

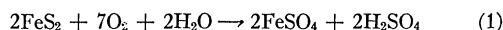
The Role of Microorganisms in Acid Mine Drainage: *A Preliminary Report*

Arthur R. Colmer and M. E. Hinkle

Agricultural and Engineering Experiment Stations, West Virginia University

SOME WATERS ISSUING FROM BITUMINOUS coal mines are near neutral or alkaline in reaction, and, as such, are not considered to be of great importance in the problem of stream pollution. This is not the case with acid mine waters. Waters of this class are objectionable, and their disposal has engaged the attention of the many agencies concerned with water conservation and with the abatement of stream pollution. Carpenter and Herndon (1) and Hodge (2) have reported analyses of this type of mine drainage. Analyses of these waters vary widely from mine to mine and from coal seam to coal seam. One sample of acid mine water had a pH of 2.9, and the total acidity, sulfates, and total iron were 1,375, 3,560, and 460 ppm, respectively. The comparable figures for another acid drainage were: pH 1.4; total acidity, 47,800 ppm; sulfates, 41,700 ppm; and total iron, 12,270 ppm. Hoffert (3) has reviewed recently the status of acid mine drainage as one of the important industrial wastes.

Attempts at solving the acid mine drainage problem. In the past, extensive programs for air sealing acid-producing abandoned coal mines have been undertaken with the purpose of reducing both the volume of the acid waters issuing from the mines and the amount of soluble acidic materials dissolved in them. It has been assumed that if the volume of surface waters gaining admission to such mines could be reduced, less of the objectionable acid drainage would be formed. It has also been assumed that if atmospheric oxygen could be cut off from the mine, or if the concentration of the oxygen present in the mine could be markedly reduced, less oxidation of the sulfur and sulfur-bearing compounds of the coal beds would result, and thus smaller amounts of acid compounds would be available for solution in the water. The following reaction indicates what is assumed to take place when the iron disulfides are oxidized:

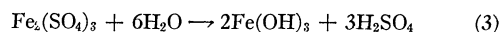
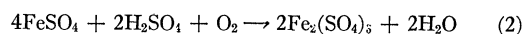


In addition to attempts to limit the acid mine drainage at its source, work has been done on modifying the acid drainage issuing from bituminous coal mines. Chemical treatments have been studied to rid these waters of undesirable constituents or to modify their composition so that they would no longer be considered objectionable.

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However, because of the cost of such chemical treatments and the unsolved problem of disposing of the large quantity of sludge which would be produced, no feasible process has been devised.

Normal changes in acid mine drainage after its formation. It has been assumed generally that the change of clear acid mine water to the characteristic amber or rusty color, which the water soon acquires after exposure to the air and which aids in making this type of pollution so objectionable, is due to the atmospheric oxidation of ferrous to ferric sulfate. The subsequent hydrolysis of the latter compound yields ferric hydroxide. This compound and hydrated iron oxides cause the marked rusty-colored water and precipitates in the water courses. The formation of the ferric sulfate and its hydrolysis may be shown by the following equations:



When bottles of clear acid water, taken from abandoned areas of bituminous coal mines (Pittsburgh seam) in Monongalia County, West Virginia, were stoppered so that no air space existed, the water remained clear even after weeks of storage. However, if a bottle was only half filled with water so that air was readily available, the amber color was formed within 24 to 48 hours, the deeper color being formed upon longer standing. Also, if clear acid mine water was placed in a container and carbon dioxide allowed to bubble through it, the amber color was not formed after a week; yet, if another container of the same water was exposed freely to the air, the color formation was rapid. If the passage of the carbon dioxide was stopped and that container then exposed to the air, the amber color developed in that water. In other words, air influences the characteristic change from the clear to the amber-colored water.

While it has been known that some members of the *Chlamydothales* have an iron compound in their sheath after growing in iron-bearing water, the role that microorganisms might have in inducing changes in the iron compounds of acid mine drainage appears to have been overlooked. The purpose of this preliminary report is to show that microorganisms do have an active role in the above interrelationship and to propose that the formation of sulfuric acid within the mine may be due to microbial action.

The role of microorganisms in the oxidation of iron in acid mine drainage. That the presence of air is not the sole criterion of whether the water's ferrous sulfate will change to ferric sulfate will be seen from the following simple experiments. If mercuric chloride, phenol, or formaldehyde—known disinfectants—are added to clear acid mine water in concentrations varying from one part per thousand to one part per hundred billion, the amber color starts to form in the water containing the smallest concentration of the chemical and in the control. It has been noted that for any chemical the appearance of this color varied with the length of incubation. Some of the waters containing the smaller concentrations of the chemicals may be clear after a week, but upon continued incubation they become amber. However, it was found that a concentration of one part per million of mercuric chloride, one part per thousand of phenol, and one part per ten thousand of formaldehyde prevented any formation of the amber color, irrespective of the length of holding.

In another test toluene was added to a half-filled bottle of clear acid mine water and the container closed. There was no amber color formation. Again, if clear mine water is filtered through an "E.K." germicide pad of a Seitz filter and this sterile water, in sterile cotton-stoppered containers, is exposed to the air, no amber color is produced. Too, if a synthetic mine water, made from distilled water and the iron disulfide (pyrite and marcasite) contained in the "sulfur balls" from acid-producing bituminous coal mines, is treated similarly, no amber color is produced in it after weeks of air exposure.

Thus, since the addition of bactericides such as mercuric chloride, formaldehyde, and phenol and a bacteriostatic agent such as toluene prevents the change from clear water to the amber color of these air-exposed acid mine waters, and since a Seitz-filtered water, where particles of microscopic size are removed, behave similarly, there is strong evidence that atmospheric oxygen is not the only agent involved in the formation of the undesirable hydrated oxides of iron. That this view is valid may be demonstrated by inoculating a Seitz-filtered portion of acid mine water or a Seitz-filtered portion of synthetic mine water with raw, untreated acid mine water. The characteristic amber color is then produced in the inoculated water.

To determine the nature of the microorganism or microorganisms concerned, 1 ml. of the raw water which had just turned amber was inoculated into 10 ml. of clear acid water sterilized by Seitz filtration. When this became amber, it served as the inoculum for a second tube of this sterile water. After such serial transfers had served to enrich the agent or agents present in the colored water, a portion of the liquid from the last amber tube was streaked on the surface of a poured agar plate composed of equal volumes of sterile mine water and 3 per cent agar-agar. After 6–10 days of incubation at room

temperature (22–28°C.), an amber color appeared in the agar, and very small colonies were apparent in and around the colored areas. Pure cultures of the bacteria were secured by repeated replating upon the acid-water agar. Microscopic examination of the organisms of the colonies proved the presence of gram-negative, non-sporeforming rods approximately $0.4\ \mu$ wide by 0.8 – $1.0\ \mu$ long. When either sterile, raw acid mine water or synthetic acid mine water was inoculated with this bacterium, the waters became amber and formed the typical rusty precipitate, whereas uninoculated sterile control tubes of the same waters remained clear. Thus, a bacterium has been isolated in pure culture which is capable of growing in acid mine water and in bringing about the oxidation shown in Equation 2. One of the acid waters commonly used as the substrate in the tests had a pH of 2.2 and a total acidity of 7,938 ppm as calculated from its titration with N/50 NaOH in hot solution to the phenolphthalein endpoint. Attempts to grow these iron-oxidizing bacteria in the synthetic sulfur medium of Waksman and Starkey (12) (which serves as an excellent substrate for *Thiobacillus thiooxidans*, a well-known sulfur oxidizer acting at low pH) have failed. Further morphological, cultural, and physiological studies are being made on the organism.

Besides the bacterium mentioned above, several fungi have been isolated from acid mine drainage. None of these seems to be an agent causing rapid oxidation of ferrous iron to ferric iron. One of the isolates has been tentatively placed in the genus *Spicaria*.¹ Microscopic examination of wet mounts of it growing in this water shows a compound of iron to be precipitated on the fungal spores and mycelium. On the solid substrate of this water the characteristic amber color is formed.

Are microorganisms involved in the formation of acid mine drainage? Equation 2 shows the change which takes place in the oxidation state of the iron of the acid mine water after this water has been produced. The finding that microbial activity is involved in this oxidation, and that atmospheric oxidation is not the sole factor, is an interesting one, yet in itself it is a decidedly minor part in the major problem concerned in this type of industrial waste.

The major problem deals with the actual production of acid water in the mine. Several questions now arise: Is Equation 1, the accepted reaction for the formation of the offensive materials of the acid drainage, solely an atmospheric oxidation phenomenon, or are microorganisms involved there also? Are the abandoned areas of the acid-producing bituminous coal mines capable of supporting microbial growth? Are there any microorganisms known which can use the substances present in these areas in their life activities and produce, as by-products, the offending materials found in acid mine drainage?

¹ We are indebted to H. L. Barnett for aid in identifying this fungus.

Carpenter and Herndon (1), in studying the sterilizing effect of acid mine waters on domestic sewage, noted the growth of an organism on their plates of nutrient agar at or near a pH of 3.0 which they identified as "*Thiobacillus thiooxidans* or a very closely related species." None of the tests used by them to arrive at such a conclusion was given. Their attempts to find the organism responsible for the acidity of the acid mine water were unsuccessful. Various coals were examined by these workers for the possible role of microorganisms in the change of the sulfur content. By comparing sterilized with nonsterilized portions they concluded that at least part of the sulfate produced in acid mine water can be attributed to bacteriological action.

In our laboratory some exploratory tests made on acid drainage have given interesting results with regard to the possible significance of bacteria in the formation of the sulfuric acid in the mine. Although numerous water samples have been studied, the sources of four typical samples are given. In one instance the water sample was taken outside of the mine from the flume into which pumps had emptied the drainage taken from an abandoned area several hundred feet below the earth's surface. In another the water was taken inside the mine as it ran out of an abandoned area and flowed along the main haulage way. A third sample came directly from an abandoned area and was caught as it issued from a bore hole in the coal. A fourth was taken from a strip mine where the water was running out of a spoil bank.

All the waters were inoculated into the synthetic medium of Waksman and Starkey (12), used for the culturing of *Th. thiooxidans*.² Growth appeared in all the inoculated flasks within 7 to 10 days, and, at that time, subcultures were made into fresh media. Repeated transfers have always yielded an active culture. Material from the flasks was streaked on the surface of the solid sodium thiosulfate medium of Waksman (11), and minute straw-yellow to cream-colored colonies were produced. Thus, a second bacterium has been isolated from acid mine drainage. Whereas the first bacterium was instrumental in the change of ferrous to ferric iron, attempts to grow it in the sulfur medium have failed. The second, however, will rapidly oxidize sulfur to form sulfuric acid, but attempts to have it oxidize the ferrous iron of the acid water have failed.

Growth taken from the liquid or solid substrate was morphologically similar to the published descriptions of *Th. thiooxidans*. Physiologically, the organism fits the description of the known sulfur oxidizer. It can satisfy its carbon requirements from the carbon dioxide of the air; its nitrogen can come from ammonium sulfate; and its energy can be derived from the oxidation of elemental sulfur or thiosulfate. The pH of the liquid medium varies from 1.5 to 0.9 after one to two weeks of growth.

² A pure culture of *Th. thiooxidans* was obtained from R. L. Starkey, to whom we are indebted for this favor.

Consideration of environment where acid mine drainage is produced with reported activities of Th. thiooxidans. It is felt that since all of the typical acid mine waters sampled have yielded organisms which fit the description of *Th. thiooxidans*, it is worth while to consider the possibility of its acting as the agent in the formation of sulfuric acid in the abandoned areas of bituminous coal mines. If one considers the actual environmental conditions existing in such areas, the possibility of this organism functioning there is quite striking.

Two gas analyses, made on abandoned areas from which one of the samples of water was taken, showed the absence of carbon monoxide in both. The methane values were 12.4 and 18 per cent; the oxygen concentrations were 3.2 and 9.4 per cent; whereas the carbon dioxide values were 3.5 and 3.8 per cent. The work of Vogler and Umbreit (10) indicates that *Th. thiooxidans* would not find some of these gaseous components of this area prohibitive to its action. These workers ran experiments using pure cultures of *Th. thiooxidans* in which the gaseous environment varied from 10 to 30 per cent of oxygen and from 0.01 to 10 per cent of carbon dioxide. By using the sulfate sulfur, formed by the oxidation of elemental sulfur, as a measure of the activity of the organisms under the experimental conditions, they felt their data showed that "a small amount of variation is evident, but that variation bears no consistent relation to either oxygen or carbon dioxide pressures. Significantly, the oxygen can be decreased to half its value in air without detectable influence upon the rate of sulfate formation." A study of the work of Vogler (8) brings out the striking ability of this organism to modify its activities under the influence of carbon dioxide. Indeed, he postulates that the "carbon dioxide may act as an oxidizing agent for sulfur."

It is well known that the waters issuing from these abandoned areas of acid producing mines are quite acid and have a high sulfate content. Waksman and Starkey (12) long ago called attention to the unusual property possessed by *Th. thiooxidans* of being able to grow when the medium contained 0.5 M concentration of sulfuric acid, equivalent to nearly 5 per cent concentration of the acid. Starkey (6) found that oxidation proceeded most rapidly between pH 2.5 and 3.5, although oxidation of sulfur is very rapid over a very much wider range. Above 6.0 there is practically no oxidation of sulfur in the culture medium by *Th. thiooxidans*. Vogler, LePage, and Umbreit (9) reported that above a pH of 5.0 there is a marked inhibition of sulfur (and thiosulfate) oxidation, but there is little effect upon the endogenous respiration in the absence of sulfur. These workers felt that the organism is adapted to just that range of H-ion concentration in which free carbon dioxide is dissolved in the medium as a gas. Waksman and Starkey (12) and Starkey (6) have noted that *Th. thiooxidans* is quite tolerant to the sulfate ion. The latter states: "However, the end product of sulfur oxidation (sulfate) is tolerated by

Thiobacillus thiooxidans in as high concentration as 20 per cent in the form of magnesium sulfate."

The temperature of the acid waters issuing from the abandoned areas tends to be below 20°C. Vogler, LePage, and Umbreit (9) found that sulfur oxidation proceeded over a temperature range of 7° to 45°C.

Thiessen (7) reported that sulfur in all coals is "present in amounts ranging from traces to as high as 10 or more percent, commercial coals of the Eastern United States contains from 0.5 to 1.5 percent of sulfur." Thiessen stated that sulfur does not occur as such in coal but is present in organic combination as part of the coal substance and in inorganic combination as pyrite or marcasite and, especially in weathered coals, as calcium sulfate. Mellor's (5) compilation is a rich source of information dealing with the iron disulfides, pyrite and marcasite. Two of the many items of interest concerning these inorganic combinations of iron and sulfur are the statements concerning the weathering or the ready breakdown of marcasite under the influence of moist conditions and the finding of free sulfur in the naturally-occurring iron disulfides.

Kirner (4) reports that bituminous coal has a nitrogen composition ranging from 0.6 to 2.8 per cent. The complete story of the forms in which this nitrogen may appear in the coals and, indeed, whether such compounds are available to microorganisms is not known. Starkey (6) found that with *Th. thiooxidans* there is some oxidation in media receiving no nitrogen other than that which may have been absorbed from the air or introduced as impurity in the non-nitrogenous compounds incorporated in the medium. Of the nitrogen sources investi-

gated, ammonium nitrogen is the only one that has been found to be available to the organism.

In this work, a bacterium, unidentified as yet, has been found in acid mine drainage which is involved in the oxidation of ferrous to ferric sulfate. A second bacterium similar, if not identical, in its morphological, cultural, and physiological characters to *Th. thiooxidans* has been isolated repeatedly from the acid mine drainage of some bituminous coal mines. It is postulated that this latter or an unknown similar organism is involved in the oxidation of the sulfur and the sulfur compounds to sulfuric acid. The increasing demands for abatement of stream pollution by acid mine drainage has intensified the efforts of many agencies to solve the problem. It is felt that the mine-sealing program has not given a complete solution and it is suggested that the study of the relation of certain microorganisms to the formation of the sulfuric acid in the mine might be a most profitable one.

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Reconnaissance Soil Survey Work in Japan

C. L. W. Swanson¹

*Department of Soils, Connecticut Agricultural Experiment Station,
New Haven, Connecticut*

THE RECONNAISSANCE SOIL SURVEY OF Japan now being conducted by American scientists is the first of its kind to be made of that country using modern survey methods for classifying and mapping soils in terms of their morphological features. Soil maps of Japan now available from Japanese sources have been made according to the old geological method of soil surveying similar to that used initially in the United States.

During the past 20 years Japan has produced only 80-85 per cent of her annual food requirements. Because of the food shortage there and in the world as a whole, the maintenance or increase of food production has now

assumed particular importance. Since modern soil maps which could be used as an aid in maximizing Japan's food production were unavailable, the survey was undertaken.

The most important agricultural areas are being mapped first, particularly the larger plains and more nearly level regions. The hilly and mountainous sections are of little importance agriculturally. A preliminary field survey was made of Japan before any reconnaissance surveys were initiated. Reconnaissance soil surveys of the Kanto Plain area near Tokyo and the island of Kyushu have been completed (Table 1). Surveys in progress include the islands of Hokkaido and Shikoku, and the Osaka Plain on the main island of Honshu. A soil map (1:250,000) printed in color and a report similar in outline to that of the Soil Survey Division, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. De-

¹ Formerly Major, Air Corps, and head of the Soils and Fertilizer Branch, Agriculture Division, Natural Resources Section, General Headquarters, Supreme Commander for the Allied Powers, Tokyo, Japan.