MICROBIOLOGICAL STUDIES OF SITES RECLAIMED WITH BACTERTICICDES

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The geochemical oxidation of pyrite may be characterized by the following reactions:

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

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

$$Fe^{3+} + 3H_2O = Fe(OH)_{3(s)} + 3H^+ \quad (3)$$

$$FeS_{2(s)} + 14Fe^{3+} + 8H_2O = 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (4)$$

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After pyrite is oxidized (equation 1), ferric iron is produced very slowly (equation 2). This second reaction, however, can be accelerated by microbial catalysis to increase the overall rate of the oxidation of ferrous iron. With the generation of ferric iron, insoluble ferric hydroxide can then form under proper elevated pH conditions (equation 3). Ferric iron can also be reduced by pyrite, resulting in the generation of more acidity and ferrous iron (equation 4).

Bacteria play an important role in equations 1 and 2, causing reaction rates to increase significantly.

Iron-oxidizing bacteria, Thiobacillus ferrooxidans, are autotrophic microorganisms which obtain their energy from the oxidation of pyrite and ferrous iron. These organisms generate acidity most actively at pH 2.0-5.0. They are indigenous to pyritic mine soils and thrive in an environment where they have access to sulfides, O_2 , water, a carbon source and acidic conditions.

Soil bacteria also play an important role in plant growth. Plants exude organic substances through their roots to the soil in order to support a healthy population of heterotrophic bacteria. In return, heterotrophs support plant life in several ways:

- Microbial cells produce organic matter (mainly polysaccharides) that helps the soil structure

and increases its water-holding capacity.

- Heterotrophs are active in the recycling of plant nutrients. Dead plants decompose, their nutrients return to the soil and become available for reuse. A portion of the CO_2 released is also utilized by the plants.
- Microorganisms in the soil provide plant nutrients by the cycling of minerals. Some heterotrophs have the ability to fix N_2 from the air; one type is symbiotic with legumes, another is free to supply all plants with fixed nitrogen. In addition, the phosphorous cycle in soils is highly dependent on microbial activity.
- Soil bacteria produce several plant growth factors and regulators that stimulate growth.
- Heterotrophs keep a good, healthy biological balance by competing with pathogenic microorganisms and preventing plant diseases.

Enumerations of the heterotrophic bacteria and the T. ferrooxidans of three coal refuse piles were done to determine the effect of bactericide treatments. The test relies on the ability of many heterotrophic soil microorganisms to grow in a semi-rich growth medium, diluted tryptic soy broth *(TSB),* under aerobic conditions. For T. ferrooxidans, a 9K medium was used. This test, like any other test for total live microbiological enumeration of natural populations, does not give the absolute total number of microorganisms. The validity of this test is in its ability to give a good estimate of the total numbers, and to compare one site to another.

Three sites were studied. Site #1 was an abandoned refuse disposal area in West Virginia covering about 25 acres. Site #2 was an abandoned refuse disposal area-in eastern Ohio covering about 5 acres. Site #3 was a coal company's refuse disposal area in southern Ohio covering approximately 4.5 acres.

Each site was reclaimed using standard reclamation techniques (soil cover, lime, fertilizer, seed, and mulch) over the entire area with bactericide treatments added to certain segments. Thus, treated and control areas were established. Changes in acidity, solubilization of metals and microbial populations were monitored in both treated and control areas.

Site #1: West Virginia Refuse Disposal Area

Site #1 was divided into five areas (Figure 1). Four received bactericide treatment, and one was used as a control. Also, the bactericide treatments were done on the site at different times (Table 1). Area E was not used in this study because of topsoil thickness variations and topography problems that would bias the results.

Table 1. treatment Dates

Area	Treatment Date
A	Sept. 1985
B	Nov. 1984 and May 1985
C	Nov. 1984
D	Control, no treatment
E	May 1985

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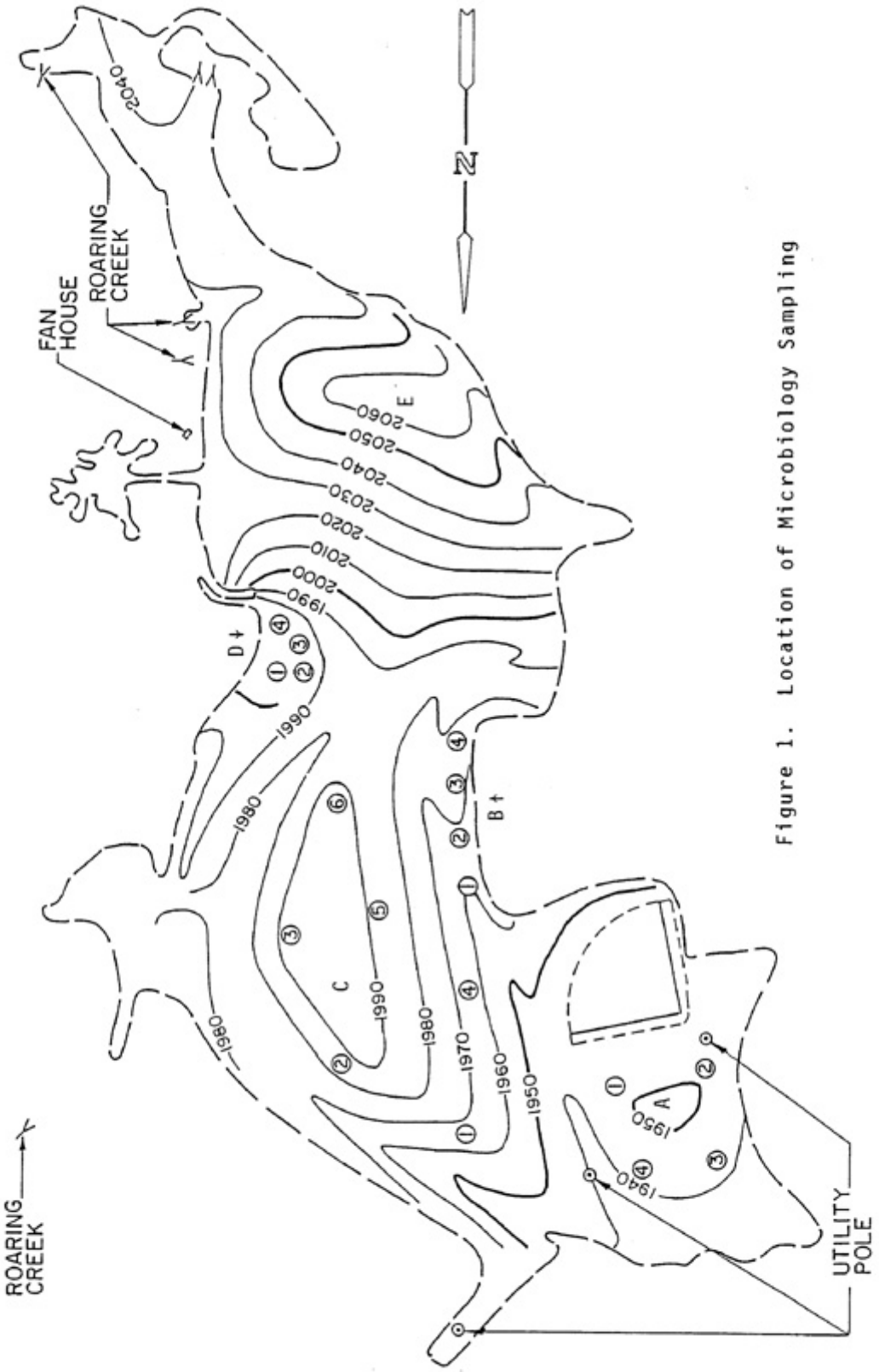

Figure 1. Location of Microbiology Sampling

Each area of the site was divided into four to six blocks. In some cases, rocky soil or inadequate cover soil made samples wholly or partially unusable. All usable samples from the same block were blended to produce one composite sample of a given level of soil per block. Blending or compositing several soil samples in this way helps produce a much more representative picture of microbial

activity in each of several blocks in each area. The cores were divided into two levels of interest. Level I represented the lower part of the cover soil which was directly above the refuse material (soil). Level 2 represented the upper level of the refuse material (refuse).

Samples were blended in 1/3X mineral salt medium BHB (Horowitz et. al., 1985) and brought to the lab for further processing. Enumeration was done using the most probable number method MPN) in which three rows of wells for growth were used. The samples were further diluted-in a thiobacilli specific growth medium (9K) in 10:1 ratios. Results were evaluated for the MPN of the microorganisms in each block-level of an area (Amer. Public Health Assoc., 1985). The MPN values were converted to estimates of MPN microbes per gram of dry soil used in each block-level evaluated for each area.

Three statistical analysis methods were used. Student's t-test was used to compare one set of results with another. Analysis of variance and multiple regression were employed for more involved situations. Each technique provided an estimate of error.

Average MPN values for T. ferrooxidans in Areas A through D in the soil and the refuse are shown in Table 2. Area D, the untreated area, has far more T. ferrooxidans present than any other area. While Areas A-C are not significantly different from each other, the difference between Areas B and C approach this condition. Note that Area B was treated twice, on 11/84 and on 5/85, while Area C was treated only once on 11/84 (Table 1)

Table 2. Average Values of T. ferrooxidans Population Desity for Soil and Refuse in Areas A - D

	T. ferrooxidans (MPN/g)			
Samples	Area A	Area B	Area C	Area D
Soil	0.22	0.11	0.11	28.0
Refuse	6.80	0.40	18.80	1925.0

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Ratio analysis of T. ferrooxidans data from composited soil samples (Table 3) indicates that 401 times as many T. ferrooxidans remained in the untreated area compared to the treated area in the refuse. This low population density of T. ferrooxidans in the treated area indicates the effectiveness of the bactericide treatment; therefore, the ability of the treated area to generate acid has been significantly reduced.

Table 3. Ratio of T. ferrooxidans in Composited Samples from Treated and Untreated Areas

	T. ferrooxidans (MPN/g)		Ratio
Samples	Treated	Untreated	Treated/Untreated
Soil	0.13	28	1:215
Refuse	4.80	1925	1:401

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Samples	T. ferrooxidans (MPN/g) Treated	Untreated	Ratio Treated/Untreated
Soil	0.13	28	1:215
Refuse	4.80	1925	1:401

Site #2: Eastern Ohio Refuse Disposal Area

The site (Figure 2) was divided into approximately two halves. After grading and contouring, one half of the site was treated with bactericide in spray and long-term, controlled release pellet form; the other half was not treated and was left to serve as a control.

The treated side was covered immediately following the bactericide application with an average of 6 inches of topsoiling material; the untreated or control side was covered with an average of 7 inches of topsoil. The site was then limed, fertilized, seeded, and mulched using standard reclamation practices.

Three test levels were established: Level 1 (topsoil) ranged from 4.5 to 1.5 inches above the soil cover-refuse interface; Level 2 (soil-refuse) from 1.5 inches above to 1.5 inches below the interface; and Level 3 (refuse) from 1.5 to 4.5 inches below the soil cover-refuse interface.

Soil samples were taken with a soil corer from both treated and untreated areas of the site. The treated and untreated areas were sampled according to a grid pattern. Each area was divided into four blocks, avoiding the border zone between treated and untreated parts. In addition, peripheral areas were avoided.

Five samples were taken from each block, totaling 20 samples per area. Each block was combined to yield a total of four composite samples per area. This reduced the variability between samples that originated from the same area, reduced the number of dilutions needed for testing, and enhanced the statistical significance of the test.

Each sample was used for both heterotrophic and T. ferrooxidans counts. Heterotrophic counts were taken for the topsoil and the soil-refuse zones only. It was shown in an earlier study (Horowitz et. al., 1985) that very few heterotrophic bacteria were present in the refuse. It is assumed that these bacteria have reached this zone during the coring and do not reside in the refuse naturally. The T. ferrooxidans counts were taken only from the soil-refuse and the refuse zones, because it was assumed that T. ferrooxidans would not be present in large numbers in the topsoil.

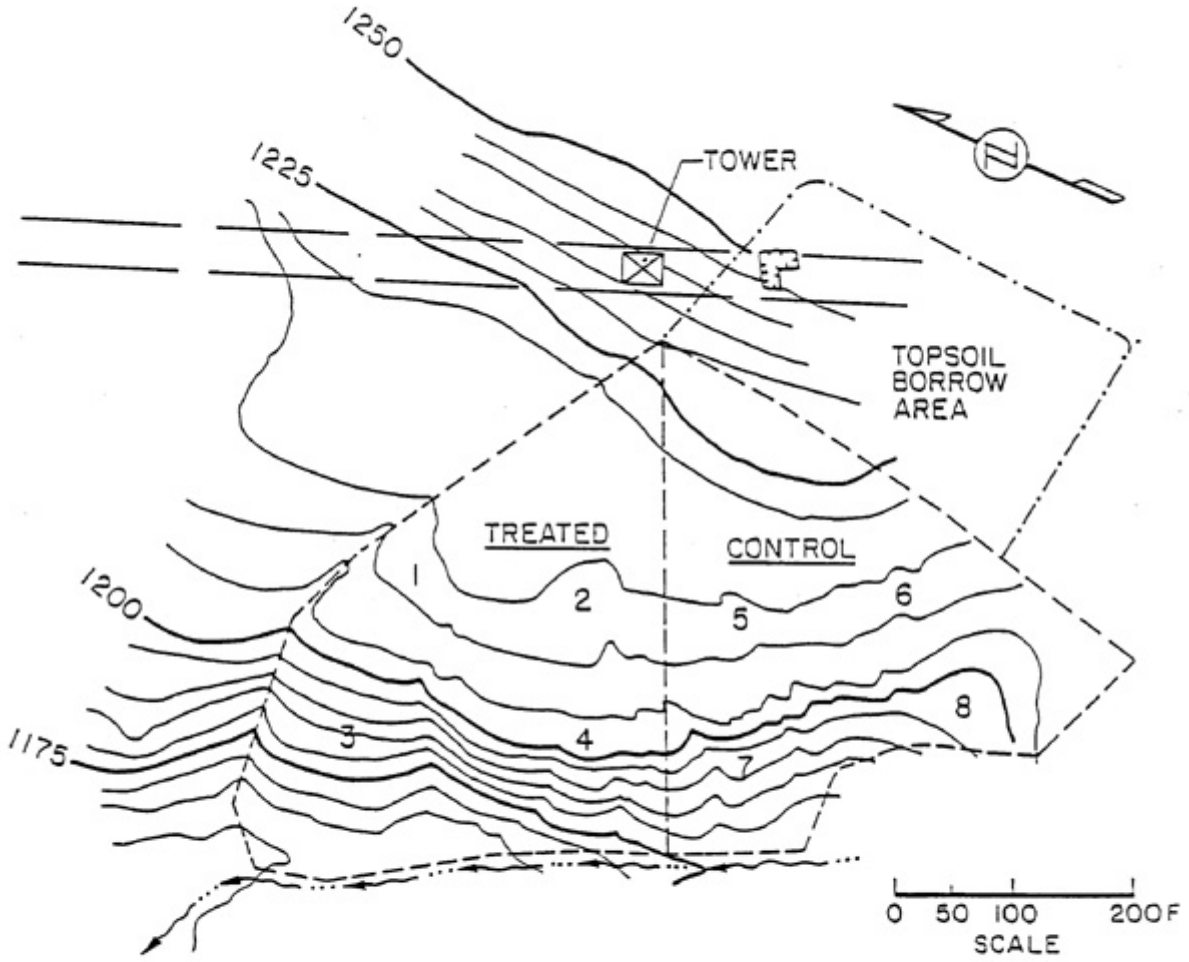

Figure 2. Site Map Showing Locations of Sampling Blocks for Microbiology Study

Two studies were conducted. The first was a study of the growth of heterotrophic microorganisms in the topsoil of the bactericide-treated refuse versus the topsoil of the untreated refuse. This comparison was conducted for composite samples of the topsoil and for composite samples of the soil-refuse zones. The second study was a ratio analysis of T. ferrooxidans to total heterotrophic bacteria. Tests of significance were made using the same statistical methods as previously detailed for Site #1.

Table 4. Ratio of Heterotrophic Bacteria in Composited -Samples from Treated and Untreated Areas

	Heterotrophs (MPN/g)		Ratio
Samples	Treated	Untreated	Treated/Untreated
Topsoil	16,582,000	384,326	43:1
Soil-Refuse	457,846	6,714	68:1
Combined	17,039,846	391,040	44:1

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The results of the heterotroph growth study (Table 4) indicate that the topsoil from the treated area clearly has more heterotrophic microoganisms than topsoil from the untreated area. The soil refuse data indicate a strong trend toward heterotrophic development. Further, the topsoil from the treated area has more total microorganisms than the topsoil from the untreated area.

Table 5. Ratios of T. ferrooxidans to Heterotrophic, Bacteria in the Soil-Refuse Zone

Area	T. ferrooxidans (MPN/g)	Heterotrophs (MPN/g)	Ratio
Treated	158,154	457,846	1:3
Untreated	6,782	6,714	1:1

Table 5. Ratios of T. ferrooxidans to Heterotrophic Bacteria in the Soil-Refuse Zone

Area	T. ferrooxidans (MPN/g)	Heterotrophs (MPN/g)	Ratio
Treated	158,154	457,846	1:3
Untreated	6,782	6,714	1:1

The ratio of T. ferrooxidans to heterotrophs in the soil-refuse zone is much lower in the treated area when compared to the ratio in the untreated area (Table 5). The treated area has a denser vegetative cover and is producing much less acidity than the untreated area, although both have approximately the same amount of cover soil.

Site #3: Southern Ohio Refuse - Disposal Area

This site was divided into two areas. Area A was the untreated or control area. Area B was treated with bactericide spray and long-term, controlled-release pellets.

Three core samples were drawn from each area. Each sample "as divided into three levels. Level 1 (topsoil) included only the top or surface soil, Level 2 (soil-refuse) contained the interface between surface soil and the refuse layer, and Level 3 (refuse) contained refuse material only. The three samples from each area were composited for each level.

The depth of cover soil was measured for each core sample to make it possible to determine whether differences in cover soil depth have any effect on the microbial population. The weight of each composite sample was determined in order to normalize the MPN numbers and relate them to the dry weight of the soil. Each MPN number was expressed per gram of dry weight soil.

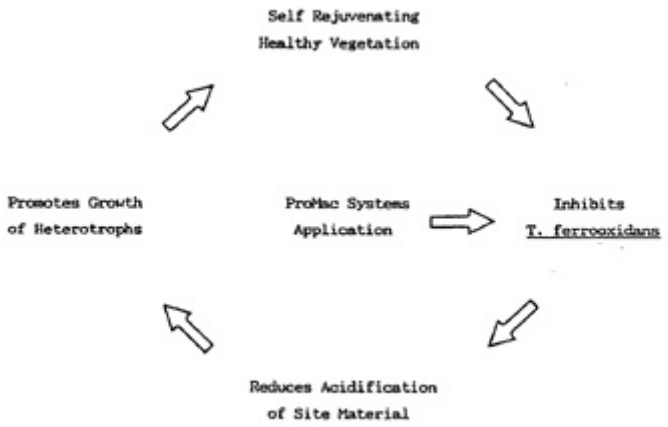
Laboratory procedures and statistical analysis methods were discussed for Site #1.

A study of the heterotrophs in the soil-refuse zone shows significantly more heterotrophs in the treated area when compared to the untreated area. Ratio analysis (treated:untreated) shows that there is 40 times more heterotrophic bacteria in the treated area (Figure 3).

In the refuse zone, the population of T. ferrooxidans in the treated area is far less than in the control or untreated area. The ratio of treated:untreated areas shows 33 times more T. ferrooxidans in the untreated area (Figure 4). Both enumeration studies indicate the bactericide treatment was effective.

Conclusions

1. At Site #1, two major improvements were noted: (A) The ProMac systems treatment had greatly reduced the population of T. ferrooxidans in refuse which will result in a large decrease in the amount of acidity being produced, and (B) The site has been stabilized with a luxuriant growth of vegetation indicating the ProMac systems treatment has already started the site back on its road to full recovery by providing the first part of nature's biological cycle.



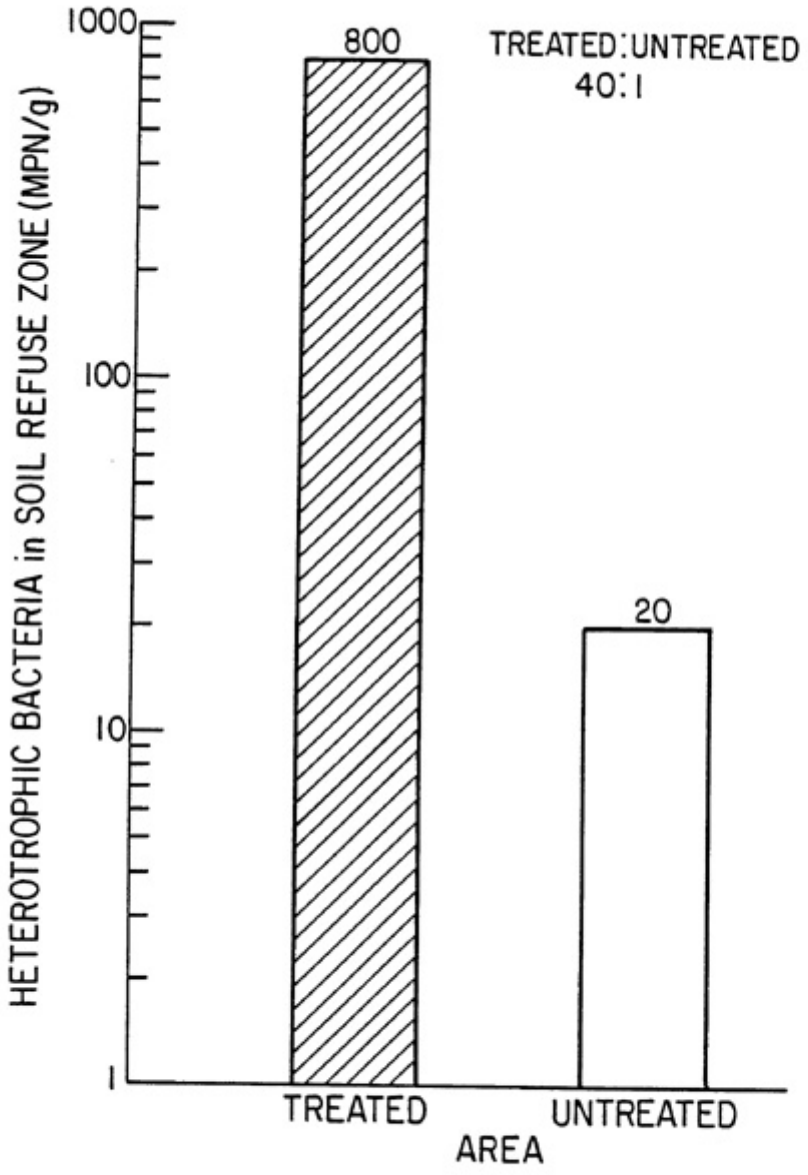


Figure 3. Ratio Analysis (Treated and Untreated) of Heterotrophic Bacteria in the Soil-Refuse Zone

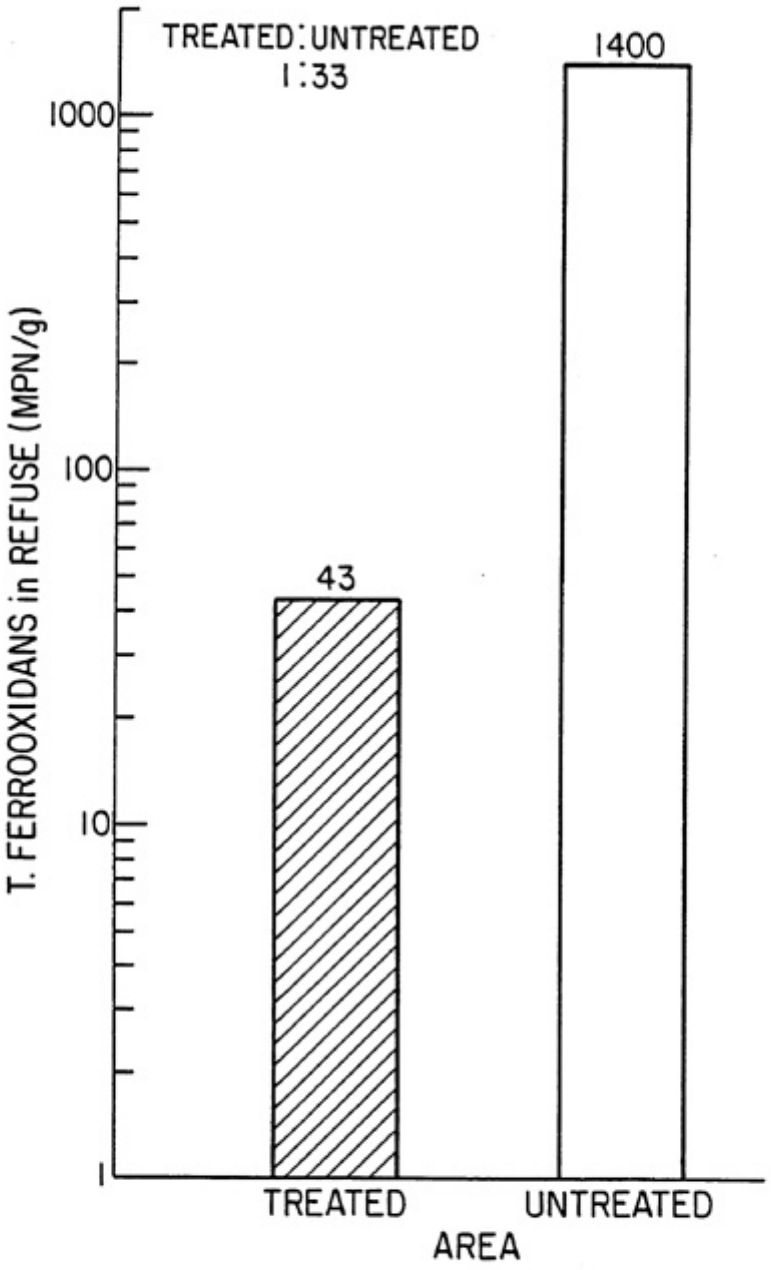


Figure 4. Ratio Analysis (Treated and Untreated) of T. ferrooxidans in the Refuse Zone

2. Topsoil and soil-refuse zones of bactericide-treated area showed higher numbers of heterotrophic microoganisms in comparison to the untreated area at Site #2.
3. Counts of heterotrophic microoganisms correlated with the depth of the sample, with higher numbers in the topsoil and lower numbers in, the soil-refuse zone. This relationship was observed for the treated and untreated areas of coal mine refuse at Site #2.
4. Treatment with bactericides increased the numbers of hetero- trophic microorganisms at Site #2 and Site #3.
5. Because the soil cover depth for treated and untreated areas in each study were essentially the same, ProMac systems treatment is effective in reducing T. ferrooxidans population and increasing heterotrophic bacteria. Thus, good vegetative growth and a non-polluting landform can be achieved with minimal soil cover.

A summary of all studies indicates that disturbed areas can be rehabilitated to develop an improved vegetative growth environment by treatment with bactericides. Self-regenerating, healthy vegetation is promoted by suppressing T. ferrooxidans activity and, at the same time, encouraging heterotroph development.

References

Horowitz, A., M. Shellhorn, and A. M. Fairlie. Microbiological Counts in Reclaimed Coal Mine Refuse. BFGoodrich Technical Report, Dec. 5, 1985, accession #25-6272.

American Public Health Assoc. Standard Methods for Examination of Water and Wastewater. Sixteenth edition, Boyd, New York, 1985