A MICROBIAL STUDY OF AN ARTIFICIAL COAL SPOIL

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The artificial coal spoil maintained in our laboratory was prepared from material gathered from the site of a former strip mine operation in Craig County, Oklahoma. The spoil is saturated with water, which accelerates the weathering process promoted by microbes over the rate seen in nature, where dry periods occur (2). The significant products of bacterial metabolism which contribute to weathering the spoil are sulfuric acid and inorganic sulfates. The bacterial populations and the composition of the leached sulfates were monitored over a timed course of study.

INTRODUCTION

When elemental sulfur or reduced sulfur compounds are exposed to the elements of nature, they become oxidized to sulfuric acid. An area where such acid production occurs is called a spoil. This acid production will tend to concentrate inorganic sulfates in the immediate area. Spoils are often found in association with mines or mine water drainage because reduced forms of sulfur are exposed to the air during the mining process. Sulfur-oxidizing bacteria have been previously shown to be associated with acid formation in the spoil environment (5,9). The acid produced in a spoil environment may have a harmful effect on plant and animal life.

The bacterial population in soil represents many diverse species which include aerobic and anaerobic organisms, as well as heterotrophic and autotrophic organisms. The soil organisms of primary concern in this study are aerobic heterotrophic bacteria, sulfur-oxidizing autotrophs, and iron-oxidizing autotrophs. The sulfur-oxidizers and iron-oxidizers are important because these are the organisms which primarily produce H_2SO_4 in the spoil environment. The heterotrophs were monitored to follow the effect of acid production on their population.

Thiobacillus species are responsible for acid production because of large-scale biological oxidation of sulfur to sulfate, as H_2SO_4 (7). The two primary substrates in coal available for metabolic oxidation by *Thiobacillus* species are pyrite (FeS_2) and elemental sulfur. Elemental sulfur is utilized by the thiobacilli according to the equation:

$$S^0 + 4H_2O \longrightarrow SO_4^{2-} + 8H^+ + 6e^-$$

Pyrite is oxidized by sulfur-oxidizing *Thiobacillus* species and *Thiobacillus ferrooxidans*, an iron-oxidizing species. The sulfur-oxidizing species attack the sulfur moiety of the mineral and oxidation occurs at a low rate. *T. ferrooxidans* can oxidize sulfur and iron simultaneously (4,6), with relatively more iron oxidized. The bacterial oxidation of pyrite proceeds by the following equation:

$$4FeS_2 + 15O_2 + 2H_2O \longrightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$$

The ferric sulfate that is produced may react with pyrite non-enzymatically yielding elemental sulfur and ferrous sulfate, both of which can be oxidized directly by the bacteria. The non-enzymatic reaction is known as the indirect mechanism of bacterial oxidation (8). The redox reactions involving iron establish a self-feeding cycle in the spoil, where the ferric ion oxidizes pyrite to liberate ferrous ion and sulfur for direct microbial oxidation.

MATERIALS AND METHODS

The artificial spoil was established in January, 1982 and has been maintained since that time. The spoil was prepared from excavated coal and overburden which contained some shale. The site of the strip mine from which this material was collected is five miles northeast of Centralia, Craig County, Oklahoma (Fig. 1). Mining was stopped in this area in 1980 because the sulfur content of the coal was too high for clean combustion. The materials for preparation of the spoil was crushed and sifted through a 1-cm sieve to remove coarse material. The sifted mix was then placed in a 4' by 4' wooden box lined with plastic. A conical mound 65 cm in diameter and 25 cm high was formed. Distilled water was

decanted into the container around the base of the mound in a manner which did not disturb the spoil material. Overnight the water rose two thirds of the distance to the summit. By 36 hr the entire mound was moist. Water depth was maintained at 2.5 cm and the temperature remained at approximately 25 C (±2) throughout the study.

Beginning 3 days after the addition of water to the mound, plate counts were performed to determine the types and numbers of organisms in the spoil. One-gram soil samples were taken from a depth of 1-2 cm and used as the source of inoculum for these counts. Heterotrophs were enumerated on tryptic soy agar supplemented with 2.5% glucose. Sulfur-oxidizing autotrophs were enumerated using thiosulfate agar at pH 5.0, modified from the media of Vishniac and Santer (9), with the following compostion, per liter: 10 g of $Na_2S_2O_3 \cdot 5H_2O$, 4 g of KH_2PO_4, 4 g of K_2HPO_4, 1 g of NH_4C1, 0.4 g of $MgCl_2 \cdot 6H_2O$, and 0.5 m*l* of trace metal solution. Iron-oxidizing autotrophs were enumerated with ferrous sulfate/silica gel medium at pH 2.5 (S. Yunker, M.S. thesis, Univ. of Okla., 1982). This medium consists of silica gel impregnated with mineral salts and overlayed with 5.0 m*l* of 25% ferrous sulfate at pH 2.5. The sampling site was located near the summit of the mound. Plate counts were performed on a weekly basis for the first 8 weeks. From the eighth week until the twentieth week, plate counts were conducted on a biweekly basis. Regular counts were not performed on three populations simultaneously until the fortieth week of the study. Random counts near the time of the fortieth week show that the figures represent the stable population values at this time.

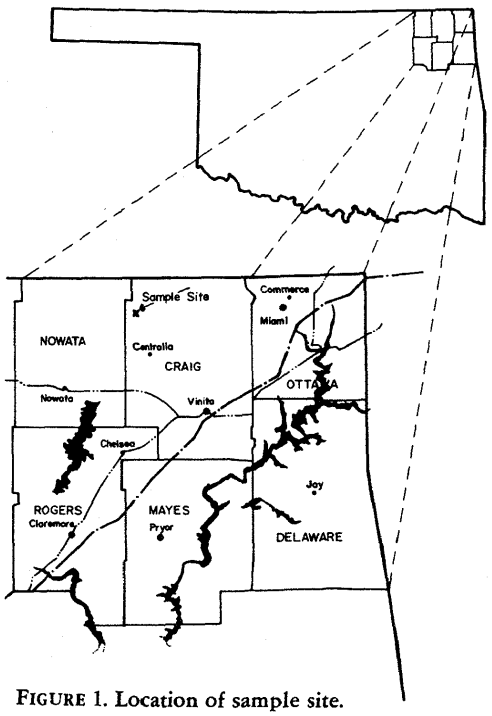

FIGURE 1. Location of sample site.

The inorganic sulfate salts that accumulated on the surface of the mound were collected and analyzed at intervals of three, six, and nine months. They were examined visually prior to x-ray analysis with an ETEC Autoscan Scanning Electron Microscope equipped with a Princeton Gamma-Tech x-ray analyzer. Samples appeared to be uniform in color and composition at the time of collection. The x-ray spectra from the samples yielded information about the elemental composition of the crystals and the type of bacterial metabolism occurring at the collection site.

RESULTS

The results of the bacterial population enumerations are summarized in Figure 2. The heterotroph population peaked in the fifth week of the study and declined steadily thereafter. The population included both gram-negative and gram-positive organisms, with coccoidal and bacillary morphologies noted. The sulfur-oxidizing autotrophs increased by approximately one thousand-fold by the twentieth week, when their population reached a maximum. Their numbers remained at or near this level for the remainder of the study. Iron-oxidizers were not detectable until the tenth week of the study. Their population rose steadily after that time. The main iron-oxidizing species has been tentatively identified as *T. ferrooxidans* (Bergey's manual, 1974). The organism isolated was a gram-negative, aerobic, non-spore-forming bacillus that grew on a completely inorganic medium at pH 2.5 using ferrous sulfate as the sole energy source.

The first inorganic crystals appeared on the surface of the mound within ten days. These were mainly the sulfates of calcium and magnesium, which accumulated near the summit of the mound. The next area to become encrusted was near the waterline.

The flakes in this area had a very high sulfur content. By the end of the fourteenth week, the entire surface of the mound was completely covered by crystal outcroppings. The most rapid accumulation of crystals was between the tenth and fourteenth weeks. The elements detected over the course of the study in the crystalline leachate were sulfur, magnesium, manganese, iron, calcium, silicon, aluminum, potassium, sodium, copper, and rhenium.

Over the course of our observations there was a succession of crystal types. The predominant species from the sixth to the fifteenth weeks appeared as long, thin, white filaments on the surface of the mound. These contained primarily sulfur, along with manganese, calcium, and magnesium as detected by x-ray analysis. Only one of the ten samples examined at the three-month sampling period contained any iron. Halides were not detected in any sample.

At the six-month sampling, no single crystal type predominated. The predominant form observed at the nine-month period was beginning to emerge at this time. This crystal type appeared at the summit of the mound. It was a medium brown crystalline array that appeared as aggregations of small spheres. The presence of sodium, magnesium, silicon, sulfur, potassium and iron was noted in such samples. In this set of ten samples, the only ones that did not contain iron were collected near the waterline. The increase in iron content of the crystals is indicative of an increase in soluble iron in the water circulating inside the mound. The presence of iron salt at the upper slopes indicates that this is the area where ferric sulfate and sulfuric acid from pyrite oxidation accumulate through the migration of the water inside the mound.

DISCUSSION

When water from the spoil pool rises inside the mound, soluble salts are leached out of the subsurface areas. The evaporation of water from the mound's surface leaves the leached salts behind as residues and provides impetus for more water to rise inside the mound by capillary action. Moisture in the mound soil aids in diffusion of soluble salts and fills the pores in the soil with water instead of air. Thus the soil gas distribution is altered by the presence of water, which displaces the insoluble gases such as N_2 and O_2. More soluble gases such as CO_2, H_2S, and NH_3 are absorbed by the water. The difference in the soil gas composition favors the growth of autotrophic bacteria.

Acid production in the mound changes the spoil environment and causes microbial succession in the spoil. The pH of the spoil material was near seven in the beginning of the study and a large population of heterotrophic bacteria grew in the pH 6.5 to 7.5 range. The spoil pH had to drop for iron oxidation to occur because iron is only weakly soluble in neutral solution and is insoluble in basic ones. The acid produced in the spoil has three potential sources: 1.) The most important of these sources is the metabolic activity of sulfur-oxidizing autotrophs which are capable of sulfur oxidation in neutral solution (pH ≈7) (9). *T. neapolitanus* and *T. thiooxidans* can oxidize sulfur in almost any oxidation form in neutral solution. These organisms may oxi-

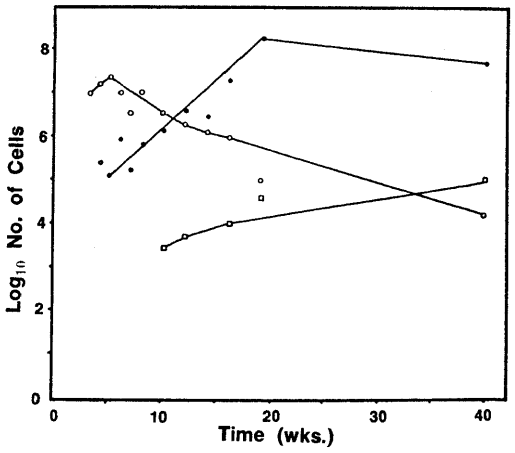

FIGURE 2. Viable counts for populations monitored from results on three different assay media. Platings were done in triplicate on the same sample on the same day.
Symbols: ●, tryptic soy agar with 5% glucose, heterotrophic bacteria
O, sodium thiosulfate agar, sulfur-oxidizing bacteria
□, ferrous sulfate/silica gel, iron-oxidizing bacteria

dize sulfur in pure culture or co-culture. In this study two distinct colony morphologies were no noted on thiosulfate medium. Both of these organisms were presumed to be in the spoil. 2.) Auto-oxidation of sulfur compounds is possible merely by atmospheric exposure. This reaction occurs at a rate only about one hundredth that of bacterially induced reaction (7). 3.) Hydrolysis of sulfate esters is caused by heterotrophic bacteria and fungi, but most likely makes an insignificant contribution to the total acidity of the spoil because these substrates are rarely found in significant amounts in soil or coal (3).

Once the pH begins to drop, the heterotroph population begins to decline. At the same time, the sulfur-oxidizing bacteria begin to flourish. It is not until the metabolic activity of the sulfur-oxidizing autotrophs lowers the pH sufficiently for the solubility of iron to increase that the presence of the iron-oxidizing bacteria becomes detectable. This occurs at about pH 5.0 (6). Growth of iron-oxidizing bacteria is inhibited in neutral solution, and they only grow when the environmental pH has been lowered (7).. The sulfur-oxidizers are not inhibited by the lowered pH and thrive in this pH range. The increased acidity is detrimental to the heterotroph population of the spoil.

Pyrite is the probable source of the iron for the iron-oxidizing bacteria. As sulfur-oxidizers utilize sulfur in the pyrite and produce acid, iron is made available for enzymatic attack by the iron-oxidizing autotrophs. The acid produced begins the enrichment process for the iron-oxidizing population. As the ferrous ion is utilized as an energy source by the iron-oxidizing bacteria, ferric ion is produced. The ferric ion aids in the further decomposition of the pyrite by chemical oxidation, establishing the iron cycle described earlier. Since both mechanisms occur simultaneously, water filling the pores in the spoil soil will become saturated with soluble iron as a function of pH. The water flux initiated by the evaporation of water from the surface of the spoil carries soluble salts through the pores in the mound. The result of this action is enrichment for *T. ferrooxidans* at the summit of the mound. This observation was supported by the fact that no iron was ever found in crystals collected from the waterline of the spoil pool. The area near the waterline was mainly inhabited by sulfur-oxidizing autotrophs.

The conclusions of the study may be summarized as follows:

1. There is an inverse relationship between the heterotrophs and the autotrophs as succession ensues.
2. The sulfur-oxidizing autotrophs lower the pH to some degree before the iron-oxidizers can flourish.
3. Shortly after the iron-oxidizers appear, there is an increase in the leaching activity and oxidation rate in the spoil. This was accompanied by a rapid thickening in the spoil's crystal accumulation between the tenth and fourteenth weeks of the study.

The artificial spoil duplicates many features of a natural spoil. The artificial spoil is a reliable source for sulfur- and iron-oxidizing bacteria which are difficult to maintain in laboratory cultures. The artificial spoil may prove useful as a test site for pollution abatement processes or for testing plant hardiness in reseeding of damaged strip mine sites.

REFERENCES

1. J. V. BECK and D. G. BROWN, J. Bact. 96: 1433-1434 (1968).
2. T. D. BROCK, Appl. Microbiol. 29: 495-501 (1975).
3. D. CASAGRANDE and K. SEIFERT, Science 195: 675-676 (1977).
4. J. LANDESMAN, D. W. DUNCAN, and C. C. WALDEN, Can. J. Microbiol. 12:957-963 (1966).
5. W. W. LEATHEN, S. A. BRALEY, and L. McINTYRE, Appl. Microbiol. 1: 65-68 (1953).
6. M. SILVERMAN, J. Bact. 94: 1046-1051 (1968).
7. M. SILVERMAN, and H. L. EHRLICH, Adv. Appl. Microbiol. 6: 153-207 (1964).
8. O. H. TUOVENEN and D. P. KELLEY, Intern. Metallurg. Rev. 19: 21-31 (1974).
9. W. VISHNIAC and M. SANTER, Bact. Review 21: 195-213 (1957).