Thiobacillus ferrooxidans and the Formation of Acidity in Simulated Coal Mine Environments

Robert L. P. Kleinmann *and* **David A. Crerar**
Department of Geological and Geophysical Sciences
Princeton University
Princeton, New Jersey 08544

Abstract Laboratory simulations have helped resolve several problems concerning the role of bacteria in producing acidic drainage from active and abandoned coal mines. It is well established that the bacterium *Thiobacillus ferrooxidans* oxidizes pyrite in synthetic liquid media and in flooded or agitated experimental simulations of coal mine environments. However, many geologists remain skeptical regarding the role of *T. ferrooxidans* in producing acidity below a near-surface belt of soil water. We have demonstrated that *T. ferrooxidans* is capable of colonizing and acidifying a near-neutral *p*H environment of crushed coal or overburden, without prior establishment of a *p*H-dependent succession of bacteria. We have suggested that *T. ferrooxidans* may accomplish this by direct oxidation of pyrite. We have also shown that *T. ferrooxidans* catalyzes pyrite oxidation in the intermediate belt of the zone of aeration, although only for a limited period of time after rainfall infiltration. *T. ferrooxidans* was not found to be significant in the simulated zone of groundwater saturation.

Acid drainage from active and abandoned coal mines is a major environmental problem. The acidity is produced by the oxidation

Geomicrobiology Journal, Volume 1, Number 4
0149-0451/79/0715-0373 $02.00/0

of iron sulfides (primarily pyrite) to SO_4^{2-} and Fe^{2+}, and by the subsequent oxidation and hydrolysis of Fe^{2+}. The oxidation of the pyrite can proceed by either of two reactions:

1. $FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+$
2. $FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$

Reaction 2 is much faster but only becomes significant when the Fe^{3+}/Fe^{2+} ratio reaches two or more (Ivanov, 1962; Morth et al., 1972). Fe^{3+} is produced by oxidation of Fe^{2+}, the kinetics of which have been well studied (Singer and Stumm, 1968; Stumm and Lee, 1961). Because of the slow rate of Fe^{2+} oxidation at *p*H below 4.5, bacterial catalysis is necessary to achieve a high ferric:ferrous ratio (Singer and Stumm, 1970). *Thiobacillus ferrooxidans,* an acidophilic chemolithotroph, is the bacterium principally involved.

However, many geologists are skeptical concerning the overall importance of bacteria in the reaction system. One objection is that past experiments usually deviated from the natural environment by using artificial culture media and agitation or forced aeration of saturated environments (Morth et al., 1972). Another argument is that *T. ferrooxidans* may occur in a thin surface horizon, well above most pyritic material (Bhappu et al., 1969; Lau et al., 1970). Finally, previous research has shown that *T. ferrooxidans* is incapable of oxidizing significant Fe^{2+} above *p*H 3.5 to 4.0 (Schnaitman et al., 1969; Bruynesteyn and Duncan, 1970). There is thus a problem in how freshly mined material is initially acidified. Walsh and Mitchell (1972b) have given an ecological explanation, proposing that reactions 1 and 2 lower the *p*H to 4.5 and that the bacterium *Metallogenium* then lowers the *p*H from 4.5 to 3.5. At this point, *T. ferrooxidans* takes over, further lowering the *p*H. Others have suggested that reaction (1), given sufficient time, acidifies the material (Singer and Stumm, 1970; Dugan, 1975).

With these problems in mind, we have simulated initial acidification in the belt of soil water and bacterial catalysis of acid formation below this zone. Our experimental conditions were selected so as to answer these questions in a manner that geologists would find acceptable. Pyritic coal was the only source of iron and reaction vessels were neither agitated nor artificially aerated.

Materials and Methods

Reagents

We used a synthetic groundwater formulation (Walsh and Mitchell, 1976) which consisted of 50 ppm $CaCO_3$, 25 ppm $MgSO_4 \cdot 7H_2O$, 5 ppm $MnSO_4$, 2 ppm $(NH_4)_2SO_4$, and 0.25 ppm KH_2PO_4. During different experimental runs, we used pyritic shale and sandstone overburden material from Kentucky (0.6 and 1.6% sulfur, respectively) and pyritic coal from southwestern and northwestern Pennsylvania, eastern Kentucky, and northern West Virginia. In the order mentioned, the coals contained 3, 4, 3.75, and 4.5% sulfur. Before use, all the coal was crushed, sieved to range in size from 0.2 to 2.0 cm, and rinsed repeatedly with distilled water. *T. ferrooxidans* was obtained from the American Type Culture Collection (no. 13728) and was cultured in 9-K medium (Silverman and Lundgren, 1959). The bacterium *Metallogenium* was isolated and cultured as suggested by Walsh and Mitchell (1972a, 1973).

Initial acidification

To test the ability of *T. ferrooxidans* to establish itself in near-neutral *p*H environments, we placed 50 g pyritic coal and 50 ml synthetic groundwater inside an ecological flask (Zajic, 1969) that was then autoclaved. Coal from West Virginia was used in 12 one-week runs; coal from other localities was used in runs of longer duration. For each run, one flask was inoculated with 0.2 ml *T. ferrooxidans* culture, another was inoculated with 0.1 ml *T. ferrooxidans* and 0.1 ml *Metallogenium,* and a third was treated with 0.2 ml sterile 9-K medium. Inoculation was by direct aseptic transfer from culture media and thus resulted in an initial iron concentration in the inoculated and sterile control flasks of approximately 35 ppm. Inoculation of 0.2 ml culture media in 50 ml synthetic groundwater resulted in an initial population of approximately 10^7 cells or approximately 5×10^5 cells/ml. In each flask, the contents were swirled and the *p*H was repeatedly adjusted with H_2SO_4 or

NaOH until stable at the desired *p*H. Hydrolysis of the iron and consequent lowering of the *p*H caused a prolonged adjustment period, but probably removed much of the already low concentration of iron.

When the *p*H was stable, the flask was tilted and carefully brought back to the horizontal plane, leaving most of the crushed material moist but slanted out of the water. The flasks were left in this manner and were not agitated until the end of the run, when each was swirled and a final *p*H reading was taken. A small sample was transferred aseptically to the 9-K medium to test for presence or absence of *T. ferrooxidans*; in the occasional instance where presence or absence did not match initial conditions, the experiment was repeated.

Activity in the zone of aeration

To test the role of *T. ferrooxidans* below the belt of soil water, we performed additional experiments in a large tubulated desiccator. Fifty grams of rinsed, crushed coal was placed in perforated polypropylene beakers that were in turn positioned so as to drain into slightly larger beakers, with the drainage out of contact with the coal (see Fig. 1). Two 0.5-cm-diam tubes inserted through the desiccator lid allowed us to control and limit contact with the atmosphere and to simulate rainfall infiltration. To simulate presence of *T. ferrooxidans* in natural mine infiltration, a total of 15 ml synthetic groundwater with a 0.1 ml inoculum was trickled onto the coal samples each week. The amount of water and of inoculum per application thus varied with the infiltration frequency, so that each application contained approximately 60 ppm Fe and 10^6 cells/ml. Each experiment consisted of at least two identical samples, one of which was treated with known inhibitors of *T. ferrooxidans* to serve as a control. Three inhibitors were compared: sodium lauryl sulfate (Dugan and Lundgren, 1964), mercuric thiocyanate, and molybdenum oxide (Imai et al., 1975).

Most experimental runs were conducted with coal that ranged in size from 0.2 to 2.0 cm. Variation within this size range from one experiment to the next did occur, but pains were taken to ensure

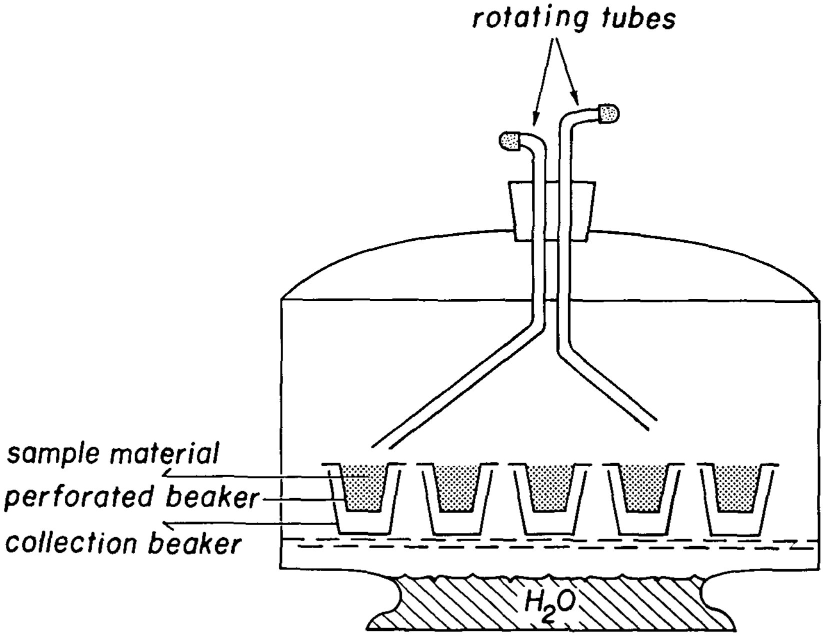

Fig. 1. Experimental set-up for simulation of the intermediate belt of the zone of aeration.

that for any one experiment, particle size distribution was random. To briefly examine the effect of particle size, two runs were conducted with material ranging in size from 0.2 to 2.0 cm and 0.02 to 0.2 cm.

At the start of each run, the double-beaker assembly containing the sample material and inhibitors was autoclaved and placed on a tray inside the desiccator. Water beneath the tray was then gently heated (approximately 45°C) for approximately 15 *min* to generate a humid atmosphere. The initial, and in some cases, only simulated infiltration was then applied by means of the glass tubes.

Up to 11-beaker assemblies could be fitted inside a desiccator, which allowed us to make duplicate runs and to compare different materials under the same experimental conditions. Every 5 to 6 days, the gentle heating was repeated to restore lost humidity.

At the end of each run, the contents of the perforated beaker were emptied into filter paper and approximately 0.5 g coal were removed and added to the 9-K medium to test for the presence of *T. ferrooxidans*. Delayed growth in the 9-K medium was interpreted as a low population of *T. ferrooxidans*; no growth indicated complete inhibition.

Drainage that had collected in the larger beaker was added to the coal and both beakers were rinsed with 100 ml deionized distilled water. This water was in turn added to the coal so that oxidation products that had not drained were included in analyses. The collected filtrate was analyzed for total dissolved iron by atomic absorption spectrophotometry and for acidity. Since acidity in acid mine drainage is in major part due to iron hydrolysis, the sample was boiled for 2 min before titration to oxidize and hydrolyze dissolved iron and to drive off dissolved carbon dioxide (Caruccio et al., 1977). After cooling to room temperature, the sample was titrated with 0.1 N sodium hydroxide solution to a *p*H 8.3 endpoint.

Activity in the zones of saturation

To simulate the role of *T. ferrooxidans* beneath the water table, we modified our desiccator experiment by eliminating the perforated beakers. Fifty grams of coal from southwestern Pennsylvania was placed in each of 6 nonperforated beakers and autoclaved. Synthetic groundwater (90 ml) was then added to each to saturate and completely cover the crushed coal. One pair of samples then received an extra 10 ml synthetic groundwater since it would receive none during the run. Inhibitors were not used; instead, sterile controls were simply not inoculated. Inoculated vessels received 0.2 ml of *T. ferrooxidans* in culture medium yielding an initial cell population of approximately 10^5 cells/ml and an initial iron concentration of approximately 18 ppm. Inoculations during the run consisted of 0.1 ml/15 ml of synthetic groundwater, at the rate of 5 ml every 2 days or 10 ml every 4 days. Sterile controls received synthetic groundwater alone. At the end of the run, contents of each beaker were treated as before except that the beakers were rinsed with the minimum amount of water necessary instead of 100 ml.

Results

Our first series of experiments in the ecological flasks simulated the approximate 30-cm well-oxygenated surface layer of coal refuse piles and abandoned, unreclaimed surface mines, where it is generally agreed that *T. ferrooxidans* is both present and active. *T. ferrooxidans* was consistently able to establish itself at *p*H values greater than 4.0, although the time necessary for acidification varied with sample material from 1 to 3.5 weeks. Similarly, given more time (up to 3 months) the *p*H of sterile material also fell below *p*H 4.0. Generally, the overburden material we tested took the

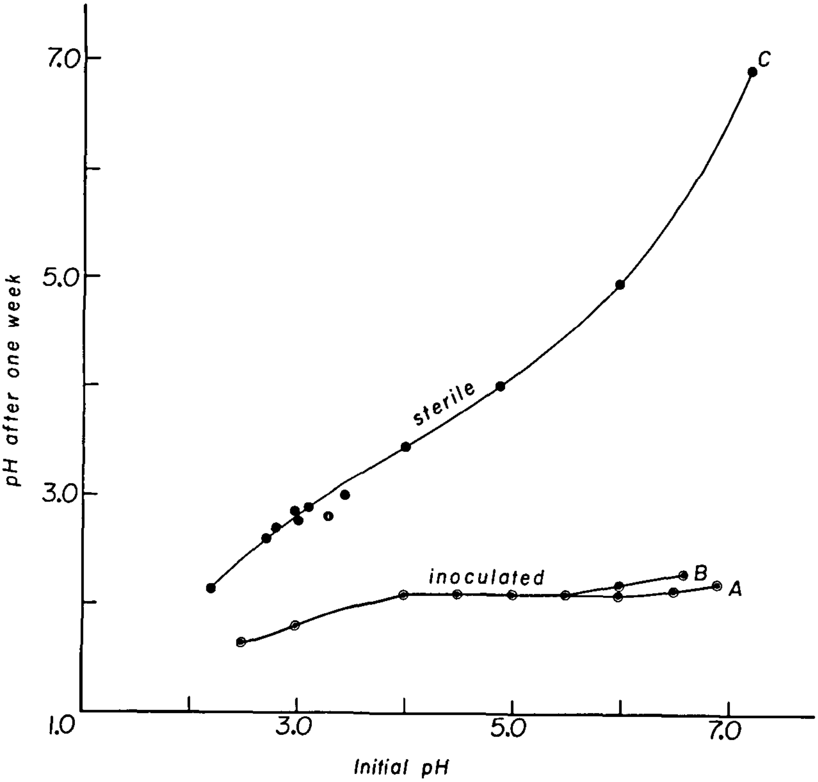

Fig. 2. Effect of bacteria on the lowering of pH in a partially saturated environment. A: Inoculated with 0.2 ml *T. ferrooxidans*; B: inoculated with 0.1 ml each of *T. ferrooxidans* and *Metallogenium*; C: inoculated with sterile 9-K culture media.

longest to go acid in both the sterile and inoculated runs, although this should not be generalized or expected to be true for other overburden material. Fastest, in both sterile and inoculated runs, was our high-sulfur West Virginia coal, which enabled us to make many 1-week runs, the results of which are shown in Fig. 2. Curve A shows that *T. ferrooxidans* is capable of colonizing and acidifying natural pyritic environments at an initial *p*H up to 6.9. By comparison, far less acid was generated in the sterile control experiments (curve C). The similarity of curves A and B indicates that the

Table 1. Effect of *T. ferrooxidans* on acid formation in simulated intermediate belt of the aeration zone in crushed pyritic coal

Duration of run	Infiltration rate		Source of coal sample	0.01 Acidity to pH 8.3 (mg/liter $CaCO_3$)		Iron		Acidity ratio Inoc.: Ster.	Iron ratio Inoc.: Ster.
(days)	Freq.[a] (days)	Amt. (ml)		Inoc.[b]	Ster.[c]	Inoc. (ppm)	Ster. (ppm)		
10	2	4.3	Ky[d]	29.7	14.1	2060	670	4.0	3.4
10	4	8.5	Ky	27.6	12.7	2280	660	3.5	3.1
13	5	10.0	Ky	30.2	17.5	1330	1080	1.7	1.2
9	9	15.0	Ky	12.4	8.9	810	540	1.4	1.5
14	14	15.0	Ky	24.8	17.6	1275	950	1.4	1.3
14	14	15.0	Ky	26.6	17.4	1500	1100	1.5	1.4
21	21	15.0	Ky	26.8	22.0	1360	1220	1.2	1.1
10	2	4.3	SW Pa[e]	9.2	2.7	470	160	3.4	2.9
10	4	8.5	SW Pa	7.7	2.5	370	145	3.1	2.6
10	10	15.0	SW Pa	5.8	4.4	155	120	1.3	1.3
21	21	15.0	SW Pa	6.1	4.5	294	235	1.4	1.3
21	21	15.0	SW Pa	5.1	4.5	271	235	1.1	1.2
13	3	6.0	NW Pa	63.8	31.8	3650	1780	2.0	2.0
13	3	6.0	NW Pa	48.2	23.2	2400	1300	2.1	1.9
13	3	6.0	NW Pa	53.0	23.8	3100	1650	2.2	1.9
13	4	8.0	NW Pa	49.6	27.0	2480	1470	1.8	1.7
13	4	8.0	NW Pa	50.2	27.0	2700	1470	1.9	1.8
13	4	8.0	NW Pa	55.0	32.2	2930	1800	1.7	1.6
12	4	8.0	NW Pa	30.5	18.7	1820	1240	1.6	1.5

[a] Freq. = frequency.
[b] Inoc. = inoculated with viable *T. ferrooxidans.*
[c] Ster. = sterile control.
[d] Ky = Kentucky.
[e] SW Pa = southwest Pennsylvania.

presence of *Metallogenium* was not a significant factor in the initial acidification process.

Our second series of experiments indicated that *T. ferrooxidans* consistently survives in the simulated zone of aeration unless an inhibitory agent is added. Table 1 shows the effect of run duration and infiltration frequency on acidity and iron concentrations for coal from Pennsylvania and eastern Kentucky. Acidity is expressed as the equivalent amount of $CaCO_3$ in milligrams per liter required to raise the *p*H to 8.3, as recommended in Standard Methods (American Public Health Association, 1975). We have found that inoculated:sterile ratios for acidity and iron allow comparison between different experimental runs by minimizing the effect of slight variations in particle size distributions and other experimental batch conditions. Although the iron and acidity values fluctuated from one experiment to the next, the iron and acidity ratios remained relatively uniform.

By comparing inoculated:sterile acidity and iron ratios of Table 1 for runs in which the simulated rainfall infiltration occurred at greater than 4-day intervals, it can be seen that *T. ferrooxidans* did not play a major role in acid formation. Also, the population of *T. ferrooxidans* was low in such runs, as evidenced by delayed growth after transfer of material to the 9-K medium. This is in contrast to runs with more frequent infiltration. Thus, it appears that *T. ferrooxidans* is highly active for approximately 3 to 4 days after rainfall infiltration, increasing acidity values 2 to 4 times over sterile controls, with sample material differences causing the variation.

It is well known that acid mine drainage can be reduced by flooding a mine and thereby decreasing the amount of available oxygen. Our third series of experiments took a brief look at the role of *T. ferrooxidans* in such a saturated environment. Acid production was in general much reduced, as can be seen by comparing the southwestern Pennsylvania results of Table 1 with Table 2. The presence or absence of *T. ferrooxidans* was probably not a significant factor, as the observed variations are small enough to be caused by the inoculated synthetic groundwater.

The importance of grain size upon the rate of acid formation has

Table 2. Effect of *T. ferrooxidans* on acidity and iron concentration in simulated zone of saturation[a]

Rate of infiltration		Acidity to pH 8.3 ($CaCO_3$, mg/liter)		Iron concentration	
Frequency (days)	Amount (ml)	Inoculated	Sterile	Inoculated (ppm)	Sterile (ppm)
2	5	85	40	70	43
4	10	75	40	55	42
10	—	55	40	47	38

[a] Crushed pyritic coal was from southwestern Pennsylvania. The run lasted 10 days.

been discussed by many researchers (Ehrlich and Fox, 1967; Torma et al., 1972; Torma et al., 1974). We looked briefly at how decreasing particle size by an order of magnitude would affect acidity in our simulated environments. In this case we discovered that there was a fairly consistent doubling of acid production and iron concentrations in both sterile and inoculated experimental runs. The results are shown in Table 3.

Table 3. Effect of particle size on acidity and iron concentration in simulated intermediate belt of the aeration zone in crushed pyritic coal[a]

Duration of run (days)	Source of sample	Experimental condition	0.01 Acidity to pH 8.3 ($CaCO_3$, mg/liter) I	II	I:II	Concentration of iron (ppm) I	II	I:II
10	W. Va	Inoc.[b]	23.6	12.4	1.9	925	500	1.9
10	W. Va	Sterile	15.6	7.25	2.5	820	370	2.2
21	Ky	Inoc.	9.9	5.1	1.9	563	271	2.1
21	Ky	Inoc.	10.1	5.0	2.0	445	203	2.2
21	Ky	Sterile	8.4	4.5	1.9	437	235	1.9

[a] Material ranged in size from 0.02 to 0.2 cm for size range I and from 0.2 to 20 cm for size range II. The samples were from West Virginia and Kentucky.

[b] Inoc. = Inoculated with *T. ferrooxidans*.

Discussion

It should first be pointed out that survival of *T. ferrooxidans* is not limited by its ability to oxidize Fe^{2+}. *T. ferrooxidans* can also produce acidity by the oxidation of compounds such as thiosulfate and elemental sulfur at *p*H values up to 6.0 (Landesman et al., 1966) and can survive even higher *p*H values. Ehrlich (1962) has demonstrated tolerance up to *p*H 6.98 in synthetic buffered media. *T. ferrooxidans* has been isolated from a Finnish river at *p*H 6.1 (Niemela and Tuovinen, 1972), and we have found it in a mine drainage-contaminated stream in southwestern Pennsylvania at *p*H 6.4. Such presence does not imply acid-producing activity, but it does indicate that the bacteria are available should a suitable environment appear.

The question is whether *T. ferrooxidans* is capable of acidifying and colonizing pyritic coal at a *p*H above 4 where it apparently does not oxidize significant amounts of Fe^{2+}. Our experiments indicate that *T. ferrooxidans* can in fact establish itself at a relatively rapid rate and that, given the condition of minimal water flow, the precursor *Metallogenium* is not necessary.

In these experiments, initial ferrous and ferric iron was limited to that included in the inoculum. Because of the rapid rate of ferric hydrolysis (Stumm and Morgan, 1970) and the extremely low solubility of $Fe(OH)_3$ at the initial pH values, Fe^{3+} concentration in the inoculated and sterile vessels was negligible ($<10^{-8}$ M). Thus, initial acidification by ferric iron in the inoculum can be discounted. Some Fe^{2+}, which did not oxidize during the initial period of *p*H adjustment, would still have been present in solution. However, since the concentrations involved are low and were present in the sterile control flasks as well as inoculated flasks, the effect of ferrous iron in the inoculum can also be disregarded.

Walsh and Mitchell (1972a, b; 1973) have suggested that *Metallogenium* normally precedes *T. ferrooxidans* in a *p*H-controlled succession of acid-producing bacteria. The discrepancy between our results and theirs probably stems from two factors: We did not enrich our synthetic groundwater with ferrous iron and we did not flood our samples with water. The latter factor suggests that *T. ferrooxidans* was able to establish acidic semi-isolated microenvironments and then spread once the local *p*H was lowered to roughly 4.0. Since ferrous iron was not added to this experiment except as part of the small inoculum, direct oxidation (Silverman, 1967) of the pyrite by *T. ferrooxidans* (as opposed to the indirect oxidation mechanism described in the introduction) probably initiated the acid-generating mechanism.

Direct oxidation of pyrite by *T. ferrooxidans* has been demonstrated by Duncan et al. (1967) and by Beck and Brown (1968). Because indirect oxidation of pyrite by *T. ferrooxidans* is approximately 7 times faster than direct oxidation (Walsh, 1976), the direct mechanism has not been considered a significant aspect of the acid mine drainage problem. But if indeed curve A of Figure 2 is controlled by direct bacterial oxidation, it would appear that it is faster than reaction 1 (curve C) as an initiator mechanism for reaction 2, providing both a low *p*H environment and a supply of ferrous ions. Reaction 1, unaided by bacteria, requires a longer time to acidify an environment to *p*H 4.0 and, since every rainfall interferes with the slow buildup of acid, the reaction rate is important. Of course, if local water is already high in ferrous iron, *Metallogenium* may provide a faster mechanism of acidifying freshly mined spoil.

We next looked at the question of whether *T. ferrooxidans* is important beneath the well-oxygenated surface layer. While this has been assumed to be true by some, important objections have been raised. Because bacterial oxidation of Fe^{2+} requires as much oxygen as does inorganic oxidation of pyrite (reaction 1), anything more than a thin film of water should significantly reduce pyrite oxidation by slowing oxygen diffusion (Lau et al., 1970). But as pointed out by Lau et al. (1970), accumulation of oxidation products in such small volumes of water might lower the local *p*H beyond even the *p*H 1 tolerance of *T. ferrooxidans* (Tuovinen and Kelly, 1974). Thus, there is the question of whether *T. ferrooxidans* is important either below the water table or in the intermediate belt of the zone of aeration.

Our results indicate that the arguments of Lau et al. are valid but that periodic flushing of the oxidation products by infiltration allows *T. ferrooxidans* to remain significant as an acid producer in the zone of aeration. We have also shown that *T. ferrooxidans* survives extended periods between rainwater infiltration. Of course, it is also likely that *T. ferrooxidans* will be washed down from the surface zone with each successive rain. In contrast, the results of our third experiment indicate that *T. ferrooxidans* is unimportant beneath the water table.

Finally, it was noted that sodium lauryl sulfate at 10 to 20 ppm was bacteriostatic rather than bactericidal in our simulated environments. At such concentrations, its effect was similar to that of extended periods without flushing; that is, *T. ferrooxidans* survived at a low population density but its role in acid production was negligible. At concentrations of 25 ppm and above, sodium lauryl sulfate was bactericidal.

Results from laboratory simulations may not truly reflect the natural mine environment. We have attempted to keep our experiments as simple as possible while maintaining necessary environmental constraints. Our work indicates that *T. ferrooxidans* is important at lower depths and higher *p*H values than previously reported. The experiments may also explain why attempts to reduce acid formation by inhibition of *T. ferrooxidans* at the mine site have failed in the past (Baker and Wilshire, 1970; Shearer et al., 1970). Due to the ease with which *T. ferrooxidans* can repopulate an area, bactericidal measures must be repeated with every rainfall. If this can be done inexpensively, acid production could be cut significantly. We are therefore in the process of developing a technique to gradually and continuously release, over a period of several years, a low level of inhibitory agent on the mine site.

Acknowledgments

We thank G. Geidel and F. Carruccio for encouragement and for supplying some sample material. A. G. Fischer and J. L. Means kindly reviewed the manuscript.

References

American Public Health Association. 1975. Standard methods for the examination of water and wastewater. American Public Health Assoc., Washington, D.C.

Baker, R. A., and A. G. Wilshire. 1970. Evaluation of *Caulobacter* as an inhibitor of acid mine drainage formation. Report to the U.S.

Dept. of the Interior. Fed. Water Quality Administration, Washington, D.C.

Beck, J. V., and D. G. Brown. 1968. Direct sulfide oxidation in the solubilization of sulfide ores by *Thiobacillus ferrooxidans*. J. Bacteriol. **96**:1433–1434.

Bhappu, R. B., P. H. Johnson, J. A. Brierly, and D. H. Reynolds. 1969. Theoretical and practical studies on dump leaching. Trans. Am. Inst. Min. Eng. **244**:307–320.

Bruynesteyn, A., and D. W. Duncan. 1970. Microbiological leaching of sulphide concentrates. Can. Metall. Q. **10**:57–63.

Caruccio, F. T., J. C. Ferm, J. Horne, G. Geidel, and B. Baganz. 1977. Paleoenvironment of coal and its relation to drainage quality. Environmental Protection Agency, Washington, D.C.

Dugan, P. R. 1975. Bacterial ecology of strip mine areas and its relationship to the production of acidic mine drainage. Ohio J. Sci. **75**:266–278.

Dugan, P. R., and D. G. Lundgren. 1964. Acid production by *Ferrobacillus ferrooxidans* and its relation to water pollution. Dev. Ind. Microbiol. **5**:250–257.

Duncan, D. W., J. Landesman, and C. C. Walden. 1967. Role of *Thiobacillus ferrooxidans* in the oxidation of sulfide minerals. Can. J. Microbiol. **13**:397–403.

Ehrlich, H. L. 1962. Observation on microbial association with some mineral sulfides, p. 153–168. *In* M. L. Jensen (ed.), Biogeochemistry of sulfur isotopes. Yale University, New Haven.

Ehrlich, H. L., and S. I. Fox. 1967. Environmental effects on bacterial copper extraction from low-grade copper sulfide ores. Biotechnol. Bioeng. **9**:471–485.

Imai, K., T. Sugio, T. Tsuchida, and T. Tano. 1975. Effect of heavy metal ions on the growth and iron-oxidizing ability of *T. ferrooxidans*. Agric. Biol. Chem. **39**:1394–1454.

Ivanov, V. I. 1962. Effect of some factors on iron oxidation by cultures of *Thiobacillus ferrooxidans*. Microbiology (Engl. Transl.) **31**: 645–648.

Landesman, J., D. W. Duncan, and C. C. Walden. 1966. Oxidation of inorganic sulfur compounds by washed cell suspensions of *Thiobacillus ferrooxidans*. Can. J. Microbiol. **12**:957–964.

Lau, C. M., K. S. Shumate, and E. E. Smith. 1970. The role of bacteria in the pyrite oxidation kinetics, p. 114–119. *In* Third symposium on coal mine drainage research. Mellon Institute, Pittsburgh.

Morth, A. E., E. E. Smith, and K. S. Shumate. 1972. Pyritic systems: A mathematical model. Environmental Protection Agency, Washington, D. C.

Niemela, S. I., and O. H. Tuovinen. 1972. Acidophilic thiobacilli in the river Sirppujoki. J. Gen. Microbiol. **73**:23–28.

Ohio State University. 1970. Sulfide to sulfate reaction mechanism. Fed. Water Pollution Control Administration, Washington, D. C.

Schnaitman, C. A., M. S. Korczynski, and D. G. Lundgren. 1969. Kinetic studies of iron oxidation by whole cells of *Ferrobacillus ferrooxidans*. J. Bacteriol. **99**:552–557.

Shearer, R. E., J. W. Mausteller, W. A. Everson, and R. F. Zimmer. 1970. Characteristics of viable anti-bacterial agents used to inhibit acid-producing bacteria in mine wastes, p. 188–198. *In* Third symposium on coal mine drainage research. Mellon Institute, Pittsburgh.

Silverman, M. P. 1967. Mechanism of bacterial pyrite oxidation. J. Bacteriol. **94**:1046–1051.

Silverman, M. P., and D. G. Lundgren. 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. 1. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. **70**:642–647.

Singer, P. C., and W. Stumm. 1968. Kinetics of the oxidation of ferrous iron, p. 12–34. *In* Second symposium on coal mine drainage research. Mellon Institute, Pittsburgh.

Singer, P. C., and W. Stumm. 1970. Acid mine drainage: the rate determining step. Science **167**:1121–1123.

Stumm, W., and G. F. Lee. 1961. Oxygenation of ferrous iron. Ind. Eng. Chem. **53**:143–146.

Stumm, W., and J. J. Morgan. 1970. Aquatic chemistry, p. 538–540. Wiley-Interscience, New York.

Torma, A. E., G. LeGault, D. Kougiomoutzakis, and R. Ouellet. 1974. Kinetics of bio-oxidation of metal sulphides. J. Chem. Eng. **52**: 515–517.

Torma, A. E., C. C. Walden, D. W. Duncan, and R. M. R. Branion. 1972. The effect of carbon dioxide and particle surface area on the microbiological leaching of a zinc sulfide concentrate. Biotechnol. Bioeng. **14**:777–786.

Tuovinen, O. H., and D. P. Kelly. 1974. Use of micro-organisms for the recovery of metals. Int. Metall. Rev. **19**:21–31.

Walsh, F. 1978. Biological control in mine drainage, p. 377–391. *In* R. Mitchell (ed.), Water pollution microbiology, Vol. 2. Wiley-Interscience, New York.

Walsh, F., and R. Mitchell. 1972. An acid-tolerant iron-oxidizing *Metallogenium*. J. Gen. Microbiol. **72**:369–376.

Walsh, F., and R. Mitchell. 1972. A pH-dependent succession of iron bacteria. Environ. Sci. Technol. **6**:809–812.

Walsh, F., and R. Mitchell. 1973. Differentiation between *Gallionella* and *Metallogenium*. Arch. Microbiol. **90**:19–25.

Walsh, F., and R. Mitchell. 1975. Mine drainage pollution reduction by inhibition of iron bacteria. Water Res. **9**:525–528.

Zajic, J. E. 1969. Microbial biogeochemistry, p. 33. Academic Press, New York.