the methods that have allowed the quantification of the number of attached bacteria, *i.e.* scanning electron microscopy, desorption, nitrogen analysis and oxygen utilisation rate. Also presented in this section are the attached bacterial concentrations reported in literature and the maximum adsorption capacities calculated in literature.

Table 4.8 summarizes the results obtained in this study by different analytical techniques for the attached bacterial concentrations in a continuous miniplant treating an arsenopyrite-pyrite concentrate. The oxygen utilisation rate results agree with the lowest result of the maximum adsorption capacity obtained from the desorption data. The wide range of maximum adsorption capacities could be due to the many interruptions to the steady state operation of the miniplant. This could result in different conditions of the ore been tested, for example the amount of jarosite associated with the ore. The desorption data does, however, give an indication of the maximum adsorption capacity. The desorption experiments should therefore be performed concomitantly with the oxygen utilisation rate measurements to give a true comparison between these methods.

Table 4.8. The average attached bacterial concentrations obtained in this study from scanning electron microscopy, desorption, nitrogen and oxygen utilisation rate experiments.

Method	Cell Number	Protein	Nitrogen	Carbon
Scanning electron micrographs - 15 day residence time	$2.45\times10^{10}\pm1.77\times10^{10}$ cells/m^2	1.27 mg/m^2	0.32 mg/m^2	1.30 mg/m^2
Desorption - > 15 day residence time	6×10^{10} cells/g	3.11 mg/g	0.78 mg/g	3.18 mg/g
	$6.4\times10^{11}\pm1.21\times10^{11}$ cells/g	33.21 mg/g	8.32 mg/g	33.92 mg/g
Nitrogen - 6 day residence time	8.37×10^{9} cells/g	0.43 mg/g	0.11 mg/g	0.44 mg/g
Oxygen utilisation rate - 15 day residence time	$5.7\times10^{10}\pm1.6\times10^{10}$ cells/g	2.96 mg/g	0.74 mg/g	3.02 mg/g
- 6 day residence time	$2.8\times10^{10}\pm0.8\times10^{10}$ cells/g	1.45 mg/g	0.36 mg/g	1.48 mg/g

The conversions used in the preparation of Table 4.8 are summarized in Table 4.9. Table 4.9 shows that the carbon and protein content of the cell are approximately 50% by mass. Also, the nitrogen content of the cell is 11% by mass. These results closely agree with the standard results for the composition of a cell (Bailey and Ollis, 1986).

Table 4.9. The conversion factors for comparison of dry weight, protein, nitrogen and carbon contents.

Dry weight per cell	1.18×10^{-10} mg/cell
Protein content per cell	5.19×10^{-11} mg/cell
Nitrogen content per cell	1.3×10^{-11} mg/cell
Carbon content per cell	5.3×10^{-11} mg/cell

It can be seen in Table 4.8 that the nitrogen analysis gave a lower result than both desorption and oxygen utilisation rate. If it assumed that all the nitrogen obtained from the difference between the nitrogen content of the unleached and leached arsenopyrite-pyrite concentrate is due to bacterial nitrogen only, then an attached bacterial concentration of 8.3×10^{9} cells/g ore was calculated. It thus appears that the nitrogen method seems to underestimate the number of attached bacteria for this mineral ore.

The scanning electron microscopy results can only be compared to the calculated maximum attachable bacteria (Section 4.10) as these results are reported as cells/m^2 and the surface per unit gram was not measured. The scanning electron microscopy results are a factor of 10 lower than the maximum attachable bacterial concentrations calculated. It is also possible to compare the attached cell density with that reported in literature. Ohmura *et al.* (1993) obtained a maximum cell density of 8.9×10^{11} cells/m^2 for attachment to pyrite by cell counts while Konishi *et al.* (1992) obtained a maximum cell density of 3.5×10^{11} cells/m^2 for attachment of *Thiobacillus ferrooxidans* to a zinc sulphide concentrate. These maximum cell densities reported are higher than the cell density (2.45×10^{10} cells/m^2) calculated from the scanning electron micrographs obtained in this study. The lower attached bacterial concentration calculated from the micrographs could be due to the inaccurate estimation of

the surface area. The surface area to which the bacteria are attached is underestimated as the micrographs are a 2-dimensional projection of a three-dimensional surface. Other reasons for the low calculated cell density estimated could be due to inaccuracies caused by detachment of bacteria from the mineral surface during sample preparation and the small fraction of the total surface area analysed.

In comparing the attached population determined by oxygen utilisation rate measurements to attached bacterial concentrations reported in literature (Table 4.10), close agreement is found. The results obtained (5.7×10^{10} cells/g and 2.8×10^{10} cells/g for a 15 and 6 day residence time, respectively) agree with results of Norris *et al.* (1988) for *T. ferrooxidans* of 4.7×10^{10} cells per gram pyrite, Karavaiko *et al.* (1986) of 4.4×10^{10} cells/g arsenopyrite and Myerson and Kline (1983) of 4.3×10^{10} cells/g coal. The results are slightly lower than the results of Gormely and Duncan (1974) of 7.67×10^{10} cells/g chalcopyrite. The oxygen utilisation rate results, also show a good correlation with the maximum adsorption capacity reported in literature (Table 4.11). This indicated that, although the results of the oxygen utilisation rate measurements are based on a hypothesis which requires further validation, this method has potential for the enumeration of the number of attached bacteria. The technique is relatively fast and requires no preliminary digestion or sample preparation before the oxygen utilisation rate can be measured. This provides a major advantage over the other methods.

Table 4.10. The attached bacterial concentrations on sulphide minerals reported in literature*.

Author	Method	Ore	Particle size μm	% Attached	Cell Number cells/g	Protein mg/g	Nitrogen mg/g	Carbon mg/g
Ohmura *et al.* (1993)	Cell counts	Pyrite	-100+40	24 %	1.92×10^{10}	0.34	0.30	1.47
Murthy and Natarajan (1992)	Protein	Pyrite	-75+63	60.4 %	4.0×10^{9}	0.08	0.06	0.30
			-1000+600	40 %	3.9×10^{9}	0.08	0.06	0.29
			-4750 +4000	25 %	4.1×10^{8}	0.008	0.006	0.03
DiSpirito *et al.* (1983)	Protein	Pyrite	-297+53	46 %	5.9×10^{11}	11.95	9.26	45.25
				56 %	2.58×10^{11}	5.15	4.05	19.79
				88 %	2.76×10^{11}	5.52	4.33	21.17
				48 %	5×10^{10}	1.0	0.78	3.83
Basaran and Tuovinen (1987)	Protein	Pyrite	-74+44	80 %	1.1×10^{11}	2.20	1.72	8.43
				17.6 %	7.75×10^{10}	1.55	1.21	5.94
Norris *et al.* (1988)	Protein	Pyrite	< 75	7 %	4.7×10^{10}	0.94	0.73	3.60
				87 %	1.94×10^{11}	3.88	3.05	14.90
Ohmura *et al.* (1993)	Cell counts	Chalcopyrite	-100+40	14 %	9.33×10^{9}	0.18	0.14	0.71
McGoran *et al.* (1969)	Nitrogen	Chalcopyrite	< 37	97 %	3.95×10^{11}	7.90	6.21	30.3
				99 %	4.03×10^{11}	8.06	6.33	30.9
Gormely and Duncan (1974)	Nitrogen	Chalcopyrite	< 37	96 %	7.67×10^{10}	1.53	1.2	5.88
Monroy Fernandez *et al.* (1995)	Cell counts	Arsenopyrite	-80+50	83 %	2.5×10^{9}	0.05	0.04	0.19
Karavaiko *et al.* (1986)	Protein	Arsenopyrite	< 44	70-95 %	4.4×10^{10}	0.88	0.69	3.37
Myerson and Kline (1983)	Protein	Coal	< 63	32 %	4.3×10^{10}	0.86	0.67	3.31
McGoran *et al.*(1969)	Nitrogen	Sulphur	-	77 %	1.36×10^{11}	2.73	2.14	10.05

* The conversions used in Table 4.10 include a dry cell weight of 1.12×10^{-10} mg/cell, a nitrogen content of 1.57×10^{-11} mg/cell and a carbon content of 7.67×10^{-11} mg/cell (Gormely and Duncan, 1974). A protein content of 2×10^{-11} mg/cell was also used (Myerson and Kline,1983) as this has been used most frequently in the literature. This corresponds to a protein content of the cell of only 17% compared to the reported values of 48-66% (Jones and Kelly, 1983). Although this value of protein per cell is low, it correlates to the protein extracted per cell over a digestion time of 15 minutes in which only a fraction of the soluble protein been released. As the 15 minutes digestion was used by these authors, the protein content per cell obtained at this digestion time was used.

Table 4.11. The maximum adsorption capacities reported in literature.

Author	Method	Ore	Particle size μm	Cell Number	Protein	Nitrogen	Carbon
Ohmura *et al.* (1993)	Cell counts	Pyrite	-100+40	1.76×10^{10} cells/g	0.35 mg/g	0.27 mg/g	1.34 mg/g
				8.9×10^{11} cells/m^2	17.8 mg/m^2	14.0 mg/m^2	68.4 mg/m^2
Asai *et al.* (1992)	Cell counts	Pyrite	25-44	6.61×10^{10} cells/g	1.32 mg/g	1.03 mg/g	5.06 mg/g
			53-63	2.5×10^{10} cells/g	0.50 mg/g	0.39 mg/g	1.91 mg/g
			63-88	1.41×10^{10} cells/g	0.28 mg/g	0.22 mg/g	1.07 mg/g
			149-177	0.91×10^{10} cells/g	0.18 mg/g	0.14 mg/g	0.69 mg/g
Konishi *et al.* (1992)	Cell counts	Zinc sulphide concentrate	-53+37	1.65×10^{10} cells/g	0.33 mg/g	0.25 mg/g	1.26 mg/g
				3.5×10^{11} cells/m^2	7.0 mg/m^2	5.49 mg/m^2	26.84 mg/m^2
Konishi *et al.* (1994)	Cell counts	Sulphur	-	4.88×10^{10} cells/g	0.97 mg/g	0.76 mg/g	3.74 mg/g

CHAPTER 5

CONCLUSIONS

5. CONCLUSIONS

The main aim of this study was to investigate various methods of enumerating the bacteria in a biooxidation system. This was investigated in the context of an arsenopyrite-pyrite containing ore with a carbon content of 1.41 mass % and a mixed bacterial population of *Leptospirillum ferrooxidans* and *Thiobacillus thiooxidans*. The methods investigated with conclusions regarding their potential and shortcomings in the enumeration of the attached bacteria are given below.

CHARACTERISATION OF THE BACTERIA

The bacteria were characterised by their elemental composition measured by microanalysis and dry weight per cell measured through the ashing experiments. The characteristics of the bacteria are summarized in Table 5.1.

Table 5.1. The characterisation of the bacterial culture.

	Mass %	Mass per cell
Carbon	46.61%	5.3×10^{-11} mg/cell
Hydrogen	7.00%	8.26×10^{-12} mg/cell
Nitrogen	10.98%	1.3×10^{-11} mg/cell
Protein	44%	5.19×10^{-11} mg/cell
Ash content of a cell	13%	1.53×10^{-11} mg/cell
Dry cell mass		1.18×10^{-10} mg/cell

From the elemental composition, an empirical formula for the bacteria of $CH_{1.8}O_{0.57}N_{0.2}$ was calculated.

ENUMERATION OF THE BACTERIA

The bacteria were enumerated by direct methods of scanning electron microscopy and desorption and indirect methods of chemical oxygen demand, ashing, protein analysis, nitrogen analysis, total organic carbon analysis and oxygen utilisation rate measurements.

Scanning Electron Microscopy (SEM)

The scanning electron micrographs showed that a mixed population was attached to the mineral ore. The bacteria sparsely populated the mineral surface in a monolayer. The scanning electron micrographs cannot be used to enumerate the attached bacteria rigorously as the micrographs are a two-dimensional projection of a three-dimensional surface. Thus, the surface area to which the bacteria are attached was difficult to determine accurately.

Desorption

The bacteria can be desorbed from the ore surface in both the presence and absence of ferrous iron in solution. A mathematical relationship between the number of cells desorbed and the desorption cycle was required to define when all the bacteria have desorbed from the ore surface.

The desorption data was found to fit the Langmuir isotherm, *i.e.* the concentration of the attached bacteria as a function of the free bacteria was characterised by a curve that monotonically approached a limiting value. The Langmuir isotherm constants gave a maximum adsorption capacity, X_{AM}, of between 6×10^{10} and 7.8×10^{11} cells/g arsenopyrite-pyrite concentrate. Thus, the desorption data can be used to determine the maximum adsorption capacity of the mineral ore.

Chemical Oxygen Demand (COD)

The chemical oxygen demand of a washed cell suspension was linearly related to the dry cell weight. Hence the chemical oxygen demand could be used to enumerate the number of free bacteria in a biooxidation system.

The chemical oxygen demand of the attached bacteria could not be measured as the mineral ore consumed the excess potassium dichromate.

Ashing

Ashing of the leached arsenopyrite-pyrite concentrate could not be used to determine the number of attached bacteria as the change in mass of the ore on ashing was not a function of the weight of the ore. In addition, the change in mass of the leached ore was less than that for the unleached ore. This implied that the ashable contents of the ore were removed during leaching. Thus, the contribution of the ore to the change in mass was uncertain.

Protein

The different protein assays investigated in this study include the Lowry, Peterson and Coomassie Blue assays. The Lowry protein assay was used to determine a protein content of $5.19\times10^{-8} \pm 0.51\times10^{-8}$ μg per cell. This correlated to a protein content relative to the dry weight of 44%. The Lowry protein assay could be used to enumerate the free bacterial concentration.

Interference was found to occur with the Lowry protein assay when used to quantify solubilised protein present after the digestion of the unleached and leached arsenopyrite-pyrite concentrate. This was attributed to the release of inorganic ions and can be seen from the result of a positive protein value of the unleached ore. In addition, the actual protein value of a digestion of leached ore with bacteria did not equal the expected value. Thus, the Lowry protein assay could not be used to enumerate the number of attached bacteria.

A lower level of interference was observed with the Coomassie Blue protein assay. In addition, mass balances with mixtures of ore and bacteria illustrated their potential to enumerate the attached bacterial population.

Nitrogen

The total nitrogen content of the leached and unleached arsenopyrite-pyrite concentrate was measured by the Kjeldahl technique. The difference between the nitrogen content of the leached and unleached ore gave an attached bacterial population of 8.3×10^{9} cells/g ore, assuming that the difference in the nitrogen content is solely due to bacterial nitrogen. The unleached ore gave a positive nitrogen content that indicated that either the ore contained nitrogen compounds or the arsenopyrite-pyrite concentrate interfered with the nitrogen analysis.

Total Organic Carbon (TOC)

The organic carbon content of a cell was measured as 5.3×10^{11} mg. The total organic carbon can be used for enumerating the number of free bacteria.

The unleached arsenopyrite-pyrite concentrate gave a positive total organic carbon reading. This suggested that either carbonate or inorganic ions released during the digestion of the mineral ore interfered with the total organic carbon analysis. A further problem was that the leached arsenopyrite had a lower total organic carbon content than the total organic carbon content of the unleached arsenopyrite. This implied that the carbonate was removed during the leaching process. Thus, the total organic carbon analysis cannot be used to enumerate the number of attached bacteria with a carbonate containing ore.

Oxygen Utilisation Rate

The oxygen utilisation rate of a washed cell suspension is dependent on the ferrous iron concentration. A critical ferrous iron concentration of 10 g Fe^{2+}/ℓ was found above which the oxygen utilisation rate remained constant. This allowed the determination of a maximum specific oxygen utilisation rate at 12 g Fe^{2+}/ℓ of 0.57 mol O_2/mol C/hr for the free bacteria. As the measured oxygen utilisation rate is a function of the cell number and the specific oxygen utilisation rate of the bacteria, the oxygen utilisation rate could be used to enumerate the free bacterial population.

It was hypothesised that the maximum specific oxygen utilisation rate of the attached bacteria is the same as that of the free bacteria. Thus, the attached population could be calculated from the measurement of the oxygen utilisation rate of an ore sample at 12 g Fe^{2+}/ℓ. The results obtained for the ore from the primary tank in the continuous miniplant indicated an attached bacterial concentration of $5.7\times10^{10} \pm 1.6\times10^{10}$ cells/g ore for a 15 day residence time and $2.8\times10^{10} \pm 0.8\times10^{10}$ cells/g ore for a 6 day residence time. Although the hypothesis requires further validation, close agreement with the maximum adsorption capacities reported in literature suggests that this method could be used to enumerate the number of attached bacteria.

A percentage of attached bacteria as a function of the total bacterial concentration of 88% for a 6 day residence time, was obtained. Hence most of the bacteria are attached to the

mineral ore.

In summary, the oxygen utilisation rate method can be used for enumerating the free and attached bacterial concentrations. However, the dependence of the maximum oxygen utilisation rate on bacterial species must be considered. The method is relatively fast compared to the other techniques such as protein and total organic carbon as it does not require any preliminary sample preparation such as digestion.

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APPENDICES

APPENDIX A: MEDIA AND BUFFERS

Sodium Phosphate Buffer, 0.2 M, pH 7.0

- Dissolve 1.42 g of Na_2HPO_4 in 50 ml distilled water
- Dissolve 0.94 g of $NaH_2PO_4.2H_2O$ in 30 ml distilled water
- Mix 30.5 ml of Na_2HPO_4 solution and 19.5 ml of NaH_2PO_4 and adjust the pH to 7.0

2.5 % glutaraldehyde in 0.1 M Phosphate Buffer

- 5 ml of sodium phosphate buffer (0.2 M, pH 7.0)
- 1 ml of commercial glutaraldehyde solution (aq., 25%)
- 4 ml of distilled water

9K media (Silverman and Lundgren, 1959)

Basal salts media:
- Dissolve 3 g $(NH_4)_2SO_4$ to a final volume of 100 ml
- Dissolve 0.1 g KCl to a final volume of 100 ml
- Dissolve $MgSO_4.7H_2O$ to a final volume of 100 ml
- Dissolve K_2HPO_4 to a final volume of 100 ml
- Dissolve $Ca(NO_3)_2.4H_2O$ to a final volume of 100 ml
- Mix the above solutions and add 200 ml distilled water

Ferrous sulphate media:
- Dissolve 83.34 g $FeSO_4.7H_2O$ in distilled water to a final volume of 500 ml
- Adjust the pH to 1.6 with concentrated sulphuric acid

Mix 700 ml of the basal salts media with 300 ml of the ferrous sulphate media and adjust the pH to 1.8.

APPENDIX B: SOLUTION PREPARATION FOR CHEMICAL OXYGEN DEMAND

0.25 N potassium dichromate solution ($K_2Cr_2O_7$)

- Dissolve 12.259 g $K_2Cr_2O_7$ in distilled water and dilute to 1000 ml

Sulphuric acid reagent

- Add 22 g silver sulphate, Ag_2SO_4, to 4 kg concentrated sulphuric acid

Ferrous ammonium sulphate titrant

- Dissolve 39 g $Fe(NH_4)_2(SO_4)_2.6H_2O$ in distilled water
- Add 20 ml concentrated sulphuric acid, allow to cool and then diluted to 1000 ml

APPENDIX C: PROTEIN ASSAYS

Lowry Protein Assay

Reagents

Reagent A: Dissolve 100 g Na_2CO_3 in 1 ℓ (final volume) 0.5N NaOH

Reagent B: Dissolve 1 g $CuSO_4.5H_2O$ in 100 ml (final volume) distilled water

Reagent C: Dissolve 2 g potassium tartrate in 100 ml (final volume) distilled water

Reagent D: Dissolve 0.3 g bovine serum albumin (Sigma Chemical Co., catalogue no. A 7906) in 1 litre (final volume) distilled water

Reagent E: Add 5 ml of 2 N Folin-Phenol (Sigma Chemical Co., catalogue no. F 9252) reagent to 50 ml distilled water and mix thoroughly

Method

All standards and samples were done in triplicate

1. Pipette 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 ml bovine serum albumin solution in test tubes for the standard protein curve
2. Pipette 1 ml of the sample into test tubes
3. Bring the total volume of all test tubes to 1 ml with distilled water
4. Mix 15 ml reagent A, 0.75 ml reagent B and 0.75 ml reagent C
5. Add 1 ml of the above mixture to each test tube and mix thoroughly
6. Incubate the tubes for 15 minutes at room temperature
7. Rapidly add 3 ml of reagent E to each tube and mix immediately
8. Incubate the sample in a dark cupboard at room temperature for 45 minutes
9. Determine the absorbance of each sample at 660 nm

Peterson Protein Assay

Reagents

Copper-tartrate-carbonate (CTC): Dissolve 100 g Na_2CO_3, 1 g $CuSO_4.5H_2O$ and 2 g potassium tartrate in 1 ℓ (final volume)

Sodium dodecyl sulphate (SDS): Dissolve 100 g sodium dodecyl sulphate in 1 ℓ (final volume)

Sodium hydroxide: Dissolve 3 g NaOH in 100 ml (final volume)

Folin-Ciocalteu phenol reagent

Bovine serum albumin solution:	Dissolve 0.5 g bovine serum albumin in 1 ℓ (final volume)
Reagent A:	Mix 100 ml of CTC, NaOH, SDS and distilled water
Reagent B:	10 ml Folin-Ciocalteu phenol reagent in added to 50 ml distilled water

Method

1. Pipette 0, 0.05, 0.1, 0.15 and 0.2 ml bovine serum albumin solution into test tubes
2. Bring the volume in the test tubes up to 1 ml with distilled water
3. Pipette 1 ml of the appropriate diluted samples into test tubes
4. Add 1.0 ml reagent A to each test tube and allow to stand for 10 minutes at room temperature
5. Add 0.5 ml of reagent B, mix and allow to stand in a dark cupboard for 30 minutes
6. Read the absorbance at 750 nm

Coomassie Blue Protein Assay

Reagents

Coomassie Blue dye:	Dissolve 50 mg Brilliant Blue G dye in 50 ml orthophosphoric acid (85%) and 25 ml 90% ethanol. The resultant solution was made up to 500 ml with distilled water.
Bovine serum albumin:	0.03 g bovine serum albumin in 1 ℓ (final volume)

Method

1. Place 0.2 ml of samples and appropriately diluted samples in test tubes
2. Add 1 ml Coomassie Blue dye reagent
3. Mix thoroughly and allow to stand for 30 minutes
4. Read the absorbance at 595 nm

APPENDIX D: CALCULATION OF q_{o2}^{max} AND ATTACHED BACTERIAL CONCENTRATIONS

Calculation of q_{o2}^{max}

Oxygen utilisation rate (measured at 12 g Fe^{2+} /ℓ) = 0.0033 ppm/s
= 11.88 μg O_2/ml/hr

Dry weight of the bacterial suspension added = 16.5 μg/ml

Therefore,

The maximum specific oxygen utilisation rate = 7.2×10^5 μg O_2/g dry weight/hr
(using carbon content 47% of the dry weight) = 1.53×10^6 μg O_2/g Carbon/hr
= 0.57 mol O_2/mol Carbon/hr.

Calculation of attached bacterial concentrations

Oxygen utilisation rate (measured at 12 g Fe^{2+}/ℓ) = 0.0070 ppms/s
= 25.2 μg O_2/ml/hr
= 7.9×10^{-7} mol O_2/ml/hr

The maximum specific oxygen utilisation rate (q_{o2}^{max}) = 0.57 mol O_2/mol Carbon/hr

Therefore,

The attached bacterial concentration = 1.4×10^{-6} mol Carbon/ml

Carbon content of a cell = 5.3×10^{-11} mg Carbon/cell
= 4.4×10^{-15} mol Carbon/cell

Therefore,

The attached bacterial concentration = 3.1×10^8 cells/ml

The solids concentration of the sample = 5.8×10^{-3} g/ml

Therefore,

The attached bacterial concentration = 5.3×10^{10} cells/g

APPENDIX E: CALCULATION OF THE MAXIMUM ATTACHED BACTERIA

Dimensions of the bacteria: 0.5×1.5 μm

Average particle size: 13.63 μm

Assuming a cubic particle (Ohmura *et al.*, 1993):

Surface area of the particle: 1115.5 μm^2

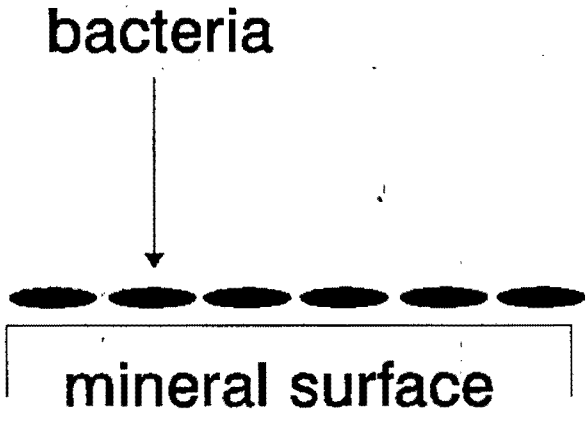
Volume of the particle: 2534.9 μm^3

Density of the particle: 2700 kg/m^3

Weight of the particle: $2.534.9 \times 10^{-18} \times 2700 = 6.84 \times 10^{-12}$ kg

Orientation of the bacteria:

A.



A

Each cell occupies 0.75 μm^2

Therefore,

maximum number of cells of the particle = 1115.5 / 0.75

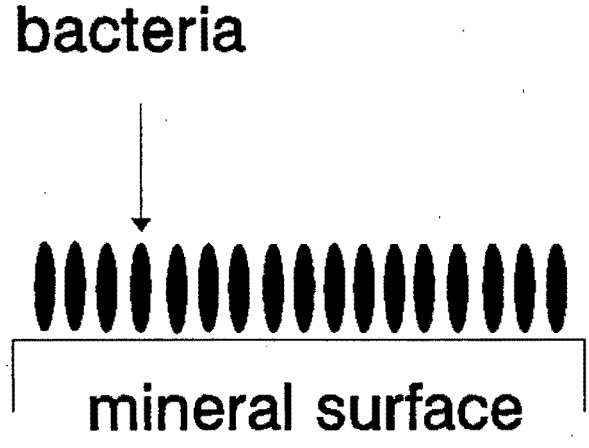
= 1487 cells

Therefore,

Number of cells/ unit mass of particle = 2.17×10^{11} cells / g ore

Number of cells/ unit area = 1.3 cells / μm^2

B.



B

Each cell occupies 0.25 μm^2

Therefore,

maximum number of cells of the particle = 1115.5 / 0.25

= 4462 cells

Therefore,

Number of cells/ unit mass of particle = 6.5×10^{11} cells / g ore

Number of cells/ unit area = 4 cells / μm^2