ISOLATION OF *Acidithiobacillus ferrooxidans* FROM SULPHUR TREATMENT PLANT, MINE WATER AND TAILINGS OF ANGLOGOLD ASHANTI CONCESSIONAL AREA AND THEIR BIOOXIDATION POTENTIAL OF REFRACTORY ORES

BY

EMELDA ADII B.Sc.(Hons) ENVIRONMENTAL SCIENCE

Dissertation Submitted to the Department of Environmental Science, College of Science, Kwame Nkrumah University of Science and Technology, In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN ENVIRONMENTAL SCIENCE

FACULTY OF BIOSCIENCE

COLLEGE OF SCIENCE

FEBRUARY, 2011

DECLARATION

I hereby declare that this work presented to the Department of Theoretical and Applied Biology in partial fulfillment for the award of MSc. Degree, is a true account of my own work except where particularly all sources of information have been acknowledge by means of references.

Signature ---------- Date ----------

NAME: EMELDA ADII

(STUDENT)

Signature ---------- Date ----------

NAME: PROF. KWASI OBIRI-DANSO

(PRINCIPAL SUPERVISOR)

Signature ---------- Date ----------

NAME: DR. B. FEI-BAFFOE

(SECOND SUPERVISOR)

Certified by:

Signature ---------- Date ----------

NAME: DR. P.K. BAIDOO

(HEAD OF DEPARTMENT)

DEDICATION

This work is dedicated to the entire Adii's family; Mr. & Mrs Emmanuel Adii, Joycelyn, Patience and Awenkanab Samuel.

ABSTRACT

The objective of the study was to find a candidate biomining organism *Acidithiobacillus ferrooxidans* in bioreactor tanks, mine water and tailings within the AngloGold Ashanti mining concession area with the aim of contributing to the development of biomining in Ghana as a sustainable mining practice. Samples from the Sulphur Treatment Plant (STP) bioreactor tanks, mine water, and soil tailings from the four geographical areas (North, South, East and West) of the Sansu dam were collected and assayed using 9K enrichment medium. Isolates were used for biooxidation over a 672 hour period and their oxidation potential assessed. A bacterium, which was acidophilic, chemolithotrophic with ferrous oxidizing potential, was isolated in ten out of twelve sample points. Isolates were also recovered from samples that were high in heavy metal (As, Fe, Cu, Pb, and Zn) concentration. The bacterial loads were higher in the bioreactor tanks (1.86×10^4-4.24×10^5) compared to mine water and tailings (6.33×10^3 -1.40×10^4) although these differences were statistically not significant (P=0.05). The isolates grew best at pH 2 and a temperature of 35^{o}C. Isolates could utilize sulphur and ferrous as energy sources. Biooxidation potential of the refractory ore was highest in isolates from the bioreactor tanks followed by mine water isolates and the tailings. Isolates from the bioreactor tank 146 showed the best biooxidation results. The presence of a candidate *A. ferrooxidans* isolate from the AngloGold Ashanti concessional area is an indication of the possibility of finding active indigenous strains of the bacterium which could serve as the basis for encouraging the massive use of the technology in Ghana's mining companies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND ACRONYMS

AMD	Acid Mine Drainage
AGA	AngloGold Ashanti
AGAED	AngloGold Ashanti Environmental Department
ANOVA	Analysis of Variance
BIOX®	Biooxidation
CFU	Colony forming Units
CIL	Carbon In Leach
EPS	Exopolysaccharide
Fe^{2+}	Ferrous Iron
Fe^{3+}	Ferric Iron
GEPA	Ghana Environmental Protection Agency
GPD	Gross Domestic Product
HiPIP	High Redox Potential Iron Oxidase
ND	New Drainage
NW-NNW	North West- North North West
PCA	Plate Count Agar
pH	Hydrogen Ion Concentration
RISCs	Reduced Inorganic Sulphur Compounds
Rpm	Revolution Per Minute
STP	Sulphur Treatment Plant
WHO	World Health Organisation

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and gratitude to God Almighty for the many blessings, strength and love. Without Him, this study would be impossible. His Name forever is Praised, Amen.

My sincere thanks and appreciation go to my supervisors, Prof. K. Obiri-Danso, Dean of International Programmes, Kwame Nkrumah University of Science and Technology (KNUST) and Dr. B. Fei- Bafour for their valuable suggestions, time and resources the sacrificed to see this work through successfully.

To my guardian family, the Akanwariwiak family, I am very grateful for your immerse support, motivation and sacrifice. I say thank you, for loving and trusting in me all this while.

I also owe the staff of the Environmental Department and Sulphur Treatment Plant of the Anglogold Ashanti Obuasi Mining Ltd, especially Uncle Peter, Mr. Osei-Owusu, and Angelina Okrah for all the support during the course of the work.

Special thanks to Dr Fei–Baffoe, Dr. Belford and Dr. John A. Larbi of the Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology (KNUST) for their assistance during the course of this research.

Special thanks to my colleges Catherine Ama Woods and Fati Aziz for their vital contribution and encouragement during those hard times of the research work. I will forever appreciate you.

Most of all, I thank the Lord for providing me and all those who helped me with whatever it took to help.

CHAPTER ONE

1.0 INTRODUCTION

The chemolithautotrophic gram-negative, non-spore forming, rod-shaped acidophilic bacterium *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*, formerly named *Thiobacillus ferrooxidans*) has attracted great interest because of its use in industrial mineral processing and its unusual physiology (Brierley, 1978; Merroun *et al.*, 2003). Various strains of the species *A. ferrooxidans* have been isolated from natural (rocks, ores and mine waters) and technological (ore concentrations and pulps of the gold and non-ferrous industries) sources. The habitats of *A. ferrooxidans* strains are geographically extremely diverse and vary in their physicochemical conditions (presence of particular sulphide minerals and their ratio, pH, temperature and the content of toxic compounds in the liquid phase). This might explain the polymorphism of *A. ferrooxidans* strains, in terms of both their physiological properties and genotypic characteristics (Karavaiko *et al.*, 2003).

The remarkable feature of the species *A. ferrooxidans* is that it derives energy from the oxidation of ferrous iron, elemental sulphur and its reduced compounds and sulphide minerals (LiveseyGoldblatt *et al.*, 1983; McCready and Gould, 1990; Rohwerder *et al.*, 2003) and uses carbon dioxide as a source of carbon. This peculiar metabolism makes *A. ferrooxidans* important in highly acidic environments (Yu *et al.*, 2007) both as an agent of Acid Mine Drainage (AMD) formation and bioremediation means for AMD clean-up, metal bioleaching (Johnson *et al.*, 1992; David *et al.*, 2001) and desulphurization of coals (Sadowski *et al.*,2003; Acharya *et al.*, 2001). This ability also makes it suitable for use in biomining to recover metals such as copper, uranium and gold (Ewart and Hugues, 1991).

The use of microorganisms to facilitate the extraction and recovery of precious and base metals from primary ores and concentrates, referred to generically as 'biomining', has developed into a

successful and expanding area of biotechnology (Rawlings and Johnson, 2007) which has gained acceptance in the sustainable mineral development concept because it offers a potentially inexpensive and nonpolluting way to pre-treat ores especially “refractory” (difficult -to -treat) and low-grade ores (Zhou and Nui, 2005).

For a long time, gold was recovered only by physical methods, namely gravity separation followed by melting, and then came amalgamation of gold with mercury. For the last 100 years, however, cyanidation has been the main process for extracting gold from ores (Morin, 1995). But with recent concerns of mining activities on environmental quality (air, water and land), priority should be given to technology where efficiency, increased availability and sustainability could be ensured because exploitation of natural resources is vital for the growth of economies.

Mineral-derived wealth is one source of capital asset which can get an economy’s ball rolling, and many of today's developed states (USA, Canada, Australia, UK, Spain) have undoubtedly benefited from mineral-generated wealth at various stages during their economic development. Less developed countries have also benefited from mineral wealth to some extent in their pursuit to develop (Petterson, 2008). For instance, the contribution of Ghana’s mining sector to the country’s Gross Domestic Product (GPD) increased from 1.3% in 1991 to an average of about 5% in recent years. Export earnings from minerals averaged 35%, and the sector stands as one of the largest contributors to Government revenues through the payment of mineral royalties, employee income taxes, and corporate taxes (Bermúdez-Lugo, 2006).

This suggests that nations, especially developing countries, must of necessity exploit their mineral reserves to help contribute to their development. This must however not come at a cost to the environment. Therefore, technologies that will make it possible to enlarge the amount of mineral deposits that can be exploited especially from refractory ores, and guarantee the mineral resources

demanded for fast economic development (Zhang Lin *et al*., 2008) while safeguarding the environment for future generations should be encouraged.

Biomining is a biohydrometallurgical technology which involves the extraction of specific metals from their ores through biological means usually bacteria (Siddiqu *et al.,* 2009). Biohydrometallurgy is no longer a promising technology but is now an established economical alternative for treating specific mineral ores. It occupies an increasingly important place among the available mining technologies (Ndlovu, 2007). Bacteria and archea have been identified as the biological tools for the process and the most frequently used ones include the iron- and sulphuroxidising *Acidithiobacillus ferrooxidans*, the sulphur-oxidising *Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus* and the iron-oxidising *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* (Clark and Norris, 1996; Leduc and Ferroni, 1994).

Although the African continent has rich mineral reserves and was the first to develop the process in South Africa, it has not benefited much from the technology. Countries such as Chile, Brazil and more recently China (Yang *et al.,* 2002) have been quick to identify the potential benefits of this technology and have established measures(especially the isolating active native bacteria) both to develop such industries and to extract value where possible (Ndlovu, 2007). For instance, here in Ghana, of the 13 registered mining companies, AngloGold Ashanti Obuasi Limited (1994) at the Sansu Creek and the Bogoso Prestea Mining Limited (2007) are the only mining companies known to use the technology even though Ghana houses the world's largest BIOX® plant (Ransford Sekyi, 2009).

One major factor that hinders the development of the technology is the availability of the bacteria for the process. The two companies that use the process in Ghana import the bacteria (Obuasi from

South Africa and Bogoso from Yellow Park in USA). One major step in the attempt to develop the technology in one's own country is to ensure the availability of the bacteria used in the processing; this research is therefore aimed at exploring the possibility of finding candidate organisms that are associated with the ores and mine waters in the Obuasi mines.

1.1 Problem Statement

The increasing growth in the mining industry world-wide including Ghana is driven in the main by socio-economic needs. Increased mining activities, however, has been taken with very little integration of the environment. Such unsustainable development has led to various forms of environmental degradation and people are now weighing in the advantages of mineral-derived wealth and the environmental degradation from mining (Aryee, 2001).

With the increasing high level of consciousness of environmental issues such as climate change (roasting of sulphur ores), water pollution (excessive cyanide use), land degradation (excavations, spills) coupled with the anticipated depletion of oxide reserves and the increasing presence of sulphide in gold ore around the world, it calls for technologies which can ensure that nations benefit from their natural resources while safeguarding their environment.

Environmentalists must therefore encourage and lobby for mining extraction best-practice for the future to maximize the recovery of minerals for sustainable economic development. One such best-practice technology which can help achieve this balance is the Biomining concept.

Also, if the environmentalists in this country are to push for the adoption of the technology by most mining companies to ensure the sustainability of the mining sector, there will be the need to find the microorganisms in our native soils that can support the process and also assess the recovery rate reachable with these indigenous microorganisms compared with those imported.

Even though this technology has been available in Ghana since 1994, not much has been done to expand the use of the process, evident in the fact that only two of the thirteen registered mining companies use this technology with the latest one starting in 2007. The polymorphic nature of the bacteria used for the process poses challenges for the development of the process. For Ghana to maximize the benefits of the process there is the need for more research to serve as a repository of knowledge for all who are interested in the use of the technology.

This will ensure that the technology is readily available for all mining groups who express interest in it but have no mother companies to support with the provision of bacteria and technical support. Hence the need for study of the biooxidation concept and assessing the possibility of finding indigenous microbes that contributes to the process.

1.2 Objectives of the Study

The aim of the study is to isolate an indigenous candidate organism from the Sulphur Treatment Plant, mine water and tailings and finding their biooxidation ability of refractory ores from a mining concession area in Ghana.

1.2.1 Specific Objectives

Specific objectives to help achieve the overall objective will consist of the following:

- To isolate *A. ferrooxidans* from the ores in the Obuasi mine site;
- Assess the heavy metal levels in their environment at the Obuasi mine site;
- To identify and characterise the bacteria using physiological and morphological features of the different isolates; and
- To use the isolates singly to assess the biooxidation potential of the isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biohydrometallurgy in Metal Extraction

The realization that the abilities of microorganisms to oxidise minerals could be harnessed in more precisely engineered operations led to the emergence of the biohydrometallurgical technology (Brierley and Brierley, 2001). Biohydrometallurgy is a natural process that use microorganisms to enhance the dissolution of metals from mineral ores especially sulphide ores, by making them more amenable to dissolution in aqueous solutions (Deveci *et al*., 2003). It has been globally applied to the recovery of base and precious metals, and is now an established industrial technology for the pre-treatment of refractory ores (Hansford and Vargas, 2001).

Biohydrometallurgy became a reality in the 1950s with the advent of copper bioleaching, although it has been known that microorganisms have contributed to the solubilisation of metal sulphides since ancient times and Romans benefited from their action long before Christ (Brierley and Brierley, 2001). This technology has been widely embraced in recent times by the mining industry because according to Ndlovu (2007) it satisfies most of the industrial requirements in terms of technical effectiveness, flexibility, robustness, ease of operation, cost effectiveness, environmental friendliness and the ability to be expanded when starting from small scale. The BIOX^{Tm} process has been a technical and economic success and offer real advantage over conventional refractory processes, such as roasting and pressure oxidation (Van Aswegen *et al.*, 2006).

Biohydrometallurgy encompasses two related microbial processes that are useful in extractive metallurgy: bacterial leaching, also known as bioleaching, and biological oxidation (biooxidation) (Acevedo, 2002). Bioleaching is leaching where extraction of metal from solid mineral into solution

is facilitated by the metabolism of certain microbes such as bacteria, archaea and eukaryote and is used today in commercial operations to process ores of copper, nickel, cobalt, zinc and uranium. On the other hand, biooxidation is an oxidation process caused by the microbes where the valuable metal remains (but becomes enriched) in the solid phase and the solution may be discarded and is used in gold processing and coal desulphurization (Brierley and Brierley, 2001). However, the two terms are often used interchangeably. Collectively, minerals Biooxidation and bioleaching are commercially proven biohydrometallurgical or Biomining processes.

Although there has been a significant research into bioleaching of zinc sulphides (Mousavi *et al.,* 2007; Olubambi *et al*., 2007; Rodriguez *et al.,* 2003), and both the sulphidic and lateritic nickel ores in the recent years (Mason and Rice, 2002; Simate and Ndlovu, 2007; Valix *et al*., 2000), current worldwide bioleaching and Biooxidation research and operations remain focused essentially on copper (Cancho *et al.,* 2007; Waitling, 2006; Sadowski *et al*., 2003) and gold production (Brierley and Brierley, 2001; Nestor *et al*., 2001).

The adaptation of the Biooxidation process (BIOX ® Process) on the African continent has proven vital because of its rich mineral deposits. The BIOX® process, which pre-treats refractory sulphide gold ores, was developed to increase gold recovery rates during the metallurgical extraction process (van Aswegen *et al*., 2006).

2.2 The African Mining Industry

The African continent is richly endowed with abundant reserves of strategic and economically important minerals (Ndlovu, 2007). These minerals hold the promise of exceptional long term social and economic benefits for the continent and have become increasingly exploited during the last couple of years thus contributing immensely to the African countries' national wealth. The continent hosts

about 30% of the planet's mineral reserves, making it a truly strategic producer of these precious metals (Coakely and Mobbs, 1999). South Africa is one of the top leading gold producers in the world whilst Ghana which ranks second after South Africa in the Continent, is the largest gold producer in West Africa and also makes a notable contribution to world gold production (www.mbendi.co.za). Natural resources development has proven vital to the economies of African nations because of its role in generating employment and foreign exchange (Ndlovu, 2007).

The historical importance of mining in the economic development of Ghana is considerable and well documented, with the country's colonial name Gold Coast reflecting the importance of the mining sector (Coakley, 1996). Gold is by far the most important mineral exploited in Ghana, accounting for 37.9% and 95.8% of total merchandise and mineral exports, respectively. Clearly, gold grossly overshadows the other minerals in terms of value generated (Bank of Ghana Annual Report, 2005).

Unfortunately, mining and mineral processing has always been a cost conscious industry and leaves us with many environmental challenges. Most of Africa's mineral industries are export oriented and thus exposed to the world market fluctuations. Even small fluctuations in mineral prices on world markets regularly lead to the closure of mines and resultant loss of employment (Ndlovu, 2007). It is therefore important to develop and utilize appropriate technologies that are simple to apply, provide low capital and operational costs, comply with environmental regulations and yet are highly productive to ensure the mineral industry is sustainable.

2.3 Sustainable Mining Concept

Over the past fifteen years there has been significant interest in the concepts of sustainable development and mining. The sustainable mining concept acknowledge that the world has an ever-growing need for minerals, which underpins a wide range of economic benefits and aims to move

mineral development forward in a consensual, strategic manner. Sustainable minerals finds a consensual way forward which:

- Generates sufficient minerals-oriented wealth to attract capitalist-oriented private-sector industries;
- Makes it essential that mining companies operate to the highest environmental standards, thus protecting the physical environment; and
- Develops methodologies and approaches which maximize lasting benefits to communities at local, regional, and national levels (Hendrix, 2005; Petterson, 2008).

Approaches that are adopted would usually encourage: maximizing resource usage and recycling; minimizing waste production; minimal negative impacts on the physical environment; local communities and economies to receive widespread and long-lasting benefits and an increased sense of responsibility towards mineral production within our sphere of influence (Petterson, 2008). The Biohydrometallurgical technology that fits such criteria and has found worldwide appreciation and application proves to satisfy the sustainable mining concept.

2.4 The Biomining Process

The process comprises of the engineered processes and the biological tools.

2.4.1 Commercial Engineered Processes and Factors Influencing Biomining

2.4.1.1 Engineering Processes

Engineering options for biomining have evolved from relatively inexpensive, partly controlled, irrigated dump or heap reactors to sophisticated, highly controlled and expensive stirred-tank reactors. Biomining is commercially employed in four different engineered processes:

- Dump bioleaching extracts copper from sulphide ores that are too low-grade to process by any other method – this process has been used since the mid 1950s

- Heap bioleaching which has been used since the 1980s, extracts copper from crushed sulphide minerals placed on engineered pads
- Heap minerals biooxidation pre-treats gold ores in which the gold particles are locked in sulphide minerals, significantly enhancing gold recovery
- Stirred-tank minerals Biooxidation enhances gold recovery from mineral concentrates in which the gold is locked in sulphide minerals and stirred-tank bioleaching extracts base metals from concentrates of metal-containing sulphide ores (Brierley, 2008).

Stirred-tank reactors consist of a series of aerated continuous-flow tanks that are used mostly in a pretreatment process for the recovery of high-value metals, such as gold, from mineral concentrates. These reactors are more expensive to construct and operate than heap reactors but allow for the precise control of parameters such as temperature, pH and aeration, all of which have a major impact on the microbial populations and metal recovery efficiency (Rawlings and Johnson, 2007b).

2.4.1.2 Effect of temperature and cooling requirement

The BIOX ™ bacterial culture is an adopted mixed culture of mesophilic bacteria .The operating temperature range for mesophilic bacteria is 30-45ºC although the reactors can be operated at temperature up to 50ºC for short period. The oxidation of sulphide mineral is extremely exothermic (Lawson, 2001). Constant cooling of the BIOX ™ reactors is therefore necessary to control the temperature to within the optimum operating temperature range.

2.4.1.3 Hydrogen Ion (pH) control

Hydrogen ion concentration (pH) is an extremely important parameter for the successful operation of the Biooxidation plant. The optimum pH range for the process is 1.1-1.5, although the process can operate over a wider pH range of 1.0-2.0. Poor pH control is often found to be the cause of the

low bacterial activity in the BIOX ™ reactors in commercial operations (Van Aswegen *et al.*, 2006).The mineralogical composition has a large influence on the acid balance during the biooxidation of the concentrates. The limestone or sulphuric acid requirement, to control pH in slurry in each reactor to be within the optimum range, will be a function of the concentrations of the various minerals in the flotation concentrate and the extent of oxidation of the minerals in the BIOX ™ reactors. pH control in BIOX ™ reactors can account for a significant portion of the operating cost for a plant (Chetty *et al.*, 2000; Van Aswegen *et al.*, 2006).

2.4.1.4 Oxygen Supply

The supply of oxygen represents the largest consumer of the power in biooxidation and is therefore a major part of both the capital and the operating cost for a biooxidation plant (Van Aswegen *et al.*, 2006). The oxygen demand is driven by the chemical oxygen demand for the oxidation of the sulphide minerals, and typical value for oxygen demand will vary from 1.8 to2.6 kg oxygen per kilogram sulphide oxidised, depending on the mineralogical composition of the concentrate and the oxidation rates achieved. The oxygen for commercial biooxidation is normally supplied by sparging compressed air into the reactors (Fraser *et al.*, 1993).

2.4.2 The Biological tools for the process

2.4.2.1 Uses of Microbes in Industry

The use of microorganisms for large scale industrial processes is not new, although it has assumed renewed emphasis in recent years. In recent years, microorganisms have found their application not only in the production of a variety of metabolites but also in the bio transformation of several chemicals. They can reduce environmental pollution through a variety of processes and other means including the following: recovery of metals from polluted waterways, elimination of sulphur from

metal ores and coal fired power plant and provide cheap and cost effective methods of mining and metallurgy. Thus microbial biotechnology will have a great impact on industry in the 21st Century (www. technoscan.com/ tracking.php).

2.4.2.2 General Physiology of Mineral-Degrading Bacteria

The most important microbes involved in the biooxidation of minerals are those that are responsible for producing the ferric iron and sulphuric acid required for the biooxidation reactions and these are the iron- and sulphur-oxidising chemolithrophic bacteria and archaea according to Rawlings, (2002). Irrespective of the type of process or temperature at which they are employed, these microbes have a number of features in common that make them especially suitable for their role in mineral solubilization. Four of the most important characteristics are:

- They grow autotrophically by fixing CO_2 from the atmosphere;
- They obtain their energy by using either ferrous iron or reduced inorganic sulphur compounds (some use both) as an electron donor, and generally use oxygen as the electron acceptor;
- They are acidophiles and grow in low pH environments (pH.1.4 to 1.6 is typical);and
- They are remarkably tolerant to a wide range of metal ions, though there is considerable variation within and between species (Dopson *et al.*, 2003).

The modest nutritional requirements of these organisms are provided by the aeration of an iron- and/or sulphur-containing mineral suspension in water or the irrigation of a heap. Small quantities of inorganic fertilizer can be added to ensure that nitrogen, phosphate, potassium and trace element limitation does not occur (Logan *et al.*, 2007).

2.4.2.3 Types of Microbes Used for Biomining

For many years, *Acidithiobacillus ferrooxidans* was the only known iron-oxidising acidophile, although since the 1970s, a number of novel, phylogenetically distinct prokaryotes with this

particular physiological trait have been described. Some of these, such as *Leptospirillum ferrooxidans* and *A. ferrooxidans*, are obligate autotrophs, while others are obligate or facultative heterotrophs, such as S*ulfobacillus spp.*, *Acidimicrobium ferrooxidans*, and "*Ferrimicrobium acidiphilum*" (Johnson *et al.,* 2003).

Iron-oxidising acidophiles have frequently been differentiated in terms of their temperature optima and initially, it appeared that all mesophilic iron-oxidising bacteria (temperature optimum < 40^{o}C) were gram-negative in contrast to moderately thermophilic species (temperature optimum 40–60^{o}C), which appeared to be gram-positive. This apparent correlation has, however, been invalidated by the isolation of mesophilic species of Sulfobacillus and other Gram-positive bacteria (Yahya *et al*., 1999), and the finding that some strains of *Leptospirillum ferriphilum* grow optimally above 40^{o}C (Coram and Rawlings, 2002). At temperatures above 60^{o}C, iron oxidising prokaryotes tend to be archaea rather than bacteria, and recently iron-oxidising archaea (*Ferroplasma spp*.) with temperature optima of 35–39 ^{o}C have been described (Edwards *et al.,* 2000; Golyshina *et al*., 2000; Okibe *et al.,* 2003).

However, the most important microorganisms are considered to be a consortium of gram-negative bacteria which include the iron- and sulphur-oxidising *Acidithiobacillus ferrooxidans*, the sulphur-oxidising *Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus* and the ironoxidising *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* (Clark and Norris, 1996; Coram and Rawlings, 2002; Foucher *et al*., 2003). Several species of fungi can also be used for Biomining (Siddiqu *et al.*, 2009).

2.4.2.4 Acidithiobacillus genus

2.4.2.4.1 Classification and Habitat

The *Acidithiobacillus genus* belongs to the γ-proteobacteria and comprises five species: *Acidithiobacillus albertensis, Acidithiobacillus caldus, Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* (Kelly and Wood, 2000) and *Acidithiobacillus cuprithermicu* (http://en.wikipedia.org/wiki/Acidithiobacillus). The genus was formed when the former *Thiobacillus* was split into the genera *Acidithiobacillus, Halothiobacillus* and *thermithiobacillus.* The members of the genus are acidophilic gram negative rods, motile by one or more flagella and comprise both mesophiles and moderate thermophiles. All four species are autotrophs capable of growth utilizing inorganic compounds such as reduced inorganic sulphur compounds (RISCs) as sole energy substrate, whereas *A. ferrooxidans* is the only representative of the genus that can also oxidise Fe^{2+} (Hamid *et al.*,2003). *Acidithiobacillus* use various RISCs or Fe^{2+} as electron donor and therefore are often found in metal sulphide deposits (Karavaiko *et al.*, 2003), fresh water associated with sulphide deposits (Gonzalez-Toril *et al.*, 2003) and seawater (Kamimura *et al.*, 2003).

2.4.2.4.2 *Acidithiobacillus* and the Iron Oxidation Pathway

Ferrous iron is readily oxidised to ferric iron and in this way it can serve as an electron donor. The Fe^{2+}/Fe^{3+} redox couple has a very positive standard electrode potential (+770 mV at pH 2), as a result only oxygen is able to act as a natural electron acceptor and in the presence of protons with the product of the reaction being water (Rawlings, 2005). The use of iron as an electron donor will therefore occur only during aerobic respiration. However, under aerobic condition, ferrous iron spontaneously oxidises to ferric iron unless the pH is low.

Therefore, extremely acidophilic bacteria are able to use ferrous iron as an electron donor in a manner that is not possible for bacteria which grow at neutral pH. Because the difference in redox potential between the Fe^{2+}/Fe^{3+} and O_2/ H_2O redox couples is small and because only one mole of

electrons is released per mole of iron oxidised, vast amounts of ferrous iron need to be oxidised to produce relatively little cell mass. These large quantities of iron are not transported through cell membrane but remain outside the cell and each ferrous iron atom simply delivers its electron to a carrier situated in the cell envelope (Ojumu *et al.*, 2005).

The mechanism of iron oxidation has been most extensively studied for the bacterium *A. ferrooxidans*. This bacterium contains a *rus* operon that is proposed to encode for the electron transport chain that is used during the oxidation of ferrous iron. The detection of rusticyanin has been linked to the growth of *A. ferrooxidans* on iron and it has been shown that the expression of the *rus* operon was 5 to 25 fold higher during growth on iron compared with sulphur. It has been suggested that rusticyanin probably functions as an electron reservoir as it readily takes up electrons available at the outer membrane and channels them down the respiratory pathway. Rusticyanin serves as a redox buffering function ensuring that the outer membrane Cyc2 electron acceptor remains in a fully oxidised state, ready to receive electrons from ferrous iron even in the presence of short – term fluctuations of oxygen. Interestingly aporusticyanin has been implicated in the adhesion of *A. ferrooxidans* cells to pyrite. Although the *rus* operon is clearly involved in iron oxidation, it is not yet known whether the components of the operon are sufficient for iron electron transport system or whether other components such as the *iro* gene for a high redox potential iron oxidase (HiPIP) might also play a role. HiPIPs might not be present in all strains of *A. ferrooxidans* and might play a bigger role in sulphur oxidation than iron oxidation (Rawlings, 2005).

2.4.3 Role of Mining Bacteria in Mineral Processing

Metal leaching is recognized as being mainly a chemical process in which ferric iron and protons are responsible for carrying out the leaching reactions. The role of the microorganisms is to generate the leaching chemicals and to create the space in which the leaching reactions take place.

Microorganisms typically form an exopolysaccharide (EPS) layer when they adhere to the surface of a mineral but not when growing as planktonic cells (McCready and Gould, 1990). It is within this EPS layer rather than in the bulk solution that the biooxidation reactions take place most rapidly and efficiently and therefore the EPS serves as the reaction space .The mineral dissolution reaction is not identical for all metal sulphides and the oxidation of different metal sulphides proceeds via different intermediates (Rawlings, 2005).

A thiosulfate mechanism has been proposed for the oxidation of acid insoluble metal sulphides such as pyrite (FeS_2). In the thiosulfate mechanism, solubilization is through ferric iron attack on the acid-insoluble metal sulphides with thiosufate being the main intermediate and sulfate the main end-product. Using pyrite as an example of a mineral, the reactions may be represented as:

$$FeS_2 + 6\ Fe^{3+} + 3\ H_2O \rightarrow S_2O_3^{2-} + 7\ Fe^{2+} + 6\ H^+ \quad (1)$$

$$S_2O_3^{2-} + 8\ Fe^{3+} + 5\ H_2O \rightarrow 2\ SO_4^{2-} + 8\ Fe^{2+} + 10\ H^+ \quad (2)$$

In the case of the polysulphide mechanism, solubilization of the acid-soluble metal sulphide is through a combined attack by ferric iron and protons, with elemental sulphur as the main intermediate. This elemental sulphur is relatively stable but may be oxidised to sulfate by sulphuroxidising microbes such as *Acidithiobacillus thiooxidans* or *Acidithiobacillus caldus* (reaction 5 below).

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5\ H_2S_n + Fe^{2+}\ (n \geq 2) \quad (3)$$

$$0.5\ H_2S_n + Fe^{3+} \rightarrow 0.125\ S_8 + Fe^{2+} + H^+ \quad (4)$$

$$0.125S_8 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (5)$$

The ferrous iron produced in reactions (1) to (4) may be re-oxidised to ferric iron by ironoxidising microorganisms such as *Acidithiobacillus ferrooxidans* or bacteria of the genera *Leptospirillum* or *Sulfobacillus*.

$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$ (6)

The role of the microorganisms in the solubilization of metal sulphides is, therefore, to provide sulphuric acid (reaction 5) for a proton attack and to keep the iron in the oxidised ferric state (reaction 6) for an oxidative attack on the mineral (Siddiqu *et al.*, 2009).

2.4.4 Role of Mining Bacteria in Acid Mine Drainage Generation

In Acid Mine Drainage (AMD) systems, there are two primary mechanisms responsible for the oxidation of pyrite and the subsequent generation of acid, oxidation of pyrite to sulfate is described by the following two end-member reactions which utilize either O_2 or Fe (III)aq as oxidants (Singer and Stumm, 1970a; Nordstrom and Alpers, 1999; Nordstrom and Southam, 1999 and Butler, 2007):

$FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2} + 2H^+$ (1)

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

The rate of reaction (1) is enhanced by the bacterium *A. ferrooxidans*. The rate of reaction (1) is limited by the availability of dissolved oxygen and therefore this reaction may represent the common reaction for pyrite oxidation under O_2 saturated conditions. Compared to oxidation by O_2, Fe (III)aq can rapidly oxidise pyrite abiotically and anaerobically via reaction (2). To maintain reaction (2), however, Fe (III)aq must be generated by the following reaction.

$Fe^{2+} + ¼\ O2 + H^+ \rightarrow Fe^{3+} + ½\ H_2O$ (3)

Under acidic conditions (pH < 3), reaction (3) can be the rate limiting step for reaction (2) and bacterial oxidation of Fe^{2+} at this low pH is several orders of magnitude faster than abiotic oxidation (Nordstrom and Alpers, 1999; Schippers and Sand, 1999). Therefore, generation of Fe (III)aq via reaction (3) is generally mediated by bacteria such as *A. ferrooxidans*, in AMD sites. This reaction

determines how much Fe (III) is available for the oxidation of pyrite at low pH and was subsequently dubbed the ‘‘rate-determining step’’ by Singer and Stumm (1970a) in AMD generation.

2.4.5 Role of Mining Microbes in Bioremediation

The processing of sulphide-rich ores in the recovery of base metals, such as copper, lead, zinc, and gold, has produced large quantities of pyrite wastes (Langmuir, 1997). When exposed to rain, this material generates acid mine drainage (AMD) which contains large amounts of sulfate, iron, arsenic, and heavy metals. Despite their toxicity, such waters host organisms, both prokaryotes and eukaryotes, which are able to cope with the pollution (Baker and Banfield, 2003).

Some of them have the capacity to modify the physicochemical conditions of the water either by detoxification or by metabolic exploitation. For example, efficient oxidation of arsenic by bacteria has been reported in AMD or in chemically somewhat similar waters like those from hot springs (Battaglia-Brunet *et al.*, 2002; Langner *et al.*, 2001; Casiot *et al.*, 2003b). Because of their elevated iron concentration, the development of iron-oxidising bacteria is favored in AMD (Hallberg and Johnson, 2003) where *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* are often observed (Baker and Banfield, 2003).

Owing to its ability to oxidise Fe, the bacterial consortium in AMD plays a major role in the immobilization of the elements that exhibit a strong affinity for solid Fe oxide phases such as Strontium (Sr), Cesium (Cs), Lead(Pb),Uranium (U) (Ferris *et al.*,2000), and As (Morin *et al.*,2003; Casiot *et al.*, 2003b). In addition, the ability of several bacterial strains in AMD to oxidise As further contributes to reduction of its toxicity in water, because As (III) is considered to be more

toxic than As (V) and because arsenate adsorbs more strongly than arsenite to Fe (III) oxides and hydroxides at acidic pH (Bowell,1994; Sadiq, 1997).

Owing to their tolerance of heavy metals and the ability of some to promote transformations that make some metals less toxic, bacteria such as *A. ferrooxidans,* in acid mine waters are useful in AMD bioremediation or that of some other industrial effluents (Sadiq, 1997).

2.5 Significance of Biomining in the 21th Century

As the worldwide high grade ore reserves reduce at appalling rate because of high metal demand, traditional techniques like pyrometellury and chemical processing are becoming more and more economically inviable (Siddiqui *et al.*, 2009). Until recently, only free-milling ores were treated by the processing companies. The refractory phases in a deposit of an otherwise cyanidable ore were simply discarded. However, with the strong demand for gold coupled with the ore depletion, interest in refractory ores has considerably increased (Morin, 1995).

The use of microbes in mineral process has some distinct advantages over traditional physicochemical methods. This process is more environmentally friendly, less consuming energy and useful for the low-grade ores. And so, it is increasingly being used because of its economical advantages (Rawlings, 2002).

Also, increased concern regarding the effect of mining on the environment is improving the competitive advantage of microbial based metal recovery processes. The enforcement of more stringent legislation to limit environmental pollution is making biomining more attractive (Siddiqui *et al.*, 2009). With the increasing regulations on the use of cyanide and the lowering of acceptable cyanide discharge levels, the gold industry faces more challenges than ever before of developing

technologies that ensure the recovery of gold from low-grade, refractory ores with the minimum cyanide use to stay in business.

2.5. 1 Refractoriness of Ores

Gold deposits can be broadly classed into two categories, primary and secondary. The secondary ore are high grade and easy to process using conventional method. However, primary deposits are sulfidic, mainly pyrite and arsenopyrite. Direct cyanide leaching of the primary deposits gives poor recoveries of 5-70%, depending on the mineralogical composition of the deposit. The difficult-to-treat concentrates are described as "refractory". Some of the causes of refractoriness are listed below:

- Gold can be encapsulated (locked up) in the mineral matrix, so that the leach reagents of cyanide and dissolved oxygen are unable to diffuse to the gold particle;
- Sulphide minerals may act as cyanicides or oxygen consumers during cyanidation, which results in insufficient cyanide and oxygen in the pulp to leach the gold;
- A protective coating may be formed on the surface of the gold by compounds such as iron oxides, antimony and lead compounds; and
- The gold may exist in solid solution ("invisible" gold) in the sulphide lattice (Dunn and Chamberlain, 1997).

These refractory ores require some form of oxidative pre-treatment in order for cyanidation to be effective in recovery of the gold to economically acceptable levels. Pre-treatment options for refractory ores include: Roasting; Ultrafine grinding; Pressure oxidation and Biooxidation (www.Wikipedia.com, 2009). But as environmental concerns on mining activities increase, the Biooxidation process has received centre attention in recent years.

2.5.2 Gold Cyanidation

Although alternative reagents have been seriously considered in place of cyanide including the halide system (Chlorine, bromine and iodine), the thiosystem (thiosulfate, thiocyanate and thiourea) and polysulphide system (S_x^{2-}), and the ammonia system (ammonia and ammonium copper-cyanide) (Wan *et al.*, 2005), it is not surprising that Cyanide is still the most common if not only universally applicable lixivant for gold bearing ores after examining the chemical characteristics and the health and safety profiles of the proposed alternatives (Hendrix, 2005). Gold cyanidation (also known as the cyanide process or the MacArthur-Forrest process) is a metallurgical technique for extracting gold from low-grade ore by converting the gold to water soluble aurocyanide metallic complex ions. It is the most commonly used process for gold extraction, but due to the highly poisonous nature of cyanide, the process is highly controversial (Wikipedia, 2008). The volatilization of cyanide as hydrogen cyanide makes it very dangerous because this gas is highly toxic; hydrogen cyanide boils at 26°C, barely above room temperature.

2.5.3 Effect of Cyanide on the Environment

Free cyanide breaks down rapidly when exposed to sunlight, although the less toxic compounds such as cyanates and thiocyanates may persist for some years. Humans are not usually the famous disaster since they can be warned not to drink or go near polluted water. However cyanide spills can have a devastating effect on rivers, killing everything for several miles downstream. Whole food chain may collapse, from phytoplankton to ospreys with fishes been the most obvious casualties (UNEP, 2000).

2.6 Ghana's Biooxidation Experience

On the African continent, South Africa and Ghana are the two countries that have adopted the technology for gold recovery. Even though Ghana prides itself in the fact that it houses the world's largest BIOX ^{Tm} plant, it leaves much to be desired.

Currently, information available at the Ghana Environmental Protection Agency Head Office indicate that, there are thirteen registered mining companies which have the requisite environmental permits for mining in Ghana. And of the thirteen, only two namely, AngloGold Ashanti Gold Limited at the Sansu creek (1994) and the Bogoso Mining Company (2007) use the BIOX technology (Ransford sekyi, 2009). A primary research amongst people involve in mining activities indicated that very few people have an idea of the technology, more interesting was the fact that a large portion of the miners have no idea about the technology.

2.6.1 The Sansu BIOX^{Tm} Plant

The installation and successful commissioning in February 1994, of the BIOX^{Tm} process for the treatment of refractory concentrates at the Sansu Sulphide Treatment Plant was a major breakthrough for the BIOX® technology. The technology was selected after intensive metallurgical test work program and was selected on the basis of reduced capital and operating cost, reduced technical risk, reduced environmental impact and for the simplicity of operation (Nicholson *et al.,* 1993).

The plant was designed to treatment nominally 720 t day^{-1}concentrate in three modules of six reactors 900m3, with a concentrate containing 11.4% sulphide (S) and 7.7% arsenic (As). The nominal treatment capacity of the plant was expanded in 1995 to 960 t day^{-1} concentrate with the addition of a fourth reactor module (Rawlings and Johnson, 2007).

The successful installation and operation of the Sansu BIOX^{Tm} plant clearly demonstrated the scale-up potential of the process using the modular design. The simplicity and ease of operation was also demonstrated, enabling the use of the technology in remote location. Process optimization and innovations have led to significant savings in operation cost while maintaining steady operation of the BIOX^{Tm} reactors (Osei-Owusu, 2001).

2.6.2 The Bogoso BIOX ® Plant

The Bogoso BIOX® Plant was completed in 2007, thirteen years after the Sansu plant was established. The Biox plant is made up of six reactors and at fully optimized production Bogoso/Prestea's sulphide circuit operates at a design capacity of 3.5 million tonnes per year of refractory sulphide ore into the crusher, equivalent to 34 tonnes per hour of concentrate through the BIOX® circuit. Overall recovery is estimated at 80%, resulting in annual gold production from the sulphide plant of between 200,000 and 235,000 ounces. The BIOX® process utilizes a combination of *Acidithiobacillus ferrooxidans, Acidithiobacillis thiooxidans*, and *Leptospirillum ferrooxidans* which are imported from the hot springs in Yellowstone National Park in the U.S. They feed on sulphurous materials, and in doing so, liberate the gold particles from the sulphide mineralization. Optimization of temperature (35 to 45 degrees Celsius) and pH (1.2 to 2.0) are the most important factors in keeping the bacteria active and vital (http://www.gsr.com/).

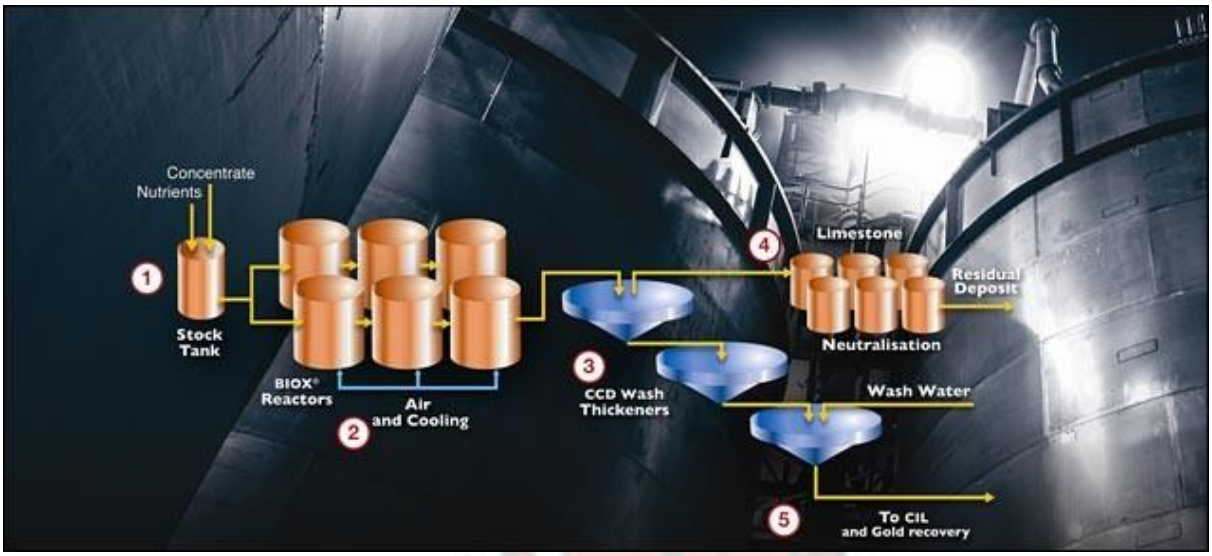

Plate 1 Flow diagram of the Bogoso BIOX® Plant

2.7 Future Prospects of Biomining

The future of biomining is challenging, as it offers advantages of operational simplicity, low capital and operating cost and shorter construction times that no other alternative process can provide. In addition, because of the minimal environmental impacts the technology causes, the use of this technology in the mining industry is set to increase. Once commercial scale hightemperature processes have been designed, the variety of minerals that will become acquiescent to biomining will increase. Although the viability of microbes that flourish at temperatures 55°C is not yet well-proven commercially, it appears that one can isolate iron- or/and sulphur-oxidising organisms for whatever temperature is required, up to at least 80°C. Therefore, where suitable microbes for mineral biodegradation at a given temperature are not yet known, they can probably be found (Rawling and Johnson, 2007; Siddiqu *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.0 Study Area

3.1

The study was carried out in the Anglogold Ashanti (AGA) Obuasi Mine located in the Obuasi Municipality of the Ashanti Region of Ghana (Fig. 1). Obuasi is about 64 km South of Kumasi the regional capital and about 200 km North West of Accra and shares boundaries with Adansi South to the East and South, Amansie Central to the West and Adansi North to the North. The

Obuasi Municipality is located

population of the Municipality currently stands at over 265,000 with about 90 % urban and 10 % rural distribution. The annual growth rate of 4% is as a result of influx of migrant workers who come to the area in search of mining and related jobs (AGA, Environmental Management Plan 2008-2011).

3.2 Geology and Mineralization of the AGA Obuasi Limited

The gold deposits at Obuasi are part of a prominent gold belt of Proterozoic (Birimian) volcanosedimentary and igneous formations. These deposits extend for a distance of approximately 300km, in a north-east/south-west trend, in south-western Ghana. Obuasi mineralisation is shearzone-related and there are three main structural trends hosting gold mineralisation namely the Obuasi trend, the Gyabunsu trend and the Binsere trend. The underground mine is situated on the Obuasi trend (AGA Environmental Management Plan, 2008-2011).

Most of the area is dominated by the Birimian metasedimentary units (Phyllites, Schists) across the Western portion of the area but in the general vicinity of the mines a few metavolcanic units (Mainly Chlorite schists) are fairly widespread and has been interpreted (Junner, 1932) to be basaltic flows and pyroclastic units. To the east, the Dampaiyau Range is underlain mainly by Tarkwaian quartzites, phyllites and minor quartz conglomerates, which are in tectonic contact with the Birimian metamorphic units. The generally coincident bedding and primary foliation planes strike to the NNE but swing sharply towards the east just north of Obuasi; dips are subvertical in the south but more moderate to the NW-NNW in the central and northern part of the area (AGA Environmental Management Plan, 2008-2011).

Two main ore types are mined, namely quartz veins and sulphide ore, other prominent minerals incl ude quartz, chlorite and sericite. The quartz vein type consists mainly of quartz with free gold in

association with lesser amounts of various metal sulphides containing iron, zinc, lead and copper. The gold particles are generally fine-grained and are occasionally visible to the naked eye. This ore type is generally non-refractory. Sulphide ore is characterized by the inclusion of gold in the crystal structure of a sulphide mineral. The gold in these ores is fine-grained and often locked in arsenopyrite. Higher gold grades tend to be associated with finer grained arsenopyrite crystals. Sulphide ore is generally refractory (AGA Environmental Management Plan, 2008-2011). The quartz veins ore which is general non-refractory can easily be processed using the traditional processes but not the sulphide ores. More of the reserved gold currently at the mine is reported to be sulphide ores (AGA Environmental Management Plan, 2008-2011).

3.3 Sampling Sites

Four major mining operation sites were selected for sampling; the Sulphide Treatment Plant (STP) bioreactors, the Sansu tailings dam and two open pits (New Drainage and Sansu) (Fig. 1). Liquid samples were taken from the STP bioreactors and the two open pits while the soil samples were taken from the Sansu tailings dam. These sampling sites were selected with the possibility of finding the bacteria due to the peculiar ecological and geological features of these areas.

3.3.1 The Sansu Tailings Dam

One of the active tailings disposal site at the AGA Obuasi mine is the Sansu tailing storage facility. The dam receives tailings from the STP and the Oxide processing sites via pipelines. The tailing is allowed to dry, collected and used in building embankments around the dam. Only few plants of *Chromolena lantara* grow some metres away from these embankments.

3.3.2 The Sulphur Treatment Plant (STP) Pit

The pit served as a dumping site for ores that were difficult to treat using the conventional method before the establishment of the Biox Plant. With the establishment of the Sulphur Treatment Plant,

ore from the old dumping has been removed and reprocessed. The old pit is being reclaimed with the introduction of grass and shrubs.

3.3.3 The New Drainage (ND) Pit

The site is a man-made small running stream that flows from a previous dug up area which is treated and allowed to flow into a restricted pond. Along the banks of the small stream are traces of dug up ore. The area is being replanted with grass and shrubs.

3.3.4 The Sulphur Treatment Plant Bioreactors

The plant consists of six equi-dimensional reactors configured as three primary reactors operating in parallel followed by three secondary reactors operating in series. The feed concentrate (bacteria/nutrients/grind ore mix) from the stock tank is fed into the primary reactors and allowed some time for the bacteria population to be established and to prevent bacteria washout before moving to the secondary reactors where sulifide (S) oxidation is completed.

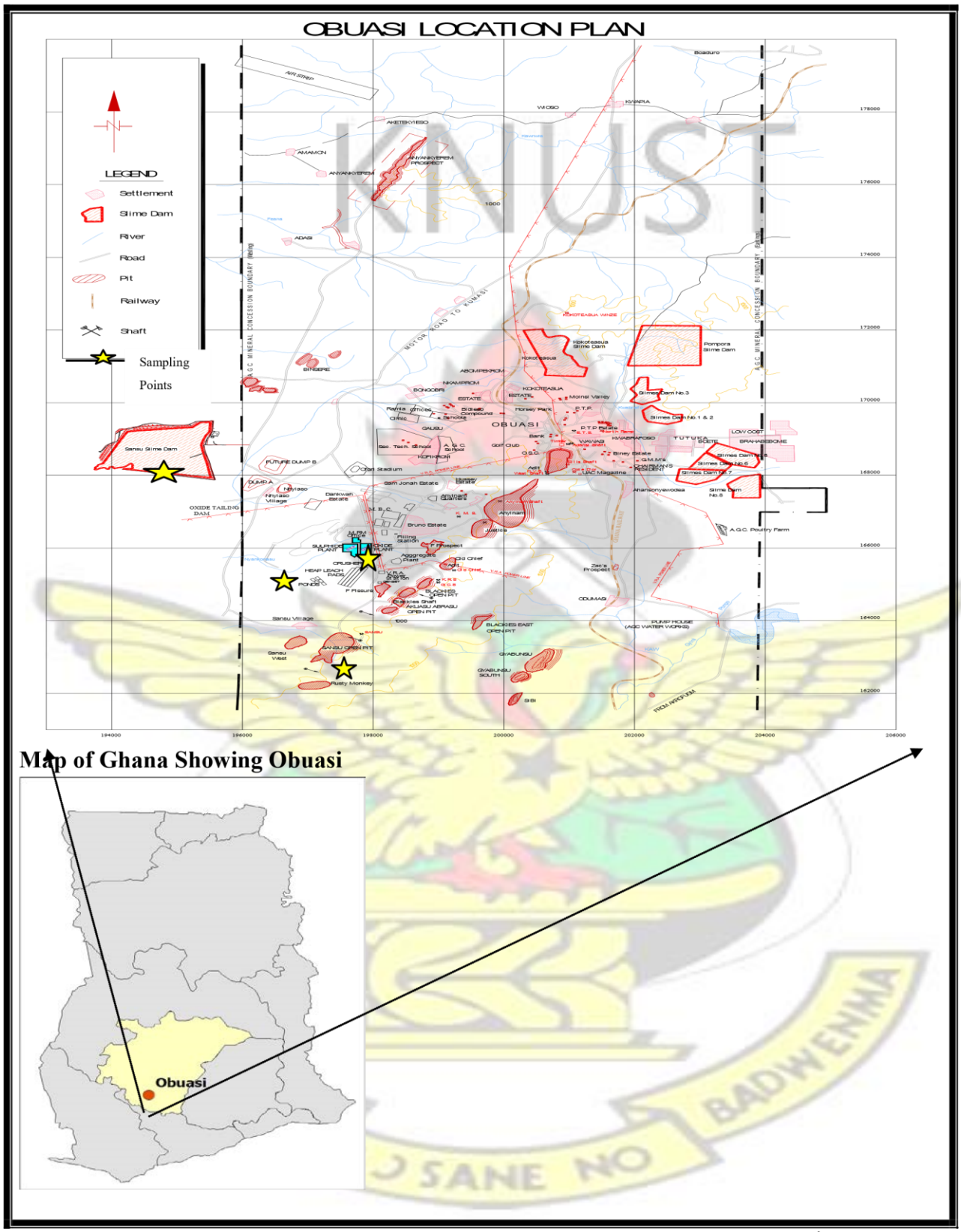

Figure 1: Map of AGA Obuasi Concession area showing the sample areas (☆) – AGAED

Plate 2 ***The Sansu Tailings Dam Site***

Plate 3 ***Physiognomy of Sampling Site for Mine Water at STP pit Showing Some Refractory Ores***

Plate 4 ***Physiognomy of Sampling Site for Mine Water at New Drainage Pit***

Plate 5 ***The Sulphur Treatment Plant (STP) Site Showing the Bioreactor Tanks***

3.4 Sample Collection

Four sites were designated within the Sansu dam area for sample collection. Two hundred (200) grams of soil samples were collected from each of the four designated sites using a sterile spatula and food bags (Plate 2). Liquid samples from the two open pits and the six bioreactors were collected into sterile 500 ml plastic bottles and transported to the laboratory in an ice chest at 4°C for analysis. The pH and temperature were determined in-situ for the liquid samples using a Hanna pH meter (model HI 83141) (Hanna Instruments). The Fe^{2+}/Fe^{3+} concentration of samples from the bioreactors were determined at the time of sampling by metric titration and potassium dichromate.

3.5 Experimental Analyses

3.5.1 Hydrogen Ion Concentration and Temperature

Hydrogen Ion Concentration and temperature of the water samples from the two pits and bioreactors were determined in-situ using a Hanna pH meter (model HI 83141, Hanna Instrument). pH of soil was determined using 1:2.5g soil: water ratio with a Hanna pH meter (model HI 83141) (Hanna Instruments).

3.5.2 Determination of Heavy Metals in Samples

Sample preparation

Soil sample was oven dry at a temperature of 105 ^{o}C for an hour and 0.2g weighed into a beaker. 3ml of concentrated HCl and 1 ml of concentrated HNO_3 were added to the beaker and heated on a hot plate at 100^{o}C for 15minutes. The solution was then topped to the 10ml mark of the beaker with de-ionized water, stirred and filtered. Filtrate was than analyzed for heavy metals.

Liquid samples were filtered to remove ore particles from the samples. The filtrate was diluted using 1:9 ratio of sample: de-ionized water.

Metal Determination

Heavy metals (As, Fe, Cu, Pb and Zn) were determined using Flame Atomic Absorption Spectrophotometer (AAS) (SPRECTRA AA 220). The instrument was first switched on and calibrated by using standard procedure set for the metals to be determined. The aspirator tube was then placed in 100ml of the prepared samples and aspired through the nebulizer and sprayed as a fine aerosol to the mixing chamber, where the sample aerosol is mixed with fuel, acetylene and carried to a burner head, which is aligned in the optical path of the spectrophotometer. The various hollow-cathode electrodes then emitted light of selected wavelength on the atoms in the free ground state in the samples. By pressing the READ key, the concentrates of the various metals were displayed on the screen.

3.5.3 Determination of Free Cyanide Concentration Using the Colorimetric method The colorimetric method designed by Merck using Microquant CN^- test kit was used in the determination of the concentration of CN^- by using a spectrophotometer set at a wavelength of 578mm.

A test tube was filled with 10 ml of the pre-treated sample. Using the microspoon provided in the cyanide kit, a spoonful of the reagent chloro-T-amine powder (white) Aldrich (CN-1A) was added and shaken to react, followed by dimethyl-1,3-barbituric acid (CN-2A). The content was shaken and three drops of reagent pyridine (CN-3A) was added to the solution in a cell. A period of about 5minutes was allowed for the reaction to complete. The concentration of cyanide in the sample was measured by comparing the colours of the treated sample with standard colours and their corresponding concentration. The concentration of the standard colour that matches with the samples gave the concentration of the sample.

Reactions:

CN^- reacts with chorine in chloro-T-amine with the formation of cyanogen chloride.

$Cl_2 + CN^- \rightarrow ClCN + Cl^-$

The cyanogen chloride reacts with pyridine to form cyano-1-pyridine.

$ClCN + C_5H_5N^- \rightarrow C_5H_5NCN + Cl^-$

The cyano-1-pyridine then reacts with dimethyl-1, 3 barbituric acid to form a complex dye, with a red blue coloration and the final colour compared to the standard colours.

3.5.4 Microbial Growth Media

3.5.4.1 Liquid Media

Iron medium ($9KFe^{2+}$)

The iron liquid medium ($9KFe^{2+}$) used in the isolation and growth of *A. ferrooxidans* was 9K mineral salts medium described by Silverman and Lundgren (1959). The 9K contains [g/L]: $(NH_4)SO_4$, 3.00; $MgSO_4.7H_2O$, 0.50; K_2HPO_4, 0.50; KCl,0.10; $Ca(NO_3)_2$ 0.01. These salts in the amount stated were dissolved in 800 ml of distilled water and the pH adjusted to 2.0 by adding 1M H_2SO_4. The basal salts solution was sterilized at 121°C for 20 minutes in aliquots.

Ferrous Sulfate ($FeSO_4 .7H_2O$) solution was prepared by dissolving 44.42g in 200 ml distilled water and the pH adjusted to 2.0 with 1 ml H_2SO_4. This was sterilized using a membrane filter with a pore size of 0.45 µm (Millipore). The sterilized ferrous sulfate solution was added aseptically to the cooled basal salts medium to give final ferrous ion concentration.

3.5.4.2 Solid Media

$FeSO_4$-Agar Plate

The $FeSO_4$-Agar medium routinely used in the study was as described by Khalid *et al.* (1993). Agar replaced Gelrite. Three separate solutions were prepared initially and mixed after sterilizing them separately.

Solution A (Ferrous sulfate solution), was prepared by dissolving 50.0g of $FeSO_4 .7H_2O$ in 200ml distilled water and the pH adjusted to 2.2 with 5M H_2SO_4. This was sterilized using a membrane filter with a pore size of 0.45 µm (Millipore).

Solution B (Mineral salts medium), was a modified form of 9K medium (Silverman and Lundgren, 1959), and contained (g): $(NH_4)SO_4$, 3.00; $MgSO_4.7H_2O$, 0.50; K_2HPO_4, 0.50; KCl,0.10; $Ca(NO_3)_2$, 0.02. All the ingredients were dissolved in 500 ml distilled water and the pH adjusted to 2.3 with 5M H_2SO_4. This solution was sterilised at 121°C for 20 minutes.

Solution C (Gelling solution), 12g of Agar (No. 1) Bacteriological [Oxoid Ltd, UK] was dissolved in 300ml distilled water for 20 minutes and sterilised at 121°C for 20 minutes.

All three solutions were allowed to cool to about 50-55°C. Solution B was mixed with solution C aseptically and solution A added to obtain a final concentration of 12% w/v agar and 5% w/v of $FeSO_4$. The solutions were stirred constantly and poured into sterilised petri dishes.

3.6 Enumeration of Acidophiles in Samples

Acidophiles present in the samples were enumerated using the Most Probable Number (MPN) method. Serial dilutions of $10^{-1}$and 10^{2} were prepared using 0.1 % Buffered Peptone Water. Aliquots of 1, $10^{-1}$and 10^{-2} ml were inoculated into test tubes containing 5ml each of 9K medium.

The tubes were incubated at 30ºC for 168 hours. After incubation, the tubes were examined for tubes with positive and negative growth.

3.7 Isolation of *A. ferrooxidans*

3.7.1 Pre-Enrichment of Samples

One millilitre of tailings liquid, mine water and STP water were inoculated separately into a 250 ml Erlenmeyer flask containing 100 ml of liquid iron ($9KFe^{2+}$) medium. The flasks were incubated under rotary conditions of 120 rpm at room temperature. Formation of a brick red solution is an indication of the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) (Plate 6).

Plate 6 The brick red color formation in some flasks showing an indication of the presence of A. ferrooxidans in a 9K medium after weeks of shaking

3.7.2 Growth of Bacteria

After shaking for 840 hours, serial dilution of each flask culture showing growth was prepared using sterile acidified 9k mineral salts as diluents. This experiment was repeated every 168 hours for 504 hours. One millilitre of each dilution was spread on already prepared $FeSO_4$ agar plates. The inoculated plates were incubated at 37ºC for about 504 to 840 hours and monitored daily until

brown rusty coloured colonies appear. Colonies were identified using colonial, microscopic and biochemical characteristics. Colonies were sub-cultured in slant tubes containing $FeSO_4$- agar medium and in flask containing 9K medium (for Biooxidation experiment).

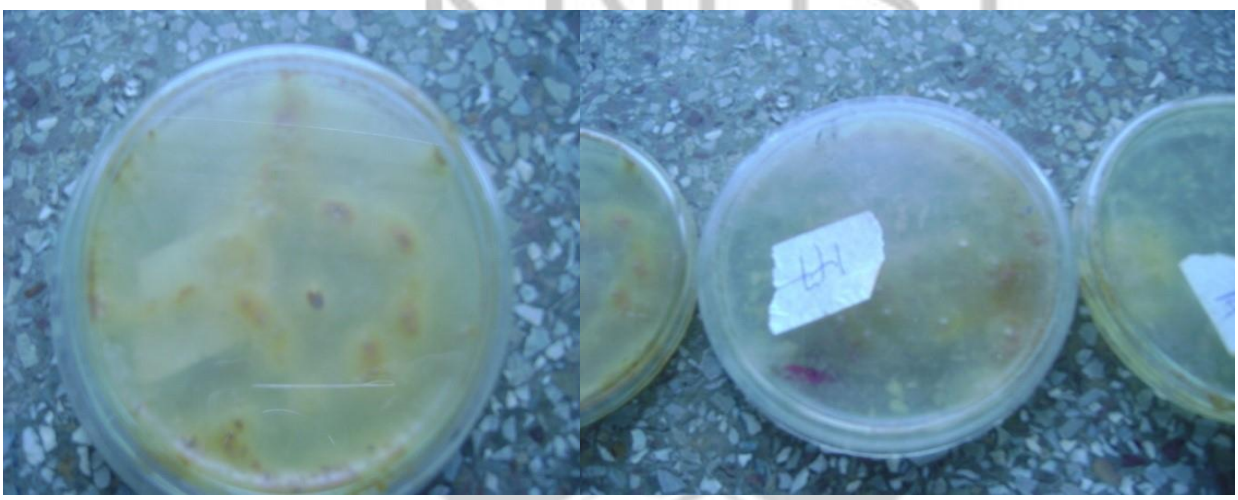

Plate 7 Brown rusty colour of colonies suspected to be A. ferrooxidans on $FeSO_4$- agar plates

3.8 Enumeration of Heterotrophic Bacteria in Sampled Sites

Serial dilutions (10^{-1} to 10^{-5}) of soil and water samples were prepared using 0.1 % Buffered Peptone water. One millilitre aliquots of each dilution were plated using Plate Count Agar (PCA). Plates were incubated at 37^{o}C for 24 hours. Colonies formed were counted and expressed as CFU 100 ml^{-1}. Single colonies were carefully removed and sub-cultured onto fresh agar slants.

3.9 Determination of Optimum pH and Temperature of Bacteria

The optimum pH and temperature values supporting the maximum growth of the isolates were determined in pH and temperature–controlled cultures. Using a sterile loop, colonies of the isolates were introduced into 50 ml of 9K medium pH values (1, 2 3, and 4) in Erlenmeyer flasks and incubated on a rotary (120 rpm) at room temperature. Using a sterilised loop, colonies of the isolates were inoculated into test tubes containing 5ml each of 9K medium with pH value of 2 and incubated at temperatures 10, 20, 30 and 40^{o}C and monitored daily for growth for 168 hours.

3.9.1 Determination of Sulfur utilization of the bacteria

The isolates were studied for the utilization of sulphur using a broth (Rajagopal and Ridar, 2007) which contained in g/L: NH_4Cl, 0.1; $MgSO_4.7H_2O$, 0.02 K_2HPO_4, 0.05; $Na_2S_2O_3.5H_2O$ 0.4 and adjusted to a pH value of 3.5 using 1M H_2SO_4. Using a sterilised loop, colonies of the isolates were inoculated into test tubes containing 5ml each of the broth and incubated at temperature of 30 ^oC and monitored daily for growth for 168 hours.

3.10 Bioleaching Experiment

Shake flask leaching experiments were carried out in twelve 250 ml Erlenmeyer flasks containing pyrite ore from the Obuasi mine and 90 ml liquid 9K medium. These flasks were inoculated with 10 ml suspension from each of the enrichment setups (bioreactor tanks, mine water and tailing isolates) containing pre-grown isolates suspected to be *A. ferrooxidans* and the flasks incubated at room temperature on a Gallenkamp Rietstra orbital shaker for 504 hours. Two controls were set up to regularise the outcomes. Samples were taken at 168 hours intervals and monitored for pH, redox potential and to analyze for the ferrous and ferric ion concentration. An aliquot of 10 ml was drawn from each flask weekly and filtered through a membrane filter with a pore size of 0.45 μm (Millipore) to remove the mineral particles. The filtrates were also analyzed for ferrous and ferric iron concentration using the complex metric titration and potassium dichromate as described by Wei-Chang (2001). Redox Potential (Eh) is a bioleaching factor which is the ratio of dissolved ferric to ferrous ions. It is calculated using the formula Redox Potential (Eh) =771 + 59log (Fe^{3+}/Fe^{2+}).

3.10.1 Control Setups

Two controls were setup to monitor and regularize the outcomes of the main experiments. The first control comprised 9K media and pyrite ore without any bacteria while the second was made of 9K media, pyrite ore and inoculum from Tank 146.

3.11 Statistical Analysis

The statistical packages GenStats Version 7.22 DE and Sigma plot were used for testing the various statistical relationships between the variables. Heavy metals and physicochemical data were analysed using a one-way completely randomized Analysis of Variance (ANOVA) tool to test any significant differences between the sample points. Graphical presentation of values was done using Microsoft Excel.

For microbiological analysis, the raw data was transformed by adding 1 to all scores in order to eliminate zero data points. Each datum point was then converted to $\log_{10}$ in order to harmonize the values for easy interpretation.

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical Characteristics of Bioreactors, Mine Water and Tailings

4.1.1 Hydrogen Ion Concentration (pH)

Average pH levels in the bioreactors were very low varying from 1.2 in the secondary tanks to 2.2 in the primary tanks. However, pH levels were relatively higher (6.2) in the tailings dam at Sansu. pH levels recorded for the Sulphur Treatment Plant (STP) pit and the New Drainage (ND) pit was 4.1 and 4.8 respectively for the mine water. Within the bioreactor tanks, the lowest pH value of 1.1 was recorded in the bioreactor tank 147 whereas the highest value of 2.2 was recorded

in tanks 11 and 13. The highest pH levels were within the tailings with the West recording 6.6 and the North 5.9 (Figure 1a).

With the exception of the west tailings (pH 6.6) which were within the Ghana Environmental Protection Agency (GEPA) and World Health Organization tolerable pH range for discharges into the environment, the other samples were predominantly acidic (Table 1). pH levels statistically varied significantly ($p < 0.05$) between sampling sites.

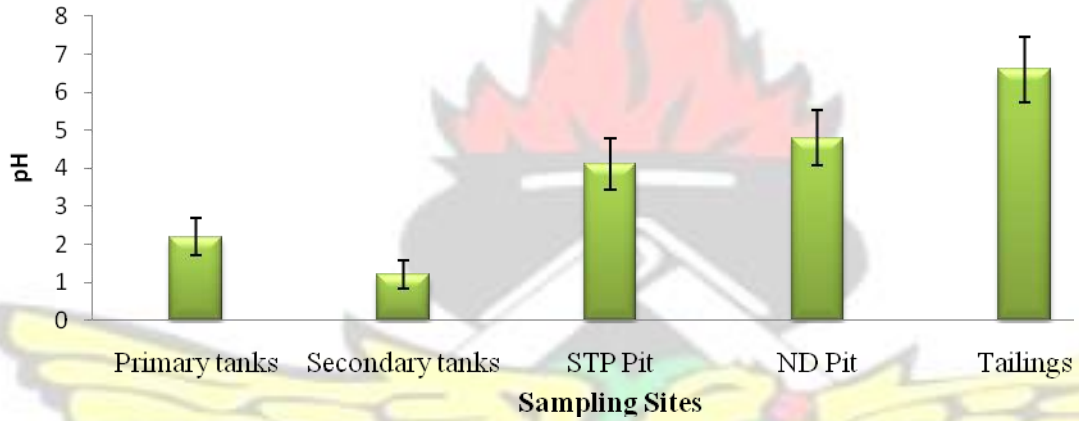

Figure 1a Average pH levels in bioreactors, mine water and tailings from the AGA mining concession area.

4.1.2 Temperature

Within the bioreactor plants, there were marked differences in temperature between the tanks with average temperatures in the primary and secondary tanks being 29ºC and 39.7ºC respectively, a difference of 10.7ºC. Average temperature variations outside the bioreactor plants were 28.4ºC in the STP pit and 26.2ºC in the ND pit with average temperature difference ranging from 0.5-2.7ºC (Figure 1b). Average temperature for the tailings dam was 28.9ºC. There were statistically significant differences between the sampling sites (P=0.001).

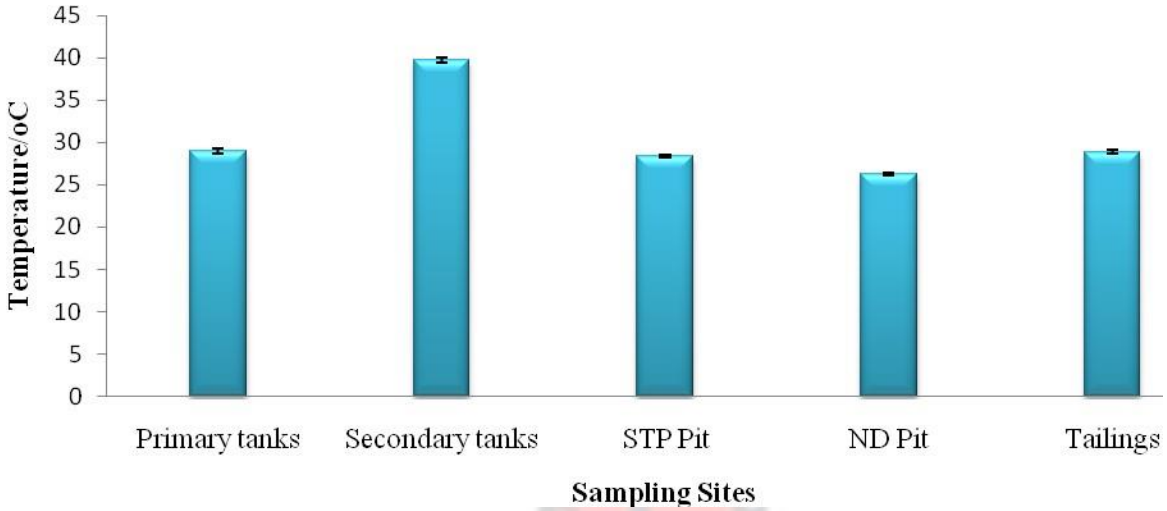

Figure 1b Average temperature levels in bioreactors, mine water and tailings from the AGA mining concession area.

4.1.3 Ferrous Iron (Fe^{2+}) and Ferric Iron (Fe^{3+}) Concentration

Concentrations of Fe^{2+} and Fe^{3+} are an indication of the biooxidation reaction processes taking place in a particular tank at the time of sampling. The results showed that the concentration of Fe^{2+} (0.005 mg/l) was the same irrespective of the tank or the period of sampling. This could be due to the size of burette used for the titration, which made it impossible to read titration volumes lower than 0.005mg/l. The Fe^{3+} concentrations however, varied from 7.33 mg/l to 12.93 mg/l in the tanks. The mean Fe^{3+} concentration in the primary tanks (12.64 mg/l) were higher than that in the secondary tanks (8.13 mg/l) (Figure 1c). The highest Fe^{3+} concentration was recorded in tank 13 (12.95 mg/l) whereas tank 146 and tank 147 recorded the lowest value of 7.33mg/l each. Also, there were statistically significant (P=0.006) differences in Fe^{3+} concentration between the tanks.

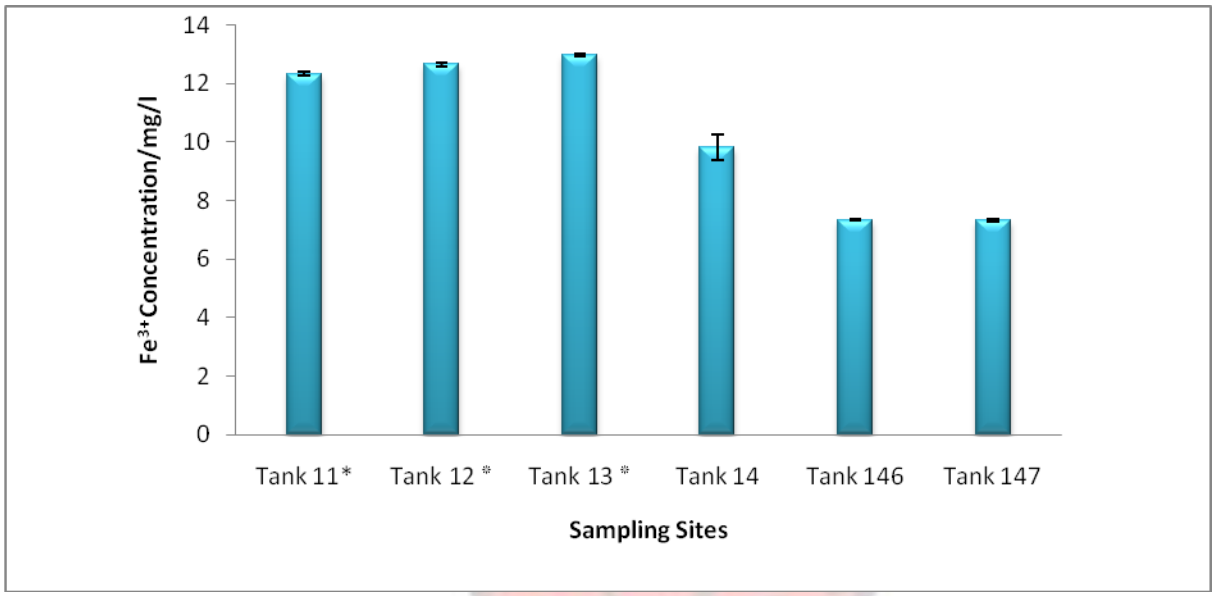

Figure 1c **Average ferric iron concentration in bioreactor tanks during the sampling period (tanks with star represent primary tanks and the others secondary)**

4.2 Heavy Metal Concentrations in Bioreactor Tanks, Mine Water and Tailings of AngloGold Ashanti mining Concession Area

4.2.1 Arsenic Concentration

Average concentrations of arsenic in the bioreactors varied from a higher concentration of 1802.7 mg/l in primary tanks to 1126.4 mg/l in the secondary tanks. Outside the bioreactor tanks, average arsenic concentrations were very low, 1.48 mg/l at the STP pit and 0.28 mg/l in the ND pit. The tailings recorded an average of 5.3 mg/g (Figure 2).

With the exception of the ND pit, arsenic concentrations in all samples were above the permissible GEPA and WHO guideline values allowable into the environment (Table 1). Statistically, there were significant ($p=0.001$) differences in arsenic levels between all the sampling sites.

Additionally, arsenic level in the primary tank 12 was statistically different from tank 11 and 13 whilst tank 147 was also statistically different from tank 14 and 146 in the secondary tanks.

4.2.2 Iron Concentration

Average iron concentration in the bioreactors varied from 1126.6 mg/l in the primary tanks to 2571.1 mg/l in the secondary tanks. However, outside the bioreactor tanks, average iron concentration was very low; 2.4 mg/l at the STP pit and 0.5 mg/l in the ND pit. Iron concentration in the tailings was 0.004 mg/g. For samples outside the bioreactors, the highest concentration of iron was recorded in the STP pit while the lowest was recorded in the tailings with values of 2.4 mg/l and 0.004 mg/g respectively (Figure 2).

Iron concentrations in the bioreactors were very much above the WHO guideline value, the mine water levels moderately above the guideline value while the tailings were within the guideline value (Table 1). Differences in iron concentrations between the sampling sites were all statistically significant (p= 0.001).

4.2.3 Copper Concentration

The recorded average copper concentrations in the bioreactor primary tanks of 80.3 mg/l were about twice that in the secondary tanks of 47.4 mg/l. Outside the tanks, the copper average concentrations were very low; 1.2 mg/l at the STP pit and 1.0 mg/l in the ND pit. Average concentration of 4.2 mg/g of copper was recorded for the tailings. The highest concentration of copper in the bioreactors (118.8 mg/l) was recorded in tank 11 whereas the lowest (42.4 mg/l) was in tank 14 (Figure 2).

With the exception of the mine water samples, all the other samples had copper concentrations above the GEPA and the WHO guideline permissible level (Table 1). Statistically, there were no significant differences (P=0.001) between the sites (Appendix 1b).

4.2.4 Lead Concentration

Average concentration of lead in the bioreactors were very low compared to the aforementioned heavy metals and varied from 0.383 mg/l in the primary tanks to 0.266 mg/l in the secondary tanks. Outside the tanks, the average concentration of lead at the STP pit was 0.103 mg/l and 0.078 mg/l in the ND pit. An average lead concentration of 0.11 mg/g was recorded in the tailings (Figure 2).

With the exception of the ND pit which had a lead concentration within the GEPA level and slightly above the WHO level, the other samples were general above the WHO and the GEPA guideline values (Table 1) (Appendix 1). There were statistically significant (P=0.001) differences in lead levels between all the sampling sites.

4.2.5 Zinc Concentration

Average concentration of zinc in the primary and secondary bioreactors plants were 51.5 and 58.7 mg/l, respectively. However, outside the tanks, the average zinc concentrations were much lower; average zinc in the STP pit was 0.9 mg/l and 0.005 mg/l in the ND pit. The average concentration in the tailings was 0.244 mg/g (Figure 2).

Zinc concentrations in the bioreactor tanks were all above the GEPA and the WHO guideline permissible levels but were below the level in the mine water and tailings (Table 1) (Appendix 1). Statistically significant (P=0.001) differences were recorded between the sampled points.

4.2.6 Cyanide Concentration

Average cyanide levels both within the bioreactor plants and outside the plant were very low; with an average of 0.004 mg/l in the bioreactor plants to a similar value of 0.004 mg/l in the STP pit and 0.35 mg/l in the ND pit. Average cyanide concentration in the tailings was 0.438 mg/g. Cyanide concentrations in all the bioreactor tanks and the STP pit were within the GEPA permissible level while the ND pit and the tailings have levels above the permissible levels (Table 1) (Appendix 1).

There were no statistically significant differences between the bioreactors. However there were statistically significant differences between the bioreactors and samples outside the bioreactors (P=0.001) (Appendix 1a).

Generally, heavy metal levels in the bioreactor tanks were well above WHO and Ghana EPA guideline values allowable into the environment while the mine water and tailings were only slightly above the guideline values (Table 1).

Comparatively, cyanide concentrations were higher in the tailings compared to samples from the mine waters and bioreactors while as iron and arsenic were higher in bioreactors and mine water samples compared to the tailings. The mean level of cyanide in the tanks was 0.004 mg/l which is within the WHO and Ghana EPA guideline values for free cyanide. For the environmental samples, 0.177 mg/l was recorded for mine water and 0.438 mg/g for the tailings which were above the WHO guideline value. However, the mine water value was within the GEPA value (Table 1).

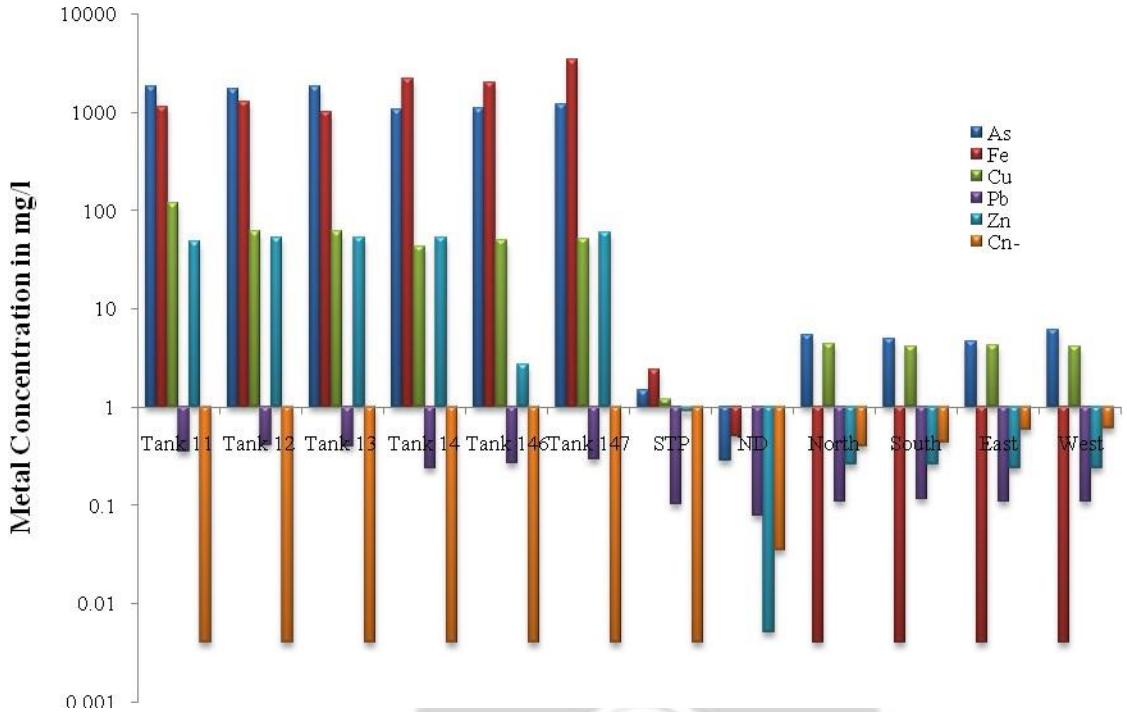

Figure 1: Elements analysis of samples points of the AngloGold Ashanti mining concession

Table 1 Mean concentration of As, Fe Cu, Pb, Zn and CN^- at the AGA environment compared to GEPA and WHO permissible levels allowable in the environment

Samples	Concentration of Metals mgl /mgg (SD)					
	As	**Fe**	**Cu**	**Pb**	**Zn**	**CN^-**
GEPA(WHO)	1.0 (0.01)	-- (0.3)	2.5 (2.0)	0.1 (0.01)	1 (3.0))	0.2 (0.07)
STP	1.48 ± (6.91)	2.40 ± (14.69)	1.20 ± (0.25)	0.103 ± (0.006)	0.900 ± (0.100)	0.004 ± (0.00)
ND	0.28 ± (0.48)	0.50 ± (7.87)	1.00 ± (0.25)	0.078 ± (0.006)	0.005 ± (0.001)	0.350 ± (0.00)
North	5.42 ± (0.62)	0.004 ± (0.001))	4.38 ± (0.09)	0.109 ± (0.006)	0.257 ± (0.006)	0.268 ± (0.23)
South	4.99 ± (1.13)	0.004 ± (0.001)	4.11 ± (0.17)	0.116 ± (0.006)	0.242 ± (0.003)	0.301 ± (0.26)
East	4.65 ± (1.02)	0.004 ± (0.001)	4.20 ± (0.01)	0.107 ± (0.001)	0.239 ± (0.006)	0.583 ± (0.03)
West	6.14 ± (0.64)	0.004 ± (0.001)	4.13 ± (0.01)	0.108 ± (0.006)	0.236 ± (0.002)	0.600 ± (0.00)

-1 -1

SD- Standard Deviation, mgl^{-1} for liquid samples and mgg-1 for solid sample

4.3 Enumeration of Acidophiles in the Bioreactors, Mine Water and Tailings

It was observed that acidophiles were present in 10 out of the 12 sampling points designated for this study irrespective of the time of sampling (Table 2). Acidophiles were however not recovered from samples collected from the East and West tailings throughout the study period.

Acidophile numbers in the bioreactor samples were generally higher than that in the mine water and tailings samples. Geometric mean counts (per 100 ml) of acidophiles in the bioreactor samples ranged between 1.86 $\times 10^4$ and 4.24×10^5 with numbers being higher in the secondary tanks compared to the primary tanks (Table 4.4). Geometric mean numbers ranged between 6.33×10^3 and 2.00×10^4 in the mine water and 4.00×10^3 and 1.50×10^4 for tailings (Table 2).

Acidophiles numbers were highest in bioreactor tank 146 (5.70×10^5) while the lowest was recorded in tank 13 (1.87×10^4). Considering samples outside the bioreactor tanks, the STP pit recorded the highest number of acidophiles (1.40×10^4) while the south tailing recorded the lowest number of acidophiles (6.33×10^3). Statistically, there were no significant differences in bacterial numbers between the sites ($P < 0.05$).

Table 2 Acidophiles Numbers in Bioreactors, Mine water and Tailings Samples from the Anglogold mining concession Area

Sample	Average Mean/ 100ml (g)	Geometric Mean/100ml (g)	Standard Deviation	Range
Tank 11	2.83×10^4	6.96×10^4	(±80.20)	2.00×10^4-3.60×10^4
Tank 12	2.27×10^4	1.87×10^4	(±46.18)	2.00×10^4-2.80×10^4
Tank 13	1.87×10^4	1.62×10^4	(±32.14)	1.50×10^4-2.10×10^4
Tank 14	2.00×10^4	2.21×10^4	(±85.44)	1.10×10^4-2.80×10^4
Tank 146	5.70×10^5	2.51×10^5	(±484.45)	1.50×10^5-1.10×10^6
Tank147	7.07×10^4	1.18×10^7	(±24.78)	4.40×10^4-9.30×10^2
STP	1.40×10^4	2.57×10^3	(±51.96)	1.10×10^4-2.00×10^4
ND	1.00×10^4	8.01×10^6	(±45.72)	6.00×10^3-1.50×10^4
North	9.33×10^3	3.72×10^6	(± 49.32)	6.00×10^3-1.50×10^4

South	6.33×10^3	2.71×10^7	(±25.16)	4.00×10^3-9.00×10^3

4.4 Growth of *A. ferrooxidans* on Solid Media

The appearance of rusty colonies on 9K solid medium is a presumptive confirmation of the presence of acidophiles although there have been numerous reports of difficulties in growing isolates on solid media (Johnson, 1995). Samples from the bioreactor plant and the mine water were all positive for acidophiles. However, samples from the West and East tailings did not show any growth on the 9K solid media.

Average $\log_{10}$ bacterial numbers (per 100 ml) in the bioreactor tanks ranged from 2.54 in tank 12 to 3.24 in tank 146. Average $\log_{10}$ counts for the mine water samples were 2.54 in the STP pit and 2.40 in the ND pit (Table 3). The North and South portions of the tailings recorded similar bacterial numbers, 2.0 (per 100g). However, the East and West portions did not show any sign of the bacteria.

Table 3 Bacteria Numbers Suspected to be *A. ferrooxidans* Colonies from Bioreactor Tanks, Mine Water and Tailings samples

Liquid Samples	CFU/100ml	Log CFU/100ml
Tank 11	6.50×10^2	2.78
Tank 12	3.55×10^2	2.54 2.60
Tank 13	4.10×10^2	
Tank 14	8.55×10^2	2.93
Tank 146	1.76×10^3	3.24
Tank 147	8.50×10^2	2.92
STP	3.50×10^2	2.54
ND	2.55×10^2	2.40

Soil Samples	CFU/100g	Log CFU/100g
North	1.50×10^2	2.00
South	1.50×10^2	2.00
East	1.00×10^0	0.00
West	1.00×10^0	0.00

4.4.1 Characteristics of the Isolates

All of the isolates obtained from either the bioreactors, mine water and tailings dam were rod shaped arranged singly or in chains and were motile. The isolates from the North tailing however, did not show any movement.

Microscopically, all the isolates were rod-shaped, gram negative and motile. Similarly, the isolates were aerobic, with a pH tolerance of 2-3 and most could utilize sulphur (Table 4). Apart from isolates in ND and South samples, isolates utilized sulphur.

Table 4 Morphological and Physiological Characteristics of Isolates

Sample	Cell stain	Gram tolerance	Motility utilisation	Respiration	pH	Sulphur morphology
Tank 11	Straight rods	-	+	Aerobic/anaerobic	2-3	+
Tank 12	Straight rods	-	+	Aerobic	2-3	+
Tank13	Straight rods	-/+	+	Aerobic	1-3	+
Tank 14	Straight rods	-	+	Aerobic	2-3	+
Tank146	Straight rods	-	+	Aerobic	2-3	+
Tank 147	Straight rods	-/+	+	Aerobic/anaerobic	2-3	+
STP	Straight rods	-	+	Aerobic	2-3	+
ND	Straight rods	-	+	Aerobic	2-[1]	(+)

[1] **.5 Heterotrophic Bacteria in the Bioreactor Tanks, Mine Water and Tailings**

The heterotrophic bacteria numbers in 100 ml of the bioreactor samples ranged from 1.49×10^9 to 7.18×10^{11} with an average of 1.93×10^{11} while that of the mine water had bacteria numbers ranging from 7.12×10^{19} to 4.49×10^{22} with an average of 2.25×10^{22}. The tailings had bacteria numbers in

North	Straight rods	-	(+)	Aerobic	2-3	+
South	Straight rods	-	+	Aerobic	2-3	(+)

(+)- Not obvious, + - obvious

4.6. Biooxidation of Refractory Ore Using the Isolates from Bioreactors, Mine Water and Tailings Samples

The change of ferrous to ferric conversion, redox potential changes and the change in pH were used to evaluate the bacterial activity and performance efficiency of the 9K shake flask tests.

After 672 hours of biooxidation, it was observed that pH reduced (from 2-1.2) while the redox potential increased (from 289 to 984.8 mV) the same period. The ferrous concentration also reduced as the ferric concentration increased with some little variation (Table 5) (Figures 3, 4, 5, and 6).

100g of soil ranging from 3.22×10^{18} and 8.76×10^{21} with an average of 2.43×10^{21} (Appendix 2c).

Table 5 Change in solution chemistry with time during 28 days of biooxidation of ore using isolates

Isolates	pH			Redox Potential /mV			Fe Concentration/mg/l			Fe $^{2+}$ / $^{3+}$ Concentration/mg/l	
	Initial	Final	%↓	Initial	Cal. Final	Change	Initial	Final	%↓	Initial	Final
Tank 11	2.00	1.50	25.0	289.00	833.6	544.6	0.30	0.10	66.67	<0.001	1.15
Tank 12	2.00	1.52	24.0	289.00	846.44	557.4	0.30	0.10	66.67	<0.001	1.90
Tank 13	2.00	1.54	23.0	289.00	867.9	578.9	0.30	0.05	88.33	<0.001	2.20
Tank 14	2.00	1.30	35.0	289.00	971.5	682.2	0.30	0.001	99.67	<0.001	2.50
Tank 146	2.00	1.40	30.0	289.00	975.1	686.1	0.30	0.001	-99.67	<0.001	2.88
Tank 147	2.00	1.30	35.0	289.00	967.4	678.4	0.30	0.001	99.67	<0.001	2.13
STP pit	2.00	1.60	20.0	289.00	838.6	549.6	0.30	0.05	83.33	<0.001	0.70
ND pit	2.00	1.67	16.5	289.00	838.6	530	0.30	0.10	66.67	<0.001	0.65
North	2.00	1.90	5.0	289.00	819	530	0.30	0.10	66.67	<0.001	0.65
South	2.00	1.88	6.0	289.00	819	511	0.30	0.15	50.00	<0.001	0.48
C	2.00	1.95	2.5	289.00	800	493	0.30	0.19	36.67	<0.001	0.30
CR	2.00	1.25	37.5	289.00	984.8	695.8	0.30	0.001	99.67	<0.001	4.20

4.6.1 Hydrogen ion concentration (pH)

Hydrogen ion concentration reduced in all the setups within the experimental period. pH reduction after 672 hours was greatest in the second control setup (2 to 1.29) (37.5%), followed by the secondary tanks (2 to 1.3) (35%), primary tanks (2 to 1.5) (25%), mine water (2 to 1.6) (20%), tailings (2 to 1.88) (6%) and then the first control setup (2 to1.95) (2.5%) (Table 5). However, after 504 hour of experimentation, pH levels in the bioreactor tanks and tailings increased slightly (Appendix 3).

The variations in pH was statistically significant (P=0.001) between the weeks (Appendix 3) but between the bioreactors set ups and the isolate outside the bioreactors, only the secondary tanks and the North and South tailing were statistically significant (P< 0.05) (Appendix 4).

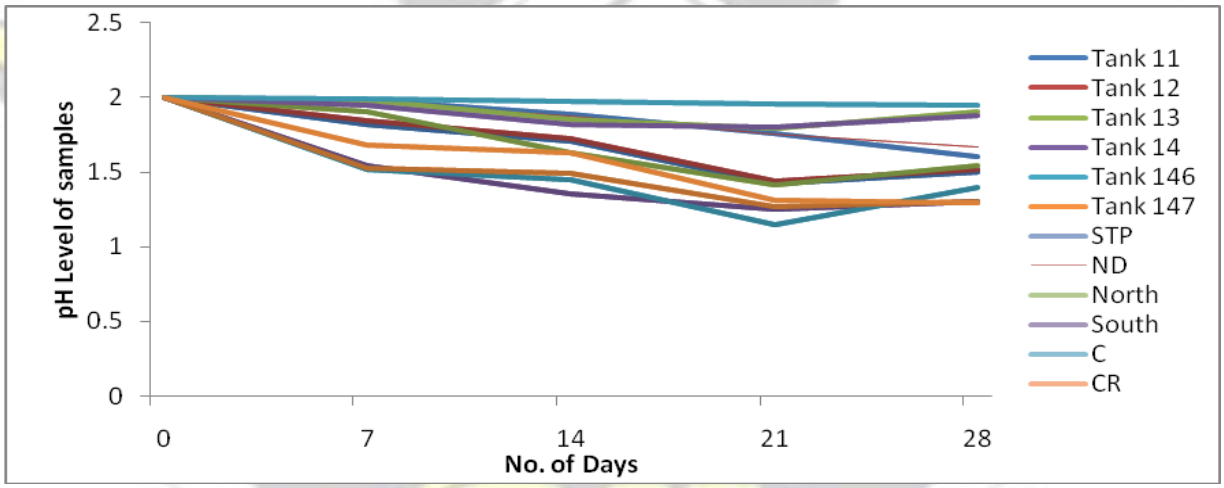

Figure 2: pH variations in biooxidation of refractory ore over a 28 days period

4.6.2 Redox Potential

Change in Redox potential values increased in all the set ups in an increasing order of; 493mV in the first control, 530 mV in the tailings, 549.6 mV for the mine water, 578.9 mV for the primary tanks, 686.1 mV for the secondary tanks and 695.8 mV for the secondary control setup.

The redox potential variation between the weeks were statistically significant (P=0.001)

(Appendix3) but not statistically significant between the bioreactors set up and other set ups (P=0.1383) (Appendix 4).

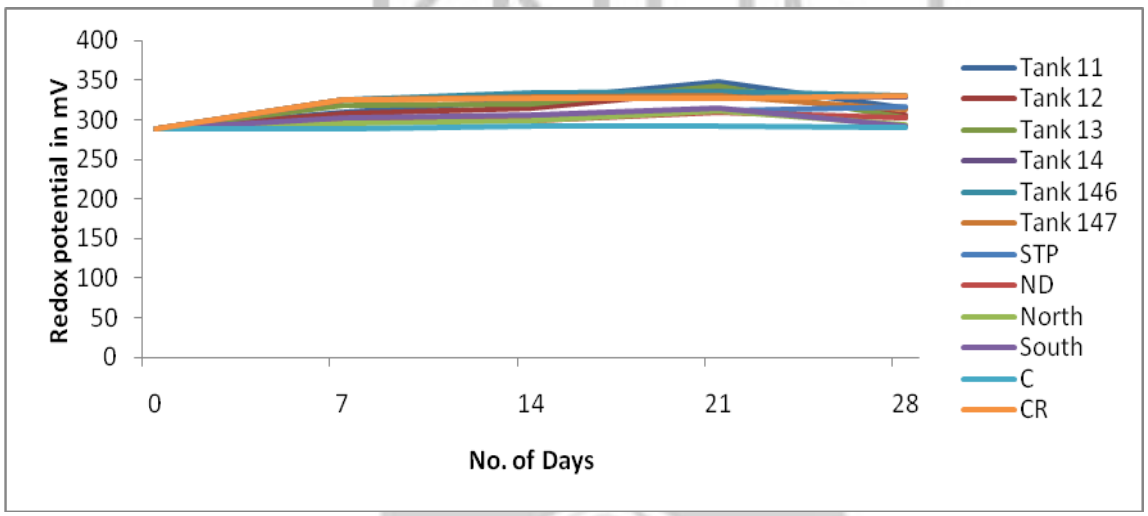

Figure 3 Redox potential variations in biooxidation of refractory ore over 28 days period

4.6.3 Ferrous Iron (Iron II) Concentrations

Ferrous concentration generally reduced over the experimentation period from 0.3 to 0.001 mg/l in secondary tanks and secondary control. Primary tanks, mine water and tailings isolates reduced ferrous iron concentrations from 0.3 to 0.1mg/l (Figure 5) whereas the first control reduced from 0.3 to.0.2 mg/l. After the experimental period of 672 hours, only traces of ferrous ions could be found in the secondary tanks and the second control experiment (Table 5).

Variations in ferrous iron concentrations within the weeks among the set ups were statistically significant (0.006) (Appendix3) but not significant between the bioreactors, mine water, tailings and the control set ups between the weeks (P=0.4593) (Appendix 4).

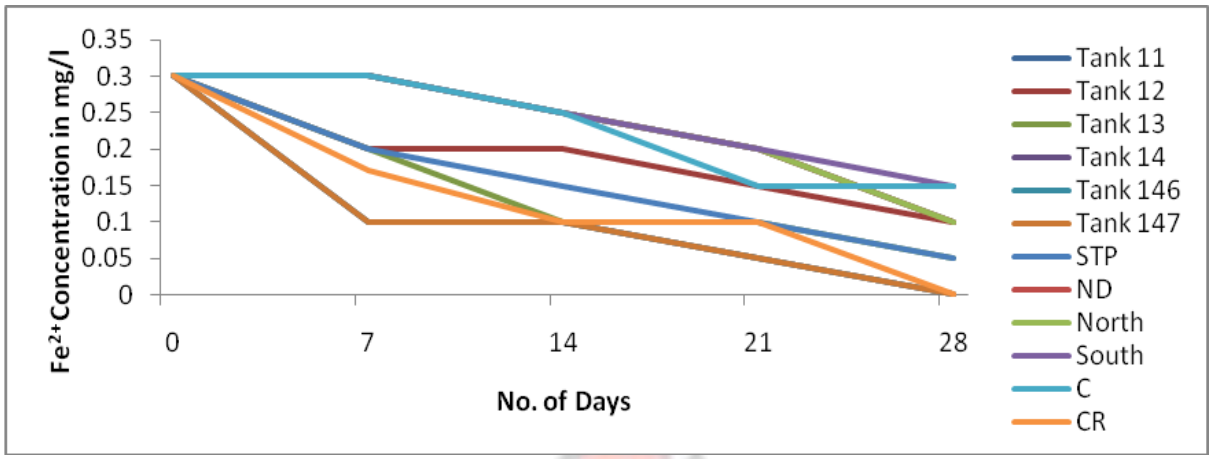

Figure 4 **Ferrous iron concentrations in biooxidation of refractory ore over 28 days period**

4.6.4 Ferric Iron (Iron III) Concentrations

The reductions in the concentration of ferrous iron translate to the increase in the concentration of ferric iron. The ferric iron values increased in all the set ups in an increasing order of; 0.3 mg/l in the first control, 0.65 mg/l for the tailings isolates, 0.7 mg/l for the mine water, 2.2 mg/l for the primary tanks, 2.88 mg/l for the secondary tanks and 4.2 mg/l for the secondary control setup (Figure 6).

After 672 hours, however, while ferric concentration in the primary tanks increased, that of the secondary tanks reduced. Ferric concentration outside the bioreactor set ups, the South tailings reduced while the other samples increased (Appendix 4).The ferric iron concentration variation among the samples within the weeks were statistically significant (P=0.001) (Appendix3). There were statistically significant differences between the bioreactor set up and the other set ups between the weeks (P=0.0065) (Appendix 4).

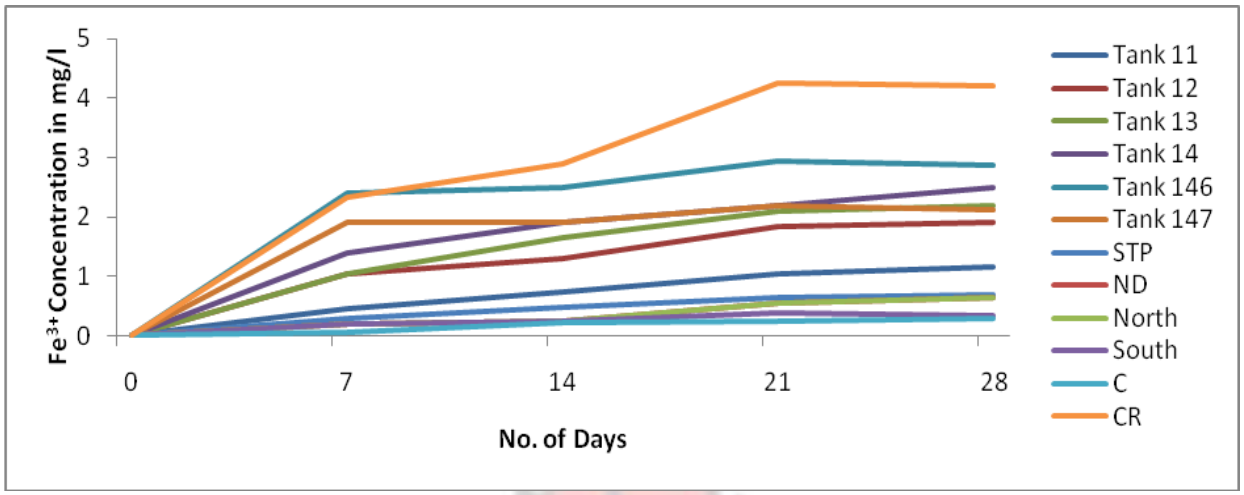

Figure 5 Ferric iron concentrations in biooxidation of refractory ore over a 28 days **period.**

4.6.5 Changes in pH and Ferric Iron Concentration

The changes in ferric iron concentrations in the set ups corresponded with the changes in the pH levels of the set ups. At low pH levels, ferric iron concentrations were high, however, in tanks 12 and 147, high pH levels recorded high ferric iron levels. The first control tank which recorded the highest pH value of 1.95 recorded the lowest ferric iron concentration value of 0.38 mg/l, whereas the second control set up which recorded the lowest pH value of 1.25 showed the highest ferric iron concentration of 4.2 mg/l (Figure 7).

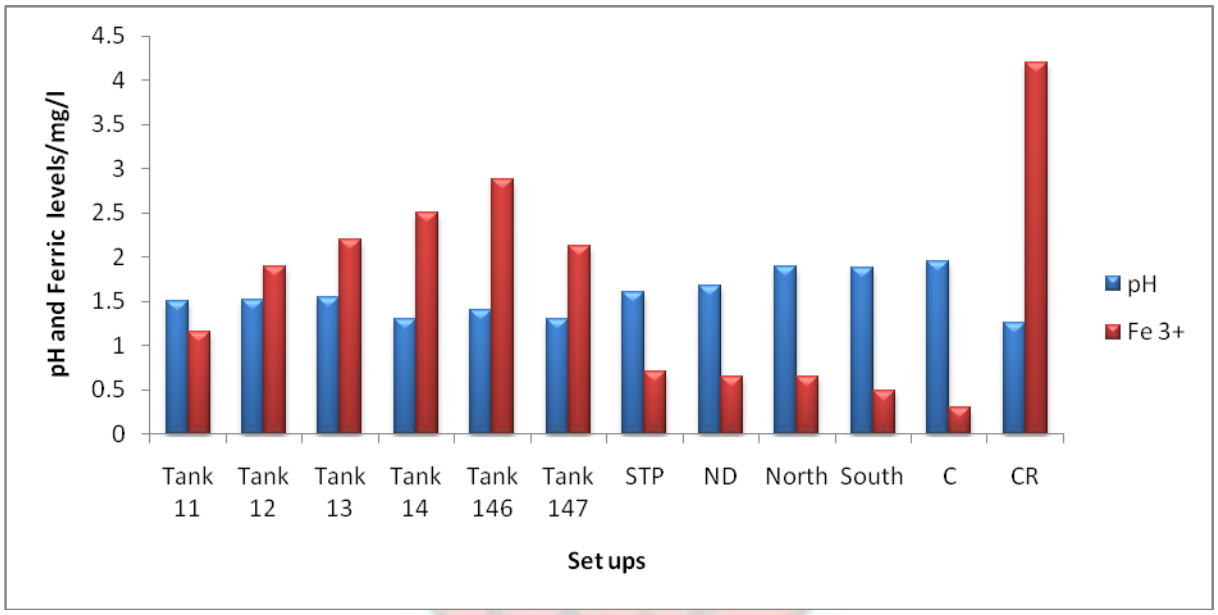

Figure 6 Ferric iron concentration and pH levels in the biooxidation set ups

4.6.6 Change in Redox Potential and Ferric Iron concentration in the Biooxidation Process

The change in ferric iron concentration in the set ups corresponded with changes in the redox potential in the set ups. Generally high redox potential corresponded with high ferric iron concentrations. Though, the redox potential values for the primary tanks, mine water and tailings were almost the same, the ferric iron concentrations of the mine water and tailings were far lower compared to the primary tanks. The highest redox potential value of 695.8 mV corresponded with the highest ferric iron concentration value of 4.2 mg/l recorded in the second control whereas the lowest ferric iron concentration 0.3 mg/l corresponded with the lowest redox potential value of 493 mV in the second control (Figure 8).

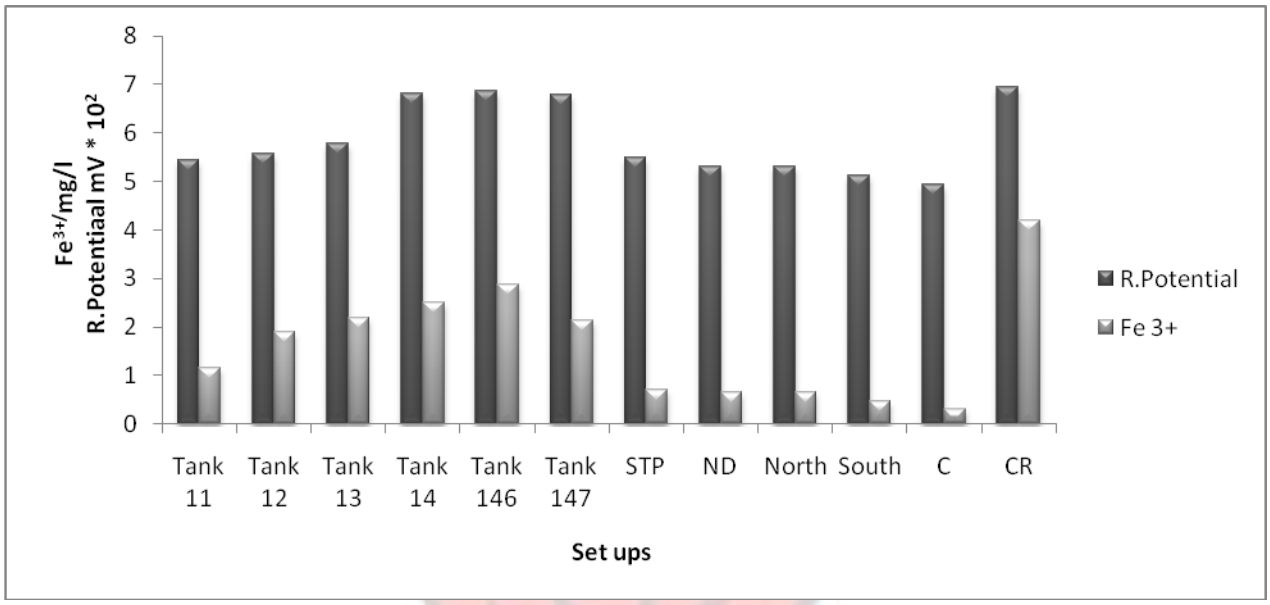

Figure 7 Concentration of ferric iron and redox potential in biooxidation set ups

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

The study revealed that bacteria isolated from the bioreactor tanks, mine water and tailings at the AGA concession area had similar characteristics to *Acidithiobacillus ferrooxidans*. The isolates from mine water and tailings were also found to be able to perform biooxidation activities comparable to that of the *A ferrooxidans* isolated from the bioreactors tanks of the AngloGold Ashanti Sulphur Treatment Plant. As biomining becomes a more practical and effective way of treating sulphide ore, the need to isolate more active strains from indigenous sources for the practical application becomes imperative. Rawlings (2007) suggest that the attempts at selecting the most suitable microorganism available and attempting to improve these by placing a selection pressure on the population is vital as indigenous bacteria turns to work more efficiently on native ore if well improved for the process.

5.1 The Effects of pH and Temperature on *A. ferrooxidans* population in Sampling Sites Ideal temperature, pH, amount of food supply and toxic substances levels provide for biomining bacteria

a suitable niche in which they can grow competitively although hydrogen ion concentration (pH) has the most marked effect upon the growth of bacteria (Klein *et al.*, 1962).

Acidophiles vary greatly in the degree to which they tolerate acidity, pH levels at the sampling sites were generally below the GEPA and the WHO levels (Figure 1a). Though these low levels are detrimental to other forms of bacteria, acidophiles grow competitively in these environments. These low levels of pH agree with the findings of Van Aswegen *et al*., (2006) that typical operating pH range in the BIOX^{TM} process is 1.2-1.8. Lower pH environment (sulphuric acid environment), provide suitable niches for the survival of acidophiles like *A. ferrooxidans* (Johnson, 2008; Brierley, 2008). The optimum pH range for the survival of acidophiles like *A. ferrooxidans* is 1-3 (Chen *et al.*, 2007; Yu *et al.*, 2007; Johnson, 2008) and this could have lead to the higher numbers of *A. ferrooxidans* in the bioreactor tanks than were present in the mine water and the tailings.

Although the pH values for the mine water and the tailings were not within the 1-3 pH range, studies by Hallberg and Johnson (2003) revealed that iron-oxidising bacteria (including *A. ferrooxidans*) have been isolated from mine drainage samples with pH above 3, a condition which could have led to the lower bacteria population observed in these sampling sites. This high pH values could be due to natural occurrences within these areas or as a result of the AGA's effort to reduce the acidity of their discharges in complying with the ISO 14001 standards. The presence of the dosing agent, lime in the ND pit (Plate 4) attests to the fact that AGA tempers with its acidic effluents.

In a typical biooxidation process, half of the retention time is spent in the primary tanks to allow a stable bacterial population to be established (Van Aswengen *et al.*, 2006) which implies that more of the bacteria population is expected from the primary tanks. However, the secondary tanks

showed more growth of the bacteria than the primary tanks. Under bioreactor conditions of pH above 2.0, the risk of killing the bacteria increase significantly which could result in the loss of the bacteria culture (Chetty *et al.*, 2000). The primary tanks at the study area operated slightly above the pH 2.0 value and also recorded the lowest numbers of bacteria among the tanks suggesting that it could be due to pH stress.

Biomining bacteria are conveniently grouped within temperature range as ambient temperature bacteria (mesophiles) (10-40 ^{o}C), moderate thermophiles (40-60 ^{o}C) and extreme thermophilic archaea (60 ^{o}C and above) (Johnson and Hallberg, 2007). In this study however, the bacteria isolated was mesophilic with temperatures ranging between 26.2-40.8 ^{o}C suggesting that the possibility of moderate thermophilic or extreme thermophilic bacteria isolates being present was very limited. This is in conformity with established fact that acidophiles that grow from ambient to approximately 40–45^{o}C appear to be widely distributed in naturally acidic environments with *A. ferrooxidans* being the principal one (Rawling and Jonhson (2007). Higher temperatures in the secondary tanks could be as a result of increased bacterial numbers leading to more activities than in the primary tanks (Figure 1b).

Although, all the tanks recorded the same amount of ferrous iron at the time of sampling, the levels of the Fe^{3+} concentrations in the primary tanks exceeded that in the secondary tanks (Figure 1c). Generally, the primary tanks recorded the least bacteria numbers compared to the secondary tanks. Also, tank 13 which recorded the highest Fe^{3+} concentrations of 12.95 mg/l recorded the least bacteria numbers of 1.84×10^4 while tanks 146 and 147 which recorded the least Fe^{3+} concentrations recorded the highest bacteria numbers of 5.70×10^5 and 7.07×10^4 respectively among the tanks. This could be due to the effect of the level of ferric iron on the growth of the bacteria.

5.2 Bacteria Ability to Tolerate Heavy Metals Concentrations

Metals are vital for the efficient physiological activities of organisms but above a certain threshold limit, it could be detrimental to organisms depending on the organisms' resistance level to these metals. Heavy metal levels in the bioreactor tanks were higher than in mine water and the tailings. The heavy metal levels in the bioreactor tanks were well above WHO and Ghana EPA guideline values allowable into the environment while that of the mine water and tailings were only slightly above the guideline values (Figure 2). During minerals biooxidation of the pyrite and arsenopyrite that occlude the gold, the ferric iron produced by the microorganisms also attacks any base-metal sulphide minerals that are present in the ore. This causes metals such as copper, zinc, lead nickel and cobalt to dissolve in the weak sulphuric acid (Brierley, 2008); this could account for the high levels of these metals in the tanks. Waste ore storage and dug up areas could be sources of high levels of heavy metals in the mine water and tailings.

The heavy metals load levels in this study are comparable to similar studies of the Goafeng Mine in China by Yu *et al.,* (2007); of As 21700 mg/l, Fe 121250 mg/l, Cu 104.45mg/l, Pb 0.95 mg/l and Zn 18500 mg/l. Whereas this loads are detrimental to other bacteria forms, species of this bacteria was able to survive under these conditions. This is so because acidophiles are remarkably tolerant to a wide range of metal ions (Dopson *et al.,* 2003).

Bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Nies, 1999). In particular, bioleaching of arsenopyrite by *A. ferrooxidans* suggests that it is tolerant to arsenic (Monroy *et al.,* 1995; Wakoa *et al.,* 1988). The arsenic resistance genes are established to be present on the chromosome of some *A. ferrooxidans* strains (Butcher *et al*., 2000).

The arsenic resistance system detoxifies the cell by active arsenite extrusion from the cytoplasm, lowering the intracellular concentration of this metalloid (Butcher *et al*., 2000, Cervantes *et al.*, 1994).

According to Rawlings and Johnson (2007) increase in resistance to metal ions in the biomining bacteria can also arise from two main sources: the occurrence of mutations in genes that are already present in the cell or the acquisition of new genes from other metal-resistance organisms, via the horizontal gene pool. Genome sequencing data on *A. ferrooxidans* suggest that metal resistance is due to a combination of both of these mechanisms (Rawlings and Johnson, 2007). The example of genes present on the chromosomes of most species of a genus are the efflux genes for arsenic (Butcher *et al.*, 2000), copper and several cations in *A. ferrooxidans* (Barreto *et al.*, 2003). Studies showed that the exposure of biomining bacteria to a metal ion results in increased resistance as a result of changes internal to a cell which account for their survival in samples with high loads of these metals (Rawlings and Johnson, 2007b).

The bioreactor tanks produced more isolates of the bacteria compared to the mine water and the tailings. The tolerance rate of bacteria from the different sampling sites could have accounted for their different survival ability and hence their populations.

Cyanide and thiocyanide reagents commonly used in gold processing have toxic or inhibitory effect on biooxidation bacteria (Van, Aswegen *et al.*, 2006). For sampling sites where cyanide levels were relatively very high especially in the East and West tailings, no bacteria were isolated, an indication of the toxic nature of cyanide on the bacteria even at low concentrations of 0.6 mg/g.

5.3 Bacteria Populations in Bioreactor Tanks, Mine Water and Tailings

The most important bacteria that catalyses biomining processes are required to live in a highly specialized growth environment and irrespective of the growth environment, the microorganism requires to grow in essentially inorganic (reduced form of sulphur or ferrous), aerobic and low pH environment (Johnson, 2008). Two classes of acidophilic microorganisms are therefore important primary agents in accelerating the dissolution of sulphide minerals at low pH. The first are those that generate acid (sulphuric) by oxidising sulphur and those that oxidise ferrous iron to ferric iron. The second group mostly heterotrophic includes eukaryotes, bacteria and archaea which have major impact on the overall process owing to their positive or negative interactions with the primary mineral sulphide-oxidising prokaryotes (Okibe and Johnson, 2004).

The environment in a bioreactor tank is highly homogenous as it operates at a set pH, temperature and controlled aeration. In an operation where a series of tanks are used as in the case of the AGA, conditions such as the concentration of soluble metals and metalloids, and often pH vary from tank to tank and this can have significant impact on the diversity and numbers of microbial species within each tank (Okibe *et al.*, 2003). This could account for the different numbers recorded in the different tanks. Limited ecological niche as a result of homogeneity in growth environment could account for the less numbers of heterotrophic bacteria in the tanks compared to the mine water and tailings. Okibe *et al.*, (2003) found that homogeneity within individual tanks in terms of pH, temperature, aeration, soluble metal and metalloid concentration results in a limited ecological niche that is often dominated by 2-4 species of acidophiles, although smaller number of other microorganisms may be present.

The natural environment which include the mining pits and tailings dams unlike bioreactor tanks, are heterogeneous both spatially or temporary in terms of pH, the presence of anaerobic pockets,

and availability of nutrients. These conditions results in a large number of micro-environments (Johnson, 2008). The variability supports a much greater diversity of mineral-oxidising bacteria and other microorganisms that colonize different zones and micro-sites within them. This could account for high numbers of the heterotrophic bacteria in the mine pits and tailings compared to the bioreactor samples.

5.3.1 Characteristic of isolates

Dew *et al.,* (1997) reported that the microbial populations in pyrite/arsenopyrite (gold) Biox culture were *L. ferrooxidans, A. ferrooxidans and A. thiooxidans*. Whereas, *A. ferrooxidans* can oxidise both ferrous and sulphur compounds, *L. ferrooxidans* oxidises only ferrous and *A. thiooxidans* oxidises only sulphur (Rawlings and Johnson, 2007). *L. ferrooxidans* is more predominant at temperatures above 45^{o}C whiles *A.thiooxidans* and *A.ferrooxidans* at temperature between 10-45^{o}C. The isolates were isolated in samples with temperatures ranging between 26 and 40^{o}C. With the exception of few variations , the isolates from the bioreactors, mine water and tailings were generally aerobic, straight rods, gram negative, motile and tolerated pH levels of 2 and 3 and could utilize both ferrous and sulphur as energy sources.

Colonies of the isolates were generally rusty brown in colour. The isolate sizes were large for the bioreactors and mine water samples but relatively small for the tailing samples. The colonies of *A. ferrooxidans* cultivated on the solid medium appeared after four to five weeks of cultivation contrary to that reported in the studies by Yu *et al.,* (2007) which appeared 48 hour after cultivation; Lavalle *et al.,* (2005) had them growing after two week of incubation and Raheb *et al*., (2007) had colonies appearing between one to eight weeks of incubation using 9K medium plates. However, the time of colonies appearance in this study was similar to that reported by Johnson *et al.,* (2005) which took three to four weeks of incubation. Comparing these

characteristics with related literatures (as in Appendix 5), the isolates could be classified *A. ferrooxidans* rather than *L. ferrooxidans* or *A. thiooxidans*.

5.4 Biooxidation of Refractory Ore

The oxidation of iron is readily assessed by monitoring changes is ferrous iron concentrations (Lovley and Phillips, 1987), while sulphur oxidation can be determined by measuring changes in sulfate concentrations (Kolmert *et al*. 2000). However, the change in pH level of the sample is a direct indication of Sulphur oxidation which can be used to measure microbial activities in leaching environment (Brierley, 2008).

The general decreased in pH values, increased redox potential values and the decreased ferrous and increased ferric iron concentrations in all set ups of the study could result from microbial activities on the refractory ore. As the sulphur in the ore is fed on by the bacteria, sulphuric acid is produced increasing the acidity of the solution, accounting for the low pH values. In the same manner, as ferrous iron is fed on, its level in the ore reduces while ferric iron is formed, increasing its concentration in the resulting solution. This agrees with Kawabe et al., (2003) who suggest that concentration of ferrous reduces as the ferric iron increased in biooxidation, however, the specific oxidation rate of ferrous ions decreased with increasing ferric ion concentration.

These reactions above results in electron transfer which accounts for the increasing redox potential of the solution. Microbes during biooxidation devour their food sources– iron and sulphur removing electrons from dissolved iron (ferrous iron) converting it to another form of iron (ferric iron) while electrons are removed from sulphur converting it to sulphuric acid (Brierley, 2008) in the process increasing the redox potential of the solution. The study revealed that as the concentration of ferric iron increased the redox potential values of the set ups also increased while

ferrous concentrations reduced. This is supported by the fact that as concentration of ferric iron exceeds the ferrous iron in set ups, the redox potential for the reaction increases (May *et al.*, 1997).

Oxidation of Fe^{2+} by *A. ferrooxidans* is possible at redox potentials of 500 up to + 850 mV, and the best leaching is achieved at 600-750 mV. It is also understood that *A. ferrooxidans* is not favored in process where the concentration of ferric iron greatly exceeds that of the ferrous iron (High redox potential of above +850) (Rawlings *et al.*, 1999).The second control tank and the secondary tanks showed redox values in the ranges of +672 - +695.8 and also showed high ferric concentrations than the other set ups. However, the redox potential for the first control was below 500 mV suggesting that the oxidation in this set up cannot be attributed to *A. ferroxidans* oxidation. Fe^{2+} oxidation of by *Leptospirillum ferrooxidans* occurs at redox potential of up to + 950 mV (Boon *et al.*, 1999), hence *L. ferrooxidans* could possibly not be responsible for the oxidation of the ore.

5.5 Conclusion

The study has shown that some strains of *A. ferrooxidans* were present in the bioreactor tanks and the mining environment of the AngloGold Ashanti concession. The bioreactor isolates were found in samples with pH 1.1 to 2.2 whilst those in the mine water and tailings were isolated from sample with pH range 4.1 to 6.0 and a temperature range of 25 to 40ºC.

The bacteria isolates were obtained from samples with high heavy metal concentration which normally would not support the growth of other microorganisms, an indication of their tolerance to a wide range of metal ion concentration.

The isolates were general gram negative, rod shaped chemolithautotrophic bacteria which could utilise both sulphur and ferrous as energy sources, which gives the indication that the bacteria isolated was *A. ferrooxidans*.

Biooxidation activity was more pronounced using isolates obtained from the bioreactor tanks compared to mine water and tailings isolates suggesting that tanks isolates were better adapted to biooxidation condition than the wild unadapted isolates from the environment.

5.6 Recommendation

- Further studies need to be conducted to identify the specific strain of *A. ferrooxidans.*
- More work on improving the working efficiency of the indigenous *A. ferrooxidans* strain found within the Anglogold concession area.
- Studies must be carried out in other mining areas in Ghana to find more indigenous species for our growing mining industry.

REFERENCE

Acevedo, F. (2002). Present and future of bioleaching in developing countries. *EJB Electron. Journal of Biotechnology*. 5:2-18.

Acharya, C., Kar, R.N. and Sukla, L.B. (2001). Bacterial removal of sulphur from three different coals. *Journal of Fuel,* 80: 2207-2216.

AngloGold Ashanti, Obuasi Environmental Management Plan 2008-2011. Prepared by African Environmental Research Consulting Company Limited. pp1-14, 25- 30, 66.

Anonymous (2009). Gold Extraction. Retrieved from www.Wikipedia.com, the free encyclopedia –(Date Accessed: 4/11/2009).

Anonymous (2008). Gold News. Retrieved from www.mbendi.co.za –(Date Accessed: 13 -06-2009).

Anonymous (2009). www.gsr.com/Operations/BogosoGallery.asp (Date accessed 7-02- 2010).

Anonymous (2008). Mining Industry in Ghana www.Wikepedia.com (Date accessed- 16-062009).

Anonymous (2008). Uses of Bacteria. Retrieved From www.Technoscan.com/ tracking.php (Date assessed 12 -11-2009).

Aryee, B.N.A. (2001). The Ghana's mining sector: its contribution to the national economy. *Resource Policy* 27: 61-70.

Baker, B. J. and Banfield, J. F. (2003). Microbial communities in acid mine drainage. *FEMS Microbiol. Ecol*. 44: 139-152.

Bank of Ghana Annual Report 2005 at www.bog.gov.gh/ private content/ file/ Research/ AnnRep 2005/Annual% 20Report (1).pdf (Date Accessed: 13 -06- 2009).

Barreto M., Quatrini R.,Bueno S., Arriagada C., Valdes J., Silver S., Jedlicki E. and Holmes D.S. (2003). Aspects of the predicted physiology of *Acidithiobacillus ferrooxidans* deduced from an analysis of its partial genome sequence. *Hydrometallurgy,* 71: 97-105.

Battaglia-Brunet, F., Dictor, M.C., Garrido, F., Crouzet, C., Morin, D., Dekeyser, K., Clarens, M. and Baranger, P. (2002). An arsenic (III)-oxidizing bacterial population: selection, characterization, and performance in reactors. *J. Appl. Microbiol*. 93: 656-667.

Bermúdez-Lugo, O. (2006)."The Mineral Industry of Ghana". In Minerals Year book. United States Geological Survey. pp. 250-259.

Boon M., Brasser H.J., Hansford G.S. and Heijnen J.J. (1999). Comparison of the oxidation kinetics of different pyrites in the presence of *Thiobacillus ferrooxidans* or *Leptospirillum ferrooxidans*. *Hydrometallurgy* 53: 57-72.

Bowell, R. J. (1994). Sorption of arsenic by iron oxides and oxyhydroxides in soils. *Appl. Geochem*. 9: 279-286.

Brierley, C. L. (1978). Bacterial leaching. *Crit Rev Microbiol.* 6: 207–62.

Brierley, C. L. (2008). Biomining-Extracting metals with Microorganisms. Brierley Consultancy LLC. Colorado. pp 1-12.

Brierley, J.A. and Brierley, C.L. (2001). Present and future applications of biohydrometallurgy. *Hydrometallurgy.* 59: 233-239.

Butcher, B. G., Deane, S. M and Rawlings D. E. (2000.) The chromosomal arsenic resistance genes of *Thiobacillus ferrooxidans* have an unusual arrangement and confer increased arsenic and antimony resistance to *Escherichia coli*. *Appl. Environ. Microbiol*. 66: 1826-1833.

Butler, T.W. (2007). Isotope geochemistry of drainage from an acid mine impaired watershed, Oakland, California. *Applied Geochemistry*. 22: 1416–1426.

Cancho, L., Blázquez, M.L., Ballester, A., González, F. and Muñoz, J.A. (2007). Bioleaching of a chalcopyrite concentrate with moderate thermophilic microorganisms in a continuous reactor system. *Hydrometallurgy* 87: 100–111.

Cervantes, C., G. Ji, Ramírez, J. L. and Silver., S. (1994). Resistance to arsenic compounds in microorganism. *FEMS Microbiol. Rev*. 15: 355-367.

Casiot, C., Bruneel, O.. Personné, J.-C., Leblanc, M. and Elbaz-Poulichet, F. (2003b). Arsenic oxidation and bioaccumulation by the acidophilic protozoan, *Euglena mutabilis*, in acid mine drainage (Carnoulès, France). *Sci. Total Environ*. 320: 259-267.

Cheng Hai-na, Hu Yue-hua, and Gao, Jian.(2007) Isolation of an Acidophilic Sulfur Oxidizing Bacterium.*Life Science Research*.11(2):105-109.

Chetty, K.R., Marais H.J. and Kruger M.J. (2000).The importance of pH control in the Biooxidation process, In Proceedings of the colloquim bacterial oxidation for the recovery of metals, Johannesburg, pp 1-2.

Clark, D.A. and Norris, P. R. (1996). Acidimicrobium ferrooxidans gen. nov., sp. nov.: mixedculture ferrous iron oxidation with Sulfobacillus species. *Microbiology*, 142: 785-790.

Coakley G. J (1996). The mineral industry of Ghana. U. S. Geological Survey Minerals Yearbook, pp. 1–7.

Coakley,G.J.and Mobbs, P.M. (1999). The mineral industries of Africa–U. S. Geological Survey Minerals Yearbook, pp. 1–4.

Coram, N.J. and Rawlings, D.E. (2002). Molecular relationship between two groups of *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. nov. dominates South African commercial biooxidation tanks which operate at 40°C. *Appl Environ Microbiol*.; 68: 838–845.

David, N., Valdivia, U. and Chaves, A. P. (2001). Mechanisms of bioleaching of a refractory mineral of gold with *Thiobacillu ferrooxidans. International Journal of Mineral Processing*, 62:187-198.

Deveci, H., Akcil, A., and Alp, I., (2003). Parameters for control and optimisation of bioleaching of sulphide minerals. In: Kongoli, F., Thomas, B., Sawamiphakdi, K. (Eds.), Process Control and Optimization in Ferrous and Non Ferrous Industry. Materials Science and Technology Symposium, Chicago, USA, pp. 9–12.

Dew, D. W., Lawson, E. N. and Broadhurst, J. L. (1997). The BIOXH process for biooxidation of gold-bearing ores or concentrates. *In* :Biomining: Theory, Microbes and Industrial Processes, Edited by D. E. Rawlings: *Springer/Landes Bioscience*. pp. 45–80.

Dopson, M., Baker-Austin, C., Ram Kopponeedi, P. and Bond, P. (2003). Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms. *Microbio*l:149:1959–1970.

Dunn, J.G. and Chamberlain, A.C. (1997). The Recovery of Gold from Refractory Arsenopyrites concentrates by Pyrolysis-Oxidation . *Mineral engineering* 9: 919-928.

Edwards, K.J., Bond, P.L., Gihring, T.M. and Banfield, J.F. (2000). An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. S*cience* 287: 1796–1799.

Ewart, D.K. and Hugues, N.H. (1991). The extraction of metals from ores using bacterial. *Adv Inorg Chem*; 36: 103–35.

Ferris, F. G., Hallberg, R. O. Lyvén, B. and Pedersen, K. (2000). Retention of strontium, cesium, lead and uranium by bacterial iron oxides from a subterranean environment. *Appl. Geochem*. 15: 1035-1042.

Fraser G.M., Kubera P.M., Schutte M.D., Weetman R.J (1993). Process/ mechanical design aspects for Lightin A315 agitators in mineral oxidation, *In*: Proceeding. Randol , Breaver creek. pp 247-253.

Foucher, S., Battaglia-Brunet, F., d'Hugues, P., Clarens, M., Godon, J.J. and Morin, D. (2003). Evolution of the bacterial population during the batch bioleaching of a cobaltiferous pyrite in a suspended-solids bubble column, and comparison with a mechanically-agitated reactor. *Hydrometallurgy* ; 71:5–12.

Golyshina, O.V., Pivovarova, T.A., Karavaiko, G.I., Kondrat'eva, T.F., Moore, E.R.B., Abraham, W.R., Lunsdorf, H., Timmis, K.N., Yakimov, M.M. and Golyshin, P.N. (2000). *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-ironoxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea. *Int. J. Syst. Evol. Microbiol.* 50: 997– 1006.

González-Toril, E., Lobet-Brossa, E., Casamayor,E.O., Amman, R. and Amils, R.(2003). Microbial Ecology of an Extreme Acidic Environment, the Tinto River. *Appl. Environ. Microbiol.* 69**:** 4853–4865.

Hallberg, K.B. and Johnson, D.B. (2003). Novel acidophiles isolated from moderately acidic mine drainage waters. *Hydrometallurgy* 71:139–148.

Hamid, Z., Seyed A.S., Rasoul, K. and Bahram, N.(2003) . Bioleaching of the copper from lowgrade ore using isolated bacteria and defined mixed cultures. *Iranian journal of Biotechnology*. 1 (3) : 1-12.

Hansford G S, Vargas T. (2001). Chemical and electrochemical basis of bioleaching processes. *Hydrometallurgy*, 59(2): 135-145.

Hendrix, J.L. (2005). Is there a green chemistry approach for leaching gold? Chemical and biomolecular engineering research and publications papers in sustainable mining. Lincoln. Pp 1-11.

Johnson, D.B, Ghauri, M.A. and Said, M.F. (1992). Isolation and characterization of an acidophilic, heterotrophic bacterium capable of oxidizing ferrous iron. *Appl Environ Microbiol*, 58(5): 14-23.

Johnson, D.B. (2008). Biodiversity and interactions of acidophiles: Key to understanding and optimizing microbial processing of ores and concentrates. *Transactions of Nonferrous Metals Society of China* 18(6): 1367-1373.

Johnson, D.B and Hallberg K.B. (2007).Techniques for Detecting and Identifying Acidophilesminerial- oxidizing microorganisms *In:* Biomining (eds), Rawlings, D. E. & Johnson, D. B. Springer. Heidelberg: Pp. 113-135.

Johnson, D.B., Okibe, N. and Hallberg, K.B. (2005).Differenciation and identification of ironoxidizing acidophile bacteria using cultivation techniques and amplified ribosomal DNA restriction enzyme analysis (ARDREA). *Microbiol Methods* 60: 299-313.

Johnson D.B., Okibe, N. and Roberto, F.F. (2003). Novel thermo-acidophilic bacteria isolated from geothermal sites in Yellowstone National Park. Physiological and Phylogenetic considerations. *Arch microbial* 180: 60-68.

Junner, N.R (1932).The geology of the Obuasi goldfield: Gold Cpast Geological Survery Memoir 2, pp 43-46.

Karavaiko, G.I., Turova, T.P., Kondrat'eva, T.F., Lysenko, A.M., Kolganova, T.v., Ageeva, S.N., Muntyan, L.N. and Pivovarova, T.A. (2003). Phylogentic heterogeneity of the species of *Acidithiobacillus ferrooxidans*. *Int J Syst Microbiol*, 53: 113-119.

Kamimura, K., Higashino, E., Moriya, S. and Sugio, T. (2003). Marine acidophilic sulphur oxidizing bacterium requiring salts for the oxidation of reduced inorganic sulphur compounds. *Extremophiles.* pp 95-99.

Kawabe, Y., Inoue, C., Suto, K. and Chida, T. (2003). Inhibitory effect of high concentrations of ferric ions on the activity of Acidithiobacillus ferrooxidans . *Journal of Bioscience and Bioengineering*, 96 (4) 375-379.

Kelly, D.P. and Wood, A.P. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *International Journal of Systematic and Evolutionary Microbiology*, 50: 511-516.

Khalid, A. M., Tariq, M.B. and Umar, M. (1995). An improved solid media for isolation enumeration and genetic investigation of autotrophic iron- and sulfur –oxidizing bacteria. *Applied Microbiology and biotechnology*. 39 (2) : 259-263.

Klein, L., Erichsen Jones, J.R., Hawkes, H.A. and Downing, A.L., (1962). River Pollution, Causes and Effects II. Butterworths and Co (Publishers) Ltd. pp 456.

Kolmert, Å., Wikström, P., Hallberg, K. B. (2000). A fast and simple turbidimetric method for the determination of sulfate in sulfate- reducing bacterial cultures. *J Microbiol Methods*, 41: 179–184.

Langmuir, D. (1997). Acid mine waters, *In* McConnin, R. (ed.), Aqueous environmental geochemistry. Prentice-Hall, Englewood Cliffs, N.J. pp. 457-478.

Lavalle, I., Chiacchiarrini, P., Polianni, C. and Donati, E. (2005). Isolation and characterization of acidophiles bacteria from patafonia Argentina, *Process Biochemistry* 40: 1095-1099.

Langner, H. W., Jackson, C. R. Mcdermott, T. R. and Inskeep, W. P. (2001). Rapid oxidation of arsenite in a hot spring ecosystem, Yellowstone National Park. *Environ. Sci. Technol.* 35: 3302-3309.

Lawson, E.N (1991). Environmental influence on BIOX $^{TM.}$ Paper presented at the 1991 bacterial oxidation colloquium, SAIMM, Johannesburg, pp 1-11.

Leduc, L.G. and Ferroni,G.D. (1994). The chemolithotrophic bacterium *Thiobacillus ferrooxidans*. *FEMS Microbiol. Rev*., 14: 103-120.

Livesey-Goldblatt, E., Norman, P. and Livesey-Goldblatt, D.R.(1983). Gold recovery from arsenopyrite/pyrite ore by bacterial leaching and cyanidation. In: Rossi, G. and Torma A. E.(editors.) Recent progress in biohydrometallurgy. Iglesias, Italy: *Associazione Mineraria Sarda*; pp. 627–41.

Logan, T. C. Seal, T. and Brierley, J.A .(2007). Whole –ore Heap Biooxidation of Sulfidic Gold Bearing Ore In *Biomining*. Rawlings, D. E. & Johnson, D. B. (eds) (2007). Springer. Heidelberg: Pp. 113-135.

Lovley, D.R. and Phillips, E.J.P. (1987) Rapid assay for microbially reduced ferric iron in aquatic sediments. *Appl Environ Microbiol* 53: 1536-1540.

Mason, L.J.and Rice, N.M. (2002). The adaptation of *Thiobacillus ferrooxidans* for the treatment of nickel–iron sulphide concentrates. *Min. Eng.* 15: 795-808.

May, N., Ralph, D. E. and Hansford, G. S. (1997). Dynamic redox potential measurement for determining the ferric leach kinetics of pyrite. *Miner Eng* 10: 1279-1290.

McCready, R.G.L. and Gould, W.D. (1990). Bioleaching of uranium. In: Ehrlich, H.L., Brierley, C.L., editors. Microbial mineral recovery. McGraw-Hill Book Co. New York: pp. 107–126.

Merroun, M.L., Geipel, G., Nicolai, R., Heise, K. H. and Selenska- Pobell, S. (2003). Complexation of uranium (VI) by three ecotypes of *Acidithiobacillus ferrooxidans* studied using time-resolved laser-induced fluorescence spectroscopy and infrared spectroscopy. *Biometals* 16: 331-339.

Monroy Fernandez, M. G., Mustin, C., de Donato, P., Barres, O., Marion, P. and Berthelin, J. (1995). Occurrences at mineral-bacteria interface during oxidation of arsenopyrite by *Thiobacillus ferrooxidans*. *Biotechnol. Bioeng*. 46: 13-21.

Morin, D. (1995). Bacterial leaching of refractory gold sulfide ores In : by Gaylardies C.C. and Videla H.A(1996), editors. Bioextraction and Biodeterioration of metals. Cambridge University press. pp 12-34.

Morin, G., Juillot, F., Casiot, C. Bruneel, O., Personné, J.-C. Elbaz-Poulichet, F., Leblanc, M. Ildefonse, P. and Calas, G. (2003). Bacterial formation of tooeleite and mixed arsenic(III) or arsenic(V)-Iron(III) gels in the Carnoulès acid mine drainage, France. A XANES, XRD, and SEM study. *Environ. Sci. Technol*. 37: 1705-1712.

Mousavi, S.M., Yaghmaei, S., Vossoughi, M., Jafari, A., Roostaazad,R., and Turunen, I.(2007). Bacterial leaching of low grade ZnS concentrate using indigenous mesophilic and thermophilic strains. *Hydrometallury* 85: 59–65.

Ndlovu, S. (2007). Biohyrometallurgy for sustainable development in the African Minerals industry. *Hydrometallurgy* 91: 20-27.

Nestor, D., Valdivia, U., Chaves, A.P. (2001). Mechanisms of bioleaching of a refractory mineral of gold with Thiobacillus ferrooxidans. *Int. J. Miner. Process*. 62: 187–198.

Nicholson H.M, Lunt D.J., Ritchie I.C., Marais H.J (1993).The design of the Sansu concentrator and BIOX^{TM} facility. Biomine; 93 conference, Adelaide, pp 138-145.

Nies, D.H. (1999). Microbial heavy metal resistance. *Appl Microbiol Biotechnol 51*: 730-750.

Nordstrom, D. K. and Alpers, C. N. (1999).Geochemistry of acid mine waters, In Logsdon, G. S. and Plumlee, M. B. (eds.) The Environmental Geochemistry of Mineral Deposits. Part, A. Processes, Methods and Health Issues, *Reviews in Economic Mineralogy,* 6: 133-160.

Nordstrom, D. K. and Southam, G. (1999) Geomicrobiology of sulfide mineral oxidation. In Banfield, J. F. and. Nealson K. H (eds).Geomicrobiology: Interactions Between Microbes and Minerals, *Mineralogical Society of America*, 35: 361–390.

Ojumu, T.V., Bello O.O., Sonibare J.A. and Solomon B.O. (2005). Evaluation of microbial systems for bioremediation of petroleum refinery effluents in Nigeria. *African Journal of Biotechnology*. 4(1): 31-35.

Okibe, N., Gericke, M., Hallberg, K.B. and Johnson, D.B.(2003). Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred tank bioleaching operation. *Appl. Environ. Microbiol*. 69: 1936-1943.

Okibe, N. and Johnson, D. B. (2004). Biooxidation of pyrite by defined mixed cultures of moderately thermophilic acidophiles in pHcontrolled bioreactors: the significance of microbial interactions. *Biotechnol Bioeng* 87, 574–583.

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

Osei-Owusu, J.(2001). Operating cost reduction initiatives at the Ashanti BIOX^{Tm} plant as at July 2001. In: Proceedings of the 4th BIOX^{Tm} Users group meeting, Wiluna, pp 39-43.

Petterson, M. G. (2008). Minerals sustainability, emerging economies, the developing world, and the 'truth' behind the rhetoric. *Estonian Journal of Earth Sciences*, 57 (2): 57-74.

Raheb, J., Khoshroo, H., Azimi, A., Nasernejad, B., Sanati, M.H., Naghdi, S. and Arabnezhad, M. (2007). Isolation and Characterization of a New *Acidothiobacillus ferrooxidans* from the Aliabad Copper Mine in Yazd Using 16s-23s Spacer Gene Nucleotide Sequencing Method. *Journal of Sciences, Islamic Republic of Iran* 18(3): 209-213.

Rajagopal, V. and Ridar. R. (2007). Isolation and characterization of sulphur oxidizing bacteria. *Journal of culture collection*. 5:73-77.

Ransford Sekyi (2009)- Personal communication with Director of Mining Department., Environmental Protection Agency, Head office, Accra- Ghana.

Rawlings, D. E. (2002). Heavy metal mining using microbes. *Annu Rev Microbiol*., 56: 65–91.

Rawlings, D.E. (2005). Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. *Microb Cell Fact* 4:13-25.

Rawlings, D.E (2007). Relevance of cell physiology and genetic adaptability of biomining microorganisms to Industrial processes. In *Biomining*. Rawlings, D. E. & Johnson, D. B. (eds) (2007). Springer. Heidelberg: Pp. 176-179.

Rawlings, D.E. and Johnson, D.B. (2007). Biomining .Springer-Verlag Berlin Heidelberg. pp 132,97-112, 199-203, 237-251, 281-302.

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

Rawlings D.E., Tributsch, H. and Hansfords G.S. (1999). Reasons why '*Leptosprillum*' –like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidising bacteria in many commercial processes of the biooxidation of pyrites and related ores. *Microbiology* 145: 5-13.

Rodríguez, Y., Ballester, A., Blázquez, M.L., González, F. and Muñoz, J.A. (2003). New information on the sphalerite bioleaching mechanism at low and high temperature. *Hydrometallurgy* 71: 57-66.

Rohwerder, T., Gehrke, T., Kinzler, K. and Sand, W. (2003). Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Applied Microbiology and Biotechnology*, 63: 239-248.

Sadiq, M. (1997). Arsenic chemistry in soils: an overview of thermodynamic predictions and field observation, *Water air and soil pollution*. 3: 1-4.

Sadowski, Z., Jazdzyk, E. and Karas, H. (2003). Bioleaching of copper ore flotation concentrates. *Minerals Engineering*, 16: 51-53.

Schippers, A. and Sand, W. (1999). Bacterial Leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. *Appl. Env. Microbiol*. 65: 319–321.

Siddiqu, M. H., Kumar, A., Kesari, K.K. and Arif J.M. (2009). Biomining - A Useful Approach Toward Metal Extraction. *American-Eurasian Journal of Agronomy* 2(2): 84-88.

Silverman, M .P. and Lundgren, D. C. (1959). Study on the chemoautotrophic iron bacterium Ferrobacillus ferrooxidans: an improved medium and harvesting procedure for securing high cell yield. *Journal of Bacteriology*, 77: 642-647.

Simate, G.S. and Ndlovu, S. (2007). Characterisation of factors in the bacterial leaching of nickel laterites using statistical design of experiments. *Adv. Mat. Res.* 2021: 66–69.

Singer, P.C. and Stumm, W. (1970a). Acidic mine drainage – the rate determining step. *Science* 167: 1121-1123.

United Nation Environmental Programme (2000). "Cyanide Spill at Baia Mare Romania, Unep / Ocha Assessment Mission; Spill of Liquid and Suspended Waste at the Aurul S.A. Retreatment Plant in Baia Mare. Geneva."

Valix, M., Usai, F. and Malik, R. (2000). Fungal bioleaching of low grade laterite ores. *Min. Eng.* 14(2): 197–203.

van Aswegen, P.C., van Niekerk, J. and Olivier, W.(2006). The BIOX™ process for the treatment of refractory gold concentrates. In: Rawlings, D.E., Johnson, D.B. (Eds.), Biomining. Springer Verlag, Berlin, Heidelberg, pp. 1–33.

Wakao, N., Koyatsu, H., Komai, Y., Shimokawara, H., Sakurai, Y. and Shiota. H. (1988). Microbial oxidation of arsenite and occurrence of arsenite-oxidizing bacteria in acid mine water from a sulfur-pyrite mine. *Geomicrobiol. J*. 6: 11-24.

Wan, R. Y., Miller, J. D., and Li, J. (2005) . Thiohydrometallurgical Processes for Gold Recovery. *Iiznovatiorzs in Natural Resource Processing, Proceedings of the Jan. X Miller Symposiunz,* Society for Mining, Metallurgy, and Exploration, Inc., Littleton, 223-244.

Watling, H.R. (2006). The bioleaching of sulphide minerals with emphasis on copper sulphidesA review. *Hydrometallurgy* 84: 81–108.

Wei-Chang, Xu (2001). Application of biotechnology in nuclear industry. Human National University of Defense Technology Press. pp 34-40.

Yahya, A., Roberto, F.F. and Johnson, D.B. (1999). Novel mineral-oxidising bacteria from Montserrat (W.I.): physiological and phylogenetic characteristics. In: Amils, R., Ballester, A. Biohydrometallurgy and the Environment toward the Mining of the 21st Century, Elsevier, Amsterdam, pp. 729– 740.

Yang, S., Xie, J., Qiu, G.and Hu, Y. (2002). Research and application of bioleaching and biooxidation technologies in China. *Min. Eng.,* 15: 361-363.

Yu Yang ,Wan Min-Xi, Shi Wu-Yang, Peng Hong, Qiu Guan-Zhou, Zhou Ji-Zhong and Liu Xue-duan (2007). Bacterial diversity and community structure in acid mine drainage from dabaoshan mine, China . *Aquatic Microbial Ecology*, 47(2): 141-151.

Zhang Lin, Qiu Guan-Zhou, Hu Yue-hua, Sun Xiao-jun, Li Jian-hua, Gu Guo-hua.(2008) Bioleaching of pyrite by *A. ferrooxidans* and *L. ferriphilum*. *Trans. Nonferrous Met. Soc*. 18: 1415-1420.

Zhou, J K. and Niu, Y.J. (2005). Advance in research of biological metallurgy of sulphide ore. *Journal of Metal Mine*, 346(4): 24-30.

APPENDICES

APPENDIX 1 Table 4.3 Mean results of As, Fe Cu, Pb, Zn and CN^- concentration at the AngloGold Ashanti mining concession area

Samples	Concentration of Metals mgl^{-1}/mgg^{-1}(SD)					
	As	**Fe**	**Cu**	**Pb**	**Zn**	**CN^-**
Bioreactor						
Tank 11	1828.7 ± (112.69)	1116.2 ± (101.4)	118.8 ±(0.55)	0.346 ± (0.100)	48.17 ± (0.49)	0.004 ± (0.00)
Tank 12	1730.3 ± (6.08)	1264.1 ± (6.32)	60.6 ± (0.60)	0.407 ± (0.011)	53.63 ± (1.21)	0.004 ± (0.00)
Tank 13	1849.3 ± (10.00)	999.4 ± (5.29)	61.5 ± (0.84)	0.397 ± (0.024)	52.80 ± (0.70)	0.004 ± (0.00)
Tank 14	1074.5 ± (9.87)	2246.4 ± (1.53)	42.4 ± (0.44)	0.239 ± (0.006)	53.44 ± (0.38)	0.004 ± (0.00)
Tank 146	1088.2 ± (5.11)	1989.1 ± (8.54)	49.2 ± (1.88)	0.268 ± (0.006)	62.70 ± (1.10)	0.004 ± (0.00)
Tank 147	1216.6 ± (55.07)	3477.7 ± (426.4)	50.6 ± (2.15)	0.291 ± (0.006)	60.08 ± (3.13)	0.004 ± (0.00)
Environment						
STP	1.48 ± (6.91)	2.40 ± (14.69)	1.20 ± (0.25)	0.103 ± (0.006)	0.900 ± (0.100)	0.004 ± (0.00)
ND	0.28 ± (0.48)	0.50 ± (7.87)	1.00 ± (0.25)	0.078 ± (0.006)	0.005 ± (0.001)	0.350 ± (0.00)
North	5.42 ± (0.62)	0.004 ± (0.001))	4.38 ± (0.09)	0.109 ± (0.006)	0.257 ± (0.006)	0.268 ± (0.23)
South	4.99 ± (1.13)	0.004 ± (0.001)	4.11 ± (0.17)	0.116 ± (0.006)	0.242 ± (0.003)	0.301 ± (0.26)
East	4.65 ± (1.02)	0.004 ± (0.001)	4.20 ± (0.01)	0.107 ± (0.001)	0.239 ± (0.006)	0.583 ± (0.03)
West	6.14 ± (0.64)	0.004 ± (0.001)	4.13 ± (0.01)	0.108 ± (0.006)	0.236 ± (0.002)	0.600 ± (0.00)

SD- Standard Deviation, mgl^{-1} for liquid samples and mgg-1 for solid sampl

APPENDIX 1A ANOVA RESULTS FOR VARIATION IN LEVELS OF HEAVY METALS IN SAMPLES

AMOMG ALL THE SAMPLE POINTS

Variate: As

Source of variation	DF	SS	MS	v.r	Fpr
Sample	11	21320966	1938270	1432.33	<.001
Residual	24	32478	1353		
Total	35	21353443			

Variate: Fe

Source of variation	DF	SS	MS	v.r	Fpr
Sample	11	183257872	16659807	1.87	<.006
Residual	24	213305613	8887734		
Total	35	396563485			

Variate: Cu

Source of variation	DF	SS	MS	v.r	Fpr
Sample	11	4.532×10^{4}	4.120×10^{3}	48290.21	<.001
Residual	24	2.05	0.08531		
Total	35	4.532×10^{4}			

Variate: Pb

Source of variation	DF	SS	MS	v.r	Fpr
Sample Residual	11	0.6178	0.05616	18534.36	<.001
	24	7.273×10^{-5}	3.030×10^{-6}		
Total	35	0.6179			

Variate: Zn

Source of variation	DF	SS	MS	v.r	Fpr
Sample	11	27329.93	2484,54	20380.71	<.001
Residual	24	2.926	0.1219		
Total	35	27332.86			

Variate: CN^{-}

Source of	DF	SS	MS	v.r	Fpr **variation**
Sample	11	1.82161	0.16560	16.30	<.001
Residual	24	0.24382	0.01016		
Total	35	2.06543			

APPENDIX 1 B LEAST SIGNIFICANT DIFFERENCE ALL PARWISE COMPARISONS TEST RESULTS FOR VARIATION IN LEVELS OF HEAVY METALS BETWEEN SAMPLE POINTS LSD All-Pairwise Comparisons Test of As by treatment

Treatment	Mean	Homogeneous Groups	SE	CTV	CV
Tank 13	1849.0	A	30.036	2.064	61.991
Tank 11	1828.7	A			
Tank 12	1730.3	B			
Tank 147	1219.9	C			
Tank 146	1086.6	D			
Tank 14	1077.8	D			
West	5.8067	E			
North	5.7843	E			
South	5.6617	E			
East	5.6527	E STP			
1.8143	E				
ND	1.4740	E			

LSD All-Pairwise Comparisons Test of Fe by treatment Treatment Mean Homogeneous Groups SE CTV CV

Tank 146	7955.8	…… A	2434.2	2.064	5023.9
Tank 147	3727.1	AB			
Tank 14	2239.8	B			
Tank 12	1267.1	B			
Tank 11	1116.2	B			
Tank 13	999.40	B			
STP	2.4737	B			
ND	0.9707	B East			
4.00E-03	B				
North	4.00E-03	B			
South	4.00E-03	B			
West	4.00E-03	B			

LSD All-Pairwise Comparisons Test of Cu by treatment

Treatment	Mean	Homogeneous Groups	SE	CTV	CV
Tank 11	118.17	A	0.2385	2.064	0.4922
Tank 13	62.033	B			
Tank 12	60.817	C			
Tank 147	52.767	D			
Tank 146	49.467	E			
Tank 14	41.563	F			
North	4.2537	G			
South	4.2043	G West			
4.1217		G			
East	4.0110	G			
STP	0.0710	H ND			
8.00E-03		H			

LSD All-Pairwise Comparisons Test of Pb by treatment Treatment Mean Homogeneous Groups SE CTV CV

Tank 11	0.4470	A	1.42 E-3	2.064	2.933E-3
Tank 12	0.4009	B			
Tank 13	0.3980	B			
Tank 147	0.2913	C			
Tank 146	0.2720	D			
Tank 14	0.2393	E			
South	0.1163	F			
North	0.1093	G West			
0.1083		G			
East	0.1070	G			
STP	0.1033	H			
ND	0.0783	I			

LSD All-Pairwise Comparisons Test of Zn by treatment

Treatment	Mean	Homogeneous Groups	SE	CTV	CVC

Tank 146	63.700	A	0.2851	2.064	0.5884
Tank 147	57.950	B			
Tank 14	53.443	C			
Tank 12	52.967	CD			
Tank 13	52.800	D			
Tank 11	48.167	E			
North	0.2570	F			
South	0.2427	F			
East	0.2393	F			
West	0.2360	F			
STP	6.33E-03	F			
ND	4.33E-03	F			

LSD All-Pairwise Comparisons Test of Cn by treatment Treatment Mean Homogeneous Groups SE CTV CV

West	0.6000	A	0.0823	2.064	0.1699
East	0.5833	A			
ND	0.3500	B			
South	0.3013	B			
North	0.2680	B			
STP	4.00E-03	C			
Tank 11	4.00E-03	C			
Tank 12	4.00E-03	C			
Tank 13	4.00E-03	C			
Tank 14	4.00E-	C Tank			
146	4.00E-03	C			
Tank 147	4.00E-03	C			

APPENDIX 1 C A pH levels variation in Samples at time of sampling One Way Analysis of Variance (Kruskal-Wallis One Way Analysis of Variance on Ranks)

Group	N	Missing	Median	25%	75%
Tank 11	**3**	**0**	2.200	2.050	2.350
Tank 12	**3**	**0**	2.000	2.000	2.225
Tank 13	**3**	**0**	2.200	2.050	2.350
Tank 14	**3**	**0**	1.300	1.225	1.375
Tank 146	**3**	**0**	1.200	1.050	1.350
Tank 147	**3**	**0**	1.000	1.000	1.225
STP	**3**	**0**	4.100	4.025	4.175

ND	**3**	**0**	4.800	4.725	4.875
N	**3**	**0**	5.900	5.825	5.975
S	**3**	**0**	6.000	6.000	6.000
E	**3**	**0**	6.400	6.250	6.550
W	**3**	**0**	6.600	6.450	6.750

H = 33.957 with 11 degrees of freedom. (P = <0.001)

All Pairwise Multiple Comparison Procedures (Student-Newman-tKeuls Method)

Comparison	Diff of Ranks	Q	P<0.05
W vs Tank 147	92.500	5.069	**Yes**
W vs Tank 146	88.000	5.254	**Yes**
W vs Tank 14	83.500	5.476	**Yes**
W vs Tank 12	64.000	4.655	**Yes**
W vs Tank 13	59.500	4.858	**Yes**
W vs Tank 11	59.500	5.536	**Yes**
W vs STP	43.000	4.650	**Yes**
W vs ND	34.000	4.389	**Yes**
W vs N	23.500	3.763	**Yes**
W vs S	17.500	3.689	**Yes**
W vs E	5.000	1.543	**No**
E vs Tank 147	87.500	5.224	**Yes**
E vs Tank 146	83.000	5.443	**Yes**
E vs Tank 14	78.500	5.710	**Yes**
E vs Tank 12	59.000	4.817	**Yes**
E vs Tank 13	54.500	5.071	**Yes**
E vs Tank 11	54.500	5.894	**Yes**
E vs STP	38.000	4.906	**Yes**
E vs ND	29.000	4.64	**Yes**
E vs N	18.500	3.900	**Yes**
E vs S	12.500	3.858	**Yes**
S vs Tank 147	75.000	4.919	Yes
S vs Tank 146	70.500	5.128	Yes
S vs Tank 14	66.000	5.389	**Yes**
S vs Tank 12	46.500	4.327	**Yes**
S vs Tank 13	42.000	4.542	**Yes**
S vs Tank 11	42.000	5.422	**Yes**
S vs STP	25.500	4.083	**Yes**
S vs ND	16.500	3.479	**Yes**
S vs N	6.000	1.852	No
N vs Tank 147	69.000	5.019	Yes
N vs Tank 146	64.500	5.266	Yes
N vs Tank 14	60.000	5.583	Yes

N vs Tank 12	40.500	4.380	Yes	
N vs Tank 13	36.000	4.648	Yes	
N vs Tank 11	36.000	5.765	Yes	
N vs STP	19.500	4.111	Yes	
N vs ND	10.500	3.240	**Yes**	
ND vs Tank 147	58.500	4.777	**Yes**	
ND vs Tank 146	54.000	5.025	**Yes**	
ND vs Tank 14	49.500	5.353	**Yes**	
ND vs Tank 12	30.000	3.873	**Yes**	
ND vs Tank 13	25.500	4.083	**Yes**	
ND vs Tank 11	25.500	5.376	**Yes**	
ND vs STP	9.000	2.777	**Yes**	
STP vs Tank 147	49.500	4.606	**Yes**	
STP vs Tank 146	45.000	4.867	**Yes**	
STP vs Tank 14	40.500	5.229	**Yes**	
STP vs Tank 12	21.000	3.363	No	
STP vs Tank 13	16.500	3.479	Do Not Test	
STP vs Tank 11	16.500	5.092	Do Not Test	
Tank 11 vs Tank 147	33.000	3.569	No	
Tank 11 vs Tank 146	28.500	3.679	Do Not Test	
Tank 11 vs Tank 14	24.000	3.843	Do Not Test	
Tank 11 vs Tank 12	4.500	0.949	Do Not Test	
Tank 11 vs Tank 13	0.000	0.000	Do Not Test	
Tank 13 vs Tank 147	33.000	4.260	Do Not Test	
Tank 13 vs Tank 146	28.500	4.564	Do Not Test	
Tank 13 vs Tank 14	24.000	5.060	Do Not Test	
Tank 13 vs Tank 12	4.500	1.389	Do Not Test	
Tank 12 vs Tank 147	28.500	4.564	Do Not Test	
Tank 12 vs Tank 146	24.000	5.060	Do Not Test	

Tank 12 vs Tank 14	19.500	6.018	Do Test	Not	**Tank 14 vs Tank 146**
Tank 14 vs Tank 147	9.000	1.897	Do Test	Not	4.500 1.389 Do Not Test
Tank 146 vs Tank 147	4.500	1.389	Do Not Test		

B. Temperature variation in sample points One Way Analysis of Variance (Kruskal-Wallis One Way Analysis of Variance on Ranks)

Group	**N**	**Missing**	**Median**	**25%**	**75%**
Tank 11	**3**	**0**	28.500	28.125	28.875
Tank 12	**3**	**0**	29.800	29.725	29.875
Tank 13	**3**	**0**	28.800	28.650	28.950
Tank 14	**3**	**0**	38.700	38.550	38.850
Tank 146	**3**	**0**	40.800	40.650	40.950
Tank 147	**3**	**0**	39.500	39.125	39.875
STP	**3**	**0**	28.400	28.250	28.550
ND	**3**	**0**	26.200	26.050	26.350
N	**3**	**0**	29.000	28.250	29.750
S	**3**	**0**	28.400	28.100	28.700
E	**3**	**0**	29.500	29.125	29.875
W	**3**	**0**	28.900	28.825	29.725

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method)

Comparison	**Diff of Ranks**	**Q**	**P<0.05**
Tank 146 vs ND	99.000	5.425	Yes
Tank 146 vs STP	78.000	4.657	Yes
Tank 146 vs S	77.500	5.083	Yes
Tank 146 vs Tank 11	71.500	5.201	Yes
Tank 146 vs Tank 13	61.000	4.981	Yes
Tank 146 vs N	55.500	5.164	Yes
Tank 146 vs W	49.000	5.299	Yes
Tank 146 vs E	39.500	5.099	Yes
Tank 146 vs Tank 12	36.000	5.765	Yes
Tank 146 vs Tank 14	18.000	3.795	Yes
Tank 146 vs Tank 147	9.000	2.777	Yes
Tank 147 vs ND	90.000	5.374	Yes
Tank 147 vs STP	69.000	4.525	Yes
Tank 147 vs S	68.500	4.983	Yes
Tank 147 vs Tank 11	62.500	5.103	Yes
Tank 147 vs Tank 13	52.000	4.839	Yes
Tank 147 vs N	46.500	5.029	Yes

Tank 14 vs STP	60.000	4.364	No
Tank 14 vs S	59.500	4.858	Do Not Test
Tank 14 vs Tank 11	53.500	4.978	Do Not Test
Tank 14 vs Tank 13	43.000	4.650	Do Not Test
Tank 14 vs N	37.500	4.841	Do Not Test
Tank 14 vs W	31.000	4.964	Do Not Test
Tank 147 vs W	40.000	5.164	Yes
Tank 147 vs E	30.500	4.884	Yes
Tank 147 vs Tank 12	27.000	5.692	Yes
Tank 147 vs Tank 14	9.000	2.777	Yes
Tank 14 vs ND	81.000	5.312	Yes

Tank 14 vs E	21.500	4.533	Do Not Test
Tank 14 vs Tank 12	18.000	5.555	Do Not Test
Tank 12 vs ND	63.000	4.583	Do Not Testes
Tank 12 vs STP	42.000	3.429	Do Not Test
Tank 12 vs S	41.500	3.862	Do Not Test
Tank 12 vs Tank 11	35.500	3.839	Do Not Test
Tank 12 vs Tank 13	25.000	3.227	Do Not Test
Tank 12 vs N	19.500	3.122	Do Not Test
Tank 12 vs W	13.000	2.741	Do Not Test
Tank 12 vs E	3.500	1.080	Do Not Test
E vs ND	59.500	4.858	yes
E vs STP	38.500	3.582	Do Not Test
E vs S	38.000	4.110	Do Not Test
E vs Tank 11	32.000	4.131	Do Not Test
E vs Tank 13	21.500	3.443	Do Not Test
E vs N	16.000	3.373	Do Not Test
E vs W	9.500	2.932	Do Not Test
W vs ND	50.000	4.652	Yes
W vs STP	29.000	3.136	Do Not Test
W vs S	28.500	3.679	Do Not Test
W vs Tank 11	22.500	3.603	Do Not Test
W vs Tank 13	12.000	2.530	Do Not Test
W vs N	6.500	2.006	Do Not Test
N vs ND	43.500	4.704	Yes
N vs STP	22.500	2.905	Do Not Test
N vs S	22.000	3.523	Do Not Test
N vs Tank 11	16.000	3.373	Do Not Test
N vs Tank 13	5.500	1.697	Do Not Test
Tank 13 vs ND	38.000	4.906	Yes
Tank 13 vs STP	17.000	2.722	
Tank 13 vs S	16.500	3.479	Do Not Test
Tank 13 vs Tank 11	10.500	3.240	Do Not Test
Tank 11 vs ND	27.500	4.404	Do Not Test
Tank 11 vs STP	6.500	1.370	Yes
Tank 11 vs S	6.000	1.852	Do Not Test
S vs ND	21.500	4.533	Do Not Test
S vs STP	0.500	0.154	Yes
STP vs ND	21.000	6.481	Do Not Test

C. Fe^{3+} concentration variation in Bioreactor tanks during sampling period

One Way Analysis of Variance (Kruskal-Wallis One Way Analysis of Variance on Ranks)

Group	N	Missing	Median	25%	75%
Tank 11	**3**	**0**	12.330	12.315	12.345
Tank 12	**3**	**0**	12.650	12.613	12.688
Tank 13	**3**	**0**	12.950	12.913	12.987

Tank 14	**3**	**0**	9.830	9.815	9.845
Tank 146	**3**	**0**	7.330	7.315	7.345
Tank 147	**3**	**0**	7.330	7.315	7.345
STP	**3**	**0**			
ND	**3**	**0**			
N	**3**	**0**			
S	**3**	**0**			
E	**3**	**0**			
W	**3**	**0**			

H = 16.155 with 5 degrees of freedom. (P = 0.006)

Comparison	**Diff of Ranks**	**Q**	**P<0.05**
Tank 13 vs Tank 147	40.500	4.380	Yes
Tank 13 vs Tank 146	40.500	5.229	Yes
Tank 13 vs Tank 14	27.000	4.323	Yes
Tank 13 vs Tank 11	18.000	3.795	Yes
Tank 13 vs Tank 12	9.000	2.777	Yes
Tank 12 vs Tank 147	31.500	4.067	Yes
Tank 12 vs Tank 146	31.500	5.044	Yes
Tank 12 vs Tank 14	18.000	3.795	Yes
Tank 12 vs Tank 11	9.000	2.777	Yes
Tank 11 vs Tank 147	22.500	3.603	No
Tank 11 vs Tank 146	22.500	4.743	Do Not Test
Tank 11 vs Tank 14	9.000	2.777	Do Not Test
Tank 14 vs Tank 147	13.500	2.846	Do Not Test
Tank 14 vs Tank 146	13.500	4.166	Do Not Test
Tank 146 vs Tank 147	0.000	0.000	Do Not Test

APPENDIX 2A

Bacteria Analysis

Group	N	Missing	Medium	25%	75%	
Tank 11	3	0	2.90×10^4	2.225×10^4	3.425×10^4	H =
Tank 12	3	**Tank13** 3	0	2.00×10^4	2.000×10^4	2.600×10^4
Tank14	3	0	2.00×10^4	1.625×10^4	2.075×10^4	with 9
Tank146	3	0	2.00×10^4	1.350×10^4	2.625×10^4	degrees
Tank 147	3	0	4.60×10^5	2.275×10^5	9.400×10^5	of
STP	3	0	7.50×10^4	5.175×10^4	8.850×10^4	freedom.
ND	3	0	1.10×10^4	1.100×10^4	1.775×10^4	(P =
North	3	**South**	0	9.10×10^3	6.775×10^3	1.353×10^4
3		0	7.00×10^3	6.251×10^3	1.300×10^4	The
		0	6.00×10^3	4.501×10^3	8.251×10^3	

0.003)

Tank 146 vs. Tank 13	1.360	6	11.390	Yes
Tank 146 vs. Tank 14	1.356	5	11.358	Yes
Tank 146 vs. Tank 12	1.277	4	10.692	Yes

differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003)

Log bacteria analysis Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	9	7.721	0.858	20.048	<0.001
Residual	20	0.856	0.0428		
Total	29	8.577			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001). Power of performed test with alpha = 0.050: 1.000

APPENDIX 2B

All Pairwise Multiple Comparison Procedures (Duncan's Method)

Comparison	Diff of Means	P	Q	P <0.050
Tank 146 vs. South	1.849	10	15.478	Yes
Tank 146 vs. North	1.694	9	14.181	Yes
Tank 146 vs. ND	1.656	8	13.863	Yes
Tank 146 vs. STP	1.499	7	12.549	Yes

Tank 146 vs. Tank 11	1.187	3	9.937	Yes
Tank 146 vs. Tank 147	0.798	2	6.680	Yes
Tank 147vs. South	1.051	9	8.798	Yes
Tank 147vs. North	0.896	8	7.501	Yes
Tank 147vs. ND	0.858	7	7.183	Yes
Tank 147vs. STP	0.701	6	5.870	Yes
Tank 147 vs. Tank 13	0.563	5	4.710	Yes
Tank 147vs. Tank 14	0.559	4	4.678	Yes
Tank 147 vs. Tank 12	0.479	3	4.013	Yes
Tank 147 vs. Tank 11	0.389	2	3.258	Yes
Tank 11 vs. South	0.662	8	5.540	Yes
Tank 11 vs. North	0.507	7	4.243	Yes
Tank 11 vs. ND	0.469	6	3.925	Yes
Tank 11 vs. STP	0.312	5	2.612	No
Tank 11 vs. Tank 13	0.173	4	1.452	Do Not Test
Tank 11 vs. Tank 14	0.170	3	1.420	Do Not Test
Tank 11 vs. Tank 12	0.0902	2	0.755	Do Not Test
Tank 12 vs. North	0.572	7	4.785	Yes
Tank 12 vs. South	0.417	6	3.488	Yes
Tank 12 vs. ND	0.379	5	3.170	No
Tank 12 vs.STP	0.222	4	1.857	Do Not Test
Tank 12 vs. Tank 13	0.0833	3	0.697	Do Not Test
Tank 12 vs. Tank 14	0.0795	2	0.665	Do Not Test
Tank 14 vs. South	0.492	6	4.120	Yes
Tank 14 vs. North	0.337	5	2.823	No
Tank 14 vs. ND	0.299	4	2.505	Do Not Test
Tank 14 vs. STP	0.142	3	1.192	Do Not Test
Tank 14 vs. Tank 13	0.00381	2	0.0319	Do Not Test
Tank 13 vs. South	0.488	5	4.088	Yes
Tank 13 vs. North	0.333	4	2.791	Do Not Test
Tank 13 vs. ND	0.295	3	2.473	Do Not Test
Tank 13 vs. STP	0.138	2	1.160	Do Not Test
STP vs..South	0.350	4	2.928	No
STP vs. North	0.195	3	1.631	Do Not Test
STP vs..ND	0.157	2	1.313	Do Not Test
ND vs. South	0.193	3	1.615	Do Not Test
ND vs. North	0.0380	2	0.318	Do Not Test
North vs. South	0.155	2	1.297	Do Not Test

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Medium	25%	75%
Tank 11	3	0	8.13×10^4	2.798×10^4	3.256×10^5
Tank 12	3	0	1.02×10^4	1.020×10^4	4.950×10^4
Tank13	3	0	1.29×10^4	1.088×10^4	2.753×10^4
Tank14	3	0	$1.29\ 1 \times 0^4$	1.290×10^4	5.168×10^4
Tank146	3	0	1.26×10^5	3.387×10^4	2.988×10^7
Tank 147	3	0	2.57×10^6	6.449×10^5	1.530×10^{11}
STP	3	0	1.29×10^3	1.290×10^3	7.972×10^3
ND	3	0	2.57×10^6	3.152×10^6	9.453×10^9
North	3	0	1.26×10^7	3.174×10^6	9.765×10^7
South	3	0	1.26×10^7	3.245×10^6	9.453×10^9

Antilog bacteria Analyses

H = 12.412 with 9 degrees of freedom. (P = 0.191)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.191)

APPENDIX 2C Heterotrophic Bacteria Numbers

Sample	Bacteria Number
Tank 11	7.18×10^{11}
Tank 12	9.40×10^{10}
Tank13	1.49×10^{11}
Tank14	1.65×10^{11}
Tank146	3.26×10^{10}
Tank 147	1.49×10^9
STP	4.49×10^{22}
ND	7.12×10^{19}
North	8.76×10^{20}
South	7.87×10^{19}
East	8.76×10^{21}
West	3.22×10^{18}

APPENDIX 3

Table 4.8 Biooxidation experiment using the isolates and refractory ore over 28 days period

Days	Sample	pH	Redox	Fe^{2+}	Fe^{3+}
7	Tank 11	1.82	308.6	0.30	0.45
	Tank 12	1.84	307.0	0.20	1.05
	Tank 13	1.90	318.7	0.20	1.05

Tank 14 1.54 324.5 0.10 1.40 Tank 146 1.52 325.3 0.10 2.40
Tank 147 1.53 325.4 0.10 1.90 STP 1.98 299.3 0.20 0.30 ND 1.97 301.2 0.30 0.20
North 1.98 295.0 0.30 0.20
South 1.95 302.7 0.30 0.20
Control
C 1.99 289.3 0.30 0.05 CR 1.68 325.2 0.17 2.33

14 Tank 11 1.71 325.2 0.25 0.75 Tank 12 1.72 314.2 0.20 1.30
Tank 13 1.63 319.9 0.10 1.65
Tank 14 1.35 330.1 0.10 1.90
Tank 146 1.45 334.2 0.10 2.5
Tank 147 1.49 329.5 0.10 1.90 STP 1.89 300.4 0.15 0.48 ND 1.87 299.4 0.25 0.25
North 1.85 298.7 0.25 0.25
South 1.82 305.1 0.25 0.25
Control
C 1.97 291.2 0.25 0.23 CR 1.63 326.3 0.10 2.90

21 Tank 11 1.42 348.0 0.20 1.05 Tank 12 1.44 337.0 0.15 1.85
Tank 13 1.41 342.9 0.10 2.10
Tank 14 1.25 331.6 0.05 2.20 Tank 146 1.15 336.4 0.05 2.95
Tank 147 1.27 330.6 0.05 2.20 STP 1.76 312.0 0.10 0.65 ND 1.75 309.7 0.20 0.55
North 1.79 310.5 0.20 0.55
South 1.80 313.9 0.20 0.38
Control
C 1.96 292.0 0.15 0.25 CR 1.31 327.1 0.10 4.25

28 Tank 11 1.50 314.6 0.10 1.15 Tank 12 1.52 306.2 0.10 1.90
Tank 13 1.54 302.0 0.05 2.20
Tank 14 1.30 329.3 0.001 2.50 Tank 146 1.40 329.9 0.001 2.88
Tank 147 1.30 312.3 0.001 2.13
STP 1.60 316.8 0.05 0.70 ND 1.67 303.3 0.10 0.65 North 1.90 293.0 0.10 0.65
South 1.88 291.2 0.15 0.35
Control
C 1.95 290.0 0.15 0.30
CR 1.29 330.5 0.001 4.20

C (ore + 9K) CR (ore + 9k + original sample from tank 146 (Bacteria)) TR(trace)

ANOVA RESULTS FOR BIOOXIDATION EXPERIMENT WITHIN SAMPLES PER WEEK

pH

Variate: pH week 1

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	1.1862555	0.1078414	245.71	<.001
Residual	24	0.0105333	0.0004389		
Total	35	1.1967889			

Variate: pH week 2

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	1.2350222	0.1122747	158.51	<.001
Residual	24	0.0170000	0.0007083		
Total	35	1.2520222			

Variate: pH week 3

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	2.3013639	0.2092149	348.69	<.001
Residual	24	0.0144000	0.0006000		
Total	35	2.3157639			

Variate: pH week 4

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	1.713489	0.155772	120.60	<.001
Residual	24	0.031000	0.001292		
Total	35	1.744489			

Redox

Variate: Redox week 1

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	5643.167	513.015	208.64	<.001
Residual	24	59.013	2.459		
Total	35	5702.180			

Variate: Redox week 2

Source ofvariation	DF	SS	MS	V.R	Fpr
Sample	11	15299.4	1390.9	9.52	<.001
Residual	24	3505.8	146.1		.
Total	35	18805.3			.

Variate: Redox week 3

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	7601.09	691.01	45.75	<.001
Residual	24	362.49	15.10		
Total	35	7963.58			

Variate: Redox week 4

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	4747.43	431.58	26.78	<.001
Residual	24	386.78	16.12		
Total	35	5134.21			

Ferrous iron

Variate: Fe^{2+} week 1

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	0.2141667	0.0194697	35.05	<.001
Residual	24	0.0133333	0.0005556		
Total	35	0.2275000			

Variate: Fe^{2+} week 2

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	0.1540972	0.0140088	25.22	<.001
Residual	24	0.013333	0.0005556		
Total	35	0.1674306			

Variate: Fe^{2+} week 3

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	0.1423222	0.0129384	25.73	<.001
Residual	24	0.0120667	0.0005028		
Total	35	0.1543889			

Variate: Fe^{2+} week 4

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	0.0991417	0.0090129	79.14	<.001
Residual	24	0.0027333	0.0001139		
Total	35	0.1018750			

Variate: Fe^{3+} week 1

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	24.928889	2.266263	362.44	<.001
Residual	24	0.150067	0.006253		
Total	35	25.078956			

Variate: Fe^{3+} week 2

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	30.521964	2.774724	949.58	<.001
Residual	24	0.070133	0.002922		
Total	35	30.592097			

Variate: Fe^{3+} week 3

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	45.405275	4.127752	473.55	<.001
Residual	24	0.209200	0.008717		
Total	35	45.614475			

Variate: Fe^{3+} week 4

Source of variation	DF	SS	MS	V.R	Fpr
Sample	11	45.842431	4.167494	549.76	<.001
Residual	24	0.181933	0.007581		
Total	35	46.024364			

APPENDIX 4

ANOVA RESULTS FOR BIOOXIDATION EXPERIMENT FOR PRIMARY, SECONDARY, STP & ND AND NORTH & SOUTH BETWEEN THE WEEKS

pH LEVELS

ANOVA Table	SS	df	MS	p-value
Treatment (between columns)	0.4707	3	0.1569	0.0384
Residual (within columns)	0.7074	16	0.04421	
Total	1.178	19		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Primary vs Secondary	0.1933	2.056	No	ns	-0.1871 to 0.5738
Primary vs STP & NDP	-0.1513	1.609	No	ns	-0.5318 to 0.2291
Primary vs N & S					
Secondary vs STP & N & S					
Secondary vs N & S	-0.3937	4.186	Yes	*	-0.7741 to -0.01320
STP & NDP vs N & S	-0.04900	0.5211	No	ns	-0.4295 to 0.3315

REDOX POTENTIAL

ANOVA Table	SS	df	MS	P-Value
Treatment (between columns)	1673	3	557.7	0.1338
Residual (within columns)	4216	16	263.5	
Total	5889	19		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Primary vs Secondary	0.5400	0.07438	No	ns	-28.83 to 29.91
Primary vs STP & NDP	16.88	2.325	No	ns	-12.50 to 46.25
Primary vs N & S	19.98	2.752	No	ns	-9.396 to 49.35
Secondary vs STP & NDP	16.34	2.250	No	ns	-13.04 to 45.71
Secondary vs N & S	19.44	2.677	No	ns	-9.936 to 48.81
STP & NDP vs N & S	3.100	0.4270	No	ns	-26.27 to

	32.47

FERROUS ION ANOVA Table	SS	df	MS	p-value	
Treatment (between columns)	0.02119	3	0.007064	0.4593	
Residual (within columns)	0.1164	15	0.007758		
Total	0.1376	18			

Tukey's Multiple Comparison Test	Mean Diff.	Q	Significant? P < 0.05?	Summary	95% CI of diff
Primary vs Secondary	0.05250	1.257	No	Ns	-0.1178 to 0.2228
Primary vs STP & NDP	-0.005000	0.1269	No	Ns	-0.1656 to 0.1556
Primary vs N & S	-0.0450	1.142	No	Ns	-0.2056 to 0.1156
Secondary vs STP & NDP	-0.0575	1.376	No	Ns	-0.2278 to 0.1128
Secondary vs N & S	-0.0975	2.334	No	Ns	-0.2678 to 0.07280
STP & NDP vs N & S	-0.04000	1.015	No	Ns	-0.2006 to 0.1206

FERRIC ION

ANOVA Table	SS	df	MS	p-value
Treatment (between columns)	7.520	3	2.507	0.0065
Residual (within columns)	6.789	16	0.4243	
Total	14.31	19		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? < 0.05?	Summary P	95% CI of diff
Primary vs Secondary	-0.7007	2.405	No	ns	-1.879 to 0.4780

Primary vs STP & NDP	0.7220	2.478	No	ns	-0.4566 to 1.901
Primary vs N & S	0.8170	2.805	No	ns	-0.3616 to 1.996
Secondary vs STP & NDP	1.423	4.884	Yes	*	0.2440 to 2.601
Secondary vs N & S	1.518	5.210	Yes	**	0.3390 to 2.696
STP & NDP vs N & S	0.0950	0.3261	No	ns	-1.084 to 1.274

APPENDIX 5

Table 1
Physiological characteristics of iron-oxidizing acidophilic bacteria

	Cell morphology	Motility	Gram stain	Endospores	Sulfur oxidation	Utilization of yeast extract	Growth at 45 °C	Growth on	
								Feo	FeSo
At. ferrooxidans	Straight rods	(+)	–	–	+	–	–	+	+
"*T. ferrooxidans*" m-1	Straight rods	–	–	–	–	–	–	+	+
L. ferrooxidans	Curved rods and spirilla	+	–	–	–	–	–	+	(+)
L. ferriphilum	Curved rods and spirilla	+	–	–	–	–	+[a]	+	(+)
"*Fm. acidiphilum*"	Straight rods and filaments	+	+	–	–	+	–	+	–
"*Sb. montserratensis*"	Straight rods	+	+	+	+	+	–	–	+
Sb. thermosulfidooxidans	Straight rods	–	+	+	+	+	+	(+)	+
Sb. acidophilus	Straight rods	(+)	+	+	+	+	+	(+)	+
"*Sb. yellowstonensis*"	Straight rods	(+)	+	+	+	+	+	(+)	+
Am. ferrooxidans	Straight rods and filaments	+	+	–	–	+	+	–	+
Isolate GSM	Straight rods	–	+	+	+	+	+	–	+
SLC group	Straight rods	–	+	+	–	+	–	–	+

(+) indicates limited motility and/or growth.
[a] Not all strains are capable of growth at this temperature.

Table 2
Presumptive identification or iron-oxidizing acidophiles based on their phenotypic characteristics

Solid medium	Colony morphologies	Cellular morphologies	Tentative identification
Mesophiles			
Feo	Small–large Bronze/rusty orange-brown Variable (entire, irregular)	Straight rods Variable motility Single cells or short filaments No spores	*At. ferrooxidans*
	Small (often very small) Orange Entire	Straight rods Nonmotile Single cells No spores	"*T. ferrooxidans*" m-1
	Small (often very small) Orange Entire	Vibrioid cells and spirilla Highly motile No spores	*Leptospirillum* spp.
	"Fried egg"[a]	Straight rods Motile Single cells and filaments No spores	"*Ferrimicrobium*" spp.
FeSo	Rusty orange-brown colonies becoming increasingly bleached with time	Straight rods Variable motility Single cells or short filaments No spores	*At. ferrooxidans*
	"Fried-egg"[a]	Straight rods Single cells Spores present	*Sulfobacillus* spp. or SLC-group
Moderate thermophiles			
Feo	Small (often very small) Orange Entire	Vibrioid cells and spirilla Highly motile No spores	*L. ferriphilum*
	Small (often very small) Orange Entire	Pleomorphic (often coccoid) Nonmotile No spores	*Ferroplasma* spp.
FeSo	"Fried egg"[a]	Straight rods Limited or nonmotile Single cells Spores present	*Sulfobacillus* spp. or isolate GSM
	"Fried egg"[a]	Straight rods Motile Single cells and filaments No spores	*Am. ferrooxidans.*

[a] Yellow/orange centered with white/off-white peripheries (see Supplemental material for colony morphologies).

Adopted from: Johnson et al., 2005