İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

INVESTIGATION OF BIOGEOCHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF ACID MINE DRAINAGE IN BALIKESIR-BALYA REGION BY MOLECULAR BIOLOGY TECHNIQUES

M.Sc. Thesis by
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Department : Advanced Technologies

Programme : Molecular Biology Genetics and Biotechnology

JANUARY 2011

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BALIKESİR-BALYA BÖLGESİNDEKİ ASİT MADEN DRENAJININ MOLEKÜLER BİYOLOJİ TEKNİKLERİ İLE BİYOJEOKİMYASAL VE MİKROBİYOLOJİK ÖZELLİKLERİN İNCELENMESİ

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FOREWORD

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Molecular Biolog

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ABBREVIATIONS

AMD **:** Acid Mine Drainage
EC : Electrical Conductivity
IC **:** Ion Chromatography
ICP : Inductively Coupled Plasma
IOB **:** Iron Oxidizing Bacteria
MPN : Most Probable Number
SOB **:** Sulfur Oxidizing Bacteria
UARR : Universal Amplified Ribosomal Region

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INVESTIGATION OF BIOGEOCHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF ACID MINE DRAINAGE IN BALIKESIR-BALYA REGION BY MOLECULAR BIOLOGY TECHNIQUES

SUMMARY

Sulfur, which is stable in ore and coal, starts to oxidize when exposed to atmospheric oxygen and water during surface and ground mining activities. As a result of these oxidation processes, acid mine drainage (AMD) which has high sulfate, metals and low pH water form. The acidity in turn can promote solubilization of heavy metals such as copper, zinc, nickel, cobalt and arsenic from the rocks. The most important characteristics of AMD known as the most common environmental problem related to mining activities is its high metal concentrations . These waters with high metal content affect ecological life in many different aspects such as plant growing in these waters can be toxic to whole food cycle. Microorganisms play a major role in oxidation of sulfur ores. The most common and important microorganism is *Acidithiobacillus* species. *Acidithiobacillus* can oxidize sulfur minerals utilizing molecular oxygen and Fe(III) as a electron acceptor. In these reactions bacteria act as catalyst and cause the reactions proceed faster, as a result, formation of AMD is largely controlled by bacterial reactions. Therefore, understanding and elucidating oxidation mechanisms of sulfide minerals such as galena, sphalerite and pyrite in any region need to take microbial reactions into account. The main goal of the research was to investigate microbial community composition of abandoned Pb-Zn mine and their influences on geochemical reactions in the region. For our goal, we used laboratory and field approaches. For laboratory studies, we designed biological and abiotic oxidation experiments with galena, sphalerite and pyrite under various conditions (pH (2-4), temperature (4-25^{o}C)) which mimics the field conditions. The biological experiments were conducted by using the acidophilic sulfur oxidizing bacteria, *Acidithiobacillus thiooxidans* bacterium (14887). Chemical control experiments were carried out under identical conditions as the biological experiments except addition of the bacterial culture to determine the role of bacteria on sulfur mineral oxidation rate. Sediment and water samples from the various waste rocks were collected during the field excursion in August 2010. Chemical properties (pH, temperature, Eh, EC) of the water samples (temporary tailing pools and the creek) were determined in the field using portable instrument (WTW). High concentration of Pb, Zn, Cu, Fe, Co, As and Cd in sediments and water were determined. 16S rRNA gene sequence analysis was performed in the sediment and water samples for identification of bacterial population in the mine tailing area. Laboratory oxidation experiments with galena and sphalerite at 25^{o}C showed high oxidation rate with *A. thioxidans* compared to chemical control experiments. Experiments under suboptimal temperature (4, 10^{o}C) indicated that *A. thioxidans* was still active even under 4^{o}C although the oxidation rate of galena and sphalerite were significantly lower compared to 25^{o}C. Unlike galena and sphalerite, oxidation of pyrite with bacteria and

without bacteria did not show significant reaction rate. Our sequence analysis indicated high bacterial diversity in the Balya Pb-Zn tailings, most prevalent sulfur - Fe(II) oxidizer along with Fe(III) reducer bacterium. Our sequence and geochemical analysis suggest that sulfur oxidation is mostly mediated by complex microbial reactions by sulfur –oxidizer bacterium in the tailings.

BALIKESİR-BALYA BÖLGESİNDEKİ ASİT MADEN DRENAJININ MOLEKÜLER BİYOLOJİ TEKNİKLERİ İLE BİYOJEOKİMYASAL VE MİKROBİYAL ÖZELLİĞİNİN İNCELENMESİ

ÖZET

Yüzey ve/veya yeraltı madencilik işlemleri sırasında, sülfürlü cevher ve kömürün içerisinde dingin durumdaki kükürt, kaya parçalanıp ufalandığından atmosferik oksijen ve su ile tepkime olanağı bularak oksitlenmeye başlar. Bu oksidasyon sonucunda, yüksek sülfat, metal ve düşük pH'lı (<3) asit maden suları/sahaları (AMS) oluşur. Düşük pH'lı bu asidik sular, kaya parçalarındaki ağır metallerin (Cd,Co, Pb, As, Zn) çözülmesini tetikler. Dünyada en yaygın çevre problemlerinden bir olan AMS'nin en karakteristik özelliği yüksek metal ve mineralojik içeriğe (galen, sfalerit, pirit) sahip olmasıdır. Yüksek metal içerikli bu sular, burada yetişen bitkilerin tüm gıda döngüsü için toksik olması gibi bir çok açıdan ekolojik yaşamı etkiler. Mikroorganizmalar sülfürlü cevherlerin oksitlenmesinde ana rol oynarlar. Bu mikroorganizmaların içinde en yaygın ve önemlilerinden biri *Acidithiobacillus* türüdür. *Acidithiobacillus* bakterisi moleküler oksijen ve Fe(III) iyonunu elektron alıcı olarak kullanarak sülfür minerallerini oksitlerler. Bu reaksiyonlar sırasında bakteri katalizör görevi üstlenerek, reaksiyonların hızla gelişmesine neden olur. Bu nedenle, herhangi bir bölgedeki pirit, galen ve sfalerit gibi sülfür minerallerin oksidasyon mekanizmasının anlaşılması ve açıklanması için mikrobiyal reaksiyonların dikkate alınması gerekir. Burada sunulan araştırmanın esas amacı, terk edilmiş Pb-Zn maden bölgesindeki mikrobiyal kompozisyonun belirlenmesi ve jeokimyasal reaksiyonlardaki etkisinin incelenmesidir. Hedefimiz için laboratuar ve arazi çalışmaları yapılmıştır. Laboratuar çalışmaları için, arazi şartlarına benzeyen birçok değişik şartlarda (pH (2/4), sıcaklık (4-25°C)) galen, sfalerit ve piriti kullanarak biyolojik ve kontrol oksidasyon deneyleri dizayn ettik. Biyolojik deneyler, asidofilik sülfür okside eden *Acidithiobacillus thiooxidans* (14887) bakterisi kullanılarak yürütülmüştür. Bakterilerin sülfür mineral oksidasyon oranındaki etkisini anlamak amacıyla kimyasal kontrol deneyleri bakteri kültürünün ilave edilmeden biyolojik deneylere benzeyen şartlarda yapılmıştır. Değişik atık kayalardaki sediment ve su örnekleri ağustos 2010'daki arazi çalışmasında toplanmıştır. Su örneklerinin (geçici atık havuzları ve dere) kimyasal özellikleri (pH, sıcaklık, Eh, EC) arazide taşınabilir cihaz (WTW) ile belirlenmiştir. Sedimentlerde ve suda yüksek konsantrasyonlarda Pb, Zn, Cu, Fe, Co, As ve Cd tespit edilmiştir. Maden atık sahasındaki bakteri populasyonunu belirlemek için sediment ve su örnekleri ile 16S rRNA gen sekans analizi yapılmıştır. 25°C'de galen ve sfalerit ile yapılan laboratuar oksidasyon denemeleri kimyasal kontrol deneylerine nazaran A.thiooxidans bakterisi ile yüksek oksidasyon oranı göstermiştir. Uygun sıcaklığın altındaki (4, 10°C) deneyler, galen ve sfalerit oksidasyon oranının 25°C'ye nazaran çok daha az olmasına rağmen, *A.thiooxidans* bakterisinin 4°C'nin altında hala aktif olduğunu gösterir. Galen ve sfaleritin aksine piritin kayda değer biyolojik ve kimyasal oksidasyonu görülmemiştir. Sekans analizlerimiz Balya Pb-Zn atıklarında

sülfür-Fe(II) okside eden ve Fe(III) indirgeyen bakteriler yaygın olmak üzere yüksek bakteri çeşitliliğinin olduğunu göstermiştir. Sekanslama ve jeokimyasal analizlerimiz sülfür oksidasyonun çoğunlukla kompleks mikrobiyal reaksiyonlarla gerçekleştirildiğini ve atıklardaki ikincil mineral oluşumunun sülfür okside eden bakterilerle yapıldığını ileri sürmüştür.

1. INTRODUCTION

1.1 Acid Mine Drainage (AMD)

Sulfur which is stable in the ore and coal are exposed to atmospheric oxygen and water, start to oxidize during surface and ground mining activities (Fig 1.1). As a result of these oxidation processes, acid mine drainage which has high sulfate, metals and low pH water form [1, 2]. The acidity in turn can promote the solubilization of heavy metal contaminants such as copper, zinc, nickel, cobalt and arsenic which appear in non-compliant concentrations in water bodies downstream of the mining activities. The most important characteristics of AMD which is known as the most common environmental problem related to mining activities is its high metal concentrations. These environmental problems have received increasing attention due to their long term environmental effects since AMD contain heavy metals such as copper, silver, arsenic, zinc, lead and mercury in ground or surface water. These waters with high metal content affect ecological life in many different aspects such as plant growing in these waters can be toxic to whole food cycle [2].

AMD can be formed by a series of complex biological and chemical reactions. One of the most intriguing aspects of the AMD problem is that the majority of AMD formed is the direct result of microbial activity [3, 4]. The most common and important microorganism is *Acidithiobacillus* species. *Acidithiobacillus* can oxidize sulfur minerals utilizing molecular oxygen and Fe(III) as a electron acceptor shown in the reactions 1 and 2, respectively. In these reactions bacteria act as catalyst and cause the reactions proceed faster. The oxidation rates of reactions 1 and 2 depend on the concentrations of molecular oxygen and Fe(III) in the environments. Below a pH of 3, reaction 3 is thought to be the rate-limiting step and proceeds primarily by mediation with bacteria [5].

$$MeS_2 + 7/2O_2 + H_2O \rightarrow Me^{2+} + 2SO_4^{2-} + 2H^+ \quad (\mathbf{1.1})$$

$$MeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Me^{2+} + 2SO_4^{2-} + 16H^+ \quad (\mathbf{1.2})$$

$$Fe^{2+} + 1/4O_2 + H+ \rightarrow Fe^{3+} + 1/2H_2O \quad (\mathbf{1.3})$$

The rate and degree by which acid-mine drainage proceeds can be increased by the action of certain bacteria. Colonies of bacteria and archaea greatly accelerate the decomposition of sulfide minerals, although the reactions also occur in an abiotic environment [4]. These microbes, called extremophiles for their ability to survive in harsh conditions, occur naturally in the rock, but limited water and oxygen supplies usually keep their numbers low. Special extremophiles known as acidophiles (e.g. *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans*) especially favor the low pH levels of abandoned mines [6, 7, 8].

Acid Mine drainage is worldwide problem related to mining activities and exist in various places such as Pennsylvania, West Virginia, Ohio, Kentucky, Maryland, Indiana/USA, Balya, Balikesir/Turkey [9]. Pennsylvania and Northern West Virginia, the two most extensively mined states in Appalachia, lack limestone formations, and as a result experience severe acid drainage pollution [10]. In the Appalachian Mountains of the eastern United States alone, more than 7,500 miles of streams are impacted [11]. There have been no systematic studies related to AMD formation in Turkey. With increasing energy demand, mining activities will increase not only in our country but also in the world. Research associated with elucidating and understanding mechanisms of AMD formation is crucial in order to develop proper remediation.

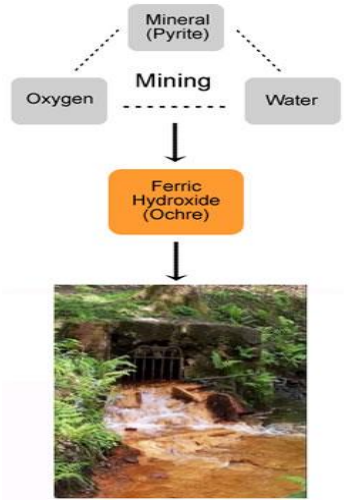

Figure1.1: Acid mine drainage formation

1.2 Sulfur Cycle in AMD Sites

Sulfur is the 8th most abundant element in the solar atmosphere and the 14th most abundant element in the earth's crust [12] and is an essential element for the growth of plants and animals. Sulfur, exist as a gas, liquid, or solid, in organic or inorganic forms, found in oxidation states ranging from +6 in sulfate to -2 in sulfides allowing it to either give or receive electrons depending on its environment [13]. Its cycling is a natural environmental process that prevents the accumulation of all of the earth's sulfur into one specific compound within a single ecosystem. The transformation reactions involved a continuous flow of sulfur containing compounds among the various components of the earth [14]. The reaction of sulfur cycle alters the chemical, physical, and biological states of sulfur and its compounds so that sulfur cycling can occur. Besides cycling among the major components of the earth's environment, there is also an internal cycling within each of the major component. Many reactions of the sulfur cycle are mediated by microorganisms [15] (Fig 1.2).

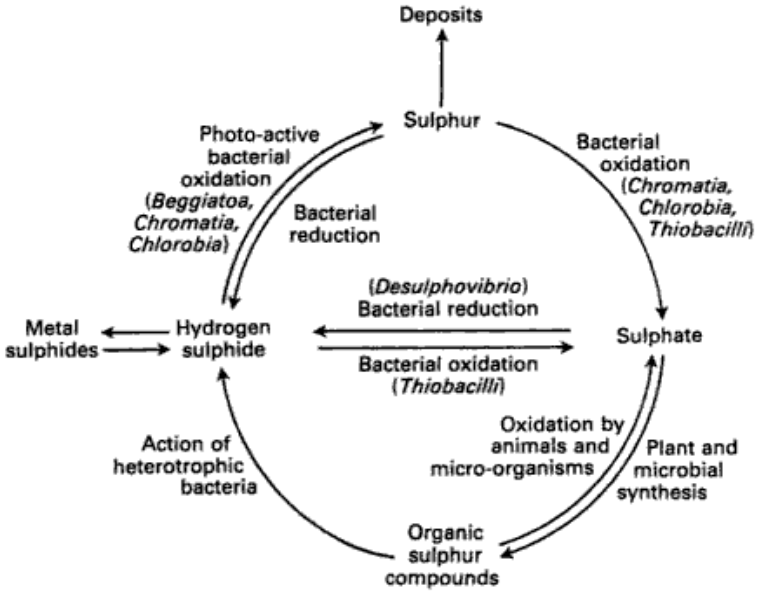

Figure 1.2: Biological sulfur cycle [16]

The biological sulfur cycle consist of a continuous oxidation and reduction of sulfur compounds by microorganisms or plants. On the left hand side of the cycle in figure 2, the most reduced sulfur compound is shown (sulfide) and on the right hand side the most oxidized form of sulfur (sulfate). Sulfur oxidizing bacteria such as

Acidithiobacillus thiooxidans used in this project cover the oxidation of sulfide to sulfate with intermediate sulfur compound such as sulfur, thiosulfate, and tetrathionate.

1.3 Sulfur Oxidizing Bacteria in AMD Sites

Sulfur oxidizing bacteria use reduced sulfur compounds in their energy transformations. These microorganisms are primarily gram negative bacteria and currently classifield as species of *Acidithiobacillus, Thiomicrospira* and *Thiosphaera*. *Paracoccus, Xanthobacter, Alcaligens* and *Pseudomonas* can also exhibit chemolithotrophic growth on inorganic sulphur compounds [17, 18]. Two clear metabolic types exist in this group: The obligate chemolithotrophs, which can only grow when supplied with oxidizable sulphur compounds (and CO_2 as the source of metabolic carbon) and heterotrophs that can also use the chemolithoautotrophic mode of growth [17, 19]. The obligate chemolithotrophs include *A.thioparus, A.neapolitanus, A.denitrificans* (facultative denitrifier), *A.thiooxidans* (extreme acidophile), *A.ferrooxidans* (acidophilic ferrous iron-oxidizer), *A.halophilus* (halophile) and some species of *Thiomicrospira*. Several *Acidithiobacillus* species are able to utilize mixtures of inorganic and organic compounds simultaneously, often referred to as mixotrophic growth [18, 20]. Of the 13 species of the genus Acidi*thiobacillus* recognized, occurring in diverse habitats, only five species are important in sulphur oxidation in soil [21]. Four of these *Acidithiobacillus thiooxidans, A.ferrooxidans, A.thioparus* and *A.denitrificans* are obligate chemoautotrophs while *A.novellus* is considered a facultative chemoautotroph [22].

Acidithiobacillus thiooxidans is a genus of proteobacteria and (syn. *Thiobacillus thiooxidans, Thiobacillus concretivorus*) is the most important genus of chemolithotrophs that metabolize sulfur [23, 24]. *A. thiooxidans* is an acidophilic, obligately autotrophic bacterium and derives its energy by oxidizing reduced or partially reduced various sulfur compounds, (e.g. elemental sulfur, thiosulfate, and tetrathionate) and sulfur minerals to sulfate [25] (Fig 1.3). Growth on the following metal sulfides has been reported: covellite, galena, sphalerite, wurtzite [26]. *A. thiooxidans* is motile rod shaped cells and can be isolated from rivers, canals, acidified sulfate soils, mine drainage effluents and other mining areas [23]. They are adapted to wide variations of temperature and pH. Most species are acidotolerant,

some even extremely acidotolerant and acidophilic. Some grow best at pH 2 and may grow at pH 1 or even at pH 0.5 [24, 25]. Most species are tolerant against heavy metal toxicity [26].

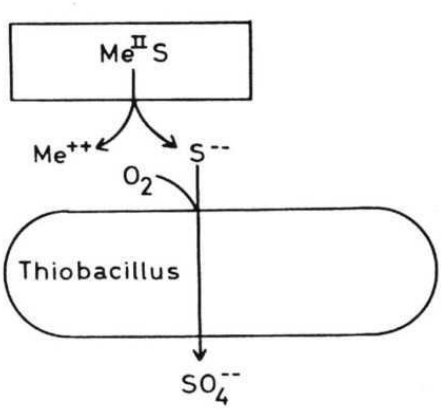

Figure 1.3: Sulfur mineral oxidation by *Thiobacillus* [27]

The strain is able to live in inorganic, acidic environments and is present in large numbers in coal mine drainage and in mineral ores. *A. thiooxidans* has been used industrially in metal leaching from mineral ores and in the microbial desulfurization of coal in combination with *A. ferrooxidans* [15, 28]. Although *A.thiooxidans* has been well studied physiologically, very little is known about its genetics. The oxidation of sulfur to sulfuric acid by *A. thiooxidans* with sulfite as the key intermediate was proposed [29, 30] on the basis of the sulfur-oxidizing enzyme and sulfite-oxidizing enzyme systems:

$$S^{o} + O_2 + H_2O \longrightarrow H_2SO_3 \qquad (\mathbf{1.4})$$

$$H_2SO_3 + \tfrac{1}{2}O_2 \longrightarrow H_2SO_4 \qquad (\mathbf{1.5})$$

This process yields 236 kcal of energy. The reduced sulfur compounds are complexed with a sulfhydryl group on a tripeptide glutathione. It is then oxidized to sulfite with the help of the enzyme sulfide oxidase [29, 31]. There is an apparent disparity in the lifestyles of these organisms. It lies in the fact that they produce sulfuric acid, but find it toxic.

1.3 The Study Area- Balikesir-Balya Pb-Zn Mine Region

The Balya Pb-Zn mine are located in Northwest Anatolia, in Balya, near Balikesir, Turkey. It is approximately 50 km away from the Balıkesir and the largest Pb-Zn mine in the region [32] (Fig 1.4, Fig 1.5, Fig 1.6, Fig 1.7, Fig 1.8).

Figure 1.4: Balıkesir-Balya Pb-Zn tailing area

Figure 1.5: Balya Pb-Zn tailing area

This Pb-Zn-Ag deposit is mined between 1880 and 1935 and was abandoned as their silver and lead content decreased. Although there are no technologies that existed about a century ago, there are about 1 million tons of gravity and flotation tailings and slags containing considerable amounts of lead and zinc [33, 34]. The local geology consists of Permian, fossil-rich massive limestones and Triassic sedimentary rocks (a series of dark pelitic shale, sandstone, and calcareous conglomerate) which were folded during the Hercynian orogeny. The volcanic rocks are part of a rhyolite–dacite–andesite–basalt sequence of regional extent [35] and related to Cenozoic rifting. Ore minerals are pyrite, galena, sphalerite, marcasite, chalcopyrite, and arsenopyrite. The reserves in Balya, estimated by (Akyol 1982), are 4.4 million tons, with 2.7% Pb, 7.2% Zn, and 0.3% Cu [36]. Minor components are pyrrhotite, marcasite, bismuth, sulfosalts, arsenopyrite, tetrahedrite-tennantite, bornite, argentite, heyrovskite, hematite, magnetite, pyrolusite, orpiment-realgar, and native tellurium [37].

Figure 1.6: Acid mine water in the Balya Pb-Zn tailing area

Figure 1.7: Acidic water reaching the spring in the tailing area

Figure 1.8: Acidic water reaching the spring in the tailing area

The main purpose of this study is to examine the effect of bacterial activity on the oxidation of sulfide minerals under the conditions mimic Balya AMD site in order to elucidate biogeochemical reactions occurring in the Balya tailing area. For these purposes, different metal sulfur minerals (pyrite, galena, sphalerite), common in Balya Pb-Zn tailing area, were biologically and chemically oxidized under the conditions which represent acid mine fields (for example pH<5). Sulfate, certain metal as an oxidation products and pH values of experimental solutions were measured depending on time. Chemical control experiments were also set up under

the biological conditions except addition culture. In addition to laboratory experiments, microbial community compositions of Balya Pb-Zn mine tailings were also investigated by molecular biology techniques. Along with the molecular biology studies, heavy metals (Fe, Cu, Pb ve Zn and ions (sulfate, nitrate, nitrite and phosphate) were measured in the field samples.

2. MATERIALS AND METHODS

2.1 Materials

Materials are listed in Appendix A.

2.1.1 Laboratory equipments

Laboratory equipments are listed in Appendix B.

2.2 Methods

2.2.1 Oxidation experiments

2.2.1.1 Preparation of Sulfide minerals for the laboratory experiments

Minerals (pyrite, galena, sphalerite) used in the current study were obtained from the Wards Scientific, USA . Prior to use in all of the experiments, minerals were ground and sieved to 63 μm. For sterilization, the mineral samples were soaked with 70% ethanol and spread in a thin even layer under UV radiation in a sterile hood for ~30 minutes to decontaminate the surface. Following these treatments, the minerals were immediately placed in sterile experimental containers.

2.2.1.2 Bacterial culture preparation

The acidophilic sulfur oxidizing bacteria, *Acidithiobacillus thiooxidans* (14887) was obtained from the German Resource Centre for Biological Material (DSMZ) and used in all the biological experiments. Bacteria were cultured in a modified thiobacillus thiooxidans medium which contains the following per liter: 0.1g NH_4Cl, 3g KH_2PO_4, 0.1g $MgCl_2.6H_2O$, 0.14g $CaCl_2.2H_2O$, 10g Sulfur powdered. All ingredients dissolved, except the sulfur, in distilled water and adjusted the pH to 4 or 2 with 1M HCl, then autoclaved. The sulfur was placed in the experimental containers and sterilized by autoclaving at 112°C for 15 minutes. Before use, the sterilized sulfur is aseptically layered onto the surface of autoclaved liquid basal medium. This pure culture was incubated at 30°C for 2 weeks. Bacterial culture were then centrifuged at 14000xg for harvesting and used in all biological oxidation experiments.

2.2.1.3 Biological oxidation experiments

Biological oxidation experiments were carried out under conditions which represent acid mine fields (for example pH<5). The composition of the experimental solution used in the all oxidation experiments as follows: 0.1g NH_4Cl, 3g KH_2PO_4, 0.1g $MgCl_2.6H_2O$, 0.14g $CaCl_2.2H_2O$, Biological oxidation experiments were carried out in 500 ml Erlenmeyer flasks containing 200 ml sterile media, sterile mineral and bacterial culture incubated in an incubator shaker at 180 rpm until the end of the experiments. For pyrite oxidation experiments, 200ml of medium, 2g sterilized pyrite and 0.1g bacterial culture; for galena oxidation experiments, 200ml of medium, 8g sterilized galena and 0.1g bacterial culture; for sphalerite oxidation experiment, 200ml of medium, 3g sterilized sphalerite and 0.1g bacterial culture containing flasks were incubated at different temperatures (25, 10, 4^oC). The lists of aerobic experiments were summarized in the example of 25^oC (Table 2.1). Chemical control experiments were carried out under identical conditions as the biological experiments except addition of the bacterial culture to demonstrate the role of bacteria on sulfur mineral oxidation. Biological experiments were carried out at pH 4 and 2 as the starting pH. Initial pH of the media was adjusted to pH (4 or 2) using 1M HCl. In order to prepare bacterial culture for the mineral oxidation experiments, a culture of *A. thiooxidans*, fully grown in the modified thiobacillus thiooxidans medium was centrifuged at 2000xg for 5min to remove all of the insoluble sulfur compounds. Sulfur-free pure culture then centrifuged at 14000xg and the cells were harvested. By reading the turbidity of the culture, corresponding amount of biomass (1.00 OD_{620nm} = 0.1±0.08 g/l dry cell weight) was obtained and used in the all biological oxidation experiments. Three groups of parallel experiments were conducted for approximately 2 months. The oxidation products of sulfate, metals (Zn, Pb, Fe_{tot}) and pH values of experimental solutions were periodically monitored during the whole experimental time period. For this purpose, 10 ml samples taken from the each flask under the sterile conditions were filtered through 0.2µ filter and pH value of filtrate was measured by using pH-meter (WTW 3100). The concentrations of Pb^{2+}, Fe_{tot}, Zn^{2+} were measured with ICP-MS and SO_4^{2-} with Ion Chromatograph (Dionex ICS-1500).

Table 2.1: List of aerobic biotic and abiotic oxidation experiments in the example of 25 ^{o}C.

Biological experiments	**Pyrite (FeS_2) pH:4/2**	*A.thiooxidans* + medium (pH:4/2)+ FeS_2
	Galene (PbS) pH:4/2	*A.thiooxidans* + medium (pH:4/2)+PbS
	Sphalerite (ZnS) pH:4/2	*A.thiooxidans* + medium (pH:4/2)+ZnS
Chemical-control experiments	**Pyrite (FeS_2) pH:4/2**	medium (pH:4/2) + FeS_2
	Galene (PbS) pH:4/2	medium (pH:4/2) + PbS
	Sphalerite (ZnS) pH:4/2	medium (pH:4/2) + ZnS

2.2.2 Field studies

2.2.2.1 Sampling procedure and physicochemical determinations in situ

Water and sediments samples for microbial and geochemical analysis were collected during August 2010. Water samples were collected from a creek around the tailings and the small temporary pools located in the center of the tailing where the water chemistry varies in a particularly large range according to season (Fig 2.1). Samples for cultivation studies were put into sterile Falcon tubes and maintained at 4°C. Samples for DNA extraction were also collected in Falcon tubes, but were plunged, on site, into a dry ice-ethanol bath for flash-freezing. They were then stored in at -21°C until processing. At each sampling point, 200 ml of water were filtered through a 0.22 μm sterile Nucleopore filter and 100 ml of acidified water with 1 ml nitric acid were saved for metal analysis. The rest unacidified water was collected into a tube, kept at 4◦C for sulfate measurements. pH, EC, Eh and temperature measurements in the water samples were done in situ and presented in Table 3.11.

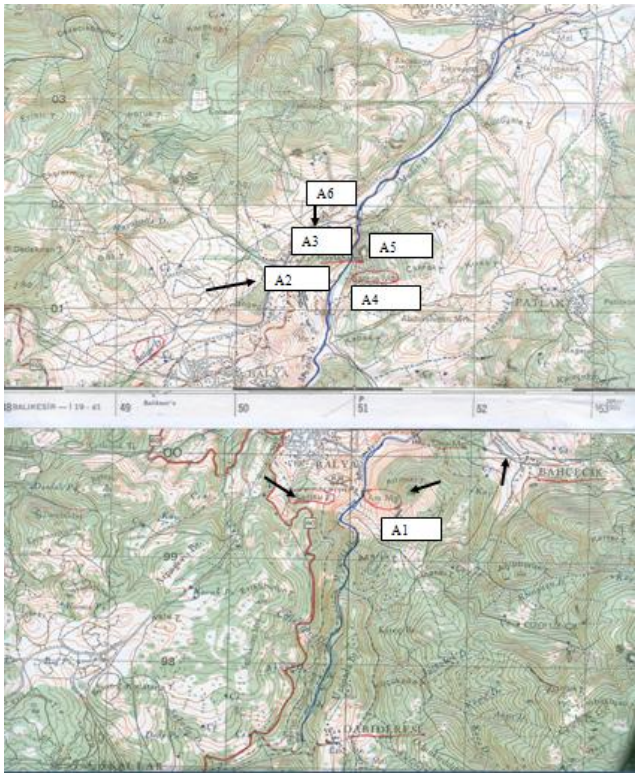

Figure 2.1: Sampling sites

Enumeration of acidophilic S-oxidizing bacteria (aSOB) and iron-oxidizing bacteria (IOB) was performed using the most probable number (MPN) technique (Table 3.8) [38]. Bacterial growth was promoted in sterile culture tubes containing 4.5 ml of selective media. Inoculation was performed by adding 0.5 ± 0.05 g of tailings and 0.5 ml of water to each of five tubes. A series of five 1:10 serial dilutions were carried out and samples were incubated at room temperature (22 ± 1 °C) for 4 weeks. Preparation of growth media and recognition of positive results followed previously described methods [39, 40].

Samples obtained from the field, were subjected to leaching operations for ICP-MS analysis. In this study, the total leaching process is performed in the laboratories of the Institute of Marine Science and management at Istanbul University. 0.5 g of dried and ground samples at 105°C were used for the analysis. Leaching method was performed in microwave oven with the mix of hydrofluoric acid, nitric acid and perchloric acid at 180°C. Solution samples stored at 4°C for subsequent ICP-MS analysis.

2.2.2.2 Genomic DNA isolation from the tailing soils and sediments samples

Fast DNA Spin Kit for Soil (Catalog # 6560-200, MPBio) were used for DNA isolation from soil sample following the protocol described below.

1. Approximately 1 g of soil and sediment sample was washed with 500 µl PBS, centrifuged at 14000 rpm for 10 min and supernatant is discarded. (Washing)
2. Up to 500 mg of washed sample added to Lysing Matrix E tube. (Sample processing)
3. 978 µl sodium phophate buffer and 128 µl MT buffer was added to the sample in the tube. (Lysis)
4. Sample containing tubes were secured in FastPrep Instrument and processed for 40 seconds at a speed of 6. (Homogenization)
5. Sample was centrifuged at 14000xg for 5 min.
6. Supernatant was transferred to a clean tube and 250 µl protein precipitation solution (PPS) was added and mixed by shaking the tube 10 times.
7. Sample was centrifuged at 14000xg for 5 min to pellet precipitate. Supernatant was transferred to a clean 2 ml tube.
8. 1 ml of resuspended binding matrix solution was added to the supernatant and mixed by inverting for 2 min to allow binding the DNA. (Binding DNA)
9. Tube is placed on a rack for 3 min to allow settling the silica matrix.
10. About 500 µl of the upper solution was discarded and the remaining mixture was resuspended.
11. 600 µl of the mixture was transferred to SPIN Filter, centrifuged at 14000xg for 1 min, catch tube was empitied, the remaining mixture was added and centrifuged again.
12. 500 µl of SEWS-M (+EtOH) was added to the fitler and centrifuged at 14000xg for 1 min to wash the DNA.
13. Centrifuge at 14,000xg for 2 minutes to “dry” the matrix of residual SEWS-M wash solution. Replace SPIN Filter in a clean catch tube.
14. SPIN Filter was air dried for 5 min.

15. Binding matrix was resuspended gently with 50 µl DNase-Pyrogen-Free Water (DES). Tube was incubated at 55 ℃ for 5 min for increasing the DNA yield.
16. A clean tube was placed under spin filter and centrifuged at 14000xg for 1 min.
17. DNA solution in the tube stored at – 20 ℃.

Agarose gel (1%) electrophoresis was performed using 5 µl of the resultant DNA solution to estimate the amount and integrity of the DNA products.

2.2.2.3 Universal amplified ribosomal region (UARR) PCR

The diversity of soil and sediment samples were investigated by 16S-rDNA methodology. Approximately 1500bp of 16S rRNA gene fragment was amplified using the universal primer pair pA-F (5'-AGAGTTTGATCCTGGCTCAG-3') and pH-R (5'-AAGGAGGTGATCCAGCCGCA-3'). PCR was performed in Biometra TProfessional Gradient Thermal cycler with 1 cycle of denaturation at 95°C for 5 min, followed by 35 cycles 95°C for 45 sec, 55°C for 45 sec and 72°C for 1 min and a final cycle of extension 72°C for 5min. PCR reactions contained 0.2 µM each primer, 10 mM dNTPs, 1 U BioTaq enzyme in the buffer and 1 µl DNA template that performed in a total volume of 50 µl. Negative controls consisted of 1 ml of molecular grade water in all PCR reactions to check for contamination.

2.2.2.4 Agarose gel electrophoresis and gel extraction

PCR products were visualized by electrophoresis on 1% (wt/vol) agarose gels including 5% ethidium bromide to check integrity and yield. All of the PCR reaction loaded into the gel to purify the PCR product. After electrophoresis, corresponding band was cut by sterile, sharp scalpel and transferred to a clean 2 ml tube. Extraction was performed by QIAGEN – QIAquick Gel Extraction Kit (Catalog # 28604, Qiagen). Gel extraction procedure is described below.

1. Weight of the gel slice was determined and 3 volumes of Buffer QG was added to 1 volume of gel. (100 mg gel was considered as approximately 100 µl)
2. Sample was incubated at 50 ℃ for 10 min. Every 2–3 min during the incubation, sample tubes were mixed by vortexing to help dissolve gel.

3. 1 gel volume of isopropanol was added to the sample and mixed by inverting the tube several times. This step increases the yield of DNA fragments <500 bp and >4 kb.
4. 800 µl sample were transferred to MinElute column and centrifuged at 13000xrpm for 1 min.
5. 500 µl QG buffer was added to the column and centrifuged at 13000xrpm for 1 min. Filtrate was discarded and MinElute column was placed back in the same collection tube.
6. 750 µl PE buffer was added to the column and centrifuged at 13000xrpm for 1 min. Filtrate was discarded and MinElute column was centrifuged again at 13000xrpm for 1 min to dry completely.
7. Column was placed on a clean tube and 10 µl EB Buffer (10 mM Tris·Cl, pH 8.5) was added to center of the membrane carefully and centrifuged at 13000xrpm for 1 min.
8. DNA solution was stored at – 20 °C until usage.

2.2.2.5 Cloning and transformation of PCR products

Cloning reaction was performed using TOPO-TA cloning kit (İnvitrogen, cat# K4500-01). 2µl of fresh PCR product, 1µl of dilute salt solution, 1µl of pCR®2.1-TOPO® cloning vector, 2µl of sterile water mixed gently in 500 µl tube and incubated for 15 minutes at room temperature (22-23°C). 2µl TOPO cloning mixture were added into a vial (50µl) of One Shot Electrocompotent *E-coli* TOP10 cells (İnvitrogen, cat# C4040-52) and mixed gently and carefully transferred solution to a 0.1 cm cuvette to avoid formation of bubbles. The sample was electroporated at 1800 voltage using electroporator (eppendorf). After electroporation, immediately 250µl of SOC medium was added and transferred the solution to a 1.5 ml snap-cap tube and shake for at least 1 hour at 37°C to allow expression of antibiotic resistant genes.

2.2.2.6 Blue/White screening and plasmid isolation

Approximately 50-100µl transformed cell were spread on a LB agar containing 100 µg/ml ampicilline and 40 µl of X-gal stock solution and incubated overnight at 37°C. White colonies on the selective plate were picked and inoculate a starter culture of

2-5 ml LB medium containing the 100 µg/ml ampicilline. It was incubated overnight at 37°C with vigorous shaking. Plasmids isolation from grown culture using Roche High Pure Plasmid Isolation Kit (Cat #1754785) were described below.

1. 2 ml of *E.coli* culture was centrifuged at 6000 x rpm for 1 min to pellet the cells and the Supernatant was discarded.
2. 250 µl Suspension+RNase Buffer was added to to the centrifuge tube containing the bacterial cell pellet.
3. 250 µl Lysis Buffer was added to the mixture and the tubes were inverted 3 to 6 times by hand and incubated for 5 min at any temperature between +15°C and +25°C.
4. 350 µl pre-chilled binding buffer was added and mixed gently by inverting the tubes 3 to 6 times.
5. Samples were incubated on ice for 5 min after mixing gently and centrifuged at 13000xg for 10 min.
6. High Pure Filter tubes were inserted into collection tubes and entire supernatant from step 5 was transferred to the filter.
7. Samples were centrifuged at 13000xg for 10 min. Flow-through was discarded.
8. Filter was washed with 500 µl Wash Buffer I and centrifuged at 13000xg for 1 min. Flow-through was discarded.
9. 700 µl Wash Buffer II was added to the filter and centrifuged at 13000xg for 1 min. Flow-through was discarded.
10. Filter tube assembly was centrifuged for additional 2 min to dry completely and transferred to a clean 1.5 ml tube.
11. 100 µl Elution Buffer was added to the filter tube and centrifuged at 13000xg for 1 min.
12. Plasmids were stored at – 20 °C until usage.

2.2.2.7 Sequence PCR and purification

Nucleotide sequences of the plasmids were determined by the following method. PCR was performed in Biometra TProfessional Gradient Thermal cycler with 1 cycle

of denaturation at 95°C for 5 min, followed by 30 cycles 95°C for 10 sec, 55°C for 5 sec and 72°C for 4 min. PCR reactions contained 2 µl ABI-RR100 dye, 2 µl ABI 5X PCR buffer, 3.2 pmole (1 µl) M13 forward/reverse primer, 4 µl dH_2O and 1 µl DNA template that performed in a total volume of 10 µl. The primer pair M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) was used in the template amplification by PCR for the subsequent cycle sequencing. The sequencing was performed with an ABI PRISM Big Dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, California) and an ABI PRISM 3700 DNA analyzer (Applied Biosystems). After completion of sequence PCR, products were purified according to the procedure below:

1. PCR products were transferred to a clean 1.5 ml tubes.
2. 1 µl sodium acetate (3 M, pH 5.2) and 25 µl cold 95 % EtOH were added to the each tubes and incubated on ice for 15 min.
3. Samples were centrifuged at 14000 x rpm for 15 min. Supernatant was discarded.
4. Pellets were washed with cold 70 % EtOH and centrifuged at 14000xrpm for 15 min. Supernatant was discarded.
5. Excess EtOH was evaporated at 95 °C.
6. DNA pellet were dissolved in 20 µl formamide and denatured at 95 °C for 3 min.
7. Tubes were stored at 4 °C until loading into sequence analyzer.

2.2.2.8 Phylogenetic analysis

Partial bacterial 16S rRNA gene sequences were subjected to a NCBI database using BLAST-N (http://www.ncbi.nlm.nih.gov/blast/) search to identify sequences of the highest similarity. Sequence library is transferred to MEGA program (Molecular Evolutionary Genetics Analysis, version 4.0) to compare with each other and phylogenetic tree is drawn.

3. RESULTS

3.1 Oxidation Experiments

3.1.1 Aerobic pyrite (FeS_2) oxidation experiments

Table 3.1 presents the results of oxidation of pyrite experiment with *A. thiooxidans* and without the bacterial culture at 25°C and two different pHs (4 and 2). In general, the biotic and abiotic experiments show very similar results regardless of pH (Figure3.1a, b). In contrast to Fe_{tot} concentration, sulfate concentration was higher at pH 4 both in the biotic and abiotic experiments. At the end of the biological experiments, sulfate was measured as 110 mg/L and the corresponding value was 100 mg/L in the non-inoculated experiments at pH 4. The concentration of Fe_{tot} was significantly higher at pH 2 compared to pH 4. Significant decrease in the pH values was only measured in the biotic experiments at pH 4 under 25^{o}C (Table 3.1 and Figure 3.1a, b).

Table 3.1: Biotic and abiotic oxidation of pyrite at 25°C.

	Biotic				**Abiotic**			
pH:4	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)
	0	4.0	0	0.3	0	4.0	0	0
	16	3.2	25	0.5	16	3.9	30	0.7
	32	3.4	35	2.0	32	3.9	22	1.3
	44	3.4	52	1.1	44	3.6	47	1.9
	54	3.1	67	1.2	54	3.5	50	1.3
	65	2.9	78	1.5	65	3.5	69	1.0
	74	3.0	95	1.5	74	3.5	94	1.1
	94	3.0	110	1.8	94	3.4	100	1.2
pH:2	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)
	0	2.0	0	11.2	0	2	0	10.2
	16	2.0	20	12	16	2.0	10	14.2
	32	2.1	25	10	32	2.1	25	15.6
	44	2.0	32	8.0	44	2.0	47.7	17
	54	2.0	40	9.5	54	2.0	55	13.7
	65	2.1	75	14	65	2.0	80.9	22.6
	74	2.0	84	22	74	2.0	74	33.1
	94	2.1	98	30.1	94	2.0	70.1	35.9

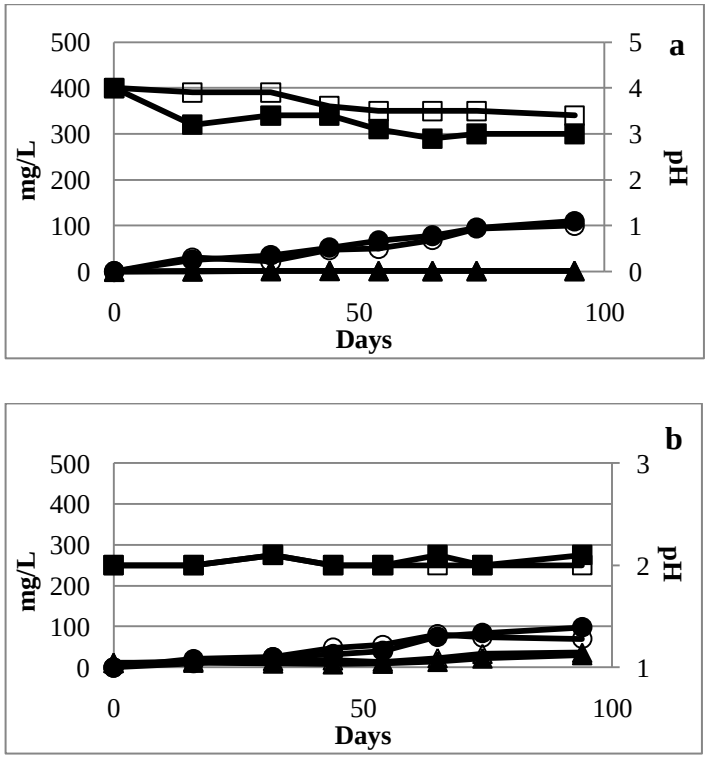

Figure 3.1: Solution chemistry during aerobic biotic and abiotic pyrite oxidation experiments at 25°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Fe concentration of biotic experiments; Δ: Fe concentration of abiotic experiments.

Consistent with the pyrite experiments at 25^{o}C, the biotic and abiotic experiments also produced similar results at pH 4 and 2 under 10^{o}C (Table 3.2). At the end of the biological experiments, sulfate concentration in both biotic and abiotic experiments shows similar values under 10^{o}C. Fe_{tot} concentration was significantly greater at pH 2 and measured as 35 mg/L and 35.7 mg/L at days 122 in biotic and abiotic experiments, respectively. At 10^{o}C, concentration of sulfate was insignificantly high relative to 25 °C at the end of 122 days although these experiments were kept longer. In contrast to low pH experiments (pH 2), the pH showed decreasing trends in the biotic experiments set up at pH 4 (Table 3.2 and figure 3.2a,b).

Table 3.2: Biotic and abiotic oxidation of pyrite at 10°C.

	Biotic				Abiotic			
	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)
pH:4	0	4.0	0	1.5	0	4.0	0	0.4
	16	3.8	0	1	16	4.0	69	0.5
	32	3.6	32	8	32	3.9	25	0.7
	44	3.5	42	5	44	3.6	47	0.6
	54	3.5	50	3	54	3.6	64	0.4
	65	3.3	77	2	65	3.6	69	0.8
	74	3.3	80	1.7	74	3.5	94	0.5
	94	3.3	127	1.6	94	3.5	110	0.3
	122	3.3	125	1.5	122	3.5	121	0.1
	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)
pH:2	0	2.0	0	7.5	0	2.0	0	11.1
	16	1.9	20	14.2	16	1.9	25	22.7
	32	2.0	60	16.3	32	2.0	62	19.7
	44	2.0	80	17	44	1.9	65	11
	54	2.1	80	18.7	54	2.2	70	25
	65	2.0	138	21	65	2.0	115	44.3
	74	1.9	146	23	74	1.9	110	42.9
	94	2.0	150	32	94	1.9	149	35
	122	2.0	150	35	122	2.2	150	35.7

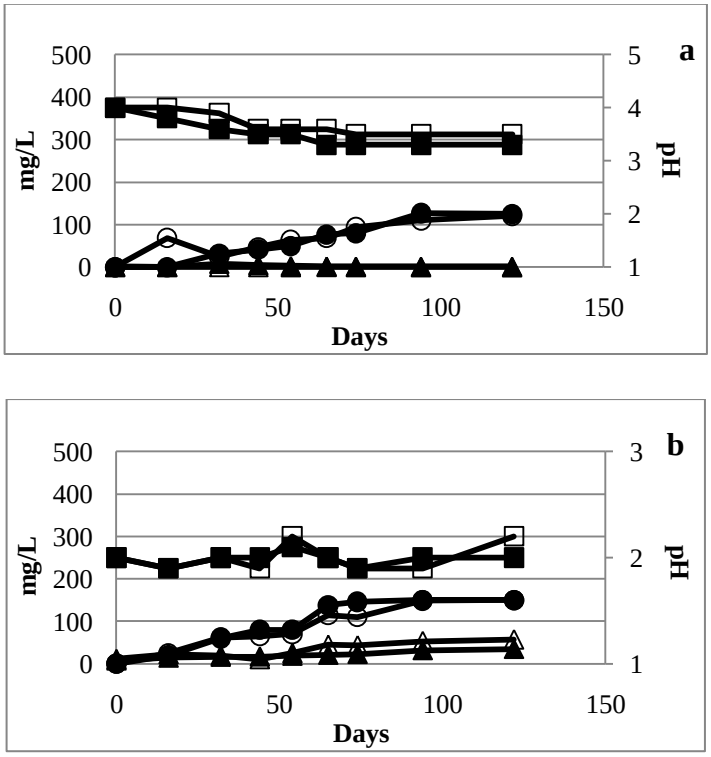

Figure 3.2: Solution chemistry during aerobic biotic and abiotic pyrite oxidation experiments at 10°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Fe concentration of biotic experiments; Δ: Fe concentration of abiotic experiments.

The biotic and abiotic pyrite experiments at pH 4 and 2 under 4°C, almost identical, produced insignificantly low sulfate and Fe_{tot} compared to 25°C and 10°C (Table3.3). Sulfate concentration was measured as 60 mg/L at pH 4 and approximately 20 mg/L at pH 2 at days 80 in both biotic and abiotic experiments (Table 3.3 and Figure 3.3a,b).

Table 3.3: Biotic and abiotic oxidation of pyrite at 4°C.

	Biotic				**Abiotic**			
pH:4	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)
	0	4.0	0	0	0	4.0	0	1.5
	10	3.5	42	1.9	10	3.5	48	1.5
	21	3.4	32	2.3	21	3.3	36	1.6
	28	3.3	42	2.7	28	3.2	40	1.8
	35	3.2	50	2.4	35	3.2	43	4.5
	44	3.0	33	2.1	44	3.2	30	1.6
	57	3.2	52	2.2	57	3.2	53	1.7
	66	3.2	51	2.3	66	3.2	52	1.3
	80	3.1	63	3.5	80	3.4	59	1.8
pH:2	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)	Days	pH	SO4 (mg/L)	Fe_{tot} (mg/L)
	0	2.0	0	23.2	0	2.0	0	20
	10	2.1	11.0	24.1	10	2.1	10.0	21
	21	2.1	16.1	24.5	21	2.1	11.4	20.4
	28	2.1	20.0	24.8	28	2.1	10.5	20.3
	35	2.1	25.6	25.1	35	2.0	9.3	20.7
	44	2.0	24.1	32.5	44	2.2	9.5	24.6
	57	2.1	21.0	32.6	57	2.1	8.9	27.4
	66	2.1	24.0	36.6	66	2.0	15	29.7
	80	2.1	25.0	37	80	2.2	18	32

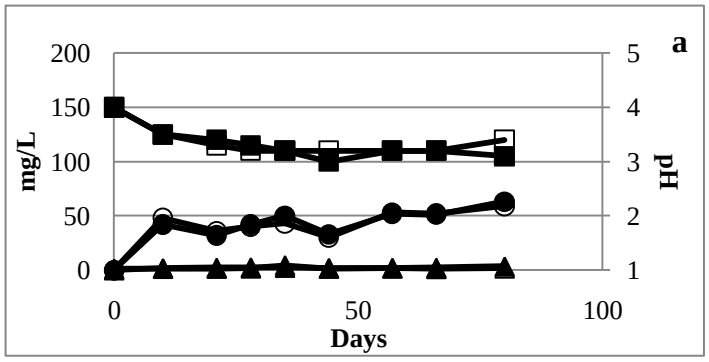

Figure 3.3: Solution chemistry during aerobic biotic and abiotic pyrite oxidation experiments at at 4°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Fe concentration of biotic experiments; Δ: Fe concentration of abiotic experiments.

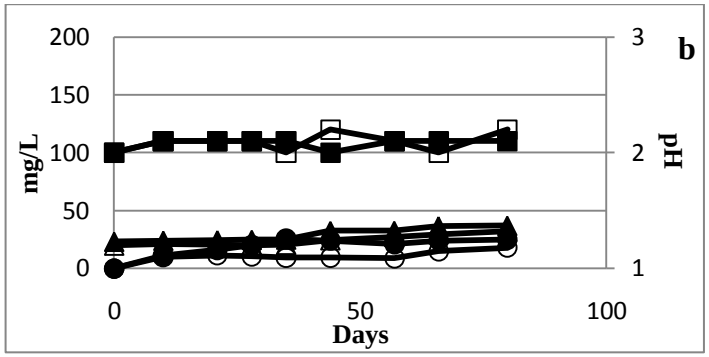

Figure 3.3 (continued): Solution chemistry during aerobic biotic and abiotic pyrite oxidation experiments at at 4°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Fe concentration of biotic experiments; Δ: Fe concentration of abiotic experiments.

3.1.2 Aerobic galena (PbS) oxidation experiments

During the oxidation of the galena with *Acidithiobacillus thiooxidans* at 25°C and 10°C, sulfate concentration increased depending on time but there was no change in control experiments (Table 3.4, Table 3.5). At the end of the biological experiments, sulfate was measured as 900 mg/L and the corresponding value was 10 mg/L in the abiotic experimets experiments at pH 4. Sulfate was increased to 600 mg/l in biotic experiments at pH:2 at 25°C. At 10°C, sulfate concentration was measured as 240 mg/L in biotic experiments and 5 mg/L in the abiotic experimets experiments at pH 4 and 140 mg/L in biotic experiments at pH:2. The concentration of Pb was significantly higher at pH 2 compared to pH 4. Significant decrease in the pH values was only measured in the biotic experiments at pH 4. The reason of pH increasing in abiotic experiment at pH 4 is formation of sulfur compounds in the solution (Table 3.4, Table 3.5, Figure 3.4a,b, Figure 3.5a,b).

Table3.4: Biotic and abiotic oxidation of galena at 25°C.

	Biotic				Abiotic			
pH:4	Days	pH	SO4 (mg/L)	Pb (mg/L)	Days	pH	SO4 (mg/L)	Pb (mg/L)
	0	4.0	0	0	0	4.2	0	0.1
	14	3.5	20	0.1	14	5.5	0	0.2
	25	2.9	75	0.1	25	6.3	0	0.1
	43	3.0	120	0.2	43	6.5	0	0
	55	3.2	200	0.3	55	6.7	0	0
	62	3.1	270	2	62	6.8	10	0.3
	78	3.2	450	3.1	78	6.8	10	0.4
	86	3.2	900	4.2	86	6.9	10	0.5
pH:2	Days	pH	SO4 (mg/L)	Pb (mg/L)	Days	pH	SO4 (mg/L)	Pb (mg/L)
	0	2.0	0	4.9	0	2.0	0	5.7
	14	2.0	20	6.2	14	2.0	0	7.1
	25	2.1	30	8.9	25	2.0	0	8.7
	43	2.1	70	9.1	43	2.1	0	8.6
	55	2.1	150	9.3	55	2.0	5	8.5
	62	2.3	260	9.4	62	2.5	0	6.9
	78	2.4	400	9.4	78	2.4	0	5.6
	86	2.4	600	9.5	86	2.4	0	9

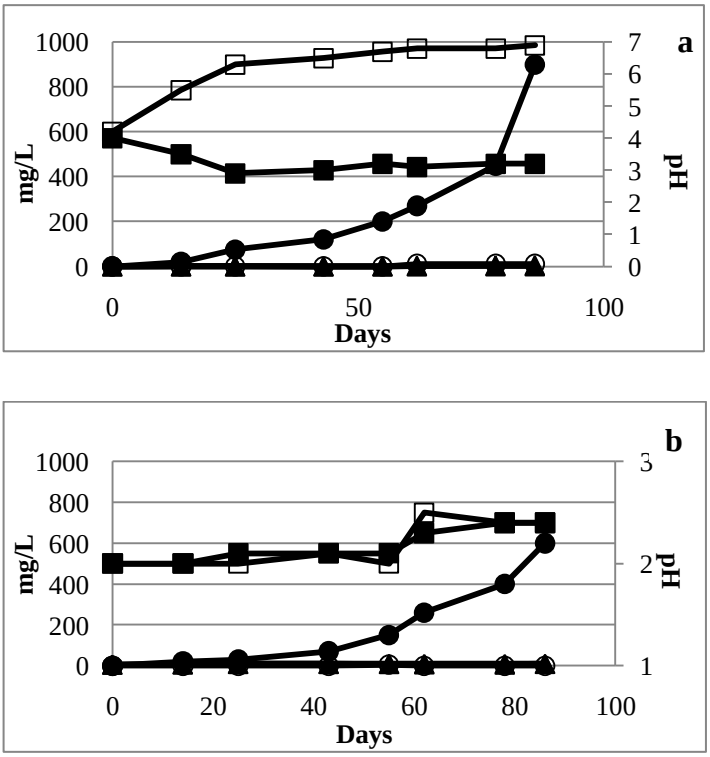

Figure 3.4: Solution chemistry during aerobic biotic and abiotic galena oxidation experiments at 25°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○:sulfate concentration of abiotic experiments; ■:pH of biotic experiments; □:pH of abiotic experiments. ▲: Pb concentration of biotic experiments. Δ: Pb concentration of abiotic experiments.

Table 3.5: Biotic and abiotic oxidation of galena at 10°C

	Biotic				Abiotic			
pH:4	Days		SO4 (mg/L)	Pb (mg/L)	Days	pH	SO4 (mg/L)	Pb (mg/L)
	0	4	0	0	0	4.0	0	0
	14	4	100	1.0	14	5	0	0.2
	22	3.9	200	1.5	22	5	0	1.5
	29	3.5	210	2.0	29	5.2	0	0.7
	36	4	200	3.0	36	5.1	0	1.1
	42	3.5	220	4.1	42	5.5	5	0.3
	55	3.6	240	4.3	55	6.5	5	0.2
pH:2	Days	pH	SO4 (mg/L)	Pb (mg/L)	Days	pH	SO4 (mg/L)	Pb (mg/L)
	0	2.0	0	0	0	2	0	0
	14	2.2	130	6.0	14	2.2	5	1
	22	2.2	155	11.0	22	2.2	10	1
	29	2.1	155	12.5	29	2.1	0	2
	36	2.1	145	13.2	36	2.1	5	3
	42	2.2	140	16.0	42	2.2	0	6
	55	2.1	140	16.0	55	2.2	0	6

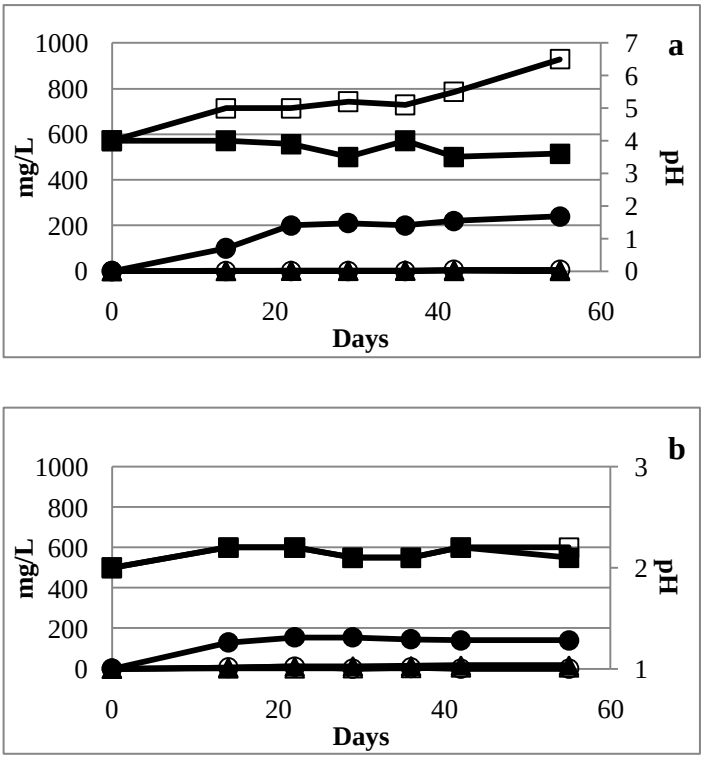

Figure 3.5: Solution chemistry during aerobic biotic and abiotic galena oxidation experiments at 10°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○:sulfate concentration of abiotic experiments; ■:pH of biotic experiments; □:pH of abiotic experiments; ▲: Pb concentration of biotic experiments. Δ: Pb concentration of abiotic experiments.

In the galena oxidation experiments with *Acidithiobacillus thiooxidans* at 4°C and pH:4, the concentration of the sulfate increased to 210 mg/L and the pH dropped down to 3.6 with the lead concentration 3.5 mg/L in 80 days. At pH 2 the concentration of sulfate increased to 85mg/L, the pH value was not changed and the concentration of the lead increased to 15 mg/L at the end of the biotic experiments (Table3.6). In the negative control experiments set up at pH 4, the pH showed increasing trend and measured as 5.9 at the end of experiments. Sulfate and Pb concentrations, although increased with time, were pretty low compared to biological experiments (Table 3.6, Figure 3.6a,b). The biotic and abiotic galena experiments at pH 4 and 2 under 4^{o}C produced insignificantly low sulfate compared to 25^{o}C and 10^{o}C. These results indicate that the sulfide oxidation rate is low at lower temperature but still oxidation reaction occurs.

Table 3.6: Biotic and abiotic oxidation of galena at 4°C

	Biotic				**Abiotic**			
pH:4	Days	pH	SO4 (mg/L)	Pb (mg/L)	Days	pH	SO4 (mg/L)	Pb (mg/L)
	0	4.0	0	0	0	4.0	0	0
	10	3.4	100	1.0	10	4.3	5.0	1.2
	21	3.3	110	1.5	21	4.3	9.0	1.5
	28	3.2	200	2.0	28	4.5	9.0	0.6
	35	3.1	225	3.0	35	4.8	9.0	0.7
	44	3.3	200	4.1	44	4.8	5.5	0.3
	57	3.6	200	3.2	57	4.9	6	0.2
	66	3.6	205	3.0	66	5.4	8.5	0.2
	80	3.6	210	3.5	80	5.9	11	0.2
pH:2	Days	pH	SO4 (mg/L)	Pb (mg/L)	Days	pH	SO4 (mg/L)	Pb (mg/L)
	0	2.0	0	0	0	2	0	0
	10	2.2	55.0	6.0	10	2.2	2.6	1
	21	2.2	60	11.0	21	2.2	4.0	1
	28	2.1	65	12.5	28	2.1	2.6	2
	35	2.1	70	13.2	35	2.1	2.0	3
	44	2.2	70	16.0	44	2.2	3.7	6
	57	2.1	82	14.5	57	2.2	3.4	5.5
	66	2.2	80	15.2	66	2.1	3.9	5
	80	2.1	85	15	80	2.2	4.0	5

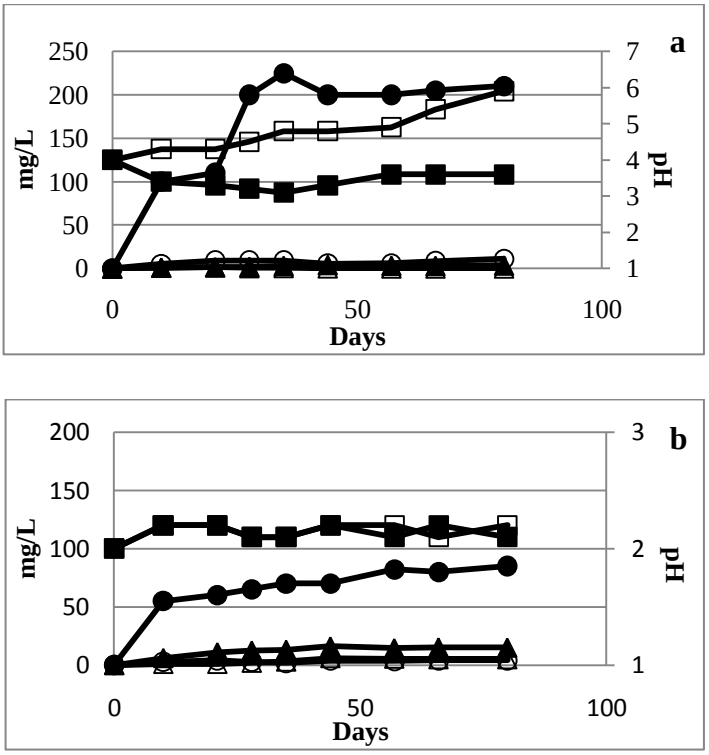

Figure 3.6: Solution chemistry during aerobic biotic and abiotic galena oxidation experiments at 4°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments;▲: Pb concentration of biotic experiments; Δ: Pb concentration of abiotic experiments.

3.1.3 Aerobic sphalerite (ZnS) oxidation experiments

The results of oxidation of sphalerite by *Acidithiobacillus thiooxidans* and without the bacterial culture at 25°C and at two different pHs (4 and 2) is presented at Table 3.8. According to oxidation results of sphalerite with *Acidithiobacillus thiooxidans* at 25°C at pH:4, the concentration of the sulfate increased to 1950mg/L, the pH dropped down to 3.83 and the concentration of the zinc increased to 12.67mg/L in 70 days. At pH:2, the concentration of the sulfate increased to 1275 mg/L, the pH was not changed and the concentration of the zinc increased to 65.05 mg/L in the end of the biotic experiments (70 days). In the abiotic experiments, oxidation rate of sphalerite is fairly low as suggested by low concentration of sulfate and zinc measured in these experiments (Table 3.7, Figure 3.7a,b).

Table 3.7: Biotic and abiotic oxidation of sphalerite at 25°C

	Biotic				Abiotic			
pH:4	Days	pH	SO4 (mg/L)	Zn (mg/L)	Days	pH	SO4 (mg/L)	Zn (mg/L)
	0	4.0	0	0.2	0	4.0	0	0.1
	13	4.1	75	6.1	13	4.9	0	0.3
	20	3.8	1000	6.9	20	5.6	0	0.4
	27	3.9	900	7.6	27	5.9	0	0.9
	55	3.9	900	11.0	55	5.8	0	0.5
	63	4.0	1300	12.0	63	5.8	0	0.5
	70	3.8	1950	12.7	70	6.2	0	0.6
pH:2	Days	pH	SO4 (mg/L)	Zn(mg/L)	Days	pH	SO4 (mg/L)	Zn (mg/L)
	0	2.0	0	0.2	0	1.9	0	0.1
	13	2.0	150	7.2	13	1.9	0	7.2
	20	1.8	925	8.1	20	2.0	0	7.7
	27	1.9	1175	7.9	27	1.9	0	8.2
	55	1.9	1125	68.9	55	1.9	0	10.3
	63	1.9	1275	63.8	63	1.9	0	10.6
	70	1.9	1275	65.0	70	2.0	50	10.8

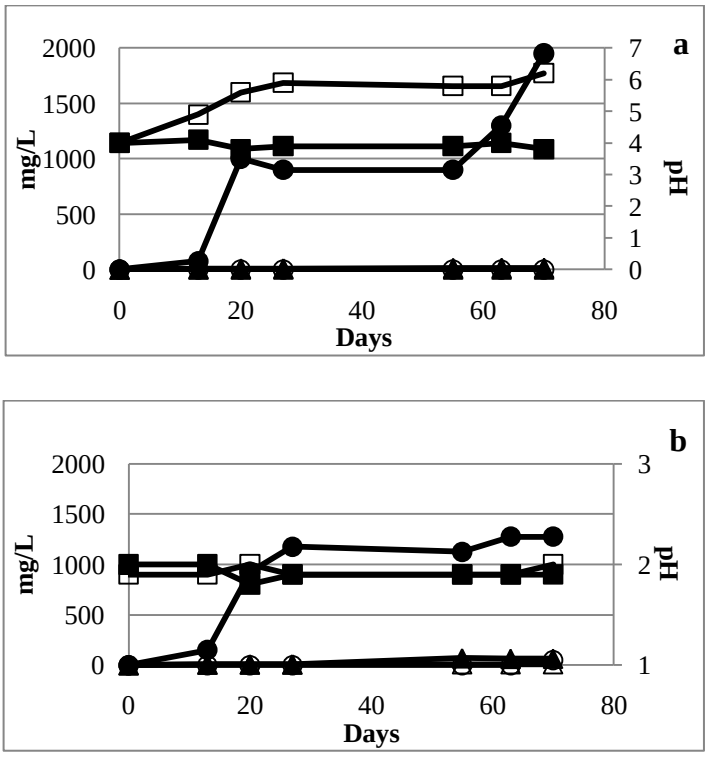

Figure 3.7: Solution chemistry during aerobic biotic and abiotic sphalerite oxidation experiments at 25°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Zn concentration of biotic experiments. Δ: Zn concentration of abiotic experiments.

10°C results, sulfate concentration was measured as 280mg/L and the concentration of zinc increased to 0.9 mg/L in biotic experiment at pH:4. At pH2, the concentration of sulfate increased to 150 mg/L, the pH was not changed and the concentration of zinc increased to 25.1mg/L in biotic experiment and 13.5 in abiotic experiments (Table 3.8 and Figure 3.8a,b).

Table3.8: Biotic and abiotic oxidation of sphalerite at 10°C

	Biotic				**Abiotic**			
pH:4	Days	pH	SO4 (mg/L)	Zn (mg/L)	Days	pH	SO4 (mg/L)	Zn (mg/L)
	0	4.0	0	0.3	0	4.0	0	0.1
	14	3.9	145	0.3	14	5.1	0	0.2
	32	3.9	200	0.4	32	5.2	0	0.2
	39	3.9	190	0.4	39	5.2	0	0.3
	46	3.9	200	0.5	46	5.1	10	0.4
	52	3.8	210	0.8	52	5.2	10	0.5
	60	3.8	280	0.9	60	5.2	0	0.7
pH:2	Days	pH	SO4 (mg/L)	Zn (mg/L)	Days	pH	SO4 (mg/L)	Zn (mg/L)
	0	2	0	0.4	0	2.0	0	0.2
	14	1.8	75	12	14	2.0	5	5
	32	1.9	120	19.5	32	1.9	20	8.8
	39	1.9	160	22	39	2.0	10	9.1
	46	1.9	150	24.2	46	1.9	0	10.8
	52	1.9	120	21	52	1.9	10	12.8
	60	1.9	150	25.1	60	2.0	0	13.5

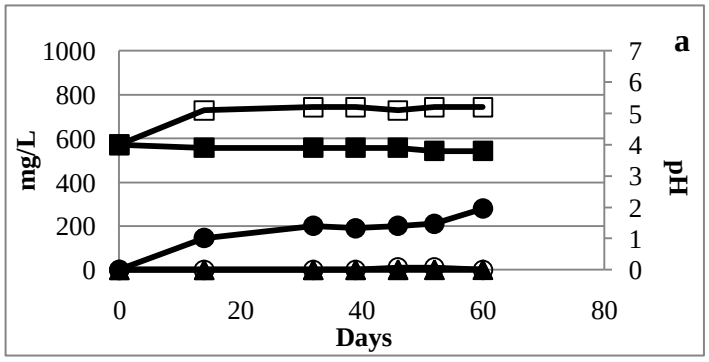

Figure 3.8: Solution chemistry during aerobic biotic and abiotic sphalerite oxidation experiments at 10°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Zn concentration of biotic experiments; Δ: Zn concentration of abiotic experiments.

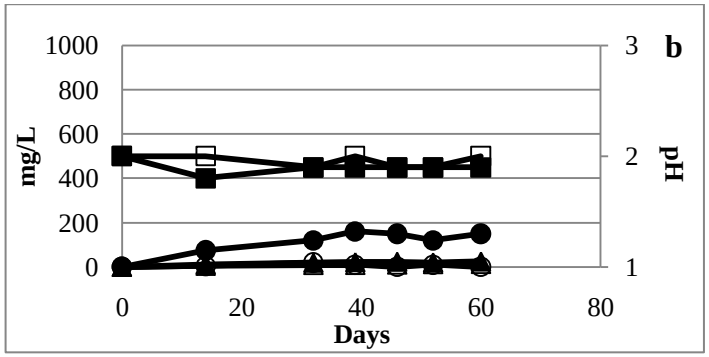

Figure 3.8 (continued): Solution chemistry during aerobic biotic and abiotic sphalerite oxidation experiments at 10°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Zn concentration of biotic experiments; Δ: Zn concentration of abiotic experiments.

At 4°C and pH 4 the oxidation of the sphalerite by *Acidithiobacillus thiooxidans* produced sulfate its concentration increased to 245mg/L, while pH dropped down to 3.8. The concentration of zinc increased to 4.1 mg/L in 82 days. At pH2, the concentration of sulfate increased to 35 mg/L, the pH was not changed and the concentration of zinc increased to 6.9 mg/L at the end of the biotic experiments (82 days). In the abiotic experiments, there was no significant change in pH, sulfate and zinc concentrations (Table 3.9, Fig 3.9a,b). Zinc concentration in both biotic and abiotic experiments at 4^{o}C is fairly low compared to 25^{o}C.

Table 3.9: Biotic and abiotic oxidation of sphalerite at 4^{o}C.

	Biotic				**Abiotic**			
pH:4	Days	pH	SO4 (mg/L)	Zn (mg/L)	Days	pH	SO4 (mg L)	Zn (mg/L)
	0	4.0	0	0.1	0	4.0	0	0
	16	3.9	145	0.8	16	5.1	0	0.5
	23	3.9	145	2.0	23	5.2	0	0.3
	43	3.9	245	2.6	43	5.2	5	0.7
	57	3.9	200	2.6	57	5.1	0	0.7
	73	3.8	245	3.6	73	5.2	0	0.7
	82	3.8	245	4.1	82	5.2	10	0.9
pH:2	Days	pH	SO4 (mg/L)	Zn (mg/L)	Days	pH	SO4 (mg/L)	Zn (mg/L)
	0	1.9	0	0.1	0	2.0	0	0.1
	16	1.8	25	4.2	16	2.0	5	0.7
	23	1.9	35	3.5	23	1.9	10	1.9
	43	1.9	40	4.4	43	2.0	10	2.6
	57	1.9	25	5.0	57	1.9	0	2.9
	73	1.9	45	6.5	73	1.9	0	3.1
	82	1.9	35	6.9	82	2.0	0	3.0

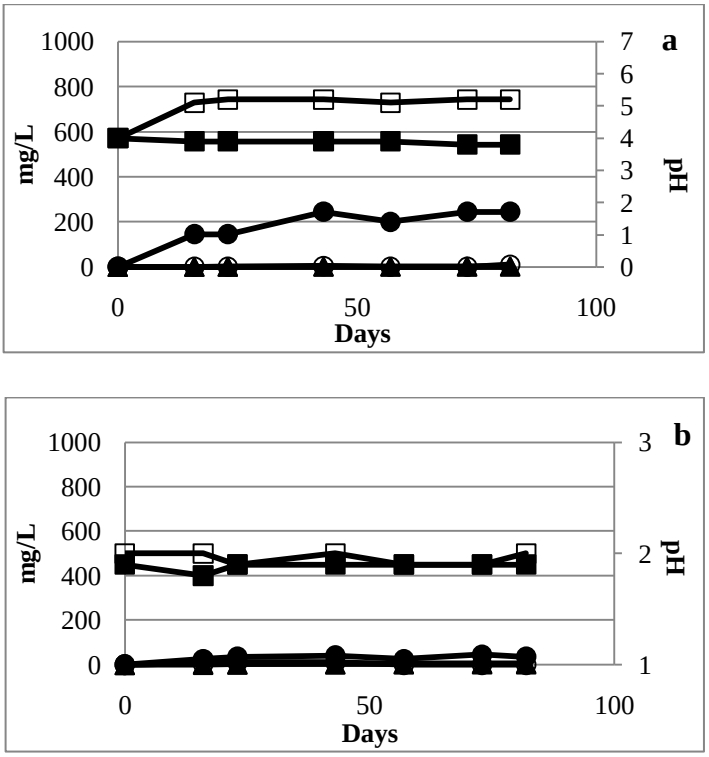

Figure 3.9: Solution chemistry during aerobic biotic and abiotic sphalerite oxidation experiments at 4°C. **a:** pH 4, **b:** pH 2. ●, sulfate concentration of biotic experiments; ○: sulfate concentration of abiotic experiments; ■: pH of biotic experiments; □: pH of abiotic experiments; ▲: Zn concentration of biotic experiments; Δ: Zn concentration of abiotic experiments.

3.2 Field Study

3.2.1 Physicochemical properties of the water samples and geochemistry of the tailings

During field excursion in August 2010, physicochemical parameters of the water samples collected from a small creek called as Maden and the temporary tailings pools are presented table 3.10 and major and trace element composition of the tailing samples in Table 3.11. The pH range from 2.8-3.8 in the spring samples and the lowest pH was measured as 2.3 in the tailing pool (data not shown). Sulfur oxidizing bacteria (SOB) and Fe oxidizing bacteria (FOB) were high in the water samples.

Table 3.10: Physicochemical properties of the creek water samples in the tailing area.

Sample	pH	Conductivity	Temperature	MPN-S[a*]	MPNFe[b*]
B1-pool	3.83	230	24	1.7×10^8	1.7×10^9
B3-creek	3.75	450	24	1.7×10^7	1.7×10^3
B4-creek	2.8	321	22	3×10^8	1.7×10^5

*a: Sulfur oxidizing bacteria(SOB); b: Fe(II) oxidizing bacteria(FOB)

Gang and ore minerals in the tailings assemblage may contain various metals and trace elements. Table 3.11 shows the element composition of the tailing samples. Occurrences of the metals from the tailings was found to follow the order Fe>Pb>Zn> Cu> Mn>Cd-Co (Table 3.11). The occurrences of Pb, Zn, Fe, Cu, As are expected based on the sulfide minerals. This order is generally agree with the tailing sulfide mineralogy. The characteristic sulfide mineral assemblage consists of galena (PbS), sphalerite (ZnS), pyrite (PbS_2), and chalcopyrite ($CuFeS_2$). Minor components are pyrrhotite ($Fe_{(1-x)}S$ (x = 0 to 0.2).), marcasite (FeS_2 with orthorhombic crystal structure), bismuth (Bi), sulfosalts ($A_mB_nS_p$; where A represents a metal; B usually represents semi-metal such as arsenic, antimony, bismuth; and S is sulfur or rarely selenium or/and tellurium.), arsenopyrite (FeAsS), tetrahedrite ($(Cu,Fe)_{12}Sb_4S_{13}$), /tennantite ($Cu_{12}As_4S_{13}$), bornite (Cu_5FeS_4), magnetite (Fe_3O_4), hematite (Fe_2O_3), pyrolusite (MnO_2), orpiment (As_2S_3) /realgar (AsS), and native tellurium (Te). The gangue mineral assemblage consists of garnet, epidote, quartz, calcite, dolomite, fluorite, tremolite, and minerals of the actinolite group [36]. The oxidation of sulfide minerals is the main source of the metals in the spring water. The relative abundance of metals and the other trace elements in the water samples generally follows the order : Fe>Pb>Zn>Cu>Cd>Co>As which indicates of sulfide minerals commonly present in the Balya mine tailing area.

Table 3.11: Element composition of the tailing samples.

	A1	**A2**	**A3**	**A4**	**A5**	**A6**
Pb %	4.47	12.31	4.3	5.35	5.4	4.4
Zn %	5.18	6.27	6.54	2.43	2.26	1.3
Fe %	9.17	15.59	6.17	10.9	13.52	11.51
Cu %	0.181	0.47	0.26	0.2	0.21	0.18
Mn %	0.12	1.19	0.28	0.15	0.11	0.1
As %	0.2	0.5	0.3	0.32	0.33	0.33
Cd	0.03	0.04	0.04	0.018	0.014	0.011
Co	0.03	0.037	0.036	0.015	0.013	0.011

3.2.2 16S rRNA analysis

Genomic DNA isolation

DNA isolation from soil sample was performed by using Fast DNA Spin Kit for Soil which include mechanic shearing method. Genomic DNA isolated from soil

samples had high molecular weight and 260/280 absorbance ratios of samples had sufficient purity for subsequent PCR amplification. *DNA isolated from 500 mg soil was loaded on a 1% agarose gel (Figure3.10).*

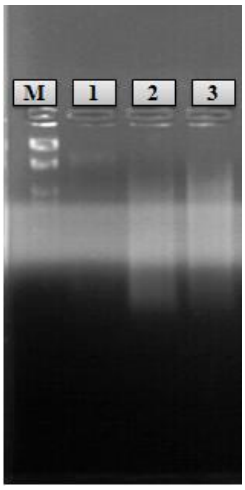

Figure 3.10: Agarose gel electrophoresis (1%) of the genomic DNA isolated from sediment samples. M, λ Hind III Marker; 1, B1 soil sample; 2, B3 soil sample; 3, B4 soil sample

UARR PCR amplification and gel extraction

Amplification of DNA from soil samples with primer pair pA/pH yielded approximately 1500bp size products when visualized on an agarose gel (Figure 3.11). PCR products were visualised by electrophoresis on 1% agarose gels including 5% ethidium bromide to check integrity and yield. All of the PCR reaction loaded into the gel to purify the PCR product.

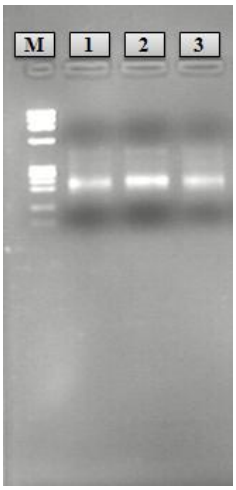

Figure 3.11: Agarose gel electrophoresis (1%) of the PCR products amplified from 16S rRNA fragments using pA/pH primer from sediment samples. M, Marker; 1, B1; 2, B3; 3, B4

Successfully produced bacterial 16S rDNA fragments of the expected size were cut by sharp scalpel and transferred to a clean tube. Aim of the DNA extraction is to get rid of undesirable extra bants. DNA extraction was performed by using QIAquick Gel Extraction Kit.

Cloning and transformation of PCR products

Ligation reaction was performed using TOPO-TA cloning kit. Cloning vectors used in the all experiments include selectable markers (ampicilline resistance markers) that allow only cells in which the vector, but not necessarily the insert, has been transfected to grow. Additionally, the cloning vectors contain color selection markers which provide blue/white screening (via α-factor complementation) on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present in the cells. Further investigation of the resulting colonies is required to confirm that cloning was successful. Following ligation, the ligation product (plasmid) is transformed into bacteria for propagation. The bacteria are then plated on selective agar to select for bacteria that have the plasmid of interest. Individual colonies are picked and plasmid isolation carried out for the following experiments.

Sequencencing and phylogenetic analyis

Nucleotide sequences of the plasmids were performed by using M13F/M13R primer pairs and PCR products were purified by ethanol precipitation methodology. Partial bacterial 16S rRNA gene sequences were subjected to a NCBI database using BLAST-N (http://www.ncbi.nlm.nih.gov/blast/) search to identify sequences of the highest similarity. Our sequence analysis indicated high bacterial diversity in the Balya Pb-Zn tailings, most prevalent sulfur, Fe(II) oxidizer and Fe(III) reducing bacterium (Table 3.12). Sequence and geochemical analysis suggest that sulfur oxidation is mostly mediated by complex microbial reactions and secondary mineral formation would be governed by sulfur oxidizer in the tailings. Sequence library was transferred to MEGA program (Molecular Evolutionary Genetics Analysis, version 4.0) to compare with each other and draw phylogenetic tree (Figure 3.12).

Table 3.12: 16S rRNA sequence results

Clone	Closest match organism or clone name	% Similarity	Phylogenetic group	Closest relative and postulated metabolism
B1-1	*Thiovirga sulfuroxydans*	97	Gammaproteobacteria	Chemolithoautotrophic sulfur-oxidizing bacterium
B1-2	*Novosphingobium* sp. Van67	95	Alphaproteobacteria	Anaerobic phenolic compound biodegradation
B1-3	*Thiobacillus thiophilus*	94	Betaproteobacteria	Obligate chemolithotrophic thiosulfate-oxidizing bacteria
B1-4	*Rhodoferax ferrireducens* T118	90	Betaproteobacteria	Fe(III)-reducing microorganism
B1-5	*Novosphingobium subterraneum*	95	Alphaproteobacteria	Aromatic-degrading bacteria
B1-6	Uncultured *Halothiobacillus* sp.	98	Gammaproteobacteria	Halophile, purple sulfur bacteria
B1-7	*Novosphingobium aromaticivorans* DSM 12444	92	Alphaproteobacteria	Aromatic hydrocarbons degradating bacteria.
B3-1	Thauera sp. MG70	99	Betaproteobacteria	*Thauera* sp. strain MZ1T is a floc-forming bacterium.
B3-2	Xanthomonas sp. T7-07	91	Gammaproteobacteria	*Xanthomonas* genus cause plant diseases
B3-3	*Acidovorax defluvii* strain BSB411	94	Betaproteobacteria	Gram negative soil bacterium
B3-4	*Ferribacterium limneticum*	92	Betaproteobacteria	Fe(III) reducing bacteria
B3-5	*Flavobacterium succinicans*	91	Bacteriodetes	-
B3-6	Uncultured perchlorate-reducing bacterium CR	91	Betaproteobacteria	Perchlorate-reducing bacterium
B3-7	*Clostridiaceae* bacterium DJF_LS13	95	Firmicutes	*Clostridiaceae* contains the *Clostridium* genus

Table 3.12 (continued): 16S rRNA sequence results

Clone	Closest match organism or clone name	% Similarity	Phylogenetic group	Closest relative and postulated metabolism
B4-1	*Thiobacillus denitrificans* ATCC 25259	97	Betaproteobacteria	Obligate chemolithoautotrophic bacterium can oxidize inorganic sulfur compounds (such as hydrogen sulfide and thiosulfate) and Fe(II)
B4-2	Uncultured *Thiobacillus* sp. clone ENR10	93	Betaproteobacteria	*Thiobacillus thioparus* is thiosulfate-oxidizing bacteria
B4-3	Uncultured *Geobacteraceae* bacterium	91	Deltaproteobacteria	*Geobacteraceae* predominates in a diversity of Fe(III)-reducing subsurface environments
B4-4	Uncultured Flavobacteria ATBLH 5952	90	Bacteriodetes	-
B4-5	*Ferrovum myxofaciens* strain P3G	90	Betaproteobacteria	Iron-oxidizing bacteria
B4-6	*Comamonadaceae* bacterium PIV81	94	Betaproteobacteria	Arsenate reducing bacterium
B4-7	*Leptothrix* sp. S1.1	93	Betaproteobacteria	Arsenite- oxidizing bacteria
B4-8	*Sphingomonas* sp. PW-1	96	Alphaproteobacteria	Gram-negative chemoheterotrophic, strictly aerobic bacteria.

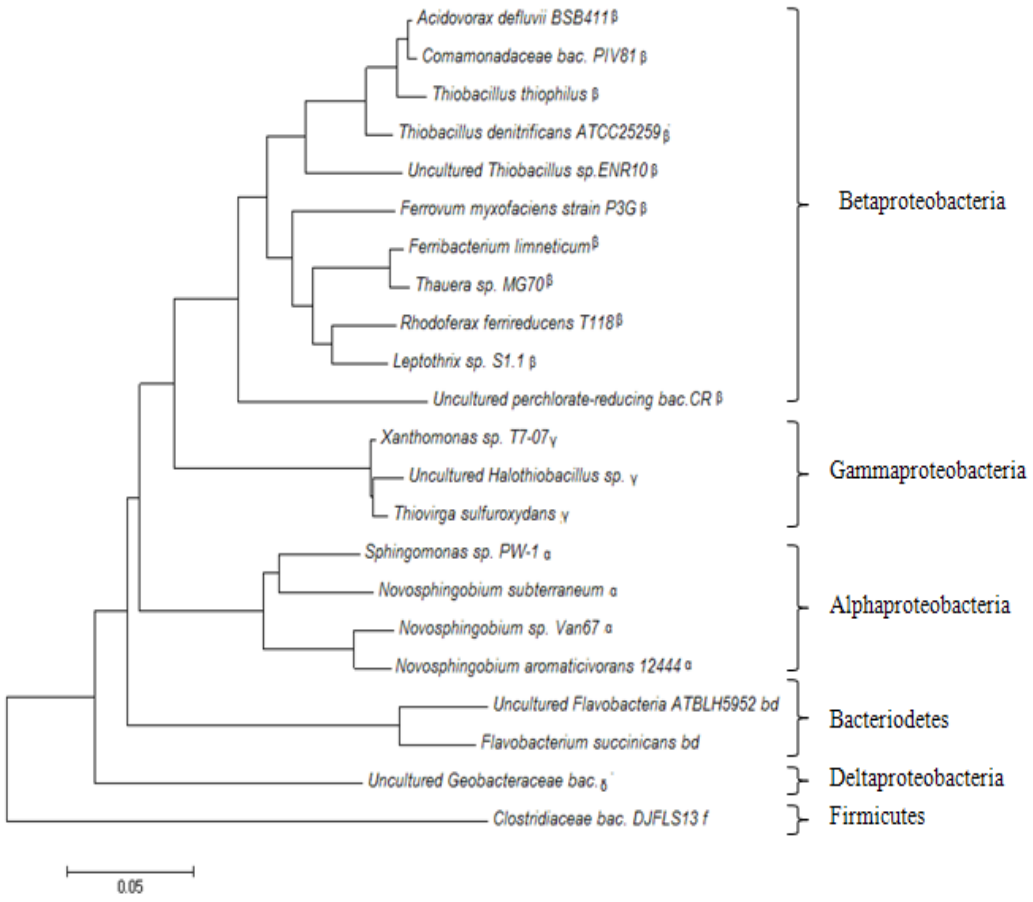

Figure 3.12: Phylogenetic tree of 16S rRNA-based clones obtained from the tailings and sediment samples

4. DISCUSSIONS

Effect of temperature, pH and bacteria on oxidation rate of sulfide minerals

Sulfur oxidizing bacteria have been extensively examined due to their influences on oxidation of sulfur minerals [23]. The other factors controlling oxidation rate of sulfide minerals are temperature and pH of the solutions that oxidation occurs. According to the experimental results carried out in this study, bacteria oxidized sulfide minerals much faster rate than those abiotic experiments irrespective of temperature and pH conditions. *A. thioxidans* is more active at 25°C relative to suboptimal temperature (10, 4°C) as result oxidation of galena and sphalerite was faster at 25°C. Therefore concentration of sulfate and Pb, Zn is much higher at 25°C. The only exception for these was pyrite experiments. Pyrite could not be oxidized by *A. thiooxidans* as suggested by previous studies [41].

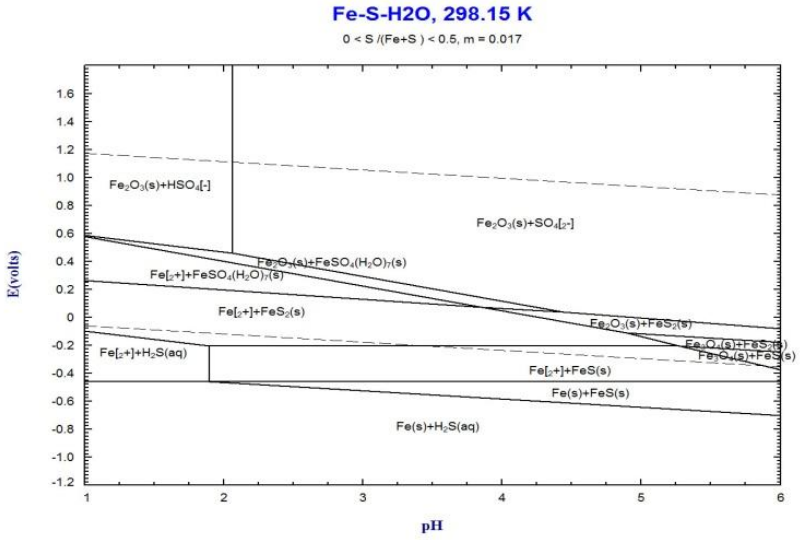

Figure 4.1: Eh-pH graphic of abiotic pyrite experiment at 25°C

Figure 4.1 explain iron sulfide attitudes according to different pHs. According to results of all pyrite experiments, metal dissolution was only occurs chemicaly at low pH values. Therefore metal concentration is remarkable higher at pH:2. These results shows that *A.thiooxidans* has no role in oxidation of pyrite.

Oxidation of sphalerite and galena by *A. thiooxidans* in the experiments may have been happen by the following steps. First step involve formation of elemental sulfur on the surface of sphalerite or galena (Reaction 1).

$$ZnS + 1/2O_2 + 2H^+ \rightarrow S^o + H_2O + Zn^{+2} \quad (\mathbf{4.1})$$

$$S^o + 2H_2O + O^*_2 \rightarrow SO_3^{2-} + H_2O^* + 2H^+ \quad (\mathbf{4.2})$$

$$SO_3^{2-} + H_2O + 1/2O^*_2 \rightarrow SO_4^{2-} + H_2O^* \quad (\mathbf{4.3})$$

$$ZnS + 2O_2 \rightarrow SO_4^{2-} + Zn^{+2} \quad (\mathbf{4.4})$$

The pH increase at the beginning of the sphalerite/galena experiments is also suggestive of the formation of surface polymeric S species which are precursors to S^o and which consume H^+ [42]. This suggestion is consistent with the proposed pathway of reactions (2-3), whereby the dissolution of sphalerite/galena is initiated by proton attack (H^+) followed by the production of S^o [43, 44, 45]. Because the solubility of S^o in the water is very low [46], continued oxidation of S^o to sulfate occurs only in the presence of sulfur oxidizing bacteria. This likely explains why the abiotic sphalerite/galena oxidation experiments produced very little sulfate compared to the biological reactions (Fig 3.5, Fig 3.6, Fig 3.7, Fig 3.8, Fig 3.9, Fig 3.10). The subsequent oxidation of S^o causes the formation of sulfite and yields protons needed for further dissolution of ZnS (Reactions 2, 3). The constant pH observed during the biological ZnS/PbS oxidation experiments is consistent with this continuous proton consumption and release (Table 3.5, Table 3.6, Table 3.7, Table 3.8, Table 3.9, Table 3.10). The oxidation pathway suggested by sphalerite and galena minerals could be valid for the temperature range studied. However, experiments under suboptimal temperature (4, 10^{o}C) indicated that the activation of *A. thioxidans* was low although the bacteria were still active even under 4^{o}C. The experimental results suggest that the main role of bacteria on oxidation of sphalerite and galena was to remove of elemental sulfur formed on the surface of minerals and thus O_2 can reach to surface of the minerals and oxidation continue. Overall, the experimental results showed that sulfate production in the abiotic incubations was significantly less than in the biotic experiments, indicating that bacteria have major effect on oxidizing sulfide minerals even under low temperature.

Distribution and identification of bacteria in the tailing and sediment samples

This work is the first to describe the microbiology of mine tailings using a combination of cultivation and molecular microbial ecology techniques for the Balya

Pb-Zn mine tailings and around. The samples taken from the tailing surface of 0-10 cm did not show any bacterial species. Different acidophiles were identified from the deeper part of the tailing (20-30 cm) (B3-B4) and sediment samples from the creek (B1). According to results of the sequence analysis, frequency of phylogenetic groups is as follows 50% Betaproteobacteria, 13.6% Gammaproteobacteria, 18.1% Alphaproteobacteria, 4.5% Deltaproteobacteria, 9.09% Bacteriodetes, 4.5% Firmicutes (Table 3.10 and Fig 3.10). It is more likely that very first part of the tailing samples (0-10 cm) were depleted in metal sulfides and thus lack of major electron donor besides being dry and therefore bacteria may have not been survived. However, 20-30 cm of the tailings sample showed the greatest number of bacteria. Fe(III) and arsenate reducing bacteria were detected in the low part of the tailing (30 cm), indicating lower dissolved oxygen concentration. Some of the acidophilic bacteria isolated from the tailing are autotrophic, and synthesize biomass by utilizing CO_2 as carbon source. They obtain energy for carbon fixation and growth by oxidation of ferrous iron and/or thiosulfate/sulfur, derived from sulfide minerals present in the tailings. These in turn support the growth of heterotrophic bacteria that live on the dissolved organic carbon (perchlorate). Two different species of iron-oxidizing bacteria were found in the tailings deposit using molecular techniques. It is interesting that *A. ferrooxidans* is not one of them. This is the most widely studied of all acidophiles, and is readily isolated from acidic sulfide-rich environments by enrichment or plating [6]. However, the fact that it was not detected in the samples suggests that it is not as significant, numerically, as other bacteria in the Pb-Zn tailings. Two sulfur and sulfur compounds oxidizing bacteria were detected in clone libraries, one highly related (> 97 % gene 16S rRNA gene sequence identity) to *Thiobacillus denitrificans* ATCC 25259, and another more distantly related (93 % identity) to *Thiobacillus* sp. clone ENR10". These two species are similar in many respects (both are known to use only sulfur compounds as energy source, are obligate aerobes and extreme acidophiles), although a differentiating feature is that *Thiobacillus denitrificans* is able to fix nitrogen. The key role of *Thiobacillus denitrificans* may provide fixed nitrogen to other sulfur oxidizing bacteria. More definitively, the sequence data results indicated that the dominant mineral oxidizing prokaryotes in the tailings deposits were *Thiobacillus spp* and Fe(II) oxidizing *Ferrovum myxofaciens*. The other important bacteria involved mineral oxidation in the tailing samples is *Leptothrix* sp. S1.1. These bacteria are known to oxidize

arsenopyrite minerals which were determined in the Pb-Zn Tailing (Table 3.13). Arsenate reducing bacteria *Comamonadaceae* bacterium PIV81 were also obtained in the tailings. The main role of *Comamonadaceae* bacterium PIV81 in the tailing samples is to reduce arsenate compounds to arsenite which then be oxidized by *Leptothrix* sp. S1.1. Arsenopyrite oxidation and reduction in the tailings is controlled by biological reactions. The sediment sample of B-1 also represent diverse bacterial groups, *Thiovirga sulfuroxydans* and *Thiobacillus thiophilus* were obtained as sulfur oxidizing chemolitotrophs. Fe(III) reducing bacteria represent 90% similarity to *Rhodoferax ferrireducens* T118. The main source for the sulfur oxidizer in the sediment sample is metal sulfur minerals carried from the tailings into the creek via rain or wind. Interestingly, number of heterotrophic bacteria use various phenolic compounds were identified in the sample. *Novosphingobium* sp. Van67 biodegrades anaerobically phenolic compound and *Novosphingobium subterraneum and Novosphingobium aromaticivorans* DSM 12444 use aromatic compounds. The source of aromatic compounds in the creek sediments come from flotation plants constructed in the tailing area.

5. CONCLUSIONS

This work demonstrated that *A.thiooxidans* can play significant role on sulfide mineral oxidation at different temperature and pH as well as showing that there was a high microbial diversity in the Balya Pb-Zn tailings. The results indicate that oxidation experiments with galena and sphalerite at 25^{o}C showed high oxidation rate with *A. thioxidans* compared to chemical control experiments. Experiments under suboptimal temperature (4, 10^{o}C) showed that *A. thioxidans* was still active even under 4^{o}C although the oxidation rate of galena and sphalerite were significantly lower compared to 25^{o}C. Unlike galena and sphalerite, oxidation of pyrite with bacteria and without bacteria did not show significant reaction rate. Combining the microbiological and geochemical data indicated high bacterial diversity in the Balya Pb-Zn tailings, most prevalent sulfur-Fe(II) oxidizer along with Fe(III) reducer and also suggest that sulfur oxidation is mostly mediated by complex microbial reactions in the tailings.

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APPENDICES

APPENDIX A: Materials

APPENDIX B: Laboratory Equipments

APPENDIX A: Materials

Acidithiobacillus thiooxidans 14887

Acidithiobacillus thiooxidans 14887 was used in this study and obtained from the German Resource Centre for Biological Material (DSMZ).

E. coli TOP10

One shot TOP10 Electrocompotent cells (İnvitrogen) were used in species identification procedures.

pCR®2.1-TOPO® Cloning vector

pCR®2.1-TOPO® Cloning vector was obtained from Invitrogen firm and used in species identification procedures.

Acidithiobacillus Thiooxidans Medium

0.1g NH_4Cl, 3g KH_2PO_4, 0.1g $MgCl_2.6H_2O$, 0.14g $CaCl_2.2H_2O$, 10g Sulfur powdered. All ingredients dissolved, except the sulfur, in distilled water and adjusted the pH to 4 or 2 with 1M HCl, then autoclaved. The sulfur was placed in bottles and sterilized by autoclaving at 112°C for 15 minutes. Before use, the sterilized sulfur is aseptically layered onto the surface of autoclaved liquid basal medium.

Luria Bertani (LB) Medium

10g tryptone (Acumedia), 5g yeast extract(Acumedia), 5g NaCl (Riedel-de-Haen) were dissolved in distilled water and completed up to 1 lt. The pH was adjusted to 7.0-7.5 with 10M NaOH and sterilized for 15 minutes under 1.5 atm at 121 °C. The medium was stored at room temperature.

LB Agar Medium

10g tryptone (Acumedia), 5g yeast extract(Acumedia), 5g NaCl (Riedel-de-Haen) were dissolved in distilled water and completed up to 1 lt. The pH was adjusted to 7.0-7.5 with 10M NaOH and sterilized for 15 minutes under 1.5 atm at 121 °C. The medium was stored at room temperature.

10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g bactoagar (XXX) were dissolved in distilled water up to 1 lt and the pH is adjusted to 7.0 with 10M NaOH. Media is sterilized by autoclaving for 15 min at 121 °C.

SOC Medium

20 g tryptone, 5 g yeast extract and 0.5 g NaCl were dissolved in distilled water. 10 ml of 250 mM KCl was added to the solution and the pH was adjusted to 7.0 with NaOH. Volume was adjusted to 1 lt with distilled water and the solution was autoclaved. 10mM $MgCl_2$ and 20 mM glucose were added just before the usage.

Ampicilline Stock Solution

100mg Ampicillin (Sigma) was dissolved in 1 ml deionized water, sterilized by filtration using 0.22µm filter. This stock solution was used in LB medium with 1/1000 dilution rate.

X-gal Stock Solution

400 mg X-gal (5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyronoside) was dissolved in 10 ml dimethyl formamide (DMF). Solution was stored in dark at -20°C.

Glycerol Stock

80 ml glycerol (Riedel-de-Haen) and 20 ml distilled water were mixed to give a 80 % (v/v) solution. It was sterilized for 15 min at 121 °C.

PBS (Phosphate Buffer Saline Buffer)

37mM NaCl (Sigma), 2.7mM KCl (Sigma), 10Mm Na2HPO4 (Fisher), 1.76mM KH2PO4 (Fisher), were prepared in distilled water and the solution was sterilized by 0.2 µm single use syringe filter. The pH was adjusted to 7.4.

Sodium Acetate Buffer

2.46 g sodium acetate (Reidel-de-Haen) was dissolved in 7 ml distilled water and pH was adjusted to 5.2. Volume was completed to 10 ml to give a 3M concentration and the solution was stored in 4 °C.

50X TAE Buffer

242 g Tris base, 57.1 ml glacial acetic acid and 18.6 g EDTA was dissolved in distilled water up to 1 lt.

Sulfur Minerals

Pyrite, galena minerals were used in this study and obtained from the Geology Museum of the Colorado School of Mines, Golden, Colorado. Prior to use in all of the experiments, minerals were ground and sieved.

APPENDIX B: Laboratory Equipments

Autoclave:	2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik.
Centrifuges:	Microfuge 18, Beckman Coulter; AvantiJ30I, Beckman Coulter
Deep freezes and refrigerators:	Ultra low freezer MDF-U4086S, Sanyo; Ultra low freezer MDF-U333, Sanyo; 1061 M refrigerator, Arcelik.
Electrophoresis equipment:	E-C Mini Cell Primo EC320, E-C Apparatus, Mini-V 8.10 Vertical Gel Electrophoresis System, Life Technologies GibcoBrl (now Invitrogen), Katalog# 21078, Horizon 11.14, Whatman, Biometra Gel Casting System, Horizon 20-25, Whatman, Biometra Gel Casting System.
Electroporator:	Electroporator 2510, Eppendorf
Gel Documentation System:	UVIpro GAS7000, UVItec Limited.
Ion chromatograpy:	Dionex ICS-1500 Ion Chromatograph, *Ion Chromatography*-Inductively Coupled Plasma-Atomic Emission Spectrometry PE Plasma 2000 (ICP-AES)
Incubator shaker:	Biolab incubator shaker, SI6-2*, ShelLab
Ice machine:	AF 10, Scotsman
Laminar flow cabinet:	Faster Laminar Flow BH-EN 2003.
Magnetic stirrer:	Heidolph Standard
Orbital shaker:	Forma orbital shaker, Thermo Electron Corporation.
pH meter:	Inolab pH level 1, Wissenschaftlich-Technische Werkstätten GmbH & Co KG
Pipets:	epResearch Pipettes, Eppendorf
Portable spectrophotometer:	DR2800-01, HACH
Thermal Cycler:	Biometra Thermal Cycler
UV sterilizer:	Vilber lourmat UV irradiation devices, VL-115.G 230V, 50/60Hz. SN: 08100091EU

Thermomixer:	Thermoshaker Ts1, Biometra.
Vortex:	SI-D256 Daigger

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