

## Chapter 11

# GEOMICROBIOLOGY OF SULFIDE MINERAL OXIDATION

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## INTRODUCTION

Current estimates on the global flux of sulfur (Berner and Berner 1996) indicate that the amount of sulfate from pyrite weathering, contributing to the average river concentration of sulfate, is about 11% of the total and that this amount is increasing because of increased mining activities. Of the rock-derived sulfate contributions to river sulfate, about two-thirds comes from evaporite bed dissolution (mostly gypsum) and about one-third comes from pyrite oxidation. The occurrence of acid mine drainage, caused by pyrite oxidation, is a major water quality problem throughout the world. Acid mine waters typically have pH values in the range of 2 to 4 and high concentrations of metals known to be toxic to aquatic organisms (Ash et al. 1951, Barton 1978, Kelly 1988, Martin and Mills 1976, Nordstrom and Ball 1985). Acid sulfate soils, most often occurring in lagoonal and estuarine environments affected by tidal cycles, cause agricultural problems from the periodic oxidation of pyrite (Van Breemen 1976, Pons et al. 1982). Occasionally the construction of highways or building structures involves excavation into pyritiferous rock that requires specially-designed engineering features to avoid rapid deterioration from the acid waters that can rapidly develop (Byerly 1996). The same process is also very important in the weathering and enrichment of sulfide ore deposits (Lindgren 1928). Biohydrometallurgists capitalize on the microbial catalysis of sulfide mineral oxidation to increase the efficiency of metal extraction (Murr et al. 1978, Lawrence et al. 1986, Barrett et al. 1993). Hence, the geomicrobiology of sulfide mineral oxidation has important ramifications from the global cycling of elements to the industrial extraction of metals.

The association of microorganisms with pyrite oxidation and the formation of acid mine drainage has a long history that begins with the discovery that microbes can utilize inorganic compounds as well as organic compounds such as metabolites. S.N. Winogradsky (1888) recognized that some microbes could derive their metabolic energy from the oxidation of inorganic compounds, such as iron and sulfur (Sokolova and Karavaiko 1968). This property affords one of the major divisions among microorganisms: the lithotrophs, e.g. *Thiobacillus* spp., that gain energy from the oxidation of inorganic compounds, and the heterotrophs (like ourselves) who gain energy from the oxidation of organic compounds. Autotrophs obtain their carbon requirements for growth through CO<sub>2</sub> fixation. Thus, *Thiobacillus* spp. have also been called lithoautotrophs. Occasionally the word chemoautotroph is used for lithoautotroph. Organisms that receive solar radiation for



their energy source and obtain cellular carbon from CO<sub>2</sub> fixation are known as photoautotrophs.

Nathanson (1902) first isolated a member of the bacterial genus *Thiobacillus*, noted for its ability to oxidize sulfur. The acidophilic bacterium, *Thiobacillus thiooxidans*, was isolated and identified by Waksman and Jaffe (1921, 1922) from soils containing free sulfur and phosphate. Rudolfs and Helbronner (1922) observed that bacteria could attack zinc sulfide. Twenty-five years later, the acidophilic autotroph *Thiobacillus ferrooxidans*, was isolated by Colmer and Hinkle (1947), implicated in the formation of acid mine drainage, and was found to oxidize both iron and sulfur. Numerous members of the *Thiobacillus* genus have now been identified and the sulfur compounds they utilize are shown in Table 1. Other acidophilic iron- and sulfur-oxidizers that have been found or implicated in the formation of acid mine waters are also shown in Table 1.

Acid mine waters have been known to contain abundant microbial life; indeed, they are often the only form of life under these conditions. Powell and Parr (1919) and later Carpenter and Herndon (1933) suggested that pyrite oxidation and the consequent acid

**Table 1.** Members of the Bacteria genera *Thiobacillus* (arranged alphabetically by species name), *Leptospirillum*, and *Sulfobacillus*; the inorganic substances they utilize (adapted from Kelley and Harrison 1984 and Barrett et al. 1993, except where otherwise indicated), and four additional Archaea spp. that are known to be associated with acid mine waters and pyrite oxidation. Acidophilic species are shown in bold type.

<b><i>Thiobacillus albertis</i></b>	H <sub>2</sub> S, S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>
<b><i>Thiobacillus acidophilus</i><sup>1</sup></b>	S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>3</sub> O <sub>6</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus denitrificans</i>	H <sub>2</sub> S, S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus delicatus</i>	S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<b><i>Thiobacillus ferrooxidans</i></b>	H <sub>2</sub> S, sulfide minerals, S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup> , Fe <sup>2+</sup>
<i>Thiobacillus halophilus</i> <sup>2</sup>	S <sup>0</sup>
<i>Thiobacillus intermedius</i>	S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus neapolitanus</i>	H <sub>2</sub> S, sulfide minerals, S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>3</sub> O <sub>6</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus novellus</i>	S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus perometabolis</i>	S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus tepidarius</i>	H <sub>2</sub> S, S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>3</sub> O <sub>6</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus thermophilica</i> <sup>3</sup>	H <sub>2</sub> S, sulfide minerals, S <sup>0</sup>
<b><i>Thiobacillus thiooxidans</i></b>	S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<b><i>Thiobacillus thioparvus</i><sup>4</sup></b>	H <sub>2</sub> S, sulfide minerals, S <sup>0</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , S <sub>3</sub> O <sub>6</sub> <sup>2-</sup> , S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>
<i>Thiobacillus versutus</i>	H <sub>2</sub> S, S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>
<b><i>Leptospirillum ferrooxidans</i></b>	Fe <sup>2+</sup> , sulfide minerals
<b><i>Leptospirillum thermoferrooxidans</i></b>	Fe <sup>2+</sup> , sulfide minerals
<b><i>Sulfobacillus thermosulfidooxidans</i></b>	Fe <sup>2+</sup> , S <sup>0</sup> , sulfide minerals
Archaea spp.	
<b><i>Acidianus brierleyi</i></b>	Fe <sup>2+</sup> , S <sup>0</sup> , sulfide minerals
<b><i>Sulfolobus solfataricus</i></b>	S <sup>0</sup>
<b><i>Sulfolobus ambivalens</i></b>	S <sup>0</sup>
<b><i>Sulfolobus acidocaldarius</i></b>	Fe <sup>2+</sup> , S <sup>0</sup>

<sup>1</sup> Also known as *T. organoparus*

<sup>2</sup> Wood and Kelly, 1991

<sup>3</sup> Egorova and Deryugina, 1963 (not a *Thiobacillus* sp.)

<sup>4</sup> Range of pH = 3 to 10



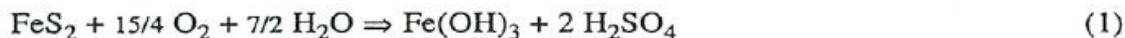
mine drainage from coal deposits may be catalyzed by bacteria. Lackey (1938) investigated 62 West Virginia streams affected by acid mine drainage, described acid slime streamers, and found flagellates, rhizopods, ciliates, and green algae. In his investigation of acidified surface waters and soils in West Virginia and Pennsylvania, Joseph (1953) observed Gram-positive and Gram-negative bacilli and cocci, actinomycetes, fungi, green algae, diatoms. In acid mine waters from a copper mine in the southwestern United States, Ehrlich (1963a) found yeasts, flagellates, and amoebas. Acid slime streamers of the type described by Lackey (1938) are a common occurrence (Temple and Koehler 1954, Nordstrom 1977, Johnson et al. 1979) and have been described in further detail by Dugan et al. (1970).

One of the most widely studied autotrophs and a key bacterium in the catalysis of pyrite oxidation is *Thiobacillus ferrooxidans*. It was isolated and identified as Gram-negative, acidophilic, and rod-shaped by Colmer and Hinkle (1947), Colmer et al. (1950), Temple and Colmer (1951), and Temple and Delchamps (1953) and shown to be essential to the production of acid mine waters (Leathen et al. 1953). Following these discoveries, there has been considerable research on both the abiotic and the microbially-catalyzed reactions for sulfide mineral oxidation. By the 1960s, the essential role of bacteria in the oxidation of pyrite, especially in coal, had been well-established (Lyalikova 1960, Silverman et al. 1961).

Two principal activities have motivated studies on microbial catalysis of sulfide minerals: the need to eliminate or reduce the deleterious effects of acid mine drainage and the advantages gained in biohydrometallurgy when bacteria are used to improve the extraction of metals from ores and mine waste materials. The reactions and the microbial ecology are complicated, rates and mechanisms are difficult to resolve, and the interactions of bacteria at sulfide surfaces are poorly understood. In this chapter we attempt to summarize the current state of knowledge of sulfide mineral oxidation with a focus on the role of bacteria in this important process.

## SUMMARY OF SULFIDE MINERAL OXIDATION REACTIONS

The three basic ingredients responsible for the formation of acid mine waters are pyrite, water, and oxygen. The overall reaction is commonly written as



where one mole of ferric hydroxide and two moles of sulfuric acid are produced for every mole of pyrite oxidized. This reaction, however, is extraordinarily simplistic compared to the realities of chemistry and the actual processes occurring in the environment. Some of the complicating features are tabulated in the following list:

- (1) Pyrite occurs over a large range of grain size and surface area. It also occurs in several different crystal forms and with large variations in defect structure, crystallinity, and trace element content. All of these properties of pyrite can affect the rate of reaction.
- (2) For each mole of pyrite oxidized in Reaction (1), 1 electron is lost by oxidation of iron, 14 electrons are lost by oxidation of disulfide, and 15 electrons are gained by the reduction of oxygen. In addition, the iron is hydrolyzed and precipitated. These reactions cannot take place in a single step. Only one or two electrons are commonly transferred at a time (Basolo and Pearson 1967). Hence, there could be 15 or more reactions with as many possible rate-determining steps to consider. To further complicate matters, other oxidizing agents have been implicated, chiefly ferric iron.



Fortunately all the intermediate reactions need not be determined to delineate the rate-controlling mechanisms involved with pyrite oxidation.

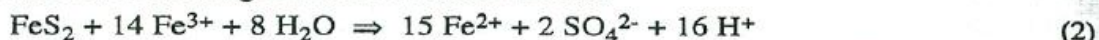
- (3) The product of pyrite oxidation is not a pure ferric hydroxide phase but a mixture of phases with variable stoichiometry, including:

goethite ( $\alpha$ -FeOOH),                      ferrihydrite ( $\text{Fe}_5\text{OH}_8 \cdot 4\text{H}_2\text{O}$ ),  
 jarosite ( $\text{KFe}_3(\text{SO}_4)_2(\text{OH})_6$ ),      schwertmannite ( $\text{Fe}_8\text{O}_8\text{SO}_4(\text{OH})_6$ ).

- (4) Products, such as elemental sulfur, have been found to form from pyrite oxidation.

Important reviews on pyrite oxidation have been published by Lowson (1982), Nordstrom (1982), and Evangelou (1995). Reviews on the oxidation of pyrite and other mineral sulfides can be found in Dutrizac and MacDonald (1974) and Nordstrom and Alpers (1997). This section highlights the major findings from these reviews and related current research.

Stokes (1901) recognized that the oxidation of pyrite by ferric iron had been known for a long time. The following balanced chemical reaction



has been confirmed by several experimental studies (Garrels and Thompson 1960, Wiersma and Rimstidt 1984, McKibben and Barnes 1986). Since ferric iron is insoluble at circumneutral pH values, Reaction (2) requires acidic conditions. Oxygen is still needed to oxidize more ferric iron from ferrous:



but the oxygen does not have to diffuse to the pyrite surfaces. Pyrite can oxidize in the absence of oxygen. Nevertheless, the overall rate of pyrite oxidation in a tailings pile, in a mine, or in a waste rock pile will largely be determined by the overall rate of oxygen transport (advection, convection, and diffusion).

Elemental sulfur may form during pyrite oxidation, but the mechanism is not well understood. At ambient environmental temperatures, little or no sulfur forms. The formation of sulfur during pyrite oxidation increases with temperature. Bergholm (1955) found that detectable sulfur appeared at 60°C and Lowson (1982) notes that sulfur formation reaches a maximum at 100°-150°C. Both authors also found that low pH, longer run times, increased ferric iron concentration, and increased oxygen concentration increase the production of sulfur.

When pyrite is attacked by acid solutions, the iron is easily leached from the surface layer, leaving a sulfur-rich surface. Buckley and Woods (1987) reviewed the X-ray photoelectron spectroscopy (XPS) literature on the surface oxidation of pyrite and discussed the effect of experimental techniques on the results. They also concluded from their own work that iron tends to be easily leached from the surface producing an iron-deficient (or sulfide-rich) surface that does not have the properties of elemental sulfur unless prolonged strong acid attack was used. Several other studies, using XPS and Raman spectroscopy, have confirmed the initial dissolution under acidic conditions first releases iron and forms a layer containing disulfide, monosulfide, and polysulfides (Sasaki 1994, Nesbitt and Muir 1994, Sasaki et al. 1995). With continued oxidation monosulfide decreases, disulfide increases proportionally, polysulfides increase, and eventually thiosulfate and sulfate form (Nesbitt and Muir 1994). The formation of the disulfide species corroborates the early work of Sato (1960) on the mechanism of oxidation of sulfide ore bodies. The degradation of this surface by either ferric iron or microorganisms, or both, is a critical and poorly understood aspect of pyrite oxidation. As Norris (1990) and Sasaki et al. (1995) mention, this sulfur-rich surface should be energetically favorable for *Thiobacillus thiooxidans* and may explain their association with *T. ferrooxidans*.



The initial oxidation and acid leaching of other sulfide minerals have also been subject to surface spectroscopy. Several studies on both air and air-saturated aqueous solutions of pyrrhotite oxidation have shown the formation of a Fe(III) oxyhydroxide by diffusion of structural iron to the surface and oxidation (Buckley and Woods 1985, Pratt et al. 1994a,b; Mycroft et al. 1995). Underneath this layer is a sulfur-enriched layer that will eventually form marcasite (Mycroft et al. 1995). The oxidation of chalcopyrite in air also leads to iron migration to the surface and formation of an Fe(III) oxyhydroxide leaving a residual  $\text{CuS}_2$  layer (Buckley and Woods 1984). Chalcopyrite oxidation in acid solution leads to the migration and dissolution of iron and a residual  $\text{CuS}_2$  surface (Yin et al. 1995). At temperatures of 80° to 100°C, most of the sulfur from the oxidation of chalcopyrite appears as elemental sulfur (Dutrizac 1990). Arsenopyrite oxidation also forms Fe(III) oxyhydroxides at the surface but arsenic also migrates to the surface and rapidly oxidizes and solubilizes when in contact with water (Nesbitt et al. 1995).

The formation of unstable sulfoxyanions of intermediate oxidation state between elemental sulfur and sulfate further complicates the chemical interpretation. One of the earliest reports was from Steger and Desjardins (1978) who found a thiosulfate compound on the surface of pyrite undergoing oxidation. Recently, a copper thiosulfate mineral has been reported from the oxidation of copper sulfides. Sulfoxyanion formation from pyrite oxidation at pH values of 6 to 9 was studied by Goldhaber (1983) who found thiosulfate, polythionate, and sulfite but only at high stirring rates. The proportions of sulfoxyanions were sensitive to the solution pH. Polythionates were dominant at low pH, thiosulfate was dominant at high pH, and some sulfite formed at the highest pH values. Moses et al. (1987) corroborated Goldhaber's results with improved analytical methods, but they found that when ferric iron replaced oxygen as the oxidant, sulfoxyanions were no longer detectable. These results implicate ferric iron as a powerful oxidant for all types of reduced sulfur species. Williamson and Rimstidt (1993) have demonstrated the rapid oxidation of thiosulfate by ferric iron.

The formation of oxidation products from sulfide minerals depends on the type and composition of the mineral. The first simple distinction is between the metal monosulfides (such as sphalerite,  $\text{ZnS}$ , greenockite,  $\text{CdS}$ , covellite,  $\text{CuS}$ , pyrrhotite (var. troilite),  $\text{FeS}$ , galena,  $\text{PbS}$ , and millerite,  $\text{NiS}$ ) and the disulfides (such as pyrite and marcasite,  $\text{FeS}_2$ , and arsenopyrite,  $\text{FeAsS}^1$ ). Minerals such as molybdenite,  $\text{MoS}_2$ , and chalcopyrite,  $\text{CuFeS}_2$  are not true disulfides because the sulfur atoms are bonded to metal atoms and not to other sulfur atoms. The monosulfides react with acid to form  $\text{H}_2\text{S}$  (Parsons and Ingraham 1970), the reactivity increasing with increasing solubility. Upon contact with air,  $\text{H}_2\text{S}$  will rapidly oxidize to form sulfur, thiosulfate, sulfite, and, ultimately but more slowly, sulfate. The disulfides, however, do not form  $\text{H}_2\text{S}$ , upon reaction with acids. Disulfides contain sulfur with an oxidation state of S(-I) intermediate between S(-II) and S(0) and a tendency to form both elemental sulfur and thiosulfate upon oxidative attack in acid solutions, depending on pH, concentration of oxidants, and temperature. Ahonen et al. (1986) have shown that pyrrhotite forms sulfur during acid dissolution at 28°C and pyrite does not. The sulfur formed from oxidation of  $\text{H}_2\text{S}$ . This distinction is fundamental to the nature of acid weathering reactions for sulfide minerals. The occurrence of hydrogen sulfide, sulfur, and thiosulfate in mine waste environments indicate either sulfate reduction or acid dissolution of monosulfides. Viable, dissimilatory sulfate-reducing bacteria have been found within the saturated zone of acidic mine tailings (Fortin et al. 1995). Stable isotope compositions of sulfur should be definitive in distinguishing these sources. Thiosulfate, sulfur, sulfite, and polythionates are not likely to be found from disulfide oxidation because the autocatalytic

<sup>1</sup> An isostructural solid solution exists between pyrite and löllingite ( $\text{FeAs}_2$ ) with arsenopyrite as a stable intermediate at exactly 0.5 mole fraction of arsenic substituted for sulfur.



oxidation on a semi-conducting surface such as pyrite is much too rapid. The results from Goldhaber's experiments cannot be reproduced without using very fast stirring rates and high shearing force at the mineral surface. The results of thiosulfate formation from pyrite oxidation in the column leaching experiments of Granger and Warren (1969) were faulty (Nordstrom and Alpers 1997) because they contaminated their columns with sodium sulfide solutions. Dissolved sulfide rapidly forms thiosulfate when exposed to the air.

Galvanic protection has been postulated to affect sulfide mineral oxidation rates during the weathering of mineral deposits (Sveshnikov and Dobychin 1956, Sveshnikov and Ryss 1964). The more electroconductive the metal sulfide, the slower its rate of oxidation whereas the less conductive sulfides exhibit increased reaction rates as long as they are in electronic contact with the more conductive sulfide. Kwong (1995) has demonstrated this process and has shown that relative rates can be predicted according to the sequence of standard electrode potentials as compiled by Sato (1992). Furthermore, Kwong et al. (1995) have shown that although bacterially-mediated oxidation increases the oxidation rates, the electrochemical sequence remains the same.

### Compilation of sulfide mineral oxidation studies

We have compiled 29 minerals for which oxidation studies have been done at temperatures below 50°C either with or without microbial participation. This list is based primarily on the reviews by Dutrizac and MacDonald (1974a), Ehrlich (1996), and Nordstrom and Alpers (1997). More information and references can be found in these reviews. The following compilation has been subdivided into four tables (Tables 2a-d) in which the iron sulfides are found in Table 2a, copper sulfides and selenides in Table 2b, arsenic and antimony sulfides in Table 2c, and the remaining sulfides in Table 2d. They have also been placed in alphabetical order within each table. A few comments are needed here. Of course, many more studies have been done on minerals like pyrite than can be referenced in this paper. The studies with valuable rate information have been preferentially included. The references themselves are given in shorthand notation and decoded at the end of all the tables. In most of these studies the surface areas were not measured. Furthermore, the effect of ferric iron concentrations were not always obtained nor distinguished from microbial action. All the necessary reaction products were usually not determined so that the stoichiometry is not always known. In that regard, Ehrlich (1964) found the pH to decrease during enargite oxidation by *T. ferrooxidans* but it increased in the abiotic control. The chemical interpretation would suggest that copper was solubilized and oxidized in both solutions but that the sulfur and arsenic were oxidized only in the presence of bacteria.

Most of the microbial studies included a sterile control but were not necessarily cited in the abiotic references. Qualitative to quantitative data is commonly available and show that microbes increase the rate of reaction when compared to the sterile control but the cause and mechanism are unclear. Many of the studies used impure minerals. These qualitative experiments were, of course, done to improve metal extraction from minerals, not to provide quantitative rate information.

### Role of bacteria

Although the association of microbes with sulfide mineral oxidation has been known for many decades, it has taken a considerable amount of research to determine the rates, mechanisms, and metabolic pathways. Discussion has focused on whether bacteria, primarily the genus *Thiobacillus*, degrade sulfides by a *direct* or an *indirect oxidation* mechanism (Silverman 1967). During direct oxidation, the bacteria attach themselves onto the sulfide mineral surface and directly solubilize the surface through hypothesized



enzymatic oxidation reactions. Indirect oxidation occurs through microbial catalysis of aqueous ferrous to ferric ion oxidation (Reaction 3) and then direct oxidation of the sulfide by the ferric ion (Reaction 2). Adsorption of either *Thiobacillus ferrooxidans* or *Sulfolobus acidocaldarius* to pyrite surfaces is rapid (Bagdikian and Myerson 1986, Chen and Skidmore 1987, 1988). Surface etch patterns that reflect bacterial attachment have been

**Table 2a.** Oxidation studies on iron sulfide minerals.

<i>Mineral / Oxidant</i>	<i>Formula</i>	<i>Abiotic reference</i>	<i>Microbial reference</i>
marcasite/Fe <sup>3+</sup>	FeS <sub>2</sub>	WR84, R94	--
pyrite/O <sub>2</sub>	FeS <sub>2</sub>	B54, BJ58, G83, MB86, M87, MH91, N94, O91	B54, BJ58, SE64, O91, P91
pyrite/Fe <sup>3+</sup>	FeS <sub>2</sub>	MB86, BJ89, M87, MH91, R94	--
pyrrhotite/O <sub>2</sub>	Fe <sub>1-x</sub> S	TC76, NS94	P91, B93

**Table 2b.** Oxidation studies of copper sulfides and selenides.

<i>Mineral</i>	<i>Formula</i>	<i>Abiotic reference</i>	<i>Microbial reference</i>
bornite	Cu <sub>5</sub> FeS <sub>4</sub>	S31, KO63, L70, D70a, KO69, D70b, U67	B54, L66
carrollite	Co <sub>2</sub> CuS <sub>4</sub>	DC64	DC64
chalcopyrite	CuFeS <sub>2</sub>	S33, BS34, I62, R94	BA57, I61, D64, T76
chalcocite	Cu <sub>2</sub> S	S30a, T67, KO69, M69,	B54, RT63, NB72, S76, B77
covellite	CuS	S30b, TI67, M71, DM74b, WR86	S76, RV78
cubanite	CuFe <sub>2</sub> S <sub>3</sub>	D70c	--
digenite	Cu <sub>9</sub> S <sub>5</sub>	T67	--
klockmannite	CuSe	--	TH72
copper-selenide	Cu <sub>2</sub> Se	K66	--
copper-telluride	Cu <sub>2</sub> Te	K66	--

**Table 2c.** Oxidation studies of arsenic, antimony, and gallium sulfides.

<i>Mineral</i>	<i>Formula</i>	<i>Abiotic reference</i>	<i>Microbial reference</i>
arsenopyrite	FeAsS	R94	E64
cobaltite	CoAsS	--	SC61
enargite	Cu <sub>3</sub> AsS <sub>4</sub>	S33, BS34, KG52	E64
gallium sulfide	Ga <sub>2</sub> S <sub>3</sub>	--	T78
orpiment	As <sub>2</sub> S <sub>3</sub>	--	E63b
realgar	AsS	--	E63b
stibnite	Sb <sub>2</sub> S <sub>3</sub>	TT63	T74, TG77
tennantite	Cu <sub>12</sub> As <sub>4</sub> S <sub>13</sub>	S33, BS34	--
tetrahedrite	Cu <sub>12</sub> Sb <sub>4</sub> S <sub>13</sub>	BS34, Y80	B54

Table 2d. Oxidation studies of other metal sulfides.

Mineral	Formula	Abiotic reference	Microbial reference
cinnabar	HgS	P39, B75	B89
cobalt sulfide	CoS	—	T71, T74
galena	PbS	H70, R94	ST74, TS74
greenockite	CdS	—	B71, T74
millerite	NiS	—	RT63, DT64, T71, T74
molibdenite	MoS <sub>2</sub>	U52,	BA57, BJ58, B65, BM73
pentlandite	(Fe, Ni) <sub>9</sub> S <sub>8</sub>	—	DM74c
sphalerite	ZnS	R94	RH22, I61, MP61, T72, KR77

Table 2e. References on oxidation studies of sulfide minerals.

B54 Bryner et al. (1954)	I62 Ichikuni (1962)	S30 Sullivan (1930b)
B65 Bhappu et al. (1965)	K66 Kholmanskikh et al. (1966)	S31 Sullivan (1931)
B71 Brissette et al. (1971)	KG52 Koch and Grasselly (1952)	S33 Sullivan (1933)
B75 Burkstaller et al. (1975)	KO63 Kopylov and Orlov (1963)	S76 Sakaguchi et al. (1976)
B77 Beck (1977)	KO69 Kopylov and Orlov (1969)	SC61 Sutton and Corrick (1961)
B89 Baldi et al. (1989)	KR77 Khalid and Ralph (1977)	SE64 Silverman and Ehrlich (1964)
B93 Bhatti et al. (1993)	L66 Landesman et al. (1966)	ST74 Silver and Torma (1974)
BA57 Bryner and Anderson (1975)	L70 Lowe (1970)	T67 Thomas et al. (1967)
BJ58 Bryner and Jamieson (1958)	M69 Mulak (1969)	T71 Torma (1971)
BJ89 Brown and Jurinak (1989)	M71 Mulak (1971)	T72 Torma et al. (1972)
BL77 Brierley and Le Roux (1977)	M87 Moses et al. (1987)	T74 Torma et al. (1974)
BM73 Brierley and Murr (1973)	MB86 McKibben and Barnes (1986)	T76 Torma et al. (1976)
BS34 Brown and Sullivan (1934)	MH91 Moses and Herman (1991)	T78 Torma (1978)
D64 Duncan et al. (1964)	MP61 Malouf and Prater (1961)	TC76 Tervari and Campbell (1976)
D70a Dutrizac et al. (1970a)	N94 Nicholson (1994)	TG77 Torma and Gabra (1977)
D70b Dutrizac et al. (1970b)	NB72 Nielson and Beck (1972)	TH72 Torma and Habashi (1972)
DC64 De Cuyper (1964)	NS94 Nicholson and Scharer (1994)	TI67 Thomas and Ingraham (1967)
DM74a Dutrizac and MacDonald (1974b)	O91 Olson (1991)	TS74 Torma and Subramanian (1974)
DM74b Dutrizac and MacDonald (1974c)	P39 Pande (1939)	TT63 Tugov and Tsyganov (1963)
DT64 Duncan and Trussell (1964)	P91 Pinka (1991)	U52 Usataya (1952)
E63b Ehrlich (1963b)	R94 Rimstidt et al. (1994)	U67 Uchida (1967)
E64 Ehrlich (1964)	RH22 Rudolphs and Helbronner (1922)	WR84 Wiersma and Rimstidt (1984)
G83 Goldhaber (1983)	RT63 Razzell and Trussell (1963)	WR86 Walsh and Rimstidt (1986)
H70 Haver et al. (1970)	RV78 Rickard and Vanselow (1978)	Y80 Yakhontova et al. (1980)
I61 Ivanov et al. (1961)	S30a Sullivan (1930a)	

observed (Bennett and Tributsch 1978). The actual mechanism of enzymatic oxidation is not entirely clear and is discussed further in the section on "Microbial Oxidation of Sulfide Minerals," below. Furthermore, it seems unnecessary to explain the rate data. We shall explore this controversy and the general interaction of bacteria with oxidizing sulfides by reviewing the empirical rate data for both ferrous to ferric oxidation and pyrite oxidation.



other sulfide mineral oxidation studies, the microbial physiology of *Thiobacillus ferrooxidans*, and the microbial ecology of mine waste environments.

### AQUEOUS Fe(II) OXIDATION KINETICS

The aqueous oxidation of ferrous to ferric ion has been firmly established for acid solutions where the rate is relatively slow. The rate increases with increasing pH but also becomes sensitive to oxygen and anionic ligand concentrations. At circumneutral pH values, the abiotic rate is so fast that bacterial catalysis has not been clearly demonstrated and it is not needed to explain the rate of aqueous iron oxidation. Singer and Stumm (1968, 1970a) showed that the rate increased rapidly with increasing pH above a value of about 4. Below this value the rate leveled out and became nearly independent of pH at about  $3 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$ . The presence of *T. ferrooxidans* increased this rate by five orders of magnitude to about  $3 \times 10^{-7} \text{ mol L}^{-1} \text{ s}^{-1}$  (Singer and Stumm 1970a,b). This catalysis is most remarkable in the enhancement of inorganic reactions and has a major effect on pyrite weathering. Measurements from microbial oxidation experiments, to study optimal growth of *T. ferrooxidans* on culture media, typically gave rates of  $2.8\text{--}8.3 \times 10^{-7} \text{ mol L}^{-1} \text{ s}^{-1}$  (Silverman and Lundgren 1959, Lundgren et al. 1964, Lacey and Lawson 1977, Noike et al. 1983). This agreement is quite good considering differences in experimental procedure and conditions.

Field determinations of the ferrous oxidation rate are very similar to those derived from laboratory experiments. Wakao et al. (1977) estimated a rate of  $3 \times 10^{-6} \text{ mol L}^{-1} \text{ s}^{-1}$  for an acid mine water in Japan but the stream velocities were not directly measured. Nordstrom (1985) measured stream velocities in some acid mine waters in California and found the oxidation rate to vary  $2\text{--}8 \times 10^{-7} \text{ mol L}^{-1} \text{ s}^{-1}$ . He found the rate in the same drainage to decrease after a rainstorm, presumably due to the flushing out and dilution of the bacterial population. For pH values of 2-4, the microbially-catalysed oxidation of ferrous iron generally has an average optimal rate of  $5 \times 10^{-7} \text{ mol L}^{-1} \text{ s}^{-1}$  whether in the field or in the lab using culture media (usually 9K medium, Silverman and Lundgren 1959) and where temperatures are about 10°-30°C. In the environment, however, ferrous iron oxidation rates are likely to be lower because of numerous environmental factors including temperature fluctuations, hydrologic conditions, nutrient limitations, and UV radiation for surface waters.

A brief note is needed here regarding the units and formalism for microbial reaction rates. In chemical kinetics, reaction rates are generally zero-order, first-order, second-order, or sometimes non-integral order. The rate equations for living entities such as bacteria are more complex than for the simpler entities of molecules and atoms. Growth cycles of microbes, expressed as population densities over time, usually follow a sigmoidal type of curve. During the early phase of growth at low cell counts, there is little change with time. The doubling process, however, is exponential (like radioactive decay in reverse) so that after a period of time with little apparent growth, known as the lag phase, there is rapid growth that can only be stopped by lack of an energy source, an essential nutrient, build-up of toxic products, or by a metabolic inhibitor. Nutrient concentrations can also become high enough that the growth rate becomes constant and independent of concentration. For a lithoautotroph like *T. ferrooxidans*, this means the ferrous iron oxidation rate can become independent of the ferrous iron concentration and the rate becomes zero order. Bacterial cell division by binary fission, is the separation of a parent cell into two daughter cells. During the lag phase and exponential growth phase, the growth is dependent on ferrous concentration and a first-order or pseudo-first-order reaction rate can be observed. The convention in microbiology is to treat this changing growth curve with the Michaelis-Menten equation that contains both a first order and a zero-order term.



The rates we are using assume nutrient-saturated conditions and zero-order rates. Most lab studies on the microbial rate of ferrous iron oxidation have shown the rate to be zero-order for a wide range of ferrous concentrations (Schnaitman et al. 1969, Okereke and Stevens 1991). The rate of sulfide mineral oxidation will now be considered.

### SULFIDE MINERAL OXIDATION KINETICS

The conventional approach to distinguish between the direct and indirect oxidation mechanisms would be to compare the rate of pyrite oxidation by iron-oxidizing bacteria with that obtained abiotically but in the presence of ferric ion. This analysis, however, is more complicated. It is known that acidophilic iron-oxidizing bacteria will generate Fe(III) from Fe(II). The question that should be addressed is: what are the relative rates among (1) oxidation of pyrite by Fe(III), (2) oxidation of Fe(II) by bacteria, and (3) oxidation of pyrite by bacteria independent of their oxidation of Fe(II)? If there is a direct mechanism of bacterial pyrite oxidation other than by regeneration of Fe(III) then for that rate to be significant, it would have to be as fast or faster than the rate of Fe(II) oxidation and there should be a plausible mechanism to explain it. Under those circumstances, the bacterial pyrite oxidation rate would have to be faster than the abiotic oxidation rate by Fe(III). With these concepts in hand, let's look at the evidence.

Nordstrom and Alpers (1997) compiled several reports on pyrite oxidation rates and summary results are shown in Table 3. The oxidation rate with ferric ion as the oxidant is faster than the rate with oxygen as the oxidant by an order of magnitude or more. Although these rates overlap when comparing different investigations, the relative rates within any one investigation show a consistently faster rate with Fe(III). Most investigators get a rate (at pH = 2, T = 25°C) close to  $1 \times 10^{-8} \text{ mol m}^{-2} \text{ s}^{-1}$  which would be less than  $9 \times 10^{-8} \text{ mol m}^{-2} \text{ s}^{-1}$  for the bacterial rate. These results would indicate an enhancement induced by microbial attachment. Unfortunately, part of this difference could be explained by sample preparation effects and experimental design problems. Furthermore, the microbial rate was obtained by assuming a surface area based on grain size which could be low by a factor of at least 2 or 3.

Table 3. Comparison of iron and pyrite oxidation rates.

Reaction	Abiotic Rate	Microbial Rate
Oxidation of Fe(II)	$3 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$	$5 \times 10^{-7} \text{ mol L}^{-1} \text{ s}^{-1}$
Oxidation of pyrite by $\text{O}_2$	$0.3 - 3 \times 10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}$	$8.8 \times 10^{-8} \text{ mol m}^{-2} \text{ s}^{-1}$
Oxidation of pyrite by Fe(III)	$1 - 2 \times 10^{-8} \text{ mol m}^{-2} \text{ s}^{-1}$	

The source of the bacterially-mediated pyrite oxidation rate study (Olson 1991) was an interlaboratory comparison implemented with sterile controls. About a 34-fold increase in rate was observed with *T. ferrooxidans* present compared to the sterile control and the control gave similar (but higher) rates to those from other studies of pyrite oxidation by oxygen. Paciorek et al. (1981) similarly found a 25-fold increase in the microbially-catalyzed pyrite oxidation rate relative to the abiotic control. Lizama and Suzuki (1989) found that the pyrite oxidation rate with *T. ferrooxidans* and Fe(III) was notably faster than the abiotic rate with Fe(III). These results, however, could be an artifact of the experimental design. When iron-oxidizing bacteria are oxidizing pyrite, they are also regenerating ferric from aqueous ferrous iron whereas the sterile control has a fixed initial ferric concentration that eventually gets used up so that the reaction rate slows down. This complication is difficult to avoid in experimental studies.



Olson (1991) reported his results in terms of the rate of formation of aqueous iron which was  $12.4 \text{ mg Fe L}^{-1} \text{ h}^{-1}$  or about  $6 \times 10^{-8} \text{ mol L}^{-1} \text{ s}^{-1}$ . This rate is about one order of magnitude less than the rate of bacterial oxidation of Fe(II) suggesting an inhibition of pyrite oxidation by the bacteria. The inhibiting effect of adsorbed bacterial cells on the pyrite surface was reported by Wakao et al. (1984) who concluded that pyrite oxidation proceeded by the indirect mechanism through the growth of free-floating ferrous-iron-oxidizing bacteria. In the experiments of Wakao et al. (1984), a surfactant was used to desorb the cells from the pyrite surface and the oxidation rate then increased again to near its former level. Contradicting the experiments of Wakao et al. (1984), Arkestyn (1979) found the rate of pyrite oxidation to decrease when he separated *T. ferrooxidans* from the pyrite surfaces by a dialysis bag. The observation that iron-oxidizing bacteria can oxidize aqueous Fe(II) faster than they can oxidize pyrite may indicate inhibition by bacterial adsorption on surfaces or, more likely, it may indicate the rate of pyrite oxidation by Fe(III) is slower than the oxidation rate of Fe(II) by bacteria. This conclusion would be consistent with the general observation that aqueous chemical reactions, especially when catalyzed, are faster than heterogeneous chemical reactions.

A review by Sand et al. (1995) summarized recent literature and provided further evidence for the indirect oxidation mechanism. They pointed out that subculturing *T. ferrooxidans* in an iron-free salt solution resulted in complete loss of measurable substrate degradation. The bacterial cells could not function in the absence of iron. Further unpublished work of theirs showed that the iron in the bacterial cells could not be removed from their host, i.e. exopolymers, by washing. The extracellular polymeric substances (exopolymers) facilitate the attachment of the bacteria to the pyrite surface. Sand et al. (1995) also emphasized the importance of thiosulfate as the first soluble oxidized sulfur species and a key intermediate during the oxidation of pyrite. Thiosulfate is oxidized to tetrathionate and hydrolysis of tetrathionate produces disulfane sulfonate which further decomposes to form a variety of products. Further elucidation of sulfoxyanion formation during bioleaching of pyrite was reported by Schippers et al. (1996). They monitored the formation of thiosulfate, elemental sulfur, polythionates and sulfate during pyrite oxidation by both *T. ferrooxidans* and *L. ferrooxidans* in solutions with pH just less than 2. The appearance of polythionates is to be expected since both Fe(III) and pyrite can catalyze the decomposition of thiosulfate to polythionates abiotically. These two studies find no evidence for the direct mechanism and substantial evidence for the indirect mechanism.

The consistency and precision of the rate data indicates the abiotic pyrite oxidation rate by Fe(III) is slower than the conversion of Fe(II) to Fe(III) by bacteria and slower than the oxidation of pyrite with bacteria and Fe(III). However, several caveats should be noted. The cited aqueous Fe(II) oxidation rate is the maximum possible rate achievable with no limiting nutrients and at optimal temperatures. Microbes in the environment are nearly always growth-limited by some necessary nutrient, predatory relationships, hydrologic conditions, or by other factors. Hence, their environmental growth rate will be slower than the values quoted above and they could be less than and probably not greater than the rate of pyrite oxidation by Fe(III). Furthermore, the oxidation rate of pyrite in the environment will be controlled by bacterial growth rates and these, in turn, will be controlled by environmental factors such as temperature, hydrologic variables (supply of water and oxygen), and nutrient limitations. The search for the rate-determining step and mechanism in the formation of acid mine drainage may be over. What we have learned from all of the relevant experimental rate studies is that both the rates of aqueous Fe(II) oxidation and the oxidation of pyrite are fast and comparable in the presence of iron- and sulfur-oxidizing bacteria. The overall rate is governed by environmental factors that affect the growth rate of the bacteria.



The link between the chemistry and the biology of sulfide mineral oxidation can be expressed as follows:

- (1) Bacteria want to be attached, or as close as possible, to sulfide mineral surfaces to maximize their efficiency of substrate utilization. The close adherence reduces the time needed for diffusion of iron between the mineral and the bacterium, it does not necessarily require a separate mechanism for sulfide mineral degradation. Attachment also changes the surface chemistry of the solid-microbe interface (see section below).
- (2) All available evidence is consistent with a chemical oxidation of sulfide minerals with aqueous catalysis by iron- and sulfur-oxidizing bacteria.
- (3) No other processes need to be invoked to explain the available data. This statement is another expression of Occam's razor.

## MICROBIAL OXIDATION OF SULFIDE MINERALS

### General physiology of *Thiobacillus ferrooxidans*

*Thiobacillus ferrooxidans* is typically known for its ability to oxidize reduced sulfur compounds and ferrous iron to produce sulfuric acid and ferric iron as by-products of its metabolism (Ingledew 1982, Harrison 1984). The general nutritional requirements of this bacterium are provided by carbon dioxide fixation (providing cellular carbon; Leathen et al. 1956), pyritic minerals as sources of energy (see Table 1) for ATP, ammonia, atmospheric nitrogen, or nitrate as its source of nitrogen (Temple and Colmer 1951, Lundgren et al. 1964, Mackintosh 1978, Stevens et al. 1986), and phosphate from acid solubilization of available phosphate minerals (Lipman and McLean 1916). Previous reports on the heterotrophic ability of *T. ferrooxidans* are actually a consequence of the presence of acidophilic heterotrophs found to co-exist with *T. ferrooxidans* (Harrison et al. 1980).

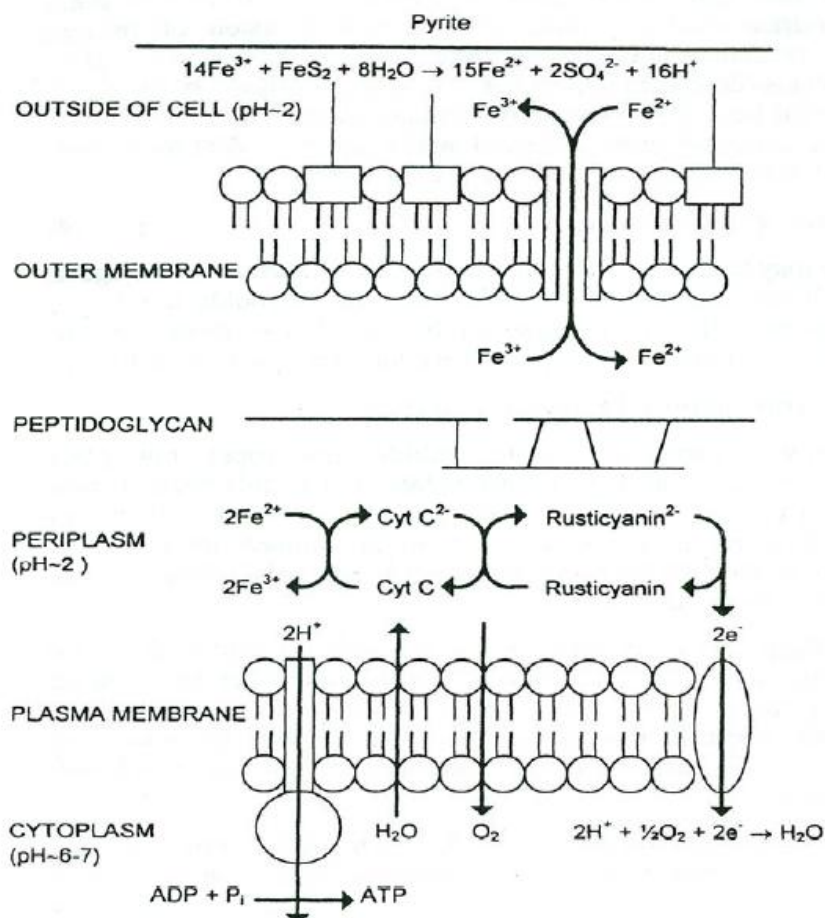
The formation of ATP occurs via a chemiosmotic mechanism (see Fig. 1). The impermeable nature of the cytoplasmic membrane to protons maintains a pH (pH ~ 2 vs. pH 6) based proton motive force, allowing protons to be transported only at sites where ATPase is anchored in the membrane (Ingledew et al. 1977). In addition to its role in general cell energetics, the ATP that is formed by this reaction is used in reverse electron transport to produce NADPH (Aleem et al. 1963) which is in turn used in the reductive assimilation (fixation) of CO<sub>2</sub> by the Calvin-Benson cycle (Maciag and Lundgren 1964).

In natural systems that contain iron, *T. ferrooxidans* may exclusively oxidize Fe<sup>2+</sup> → Fe<sup>3+</sup> to gain energy. This oxidation, yields a chemically reactive cation which can scavenge electrons from less electronegative metal species (e.g. pyrite, Eqn. 2; Mehta and Murr 1982) forming ferrous iron once again. The formation of reduced iron from the latter provides an effective bacterial electron carrier for sustained lithotrophy. Mineral dissolution then, is a chemical process enhanced by bio-catalysis (Singer and Stumm 1970, Keller and Murr 1982, Hutchins et al. 1986, Baldi et al. 1992).

The oxidation of iron yields low levels of energy. Silverman and Lundgren (1959) calculated 18.5 moles of iron need to be oxidized to assimilate 1 mole of carbon. These calculations assume 100% metabolic efficiency which is not possible in biological systems. For *Thiobacillus ferrooxidans*, metabolic efficiency has been found to be highly variable (3.2%, Temple and Colmer 1951; 4.8-10.6%, Beck and Elsdon 1958; 20.5±4.3% Silverman and Lundgren 1959; 30%, Lyalikova 1958). The poor energy yield for carbon utilization from iron oxidation remains a puzzle.

In our model (Fig. 1) of iron oxidation in *T. ferrooxidans* adapted from Ingledew et al. (1977) and Blake et al. (1992) the biological oxidation of Fe(II) to Fe(III) and the





**Figure 1.** A model (adapted from Ingledew et al. 1977, and Blake et al. 1992) for the bioenergetics of iron oxidation by *T. ferrooxidans* based on a bidirectional diffusion gradient for Fe(II/III).

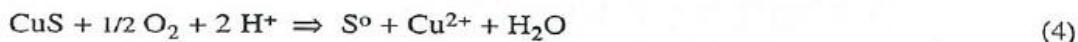
geochemical oxidation of pyrite by Fe(III) creates a bidirectional diffusion gradient which continuously drives Fe(II) into the periplasm and Fe(III) out of the periplasm. Iron will bind weakly (if at all) to the organic constituents on the cell surface and in the periplasm because they will be outcompeted by protons at pH 2. Other aspects of the model are the same as those described in the model of Blake et al. (1992).

### Direct vs. indirect oxidation of non-ferrous sulfide minerals

The biooxidation of sulfide minerals presents a special problem when iron is not a component of the mineral(s). In these systems, the direct, enzymatic oxidation of susceptible mineral sulfides has been proposed in which each bacterial cell acts as a conductor of electrons from the crystal structure of chalcocite to oxygen (Ehrlich 1996). The transfer of constituent electrons from pyrite under acidic conditions has also been described by Wiersma and Rimstidt (1984). While these models are supported by an extremely close bacterial-mineral interaction (Bagdikian and Myerson 1986, Duncan and Drummond 1973, Bennet and Tributsch 1978, Norman and Snyman 1987, Southam and Beveridge 1992), the transfer of free electrons from the crystal structure only represents a partial reaction and



cannot occur without an appropriate bacterial electron acceptor. The outer membrane (OM) of Gram-negative Bacteria, e.g. *T. ferrooxidans*, functions as a passive diffusion barrier (Beveridge 1981, 1988) and does not possess electron acceptors (Haddock and Jones 1977). Therefore, mineral sulfide oxidation must proceed via diffusion of reduced compounds across the outer membrane into the periplasm. Soluble ferrous iron will be present in iron-containing systems (described above; Fig. 1), acid dissolution of chalcocite releases soluble  $\text{Cu}^+$  (Nielsen and Beck 1972) and nonbiological, autooxidation of covellite under acid conditions produces elemental sulfur (Rickard and Vanselow 1978) most likely from oxidation of  $\text{H}_2\text{S}$  formed during acid dissolution of a monosulfide,



Uptake of this elemental sulfur may be promoted by the production of surface active agents such as those described by Jones and Starkey (1961). The low Reynolds number of bacterial-sized particles dictates that diffusion of these soluble constituents away from the bacterial cell surface will be limited (Purcell 1977), promoting continued bacterial activity.

### *Thiobacillus* spp.—mineral interaction in natural systems

*Thiobacillus* spp. can colonize naturally occurring sulfides (outcrops), low grade sulfide-containing ore (to promote acid leaching of base metals) and sulfide-bearing mine wastes (tailings) disposed of, at the earth's surface. The high surface:volume ratio created by grinding sulfides to extract base metals, creates an optimum environment for growth of *Thiobacillus* spp. For this reason, the following discussion focuses on the colonization of minerals surfaces in mine tailings environments.

The occurrence of metabolically diverse bacteria in the subsurface (Amy et al. 1996) down to at least 2.7 km (Boone et al. 1995), suggests that mineral sulfides may contain low populations of thiobacilli prior to mining. Whether or not this is true, mine tailings become quickly colonized with thiobacilli once discharged from the mill (Southam and Beveridge 1993). However, the mechanism of its colonization of tailings is not well understood (Dispirito et al. 1983).

When tailings are deposited as an aqueous slurry and allowed to drain, water is retained at the grain boundaries and in the pores between grains because of the capillary forces counteracting gravity (Nicholson et al. 1990). This area of partial water saturation, the vadose zone, provides all of the essential physical and chemical requirements for growth of *Thiobacillus* spp. In the vadose zone, the capillary border on the mineral surface supplies water to support life (i.e. protection against dessication which is deleterious to thiobacilli; Brock 1975), the pore spaces allow for the influx of gaseous oxygen (as terminal electron acceptor) and carbon dioxide (for carbon fixation) and the sulfide minerals serve as the substrate for lithotrophy (see Table 2). Drying at the surface of a sulfide tailings and freezing conditions reduce thiobacilli populations (Southam and Beveridge 1992) although near freezing conditions do not (Ahonen and Tuovinen 1992). Survival of thiobacilli during periodic wetting when microaerophilic to anaerobic environments may form (due to limited diffusion of oxygen into water saturated material) is presumably due to anaerobic metabolism (Pronk et al. 1992, Das and Mishra 1996).

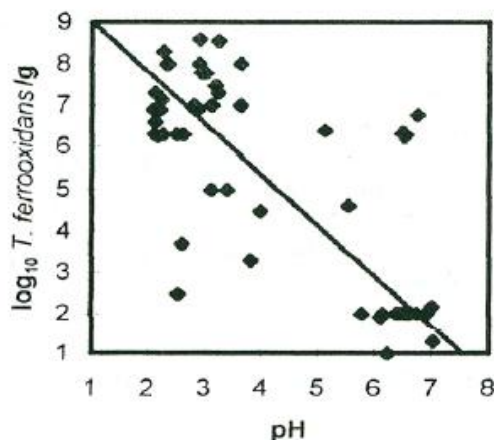
*Metallogenium* has been described as an organism potentially responsible for the transition of mineral sulfide-rich environments from neutral to acid pH conditions (Walsh and Mitchell 1972). The proper identification of this bacterium is somewhat questionable and it is not required for this transition to occur. At the Copper Rand mine tailings, an individual *Thiobacillus ferrooxidans* subspecies (an LPS chemotype) was able to transform an alkaline pH (pH 8) tailings environment down to pH 3 (Southam and Beveridge 1993). At the Kidd Creek tailings pile, Blowes et al. (1995) demonstrated the



predominant colonization with *Thiobacillus thioautotrophicus* under circumneutral pH conditions down to a pH of about 5 with *T. ferrooxidans* and *T. thioautotrophicus* becoming predominant at pH values of 4 and less. Measured pH is not, however, a unique or accurate indicator of viable populations of thiobacilli. Low populations of *T. ferrooxidans* can be recovered from the surface of acidic (pH < 3) mine tailings where evaporation produces low water activity which can inhibit or kill thiobacilli (Brock 1975, Southam and Beveridge 1992). Although the optimal pH for growth of *Thiobacillus* spp. is pH < 3 (Trafford et al. 1973, Amaro et al. 1991), viable thiobacilli can be cultured from tailings possessing neutral environmental pH values (see Fig. 2; Southam and Beveridge 1992). These thiobacilli must be employing acidic nanoenvironments because growth at neutral pH is not possible.

As mentioned previously and shown in Figure 3, growth on minerals is facilitated by close bacteria-mineral interaction (also see Sand et al. 1995). This close mineral interaction is important because the electron carriers responsible for iron oxidation and energy uptake are found in a gel-like (Hobot et al. 1984) periplasm (Blake et al. 1992, Harrison 1984, Ingledew et al. 1977). A close juncture will also promote the diffusion of soluble electron carriers e.g. soluble iron (Fig. 1) or  $\text{Cu}^+$  (Nielsen and Beck 1972) or atomic % S (Rickard and Vanselow 1978). Colonization of sulfide minerals and the resulting, lithoautotrophy begins under bulk 'neutral' pH conditions and probably occurs on the nanoscale through the development of an acidic interface between the bacteria and the mineral surfaces (compare Figs. 1, 2 and 3).

**Figure 2.** *Thiobacillus ferrooxidans* plotted against sample pH for samples from the Lemoine mine tailings (Southam and Beveridge 1993). The two clusters along the straight line are typical of what is expected for *Thiobacillus* spp. The outliers show (1) low populations at low pH where drying has caused death or decreased activity and (2) high populations at high pH indicating acidic nanoenvironments.



**Figure 3.** An unstained thin section of a tailings sample from the Lemoine mine which demonstrates the close association between *T. ferrooxidans* and a chalcopyrite mineral surface. Bar equals 0.5 μm. (Published with permission from Southam and Beveridge 1992).



*Thiobacillus ferrooxidans* binds preferentially to pyrite dispersed through a coal slurry containing abundant organic surfaces (Bagdigian and Myerson 1986, Mustin et al. 1992). Bennet and Tributsch (1978) and Norman and Snyman (1987) have demonstrated that *T. ferrooxidans* chooses to colonize fracture lines and dislocations on pyritic mineral surfaces. These fine grooves (0.2 to 0.5  $\mu\text{m}$ ) in the mineral surface eventually develop into corrosion pits, widening and enlarging until the mineral grain is destroyed (Tributsch 1976, Norman and Snyman 1987). Mineral dislocations may provide convenient physical recesses for bacterial colonization and a unique surface charge promoting the attachment of *T. ferrooxidans*. This is one way in which certain strains may exhibit mineral selectivity.

The thiobacillus surface component responsible for preferential binding to sulfide minerals and the resulting close bacteria-mineral interaction is lipopolysaccharide (LPS) because this molecule is situated on the outer membrane surface of Gram-negative Bacteria and its side chains extend beyond the usual bilayer structure of a membrane. *Thiobacillus* spp. do not possess capsular material (Shively et al. 1970, Wang et al. 1970, Vestal et al. 1973, Hirt and Vestal 1975, Rodriguez et al. 1986, Yokota et al. 1988). LPS heterogeneity (Southam and Beveridge 1993), i.e. differences in LPS O-antigen side-chains, will control the cell surface charge character and hydrophobicity which could affect the ability of different strains to colonize different sulfide mineral surfaces. Because charge character is also pH dependent (Chakrabarti and Banerjee 1991), the reduction in pH as thiobacilli colonize mine tailings material would also affect, presumably enhance, their ability to colonize tailings as acid mine drainage develops.

Lizama and Suzuki (1991) characterized a *T. ferrooxidans* strain which could oxidize either pyrite or chalcopyrite but not sphalerite and a second strain which could oxidize either pyrite or sphalerite but not chalcopyrite. The ability to differentiate between chalcopyrite and sphalerite suggests that a form of recognition, presumably reflected in LPS chemistry (Southam and Beveridge 1993), exists towards these minerals. *Thiobacillus* spp. are also known for their ability to adapt to various types of sulfide ores prior to the initiation of active microbial leaching (Suzuki et al. 1990). This adaptation mechanism is not well understood although it might have something to do with "activation" by chemical oxidation (Moses and Herman 1991). Although phenotypic switching has been demonstrated in *T. ferrooxidans*, a relationship between LPS chemistry and mineral adaptation has not been demonstrated (Schrader and Holms 1988, Southam and Beveridge 1993).

Acid mine waters from many different types of oxidizing sulfide mineral deposits typically have high concentrations of both *T. ferrooxidans* and *T. thiooxidans*, and in roughly equal proportions (Scala et al. 1982). This observation raises the question of what role *T. thiooxidans* plays in the oxidation process because *T. ferrooxidans* can oxidize both iron and sulfur. The difference between *T. ferrooxidans* and *T. thiooxidans* is not simply that *T. thiooxidans* cannot oxidize Fe(II). At Brimstone Basin, an elemental sulfur deposit in Yellowstone National Park, *T. thiooxidans* populations were 2 to 3 orders of magnitude greater than *T. ferrooxidans* (Southam, Donald, and Nordstrom, pers. comm.). Nature has selected for the most efficient sulfur-oxidizing bacterium.

Cells grown on ore are difficult to dissociate from the ore particles (Gormley and Duncan 1974, Suzuki et al. 1990, Southam and Beveridge 1992) demonstrating that a tight association must occur between *Thiobacillus* spp. and sulfide mineral surfaces. The development of a tight association between the bacteria (LPS) and sulfide minerals may proceed via a hypothetical two phase mechanism (Southam et al. 1995). First, either ionic, salt-bridging or hydrophobic interactions would reversibly attach the bacterium to the mineral surface so that an acidic interface could be established to sustain lithoautotrophy. Phase two encompasses the subsequent cementing of the bacterium to the mineral surface



with iron oxy-hydroxides (Bigham et al. 1990, Bhatti et al. 1993) forming an even more acidic nanoenvironment (i.e. precipitation of  $\text{Fe}(\text{OH})_3$  yields  $\text{H}^+$ ) to support bacterial growth and multiplication. The role of ferric oxy-hydroxides in glueing thiobacilli to mineral surfaces is supported by their release after solubilization of these precipitates (Ramsay et al. 1988, Southam and Beveridge 1993). Strong adherence of *T. ferrooxidans* to minerals via iron precipitates (Southam and Beveridge 1992, 1993) may have an important ecological role in reducing the diffusion of metabolic products (e.g.  $\text{Fe}(\text{III})$  and sulfuric acid) away from the cell-mineral interface. This would help maintain an acidic nanoenvironment at the mineral surface and provide a potential source of soluble ferrous iron through repeated chemical oxidation of the sulfide (Reaction 2) thereby promoting the growth of *T. ferrooxidans*.

### Effects of temperature

Environments where iron, sulfur, and sulfide minerals are oxidizing can have a large range of temperature from 0°C in some waste rock piles (Strömberg and Banwart 1994), to 50-60°C in some underground mines (Nordstrom and Potter 1977, Nordstrom and Alpers 1995) and waste rock piles (Hendy 1987), to boiling in hot spring waters (Bott and Brock 1969). Temperature is one of the distinctive and most important environmental parameters that influences the activity of microorganisms. It not only influences their rate of growth but it can also determine the predominant genus and species. Bacteria are thermally divided into psychrophiles, mesophiles, thermophiles and hyperthermophiles. Psychrophiles can grow at 0° with an optimum of 10°-15° and a range up to 30°C. Mesophiles tend to grow at temperatures below 40°C with an optimum temperature around 30°C. Thermophiles have an optimum temperature of about 50°-60°C and hyperthermophiles have an optimum temperature of >80°C. Both obligate and facultative thermophiles have also been observed.

Very little information is available on psychrophilic activity in mine waste environments but Strömberg (1997) has provided evidence that for a waste rock pile at Aitik, Sweden with a temperature range of 0° to 12°C and averaging 5°C, microbial catalysis is affecting pyrite oxidation. Ferroni et al. (1986) and Berthelot et al. (1993) found that species such as *T. ferrooxidans* can be psychrotrophic. They enriched mine water samples from Elliot Lake, Ontario and found that growth continued at temperatures as low as 2°C, with faster growth for the enriched mine water than that for *T. ferrooxidans* ATCC 33020. Both cultures had the same growth rates and the same optimum temperature over the range of 12° to 35°C.

Acidophilic iron- and sulfur-oxidizing bacteria are either mesophiles or thermophiles. Figure 4 shows the temperature range for growth of several important mesophiles and thermophiles (Norris 1990). The growth rates shown are not necessarily independent of pH. Norris (1990) has noted the increase in rate with an increase in pH for *T. ferrooxidans*. However, Nordstrom (1977, 1985) found that the ferrous iron oxidation rate for acid mine waters of pH 1-2 was virtually the same as that for 9K culture medium of pH 2-3. The influence of pH on microbial kinetics has not been clearly defined for sulfide mineral and ferrous iron oxidation. *Thiobacillus* spp. and *Leptospirillum* spp. are mesophiles with typical temperature optima in the range of 30° to 35°C, although the same species can have a different range and optimum as shown in Figure 4. These species have a temperature range that coincides well with the typical temperature range of most environments where pyrite is oxidizing. The new species, *Leptospirillum thermoferrooxidans*, is a moderate thermophile with a temperature optimum of 45°-50°C (Golovacheva et al. 1992). *Sulfobacillus thermosulfidooxidans* and strain TH1 are Gram-positive, thermophiles, and facultative lithotrophs, unlike *T. ferrooxidans*. They show greater morphological variation and are more elongated (Karavaiko et al. 1988).



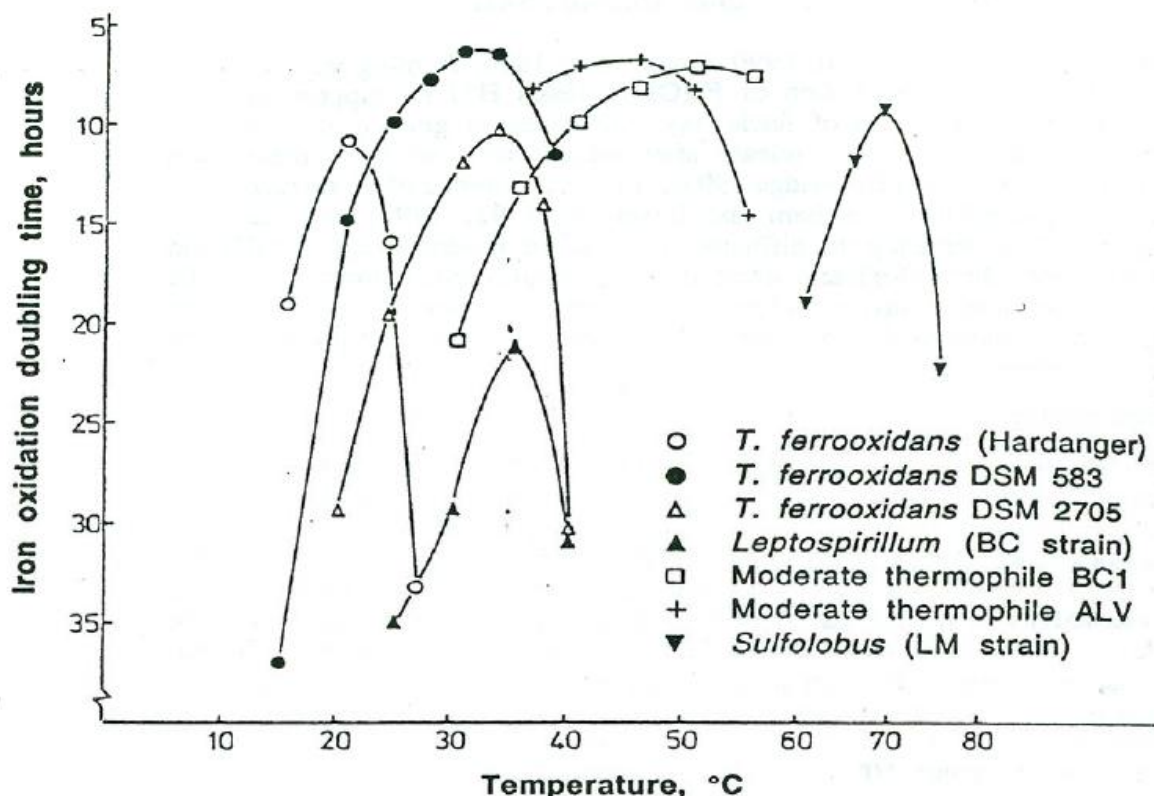
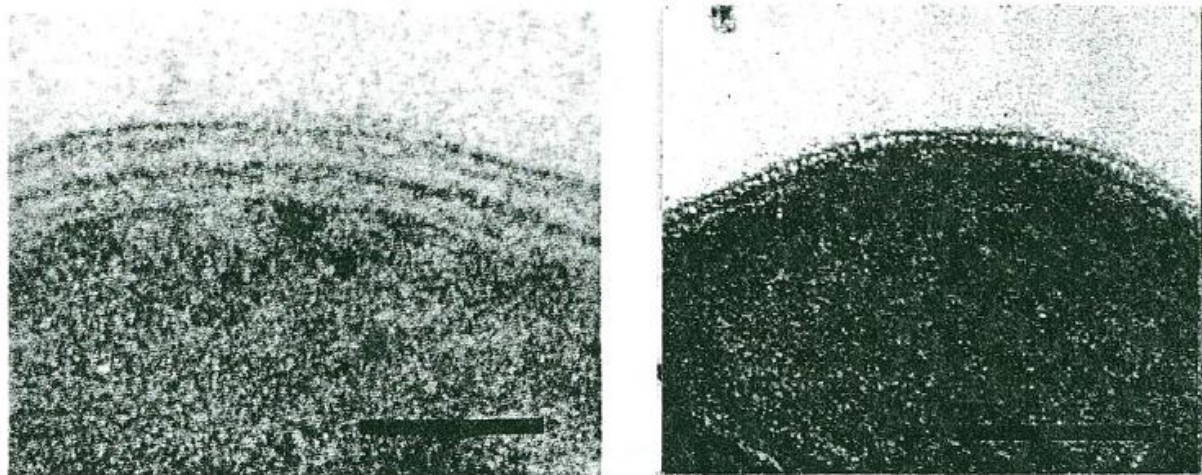


Figure 4. Temperature ranges for several acidophilic iron- and sulfur-oxidizing mesophiles and thermophiles (reproduced with permission from Norris 1990).

There are four genera of Archaea that are aerobic, acidophilic, coccoid in form, and moderate to extreme thermophiles: *Sulfolobus*, *Acidianus*, *Metallosphaera*, and *Sulfurococcus*. They oxidize reduced sulfur compounds and some oxidize  $\text{Fe}^{2+}$  and sulfide minerals. Thermophiles that oxidize iron and sulfur are *Sulfolobus* spp. and *Acidianus* spp. Since the original classification of *Sulfolobus acidocaldarius* by Brock et al. (1972), three species have been recognized: *S. acidocaldarius*, *S. solfataricus*, and *S. brierleyi* (Zillig et al. 1980). *S. brierleyi* has subsequently been reclassified as *Acidianus brierleyi*. *Sulfolobus* oxidizes arsenopyrite as well as pyrite (Ngubane and Baecker 1990). Norris and Parrot (1986) have demonstrated the catalytic effect of *Sulfolobus* on the oxidation of concentrates of pyrite, chalcopyrite, pentlandite, and nickeliferous pyrrhotite at a pH of 2 and 70°C.

As previously described, *Thiobacillus* spp. are Gram-negative Bacteria (Fig. 5) possessing LPS as their outermost cell surface component (Fig. 1, Berry and Murr 1980) while *Sulfolobus* spp. only possess an S-layer external to the cell membrane (Taylor et al. 1982; Fig. 6). An S-layer is a two-dimensional paracrystalline array of protein that functions as a diffusion barrier in cells on which they occur (see by Fortin et al., this volume). For *Sulfolobus* spp. the formation of a nanoenvironment scale diffusion gradient, which is responsible for the growth of *Thiobacillus* spp. on mineral surfaces (Fig. 1), must be accomplished without a periplasm. The same must be true for the mycoplasma, *Thermoplasma acidophilum* (see next section).





**Figure 5 (left).** A stained thin-section of *Thiobacillus ferrooxidans* ATCC 13661 demonstrating the usual gram-negative cell envelope structure in which lipopolysaccharides represent the outermost envelop component. Bar equals 50 nm.

**Figure 6 (right).** A negatively stained *Sulfolobus* spp. demonstrating the crystalline nature of the cell envelope. Bar equals 200 nm.

Based on its ability to produce bacterial endospores, *Thiobacillus thermophilica* (Egorova and Deryugina 1963) is likely a *Sulfobacillus* spp. (Golovacheva and Karavaiko 1978). Gram-positive, acidophilic iron-, sulfur- and mineral-oxidizing bacteria have not received very much attention. The fact that they are not common, or at least rarely described, may relate to the difficulty with which a Gram-positive bacterium can establish an efficient diffusion gradient.

In this review of the literature on bacterial physiology and bacterial interactions with sulfide minerals we have tried to show how *Thiobacillus* spp. function at low pH and regenerate ferric iron from ferrous. At the physiological level, the promotion of sulfide mineral oxidation by bacteria does not require their attachment to surfaces but the close proximity would certainly decrease the diffusion distance to the substrate source thereby maintaining higher concentrations of iron for growth. The overall effect is to enhance the oxidation rate. High temperature (40° to 100°C) oxidation is also enhanced by both Bacteria and Archaea iron- and sulfur-oxidizers but dominated by the Archaea at the higher temperatures.

### MICROBIAL ECOLOGY IN MINE WASTE ENVIRONMENTS

In the introduction to this chapter we mentioned that mine waste environments and acid mine waters support a wide diversity of microbial life. Autotrophic bacteria, heterotrophic bacteria, green algae, fungi, yeasts, mycoplasmas, and amoebae have all been found in acid mine waters. A study by Wichlacz and Unz (1981) reported 37 acidophilic heterotrophs that were isolated and partly characterized from acid mine drainage. Belly and Brock (1974) found numerous heterotrophic bacteria in association with coal refuse piles. In the Rum Jungle mine site, Australia, a mine dump was found to have relatively low numbers of *T. ferrooxidans* and high numbers of acidophilic heterotrophs with no apparent seasonal variations, suggesting a stable population had been established (Goodman et al. 1981). The occurrence of this rich and complex microbial ecology deserves a short discussion.



As Dugan et al. (1970), Johnson et al. (1979), and Ledin and Pedersen (1996) have astutely pointed out, autotrophic thiobacilli will leak or excrete organic compounds that may be utilized by heterotrophic bacteria. Dead autotrophic cells will provide more organic sustenance for heterotrophs. Furthermore, a commensal relationship, possibly even a symbiosis (Battacharyya et al. 1990), exists between heterotrophic and autotrophic bacteria. *T. ferrooxidans* excretes pyruvate during its growth (Schnaitman and Lundgren 1965) and pyruvate can inhibit growth. The heterotroph *Acidiphilium cryptum* uses pyruvate as a carbon and energy source and its presence can enhance the growth of *T. ferrooxidans* (Harrison 1984, Wichlacz and Thompson 1988). *Acidiphilium* spp. are common inhabitants of acid mine drainage (Harrison 1981, Wichlacz et al. 1986, Kishimoto and Tano 1987) and copper dump leaching operations (Groudev and Groudeva 1993) and they are always found in association with *T. ferrooxidans* (Lobos et al. 1986, Battacharyya et al. 1991).

Ehrlich (1996) and Ledin and Pedersen (1996) have noted that several satellite microorganisms live in close association with *T. ferrooxidans*. Some of these are other species of thiobacilli noted previously (see Table 1). Other heterotrophic microorganisms, beside *Acidiphilium* spp., include *Pseudomonas*, *Bacillus*, *Micrococcus*, *Sarcina*, *Crenothrix*, *Microsporium*, *Aerobacter*, *Caulobacter*, and the fungi and yeasts *Acontium*, *Cladosporium*, *Penicillium*, *Trichosporon*, and *Rhodotorula*. Brock (1973) demonstrated that the lower pH limit for the growth of cyanobacteria (formerly "blue-green algae") is about 4 and only green algae can tolerate lower pH conditions. Green algae are common in acid mine waters where they have access to sufficient sunlight and continuous and steady-flowing water. Nordstrom (1977) found several types of green algae at Iron Mountain, California. The most common algae occurring in acid mine waters are *Chlamydomonas*, *Chlorella*, *Ulothrix*, *Chroomonas*, and *Euglena*. Additional acidophilic or acid-tolerant algae, diatoms, and higher plants are described in Kelly (1988).

One of the most unusual microorganisms found in environments where pyrite oxidizes is the *Mycoplasma*, *Thermoplasma acidophilum* (Darland et al. 1970). *Mycoplasma* are like bacteria without cell walls. They are Gram-negative, highly pleomorphic procaryotic organisms, and yet *T. acidophilum* has a pH optimum of 1-2 and a temperature optimum of about 60°C.

The microbial ecology of environments where acid waters have developed from the oxidation of pyrite and other sulfide minerals is poorly understood. Investigations have shown the possibility for a microbial succession from autotrophs to heterotrophs to more complex organisms in a food chain. If the hydrology and climate do not have sudden and large variations (e.g. steady-state flow most of the time) there are better opportunities for growth of a stable and more mature microbial community. Those environments with highly variable and dynamic conditions (high erosion rates, onset of sudden extreme storms) should discourage the development of large and stable populations of microbes.

## SUMMARY AND FUTURE WORK

Any attempts to understand the degradation of pyrite and other sulfide minerals or to ameliorate the water quality hazards associated with mining of sulfide mineral deposits without recognizing the role of microorganisms are likely to fail. Literature on the subject is voluminous. More than a century ago, microbial interactions with sulfur and sulfide minerals have been scientifically investigated. Lithoautotrophs, such as *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, *Leptospirillum ferrooxidans*, and some of their co-habitants such as *Acidiphilium*, *Thermoplasma*, *Bacillus*, *Micrococcus*, *Chlamydo-*



*monas*, *Chlorella*, *Ulothrix*, and *Euglena* are an integral part of the environment where sulfide minerals are oxidizing.

Investigations on the geomicrobiology of sulfide mineral oxidation have clarified the mechanism of microbial catalysis, physiological features that allow them to respire and reproduce in acidic waters, and their microbial ecology. The main role of iron- and sulfur-oxidizing bacteria is in the oxidation of aqueous ferrous to ferric iron. The ferric iron can then rapidly attack the sulfide surface. These rates are comparable so that pyrite will oxidize from the ferric iron as rapidly as the microbes can regenerate the ferric iron. The bacterial catalysis of ferrous iron, however, will be a function of environmental conditions including (but not only) temperature, pH, density or total dissolved solids, chemical composition of the solution, ecology of commensal or predatory species, oxygen concentrations, hydrologic conditions, and mineralogy.

The initiation of pyrite oxidation does not require an elaborate sequence of different geochemical reactions that dominate at different pH ranges (e.g. Kleinmann et al. 1981). *Thiobacillus* spp. employ nanoenvironments to grow on sulfide mineral surfaces, usually as iron-cemented biofilms (Southam et al. 1995). These nanoenvironments can develop thin layers of acidic water that, at first, do not affect the bulk water chemistry. Eventually, with progressive oxidation, the nanoenvironment (nanometer scale) becomes a microenvironment (micrometer scale). Evidence for acidic microenvironments in the presence of circumneutral pH for the bulk water in the subsurface can be found in the occurrence of jarosite in certain soil horizons where the current soil water is neutral (Carson et al. 1982). Jarosite can only form under acidic conditions. The occurrence of isolated cubes of goethite pseudomorphs after pyrite also suggests that pH gradients probably existed near the surface of the oxidizing pyrite but may not have affected the bulk water pH. Of course, the bulk water may have been acidic in the past and currently it is neutral, however, experiments such as those of Goldhaber (1983) and Moses et al. (1987) indicate that a lot of oxidative chemistry with large chemical gradients occurs within nanoscale to microscale dimensions at the mineral-water interface.

Some of the contradictory studies regarding bacterial adsorption on sulfide surfaces and their effect on oxidation rates need to be resolved. The mechanism of *T. ferrooxidans* mineral selectivity is not known, however, it must be conferred by the nature of its lipopolysaccharide. The *Sulfolobus* mineral interface has not been studied, nor has the distribution and importance of *Sulfolobus* and *Sulfobacillus* in mine waste environments been studied. The various factors that can inhibit autotrophic growth in sulfide mineral environments and their relative importance should be clarified.

The oxidation of elemental sulfur by *Thiobacillus* spp. requires the formation of a wetting agent that reduces the surface tension. Jones and Starkey (1961) describe a proteinaceous compound which promotes growth of *T. thiooxidans* on elemental sulfur. This wetting agent is also important for dissolution of the sulfur-rich layer formed by acid leaching of base metals from pyrite and chalcopyrite. In his review of the biochemistry of inorganic sulfur oxidation by chemolithotrophs, Kelly (1982) emphasized the oxidation of thiosulfate and polythionates with almost no information on solid sulfur oxidation. Clearly more studies are needed to elucidate the mechanism of growth on elemental sulfur.

Microbial ecology and phylogeny have undergone a revolution with the application of 16S rRNA sequence analysis. However, defining phylogenetic relationships based on sequence diversity raises new problems that have yet to be addressed, such as how to reconcile conventional taxonomy based on physiology and function with differences based on sequence types. Recent work on thiobacilli have highlighted some of these difficulties (Goebel and Stackebrandt 1994). Although strain-specific molecular probes may not be



possible, the phylogenetic diversity of *Thiobacillus* spp. has been revealed with rRNA sequence analysis (Lane et al. 1985, Goebel and Stackebrandt 1994). This molecular diversity has enabled the use of 16S rRNA probe (Goebel and Stackebrandt 1994) and PCR-mediated 16S rDNA detection (Wulf-Durand et al. 1997) of acidophilic microorganisms from natural environments and bio-leaching operations. Future developments in microbial ecology of acidophilic microorganisms will likely rely on these molecular techniques.

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