Aspects of microbial sulfur cycle activity at a western coal strip mine
by Gregory James Olson

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University

Abstract:
The activity of certain groups of sulfur cycle bacteria associated with waters, sediments, and the coal bearing strata of a coal strip mine at Decker, Montana, was studied. Other mining areas of southeastern Montana and northeastern Wyoming were examined to a lesser degree.

Thiobacillus ferrooxidans, one of the major contributors to acid mine drainage, was consistently detected in the mining environment. Physiological studies of T. ferrooxidans isolates indicated that these acidophilic iron and sulfur oxidizing organisms were typical of the species in their preference for low pH and ability to oxidize pyrite. Since 1) acidic conditions were never observed at Decker, 2) the isolates died off in mine water environments, and 3) no acid could be formed from coal samples inoculated with a Th ferrooxidans isolate, it was thought that their activity was limited to microzones in the coal bearing strata where they oxidized sulfuritic material. Any acid formed was quickly neutralized by bicarbonate in the groundwaters.

Sulfate reducing bacteria also were common in the mine waters and sediments. These organisms were particularly active in the settling pond sediments as was evidenced by the rapid rate of conversion of radiolabeled sulfate to sulfide. The hydrogen sulfide produced by these organisms contributed to heavy metal precipitation in the settling pond.

Well waters sampled over a wide area of southeastern Montana contained hydrogen sulfide and sulfate reducing bacteria were detected in all but one well. Activity of these organisms in the groundwater could not be demonstrated by radioisotope experiments, however, a comparison of stable sulfur isotope ratios between groundwater sulfates and sulfides showed the sulfide was likely produced by sulfate reducing bacteria.

ASPECTS OF MICROBIAL SULFUR CYCLE ACTIVITY AT A WESTERN COAL STRIP MINE

by

GREGORY JAMES OLSON

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of the requirements for the degree

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Approved:

Chairperson, Graduate Committee

Head, Major Department

Graduate Dean

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ABSTRACT

The activity of certain groups of sulfur cycle bacteria associated with waters, sediments, and the coal bearing strata of a coal strip mine at Decker, Montana, was studied. Other mining areas of southeastern Montana and northeastern Wyoming were examined to a lesser degree.

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Well waters sampled over a wide area of southeastern Montana contained hydrogen sulfide and sulfate reducing bacteria were detected in all but one well. Activity of these organisms in the groundwater could not be demonstrated by radioisotope experiments, however, a comparison of stable sulfur isotope ratios between groundwater sulfates and sulfides showed the sulfide was likely produced by sulfate reducing bacteria.

INTRODUCTION

The Fort Union coal formation in southeastern Montana contains large deposits of low-sulfur sub-bituminous coal which occurs in seams up to 25 m thick (36). Mining operations in this area have been expanding and, with the depletion of readily available sources of low-sulfur coal elsewhere, removal of the abundant and easily accessible coal reserves in portions of the western United States by surface extraction methods has accelerated, and will likely continue.

The water resources of southeastern Montana are scant. Except for Rosebud Creek and the Tongue and Powder Rivers, little surface water is available (36). Protection of surface and groundwater quality in the face of increasing mining activity is of major importance to this region.

Microorganisms catalyzing sulfur transformations can have significant effects on surface and groundwater quality, especially in connection with coal mining. The exposure of sulfuritic minerals (chiefly pyrite and marcasite) found in association with coal deposits results in the formation of sulfuric acid and the solubilization of heavy metals (46). This process has resulted in serious water pollution problems in certain areas of the United States, adversely affecting thousands of miles of rivers and streams (32). The Ohio River alone receives the equivalent of three million tons of concentrated sulfuric acid annually from mine effluents (35). The acidophilic iron and sulfur

oxidizing bacterium Thiobacillus ferrooxidans is a major contributor to acid mine drainage (58). The oxidation of pyrite, and the resultant formation of sulfuric acid, can be accelerated several hundredfold over over the non-microbial chemical rate as a result of the activities of this organism (8). Heavy metals may be leached into groundwaters as a result of movement of these waters through mine tailings where sulfide minerals are oxidized by thiobacilli to form acid (12). Certainly, not all mines produce acidic drainage. The amount of pyrite available, its distribution and particle size, and the buffering capacity of waters coming in contact with the pyrite affect the amount of acid produced (55). Even in the Ohio-West Virginia-Pennsylvania area where acid mine drainage is most common, mine drainage is often not highly acidic (60). Acid mine drainage has not been reported in southeastern Montana but does exist elsewhere in the state (37).

Another group of bacteria important in sulfur transformations, the sulfate reducers, has been suggested by Tuttle, et al., and King, et al., as a means of combatting acid mine drainage due to the ability of this group to raise the pH of waters and precipitate heavy metals (26,65). These authors have suggested that addition of organic matter to acidic mine settling ponds or lakes will accelerate sulfate reduction and thus improve water quality in regard to pH and heavy metal content. Even in non-acidic environments, sulfate reducing bacteria have been used as a means of trapping heavy metals in settling ponds

(18). Sulfate reducing bacteria are strict anaerobes which use sulfate as a terminal electron acceptor producing hydrogen sulfide (43). The sulfide formed reacts rapidly with heavy metals that may be present, creating highly insoluble metal sulfides (42).

Sulfate reducing bacteria have also been implicated in contamination of certain groundwaters with hydrogen sulfide (15,20,30). Groundwaters over a large area of southeastern Montana have varying levels of hydrogen sulfide (29). Oftentimes these groundwaters flow through coal beds of the region which serve as important sources of groundwater (69). It is not known if mining affects hydrogen sulfide formations in these waters.

In contrast to the eastern part of the United States, microbial sulfur cycle processes important in western coal mining environments have not been well studied. The microbiology of alkaline mine drainage, in general, is poorly understood (32). This study was undertaken to describe the possible effects sulfur transformations could have on surface and groundwater quality which result from coal strip mining in the Decker area of southeastern Montana (Figure 1). Here mining operations have interrupted the normal flow of groundwater. The most important aquifers to be interrupted are the coal seams themselves (69). As a result, groundwater flows into the mine pit and is pumped out so that mining activities may continue. This altered groundwater is collected in a sump pit from which it is pumped into the mine settling

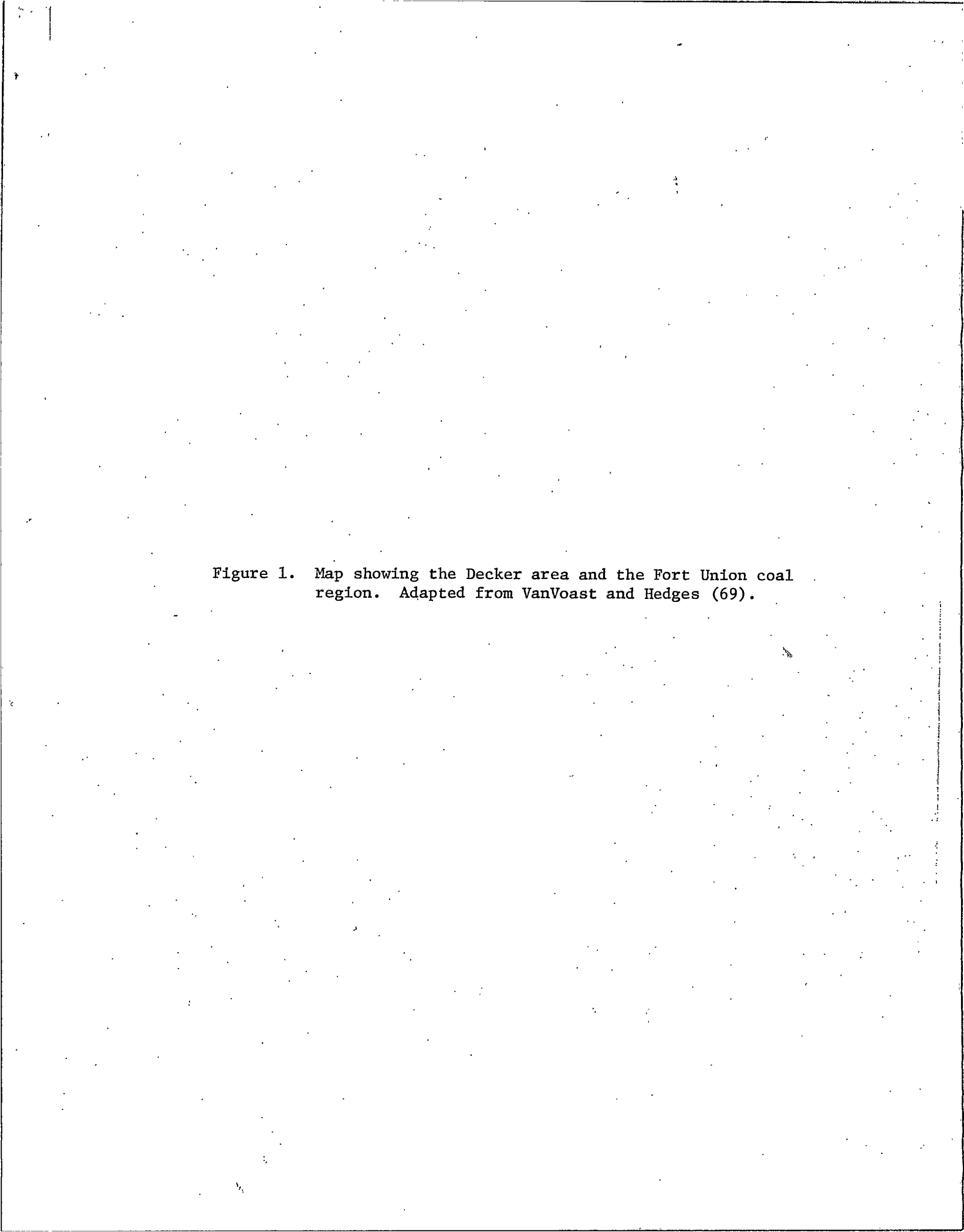
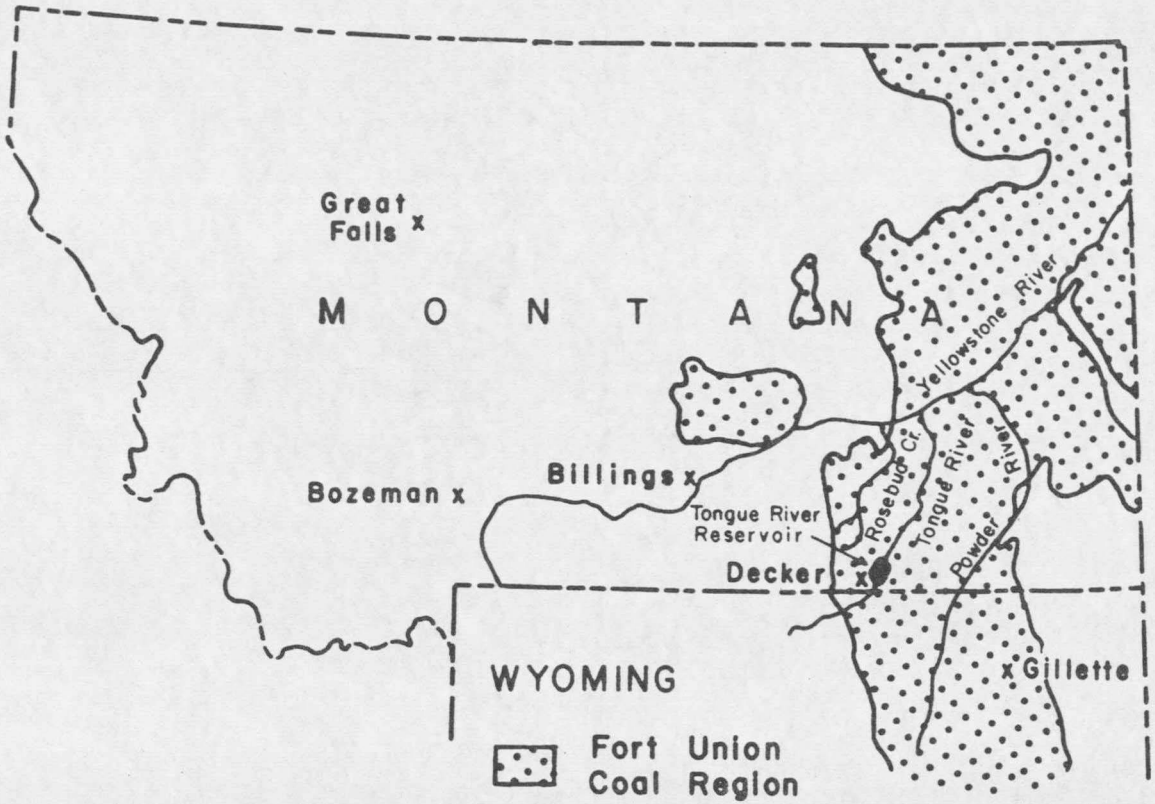


Figure 1. Map showing the Decker area and the Fort Union coal region. Adapted from VanVoast and Hedges (69).

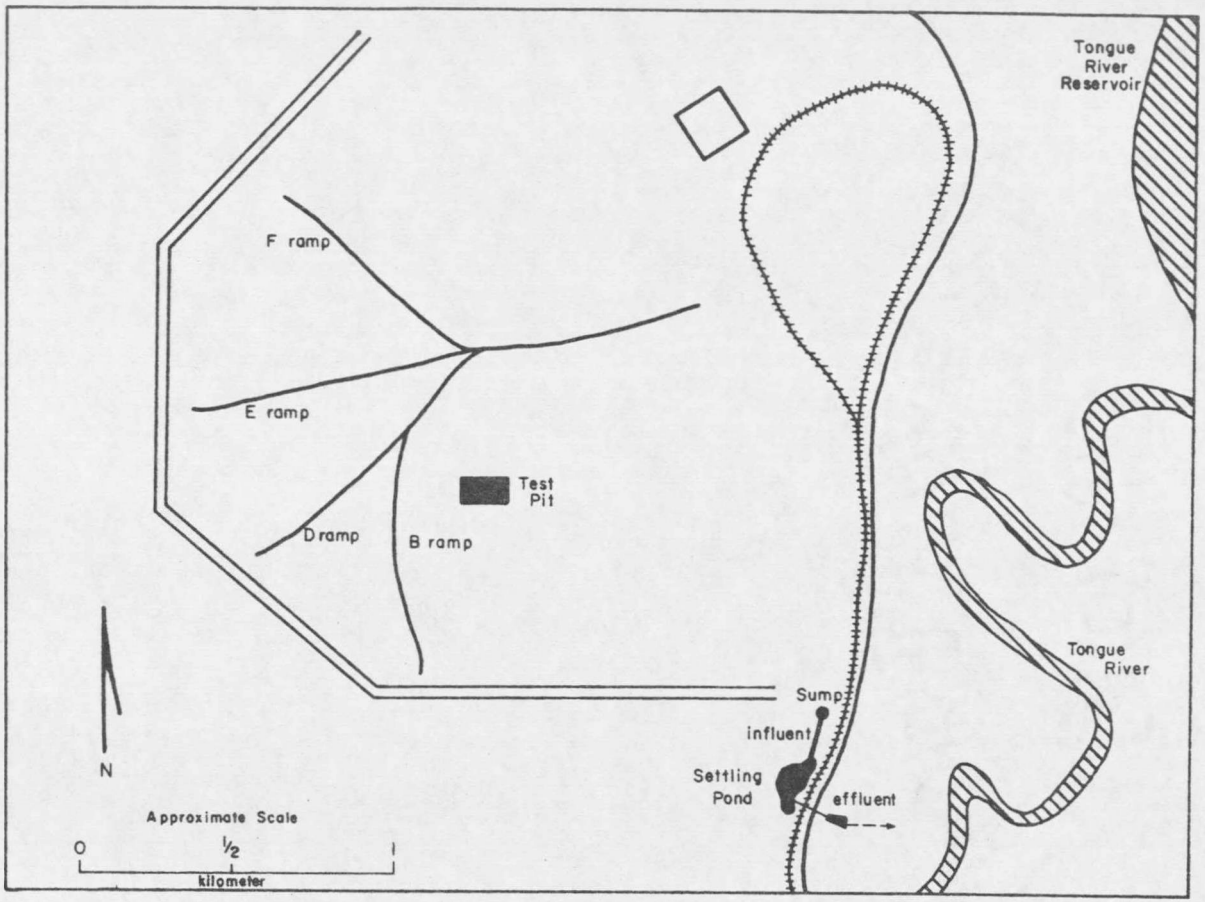

Great Falls
MONTANA
Yellowstone River
Bozeman
Billings
Tongue River Reservoir
Rosebud Ck.
Tongue River
Powder River
Decker
WYOMING
Gillette
Fort Union Coal Region

pond. Overflow water (the mine effluent) is discharged on to the Tongue River flood plain (Figure 2). When mining is completed, spoils will be replaced in the mined out area. As groundwater accumulates in the replaced spoil, significant changes in groundwater quality may result due to leaching of soluble minerals (68).

This study involves an investigation of microbial sulfur cycle processes that may be important in the aquatic environment within the mine and of the effects these transformations may have on the quality of mine discharge. This study attempts to answer the following questions: 1) are acidophilic sulfur bacteria found in connection with the Decker mine environment, and, if so, are these the same organisms implicated in the production of acid mine drainage in other areas of the country, and are they active in the coal-bearing strata at Decker; 2) are sulfate reducing bacteria found in the settling pond sediments at the Decker mine and, if so, are they active and contributing to heavy metal precipitation; and 3) are sulfate reducing bacteria present in the groundwater of the coal deposit areas and are they responsible for formation of the hydrogen sulfide which occurs there?

The answers to these questions should contribute to a better understanding of the important microbial sulfur cycle processes occurring in alkaline mine drainage of the western United States as well as in the groundwaters of mining areas in southeastern Montana.

Figure 2. Map of the Decker Mine. The double line represents the mine pit.


Tongue
River
Reservoir
F ramp
E ramp
Test
Pit
D ramp
B ramp
Tongue
River
Sump
influent
Settling
Pond
effluent
N
Approximate Scale
0
1/2
1
kilometer

The results of the study should also be of predictive value for other areas of the west which will be subjected to the expected intensification of coal strip mining in the near future.

Site description

The major study area was the Decker coal strip mine, located in southeastern Montana about 30 km north of Sheridan, WY. The Decker mine, one of the world's largest, is situated adjacent to the southern end of the Tongue River Reservoir.

As mentioned previously, strip mining operations have interrupted the normal flow of groundwater through the coal beds, resulting in water accumulation in the mine pit. The water slowly flows to the sump which is located at the lowest point of the mining area. From here the "altered" groundwater is pumped into the settling pond (Figure 2). The water flowing into the settling pond from the sump pit is termed the influent. In June, 1976 the sump was buried by the coal company under several meters of coarse, porous rock, however water still accumulated in this area, and the buried sump was still pumped intermittently. The settling pond occasionally received water pumped directly from the mine pit.

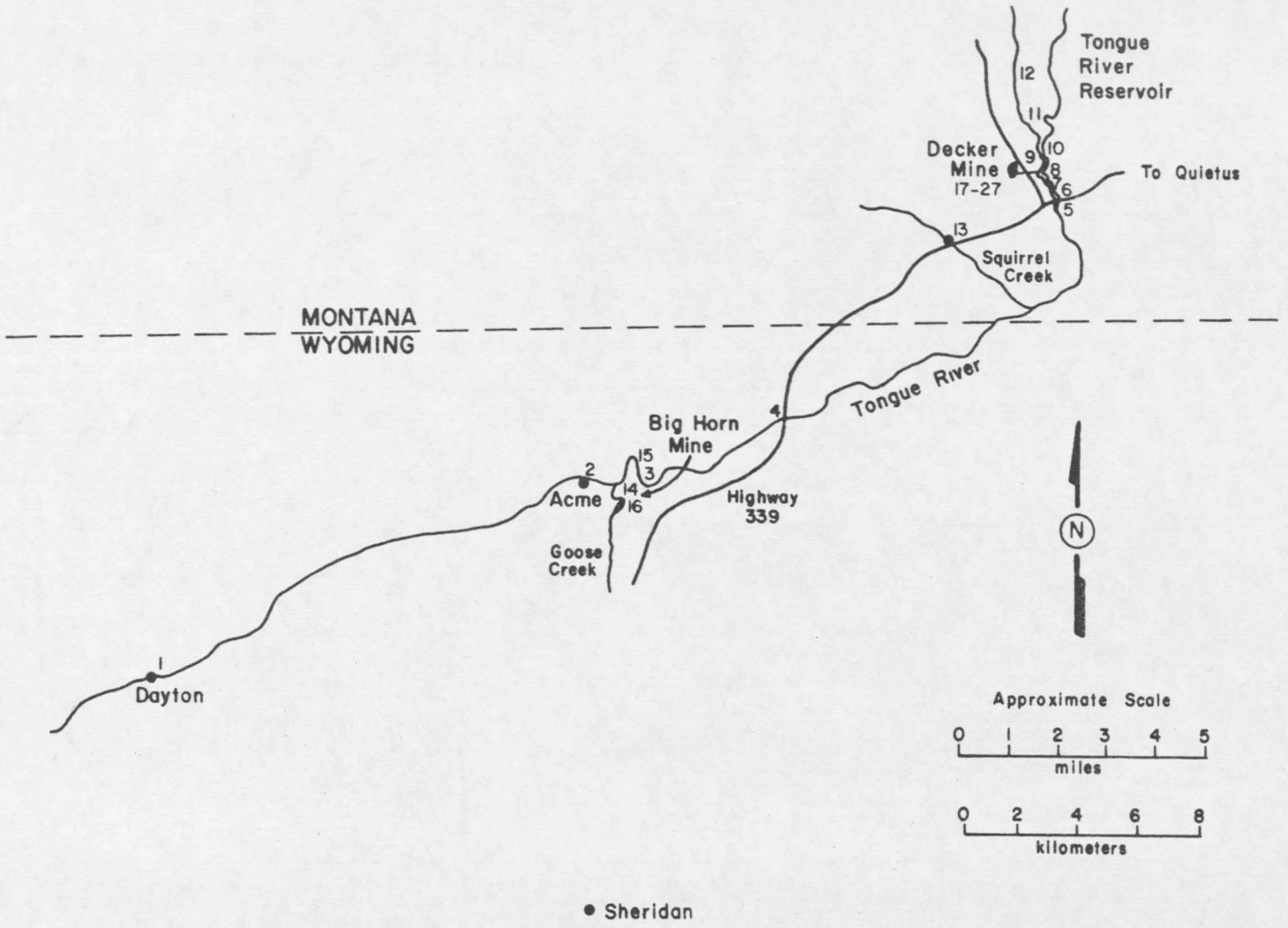
During the early part of this study the settling pond was about one meter deep, had an area of about three acres, and discharged approximately 400,000 gallons of water daily (Decker mine personnel,

personal communication). Some modifications of the pond took place during the course of the study resulting in a slight deepening and reduction in area of the pond. The effluent of the pond became intermittent after July, 1976.

Samples were also collected at the Belle Ayr mine near Gilette, WY (Figure 1) and at the Big Horn mine near Acme, WY (Figure 3). For comparative purposes, samples were collected at No Name Creek in the town of Sand Coulee, MT which is just southeast of Great Falls. This is an acid mine drainage creek of very low pH.

Well water samples were collected over a wide area of southeastern Montana ranging from the Decker area to the Ashland, MT area (about 70 km northeast of Decker) to the Colstrip, MT area (about 100 km north of Decker). Coal strip mines are in operation in the Colstrip area and are possible in the future in the Ashland area.

Figure 3. Map of the Decker-Big Horn mine region showing sampling sites for Thiobacillus ferrooxidans.


Tongue
River
Reservoir
12
11
10
9
8
7
6
5
Decker
Mine
17-27
To Quietus
13
Squirrel
Creek
MONTANA
WYOMING
Tongue River
4
Big Horn
Mine
15
3
2
14
16
Acme
Highway
339
Goose
Creek
1
Dayton
N
Approximate Scale
0 1 2 3 4 5
miles
0 2 4 6 8
kilometers
Sheridan

MATERIALS AND METHODS

ESTIMATION OF SULFUR CYCLE BACTERIA

Sampling procedures

All sediment or water samples were collected in sterile plastic containers and were kept well chilled until processing which was usually within four hours. Sediment samples were obtained using a Phleger core sampler with removable, sterilizable butyrate liners (Hydro Products, San Diego, CA) or an Ekman dredge (Wildlife Supply Co., Saginaw, MI).

Enumeration

Estimation of sulfur cycle bacterial populations was performed using the five tube most-probable-number (MPN) technique. The types of organisms enumerated, media employed, and methods of detection are listed in Table 1. These procedures are a modification of those of Tuttle, et al. (64). Only surface sediment was utilized in the sediment MPN enumerations. Sterilized mine water or salts of the appropriate medium were employed in the dilution blanks. After inoculations, the MPN tubes were allowed to incubate in the dark at room temperature (23 $\pm$ 3 C) for 21 days prior to being scored as positive or negative.

WATER CHEMISTRY

Collection vessels were acid cleaned (3 N HCl) glass or nalgene containers. After collection, samples were kept chilled in transit to the field laboratory where they were analyzed or prepared for analysis

Table 1. Types of organisms enumerated, media employed, and detection procedures involved in MPN determinations.

Physiological process	Culture medium	Method of detection
Sulfate reduction	Medium E of Postgate (44)	Blackening due to formation of ferrous sulfide
Low pH sulfur oxidation	9K salts of Silverman and Lundgren (47) plus 1% elemental sulfur	Red color upon addition of five drops of 0.4% thymol blue (acid production)
Neutral pH thiosulfate oxidation	Thiosulfate broth of Vishniac and Santer	Turbidity and yellow color upon addition of two drops of 1% brom thymol blue (acid production)
Low pH iron oxidation	9K salts of Silverman and Lundgren (47) plus 14.74 grams of ferrous sulfate per 1	Orange-brown precipitate of oxidized iron

promptly. Alkalinity was measured by potentiometric titration according to Standard Methods (1), specific conductance by a labline mho meter, model mc-1 mark IV, pH by a Beckman model 76t expanded scale pH meter, sulfate and total iron by the Hach method (14) using Sulfaver IV and ferrozine reagents, respectively, sulfide by the phenylenediamine method of Strickland and Parsons (53), dissolved oxygen by a Yellow Springs dissolved oxygen meter, model 54 (measurements made in the field), or by the azide modification of iodometric titration, adding the reagents in the field (1). Ferrous iron was measured by the permanganate titration method of Skoog and West (50).

STUDIES WITH THIOBACILLUS FERROOXIDANS

Sample collection and isolation procedures

Samples of water and sediment were collected from a variety of locations in Montana and Wyoming in an attempt to determine whether or not T. ferrooxidans was present. Water or sediment (1.0 ml) was inoculated into 50 ml of sterile 9K-iron medium (in a 125 ml flask) in the field (Decker and Big Horn area samples) or in the laboratory at MSU within 48 hours of collection (Belle Ayr and No Name Creek samples). The flasks were incubated for 15 days at room temperature. After this time, 1.0 ml was withdrawn from each flask and inoculated into nine ml of fresh, sterile 9K-iron medium. After an additional 15 days of incubation the test tubes were visually assessed for iron oxidation.

All positive tubes (dark brown precipitate) and selected negative tubes were subjected to further testing procedures. These consisted of removing 1.0 ml from the test tube and inoculating it into a 125 ml flask containing 50 ml of fresh, sterile 9K-iron medium. After six days of incubation the flasks were tested for 1) concentration of ferrous iron using the permanganate titration method of Skoog and West (50); 2) pH using a Beckman Zeromatic SS-3 pH meter with a model 39501 combination electrode (Beckman Instruments Co., Irvine, CA); and 3) presence of short rod-shaped bacteria by phase contrast microscopy. Selected cultures were subjected to purification procedures which consisted of two successive single colony isolations on Manning's ISP medium (34). The colonies were inoculated into 9k-iron broth and incubated for one week before restreaking. After the second single colony isolations the cultures were checked for heterotrophic contamination by streaking onto Sabouraud's dextrose agar and onto two tryptone glucose extract agar plates, one pH 4.5 and one at 7.0 (Difco, Detroit, MI). Plates were incubated for ten days at 28 C before discarding.

Batch growth and harvesting procedures

To secure washed cell suspensions of T. ferrooxidans isolates the following procedure was employed: One ml of a refrigerated 9K-iron culture was inoculated into 100 ml of fresh, sterile 9K-iron

medium in a 250 ml flask. This flask was placed on a rotary shaker ("Orbit", Lab-Line Instruments, Inc., Melrose Park, IL) and incubated for three days at room temperature and 140 oscillations per minute. After this time, 50 ml were transferred to a 500 ml gas washing bottle (Bellco) containing 450 ml of the 9K iron medium. The contents of the bottle were then aerated at a rate of one liter per minute by an aquarium pump hooked up to the bottle via a trap tube containing sterile glass wool. The culture was incubated for about 24 hours at room temperature. After incubation, the air source was disconnected and the contents of the flask were allowed to settle for about 30 minutes to allow large particles of oxidized iron to settle out. The liquid was then poured off into two sterile 250 ml centrifuge bottles and the cells were pelleted at 5000 x g for 15 minutes in a Sorvall RC2-B refrigerated centrifuge. To secure a washed cell suspension the method of Silverman and Lundgren (47) was employed. The final resuspension of the washed cells was in distilled water adjusted to pH 3.5 with sulfuric acid for respirometry experiments or in pH 2.6 9K salts for other experiments requiring washed cells. Cell suspensions were refrigerated and always used within 72 hours of harvesting.

Respirometry

Manometric studies were carried out on a Gilson differential

respirometer (Gilson Medical Electronics, Middleton, WI) using standard manometric techniques (67). Each reaction vessel (single side arm) contained 2.8 ml of sterile 9K salts plus an energy source, either ferrous iron (as ferrous sulfate), 50 µmoles; thiosulfate (as sodium thiosulfate), 50 µmoles; glucose, 50 µmoles; or elemental sulfur, 50 mg. The initial pH values in the reaction vessels were 2.5 to 2.7 except for the thiosulfate containing vessels which were at pH 4.0 to 4.2. The center well of all vessels contained 0.2 ml of a washed cell suspension of a T. ferrooxidans isolate containing between 51 and 158 µg protein as determined by the method of Lowry, et al (31) after cell disruption by the sonication method of Camper (4). The system was allowed to equilibrate for 30 minutes before the cells were tipped in. Incubation was at 28 C and agitation at the rate of 140 strokes per minute. The gas phase was air. To calculate QO_2 (protein) values the amount of oxygen taken up in the first hour of incubation of the vessels was used. QO_2 (protein) is defined as the oxygen uptake in µl per mg cell protein per hour.

Adaptation to other energy sources

Attempts were made to adapt T. ferrooxidans isolates to energy sources other than iron. Adaptation to sulfur was attempted using the method of Dugan and Tuttle (9). Adaptation to thiosulfate was attempted using the method of Tuovinen and Kelly (63) and adaptation

to glucose was attempted using the method of Tabita and Lundgren (56).

Studies with coal and pyrite

Coal experiments were initiated by addition of 10.0 g of 28 to 80 mesh rider seam coal from the Decker mine to 100 ml of sterile 9K salts in a 250 ml flask. Sodium azide (0.03 g) and/or ferrous sulfate (4.42 g) were added to some of the flasks. All flasks were inoculated with 0.2 ml of a washed cell suspension containing about 10^8 cells of isolate TF-1. After four hours of equilibration pH measurements and acidity determinations were made. Acidity was measured by potentiometric titration with 0.1 N sodium hydroxide to pH 8.3 (1). After 33 days of incubation at room temperature with 140 oscillations per minute on the rotary shaker, the measurements were repeated.

Pyrite experiments were initiated by the addition of 5.0 g of finer than 50 mesh pyrite to 100 ml of one-tenth strength 9K salts in a 250 ml flask. The flasks were inoculated with 1.0 ml of washed suspensions of various T. ferrooxidans isolates containing about 5×10^8 cells per ml. The flasks were allowed to equilibrate for six hours, then pH measurements, acidity titrations (done with boiling samples to the phenolphthalein end point) and sulfate concentrations were measured. Sulfate was determined using the Hach method (14) with sulfaver IV powder pillows. After 48 days of incubation at room temperature and shaking at 140 oscillations per minute, the measurements

repeated.

Survival experiments

The survival of T. ferrooxidans in mine waters was studied using MSU-VME membrane diffusion chambers developed by McFeters and Stuart (38). The chambers were fitted with 0.45 µm membrane filters (Millipore, Bedford, MA). At the Decker settling pond the chambers were immersed and suspended in a styrofoam collar to keep them at the proper position in the water for incubation and sampling. After the chambers had filled with water through the pores in the filters, 1.0 ml of a washed cell suspension was added through the injection port using a one ml plastic syringe (Pharmaseal, Glendale, CA). For experiments involving the natural populations, the chamber was filled with the natural water sample using a 30 ml plastic syringe (Jelco, Raritan, NJ). The contents of the chamber were then mixed by drawing liquid into the syringe attached to the injection port and expelling it ten times. After mixing, the zero time samples were removed and inoculated into tubes of 9K-iron medium in the MPN fashion. Dilution blanks contained sterile 9K salts. The chambers were then capped, covered with a foil hood, and left to incubate in situ.

RATE OF SULFATE REDUCTION

The techniques employed for the measurement of the rate of sulfate

reduction are modifications of the procedures of Ivanov (20).

Radioisotope

The radioisotope employed was $H_2{}^{35}SO_4$ with a specific activity of 43 Ci per mg (New England Nuclear, Boston, MA), diluted with distilled water to a concentration of 0.02 µCi per ml. Five ml aliquots of this solution were kept frozen in capped serum vials until use.

Field procedures

Sediment cores from the Decker mine settling pond were taken with the Phleger corer. Ten or 20 cm sections of the cores (approximately 100 and 200 ml, respectively) were sliced off with a sterile spatula and placed in small, sterile, acid washed, screw-capped jars, nearly filling them. The headspace of each jar was then gassed out with nitrogen (lecture bottle size, Matheson Gas Co., Joliet, IL). After a few seconds of gassing the jar was capped quickly and shaken vigorously to homogenize the contents. A sawed-off ten ml plastic syringe (Becton, Dickinson and Co., Rutherford, NJ) was then used to withdraw ten ml portions of the homogenized sediment which was dispensed into sterile, acid washed anaerobic culture "roll" tubes (Bellco). The headspace of each tube was then gassed out with nitrogen and the tubes were quickly sealed with rubber stoppers. The remaining sediment was saved for later sulfate analysis. All tubes then received an injection of a small amount of the radioisotope solution (0.2 to 0.5 ml depending

on the experiment) through the stopper by the use of a one ml plastic syringe. The controls received 1.0 ml of formalin. Some tubes received a spike (0.2 ml) of sodium lactate to make a final concentration of 100 or 300 mg lactate, depending on the experiment. The tubes were shaken, wrapped in foil, then submerged in the settling pond inside a wire basket. At the conclusion of the experiment, the contents of each tube were fixed by injection of 2.0 ml of a solution of 3.5% cadmium acetate in 4% acetic acid. The tubes were shaken, then transported on ice to the laboratory at MSU. A similar procedure was followed in attempts to quantify the rate of sulfate reduction in well water samples. After allowing the well to flow for several minutes, 14.0 ml water samples were collected in plastic syringes which were then injected immediately into sterile, gassed out (nitrogen) roll tubes. At each well, water samples were added to five tubes which received 0.8 ml of anoxic solutions of various compounds. One tube was spiked with distilled water, one with sodium sulfide (final concentration 100 mg/l), one with sodium lactate (final concentration 100 mg/l), one with sodium sulfide and sodium lactate (final concentrations 100 mg/l), and one with sodium dichromate (final concentration 2000 mg/l). The tubes were incubated at ambient temperature until returned to MSU (always within 48 hours of collection) when they were placed in an incubator and incubated at near the in situ temperature. After incu-

bation was completed, the contents of the tubes were fixed by injection of 1.0 ml of 1.0 N zinc acetate.

Laboratory procedures

Sediment or water samples from the roll tubes were washed, with a stream of anoxic water, into a 125 ml side-arm flask. The flask was connected to a nitrogen gas cylinder and to two hydrogen sulfide trapping solutions via a condenser. The traps contained 1.0 N sodium hydroxide or 3.5% cadmium acetate. The system was then purged with oxygen-free nitrogen (obtained by passing the gas through a heated copper column before it reached the reaction flask) for 15 minutes to remove oxygen from the system. After this time, sulfides were liberated from the sediment or water in the flask by injection of ten ml of a stannous chloride-HCl solution (80 g of stannous chloride in a liter of 6 N HCl). The stannous ions prevented any possible oxidation of the sulfide by ferric ions that might have been present (7). The reaction flask was then brought to a boil while the gassing continued. After 30 minutes, the traps were disconnected and an aliquot (0.2 to 1.0 ml) of trapping solution was removed to a plastic poly-Q scintillation vial (Beckman) which contained 9.0 ml of Aquasol scintillation cocktail (New England Nuclear). The vials were counted on a Beckman LS-100C liquid scintillation counter to a 5% error. Counts were corrected for background and quenching.

Sulfate content of the sediment samples was determined by obtaining the interstitial water of the sample. This was accomplished by centrifuging the sediment in an acid washed plastic centrifuge bottle at 5000 x g for ten minutes, then removing the supernatant liquid and analyzing it for sulfate by the Hach method (14).

The daily rate of sulfate reduction in mg H_2S produced per liter of sediment or water was calculated by using the formula of Ivanov (20):

$$\text{Rate} = \frac{r \cdot (S/SO4) \cdot 24 \cdot 1.06}{R \cdot t}$$

In this formula, R is the radioactivity of the initial sulfates in counts per minute per l, r is the radioactivity of evolved sulfide in counts per minute per l, (S/SO_4) is the amount of sulfate sulfur in the analyzed sediment or water in mg/l, t is the duration of the experiment in hours, and 1.06 is a correction factor for converting sulfide sulfur to hydrogen sulfide.

SEDIMENT ANALYSES

Dry weight determinations

Sediment samples were collected in screw-capped jars as described earlier. To determine the dry weights of sediment samples, 1.0 ml of the homogenized sediment was drawn into a sawed-off one ml plastic syringe and then deposited on top of a Millipore 1504700 glass fiber filter. The filter had been heated at 105 C for one hour, then cooled

in a desiccator and reweighed, and after the sediment was deposited on the filter this procedure was repeated.

Metal bound sulfides

Ten ml of sediment were removed from the homogenized sample and added to the same apparatus used in the $H_2{}^{35}S$ distillation. The sulfide trapping solution was 3.5% cadmium acetate. After distillation, the cadmium sulfide precipitate was washed into a 16x150mm test tube. The precipitate was then filtered onto a Millipore 1504700 glass fiber filter which had been preweighed in the manner described above. The filter was then heated at 70 C under a stream of nitrogen for one hour, placed in a desiccator until cool, and reweighed.

Heavy metal analysis

On one occasion sediment was extracted with hydriodic acid as described by Murthy, et al. (41). The hydriodic acid extract was then analyzed for certain heavy metals by William Brady of the Chemistry department, MSU.

ISOTOPE ANALYSIS

Approximately 20 l of well water were collected in two acid washed plastic "cubitainers" (Cole-Parmer, Chicago, IL). About ten ml of sodium hydroxide and ten ml of zinc acetate (1.0 N solutions) were added to the containers (to precipitate sulfide as ZnS) which were then

capped and transported back to the laboratory at MSU. The water in the containers was then filtered through 142 mm Millipore filter with a pore size of 0.45 μm to trap the ZnS. The sulfates in the filtered water sample were then precipitated by adding barium chloride to the water sample after it had been acidified to about pH 3 and brought to a boil. The barium sulfate was collected by filtration through a Millipore glass fiber filter. The ZnS on the filter was transferred to the previously described apparatus used in the distillation of sulfides. About ten ml of the stannous chloride-HCl solution was injected into the flask after purging the atmosphere with nitrogen and the evolved sulfides were retrapped in a 5% silver nitrate solution. The silver sulfide was then allowed to settle in the trap tube, and the supernatant liquid was drawn off with a pipet. The precipitate was washed twice with distilled water, then transferred to a small acid washed screw-capped vial where it was allowed to air dry. The barium sulfate was scraped off the glass fiber filter into a small vial, and heated at 105 C for a few minutes to dry. The samples of sulfates and sulfides were then mailed to the laboratory of Dr. I.R. Kaplan, Department of Geology, UCLA, where determination of the sulfur isotope ratios of the samples were run. Values are reported as $\delta^{34}S$, which is obtained in the following manner:

$$\delta^{34}S = \frac{^{34}S/^{32}S \text{ sample} - {}^{34}S/^{32}S \text{ standard}}{^{34}S^{32}S \text{ standard}} \times 1000$$

The standard is meteroite troilite (23).

IDENTIFICATION OF OTHER SULFUR BACTERIA

Thiothrix

Samples of a cream-colored filamentous growth lining the channels leading to the sump of the Decker mine were removed with a forceps into a plastic bag and returned to MSU. In the laboratory, a small portion of the tuft was rinsed three successive times in petri plates containing sterile distilled water, and then placed in the center of a petri plate containing 0.2% beef extract and 1% agar. The plates were incubated in the dark at room temperature and at 30 C. Cultures on the plates were checked microscopically for gliding motility after six and 18 hours.

The cell filaments were stained for lipid inclusions (sudan black) and metachromatic granules (acidified methylene blue). Filaments were also checked for sulfur granules by treatment with warm acetone and ethanol during microscopic examination.

Quantification of the elemental sulfur content of the filaments was accomplished by extracting a tuft of filaments (about 0.1 g wet weight) in boiling acetone for one hour. After this time the tuft of filaments was removed and the residue allowed to evaporate to dryness. The residue was then redissolved in 10.0 ml of acetone and a colorimetric test for elemental sulfur was performed according to the method

of Skoog and Bartlett (49).

Thiobacilli

Selected cultures from positive thiosulfate oxidation MPN tubes were subjected to purification by several successive single colony isolations (streak plate) on Vishniac and Santer's thiosulfate medium (70) containing 1% Difco purified agar. Enrichment for anaerobic sulfur oxidizers was accomplished by inoculation into the S8 medium of Hutchinson, et al. (16). Pure cultures were obtained by several successive single colony isolations on S8 medium containing 1% Difco purified agar. Plates were incubated in an anaerobe jar employing $H_2 + CO_2$ "Gas-paks" (BBL, Cockeysville, MD). Colonies were always inoculated into broth and incubated for one week before restreaking. After pure cultures had been obtained they were identified using some of the tests involved in the scheme of Hutchinson et al. for identification of thiobacilli (17).

Attempts were also made to isolate low pH thiosulfate oxidizers and low pH heterotrophs using enrichment broth inoculated with 1.0 ml of settling pond influent water. The thiosulfate broth was Vishniac and Santer's (70) at pH 4.5. Heterotrophic broth was both pH 3.0 nutrient broth (Difco) and pH 3.0 iron peptone broth (10) made up at one-half strength and filter sterilized.

RESULTS

MOST-PROBABLE-NUMBER DETERMINATIONS

In order to determine the numbers of sulfur cycle bacteria present in the mine environment, MPN enumerations were performed. Sulfate reducing bacteria were common in the sediments of the Decker mine settling pond. These organisms were also consistently found in the mine settling pond influent, effluent, and surface water (Table 2). Acidophilic iron and sulfur oxidizing bacteria and bacteria oxidizing thiosulfate at neutral pH were also found in the mine settling pond sediments as well as within other mine waters. Acidophilic sulfur oxidizers were not detected in the test pit or in settling pond surface waters. Acidophilic iron oxidizers were especially numerous in the influent waters, and generally outnumbered other groups of sulfur bacteria at this location. These organisms were also predominant in acidic No Name Creek, exceeding MPN values of the Decker mine settling pond influent waters by at least an order of magnitude. Bacteria oxidizing thiosulfate at neutral pH were not detected in No Name Creek.

As at the Decker mine, sediments in the mine pit at the Belle Ayr mine contained appreciable numbers of iron oxidizing bacteria. Sediment from Caballo Creek, just below the settling pond discharge, was negative for these organisms.

TABLE 2. MOST-PROBABLE-NUMBER DETERMINATION (IN ORGANISMS PER 100 ML) OF SULFUR CYCLE BACTERIA. VALUES GIVEN ARE RANGE (TOP LINE), ARITHMETIC MEAN (MIDDLE LINE), AND NUMBER OF DETERMINATIONS (BOTTOM LINE). ND=NOT DETERMINED.

	SULFATE REDUCTION	SULFUR OXIDATION	IRON OXIDATION	THIOSULFATE OXIDATION
SETTLING POND SEDIMENT (SOUTH END)	8.0×10^{4}->2.4×10^{7} 6.62×10^{6} 14	4.0×10^{3}-2.4×10^{5} 4.85×10^{4} 13	4.0×10^{3}-3.5×10^{6} 4.54×10^{5} 13	4.9×10^{4}-9.2×10^{6} 2.32×10^{6} 9
SETTLING POND SEDIMENT (NORTH END)	3.3×10^{5}-9.2×10^{6} 4.98×10^{6} 3	1.3×10^{4} 1	3.3×10^{4}-3.5×10^{6} 1.77×10^{6} 2	4.9×10^{5} 1
SETTLING POND SURFACE WATER	2.3×10^{2}-5.4×10^{3} 2.19×10^{3} 8	0-1.3×10^{2} 2.1×10^{1} 8	0-5.4×10^{3} 1.11×10^{3} 8	0->2.4×10^{4} 6.28×10^{3} 8
SETTLING POND INFLUENT WATER	4.6×10^{2}->2.4×10^{4} 1.61×10^{4} 5	4.9×10^{2}-7.0×10^{3} 2.53×10^{3} 4	3.5×10^{3}-3.5×10^{4} 2.05×10^{4} 5	4.0×10^{1}-3.5×10^{4} 7.95×10^{3} 5
SETTLING POND EFFLUENT WATER	3.3×10^{3}->2.4×10^{4} 1.37×10^{4} 2	0 2	0-2.2×10^{3} 1.1×10^{3} 2	2.6×10^{2}-1.1×10^{4} 5.63×10^{3} 2
TEST PIT WATER	ND	0 1	3.3×10^{3} 1	4.6×10^{3} 1
STOCK WELL WATER	1.3×10^{2}-1.7×10^{3} 9.2×10^{2} 2	0-2.0×10^{1} 1.0×10^{1} 2	2.0×10^{1}-7.0×10^{1} 4.5×10^{1} 2	3.3×10^{2}-4.9×10^{2} 4.1×10^{2} 2
NO NAME CREEK WATER	ND	1.7×10^{4} 1	>2.4×10^{5} 1	0 1

TABLE 2. CONTINUED

	SULFATE REDUCTION	SULFUR OXIDATION	IRON OXIDATION	THIOSULFATE OXIDATION
BELLE AYR MINE PIT (1)	ND	ND	$>2.4 \times 10^6$ 1	ND
BELLE AYR MINE PIT (2)	ND	ND	2.0×10^3 1	ND
BELLE AYR MINE CABALLO CREEK	ND	ND	0	ND
BELLE AYR MINE SETTLING POND	ND	ND	9.2×10^5 1	ND

WATER CHEMISTRY

Table 3 is a list of some water chemistry determinations made in the Decker mine. For comparative purposes, No Name Creek water, which is an acidic mine drainage type, was also analyzed for certain parameters. The influent to the Decker settling pond differs slightly from the settling pond and effluent waters in that it is of lower pH and of lower dissolved oxygen content, and higher in iron and sulfide. The three Decker waters differ markedly from No Name Creek water in regard to pH, sulfate and iron content.

STUDIES WITH *THIOBACILLUS FERROOXIDANS*

Sampling results and isolates obtained

Figure 3 and Table 4 show the sites where sampling for *T. ferrooxidans* was performed, and Table 5 gives the results of the primary enrichments for the organisms--the ability to oxidize large amounts of ferrous iron at an initial pH of 2.6. To confirm that *T. ferrooxidans* was present, selected flasks were subjected to further testing for pH, ferrous iron concentration, and presence of short rod-shaped cells. Using these procedures it was found that all sediment samples except for sites 1,3, and 30 were positive for *T. ferrooxidans*, however, water samples along the Tongue River were negative for the organism. Water samples within the Decker mine and in a stock well east of the mine were positive. The organism was also detected in a sample taken from an

TABLE 3. WATER CHEMISTRY DATA FOR THE DECKER MINE AND NO NAME CREEK. INFLUENT AND EFFLUENT VALUES ARE ARITHMETIC MEANS OF FOUR MONTHLY DETERMINATIONS (JULY-OCTOBER, 1976). SETTLING POND VALUES ARE ARITHMETIC MEANS OF EIGHT MONTHLY DETERMINATIONS (JULY, 1976 TO FEBRUARY, 1977). ALL VALUES ARE MG/1 UNLESS LISTED OTHERWISE. THE IRON VALUE OF NO NAME CREEK IS FERROUS IRON.

	pH	SO_4	SULFIDE	TOTAL IRON	SPECIFIC CONDUCTANCE (UMHO/CM)	TOTAL ALKALINITY (MG/1 $CaCO_3$)	DISSOLVED OXYGEN
INFLUENT	7.46	256	0.23	0.196	1338	ND	4.4
EFFLUENT	8.36	283	NONE	0.096	1471	ND	12.3
SETTLING POND	8.14	294	NONE	0.099	1865	703.0	7.0
NO NAME CREEK	2.80	15000	NONE	563	ND	ND	ND

Table 4. Sampling sites for T. ferrooxidans

Tongue River and Reservoir

Site 1 At highway 14 bridge in Dayton, WY
2 At Acme, WY power house, approximately ½ km upstream from confluence with Goose Creek
3 Approximately ½ km below Big Horn mine
4 At highway 339 bridge
5 At Munson Ranch approximately ¼ km upstream from county highway bridge
6 At county highway bridge, approximately 1 km upstream from Decker mine effluent area
7 At railroad bridge approximately ½ km upstream from Decker mine effluent area
8 At Decker mine effluent area
9 Approximately ½ km downstream from Decker mine effluent area
10 Approximately 1½ km downstream from Decker mine effluent area
11 Tongue River Reservoir-south end
12 Tongue River Reservoir-approximately 2 km north of site 11
13 Squirrel Creek near Decker post office

Big Horn mine

14 Holding basin emptying into Goose Creek-near discharge 5
15 Discharge 2 settling basin
16 Mine pit

Decker mine

17 Bottom of B ramp-mine pit
18 Bottom of D ramp-mine pit
19 Bottom of E ramp-mine pit
20 Settling pond
21 Settling pond influent
22 Settling pond effluent
23 Test pit
24 Stock well east of mine
25 Core sample, D-1 coal seam
26 Core sample, D-2 coal seam
27 Coal wall near settling pond

Table 4. Continued

Belle Ayr mine

Site 28 Mine pit
29 Mine pit
30 Caballo Creek, below sedimentation pond discharge (intermittent)
31 Sedimentation pond

No Name Creek

32 Approximately ½ km north of Sand Coulee, MT
33 Across from fire station, Sand Coulee

Table 5. Results of sampling for T. ferrooxidans

		CONFIRMATION CULTURE		
Tongue River sites	PRIMARY ENRICHMENT	FINAL pH	% IRON OXIDIZED	MICROSCOPY
1 sediment	-			-
2 sediment	+	2.15	99	+
3 sediment	-			-
4 sediment	+	2.15	98	+
5 sediment	+	2.11	99	+
water	-	2.88	0	-
6 sediment	+	2.15	99	+
7 sediment	+	2.16	99	+
water	-			-
8 sediment	+	2.16	99	+
water	-			-
9 sediment	+	2.12	98	+
10 sediment	+	2.15	99	+
11 sediment	+	2.14	97	+
water	-	2.76	0	-
12 sediment	+	2.13	98	+
13 sediment	+	2.10	97	+
Big Horn mine sites				
14 sediment	+	2.17	98	+
15 sediment	+	2.14	98	+
16 sediment	+	2.12	99	+
Decker mine sites				
17 sediment	+	2.15	98	+
18 sediment	+	2.12	99	+
19 sediment	+			
20 sediment	+			
water	+			
21 water	+			
22 water	+			
23 water	+			
24 water	+			
25 coal	-			
26 coal	-			
27 coal	+			

Table 5. Continued

	PRIMARY ENRICHMENT	FINAL pH	% IRON OXIDIZED	MICROSCOPY
Belle Ayr mine sites				
28 sediment	+			
29 sediment	+			
30 sediment	-			
31 sediment	+			
No Name Creek sites				
32 water	+			
33 water	+			
Controls				
1. Uninoculated	-	2.76	1	-
2. (+) culture plus formalin	-	2.58	0	-

exposed, wet coal wall within the Decker mine, however one core sample from each of the two principal coal beds was negative.

Selected positive enrichments were further tested to ensure a positive reaction was indicative of T. ferrooxidans. After reinoculation into fresh pH 2.6, 9K-iron medium and incubation for six days, it was found that all positive enrichment flasks showed a significant drop in pH, nearly total oxidation of the ferrous iron, and numerous rod-shaped bacteria. Selected negative flasks and controls showed little or no drop in pH, oxidation of ferrous iron, or observable bacteria (Table 5).

Some of the selected positive enrichments were subjected to bacterial purification procedures, and several isolates were obtained (Table 6). These isolates were shown to be free from heterotrophic contamination by streaking on two tryptone glucose extract agar plates at pH 4.5 and 7.0, and on Sabouraud's dextrose agar following ten days of incubation. The isolates were all gram (-) weakly motile rods.

Physiological experiments

Table 7 is a compilation of physiological characteristics of some of the T. ferrooxidans isolates. With iron as a substrate, generation times ranged from 7.7 to 15.9 hours at room temperature and respiration rates ranged from 240 to 984 µl oxygen uptake per mg cell protein per hour. All isolates were capable of growth on

Table 6. Origin of *T. ferrooxidans* isolates

ISOLATE	ORIGIN
TF-1, TF-2	Decker mine settling pond sediments
BC	Big Horn mine pit sediment
SC	Acidic No Name Creek near Sand Coulee, MT
BA-1,2,3,4	Belle Ayr mine pit sediments
TR-L	Tongue River sediment below Decker discharge
TR-M	Tongue River sediment above Decker discharge

TABLE 7. SOME PHYSIOLOGICAL CHARACTERISTICS OF I. FERROOXIDANS ISOLATES. GENERATION TIMES ON IRON WERE MEASURED AT ROOM TEMPERATURE. QO_2 (PROTEIN) = O_2 UPTAKE PER MG CELL PROTEIN PER HOUR. SUBSTRATE WAS 50 UMOLES FERROUS IRON.

ISOLATE	GENERATION TIME--9K PLUS IRON	QO_2 (PROTEIN)	GROWTH ON ELEMENTAL SULFUR	THIOSULFATE	GLUCOSE	PYRITE
TF-1	8.4 (HR)	984	+	+	-	+
BC	9.2	418	+	+	-	+
SC	10.2	674	+	+	+	+
BA-1	7.7	534	+	+	-	+
BA-3	12.9	240	+	+	-	+
TR-M	15.4	319	+	+	-	+
TR-L	15.9	323	+	+	-	+

elemental sulfur at pH 2.6. After some difficulty, all isolates were adapted to grow at the expense of thiosulfate at pH 4.1. Initial attempts to cultivate the organisms on thiosulfate after growth on elemental sulfur failed, and the method of Tuovinen and Kelly (63) was finally used to adapt the cells to thiosulfate. This technique involved harvesting the cells after growth on iron, and using a large inoculum (about 10^9 cells) for transfer to thiosulfate. All isolates were adapted to thiosulfate in this manner. In addition, all isolates formed acid from pyrite (Table 8). One of the isolates was adapted to growth on glucose (SC). The generation time when isolate SC was grown on 0.1% glucose at pH 2.7 was 18 hours (room temperature).

To investigate the likelihood of growth at the high pH of mine waters, the upper pH limits for growth of the T. ferrooxidans isolates were tested on sulfur and thiosulfate media using adapted cells. The iron medium could not be used for these experiments due to the rapid oxidation of ferrous iron above pH 4. The highest pH supporting growth on thiosulfate was 5.7 and on sulfur, 5.3. No isolate was observed to grow on either energy source after seven weeks of incubation at pH 6.3.

Leaching experiments

Leaching experiments were performed to check on the ability of T. ferrooxidans to form acid from possible sulfuritic materials present

TABLE 8. OXIDATION OF PYRITE BY T. FERROOXIDANS ISOLATES. EACH FLASK CONTAINED 5.0 G CRUSHED PYRITE AND 100 ML OF ONE-TENTH STRENGTH 9K SALTS. FLASKS WERE INOCULATED WITH ONE ML OF WASHED SUSPENSIONS OF THE APPROPRIATE CELLS (ABOUT 10^9 CELLS). FLASKS WERE ALLOWED TO INCUBATE FOR SIX HOURS BEFORE ZERO TIME MEASUREMENTS WERE MADE. SULFATE CONCENTRATIONS ARE EXPRESSED IN MG/1, ACIDITY AS MG/1 $CaCO_3$.

	pH		SULFATE		ACIDITY	
	0	48 DAYS	0	48 DAYS	0	48 DAYS
TF-1	2.61	1.81	405	5125	435	8200
BA-1	2.61	2.08	378	2125	460	4000
BC	2.60	1.87	331	7750	485	9400
SC	2.61	1.98	479	2500	485	4100
BA-3	2.52	2.20	405	1375	490	2700
TR-M	2.52	1.98	459	3500	445	4800
TR-L	2.53	1.89	392	7000	400	7800
UNINOCULATED	2.60	2.61	378	550	455	825
UNINOCULATED	2.58	2.58	446	575	540	950

in coal samples. Coal from the Decker rider seam incubated with isolate TF-1 showed no discernible acid production over the controls (Table 9). Adjustment of the pH to more favorable levels for this organism made no difference in the results. When similar experiments were carried out using pyrite, all isolates were capable of forming acid, as mentioned previously.

Survival studies

The occurrence of the obligately acidophilic T. ferrooxidans in the waters of the Decker mine prompted studies to determine the survival capability of the organism in mine waters. Early experiments showed a rapid die-off of laboratory cultivated isolates TF-1 and TF-2 when these cells were incubated in membrane chambers in the Decker mine settling pond (Figure 4). Another experiment was performed later to assess the survival of a laboratory cultivated isolate (TF-1) as well as indigenous populations of T. ferrooxidans in the settling pond and in the settling pond influent waters. Results show a more gradual die-off of laboratory cultivated TF-1 than in the June, 1977 experiment (Figure 4). There appeared to be little difference in the survival characteristics of these cells between the two incubation sites. Indigenous settling pond and influent populations of T. ferrooxidans also appeared to die off slowly and at comparable rates (Figure 5). It appeared that natural settling pond cells may have died

Table 9. Incubation of Thiobacillus ferrooxidans with coal: 10g of 28-80 mesh rider seam coal were added to 100 ml 9k salts medium. Sodium azide (0.03g), and/or iron (4.42g $FeSO_4 \cdot 7H_2O$) was added to some flasks. All flasks were inoculated with 0.2 ml of a washed suspension of Thiobacillus ferrooxidans (isolated from the mine settling pond) containing 10^8 cells/ml. After 4 hours of equilibration, pH measurements and titrations were made on 10 ml samples. All measurements were made in triplicate and the means and the 95% confidence limits are shown.

	INITIAL		33 DAYS		CHANGE	
TREATMENT	pH	ACIDITY (mg/l $CaCO_3$)	pH	ACIDITY	pH	ACIDITY (mg/l $CaCO_3$)
None	7.09±0.26	249±6	6.09±0.03	336±68	-1.00	+87
Sodium azide	7.21±0.27	221±15	6.42±0.12	307±136	-0.79	+86
None	4.47±0.09	397±37	5.32±0.10	425±65	+0.85	+28
Sodium azide	4.45±0.20	422±61	5.38±0.03	411±86	+0.93	-11
Iron	3.77±0.07	---	2.24±0.13	---	-1.53	--
Iron + Sodium azide	3.93±0.08	---	2.86±0.08	---	-1.07	--

Figure 4. Survival of laboratory grown Thiobacillus ferrooxidans isolates in settling pond and influent waters using membrane chambers. Bar indicates 95% confidence limits. TF-1/SP indicates isolate TF-1 incubated in the settling pond.

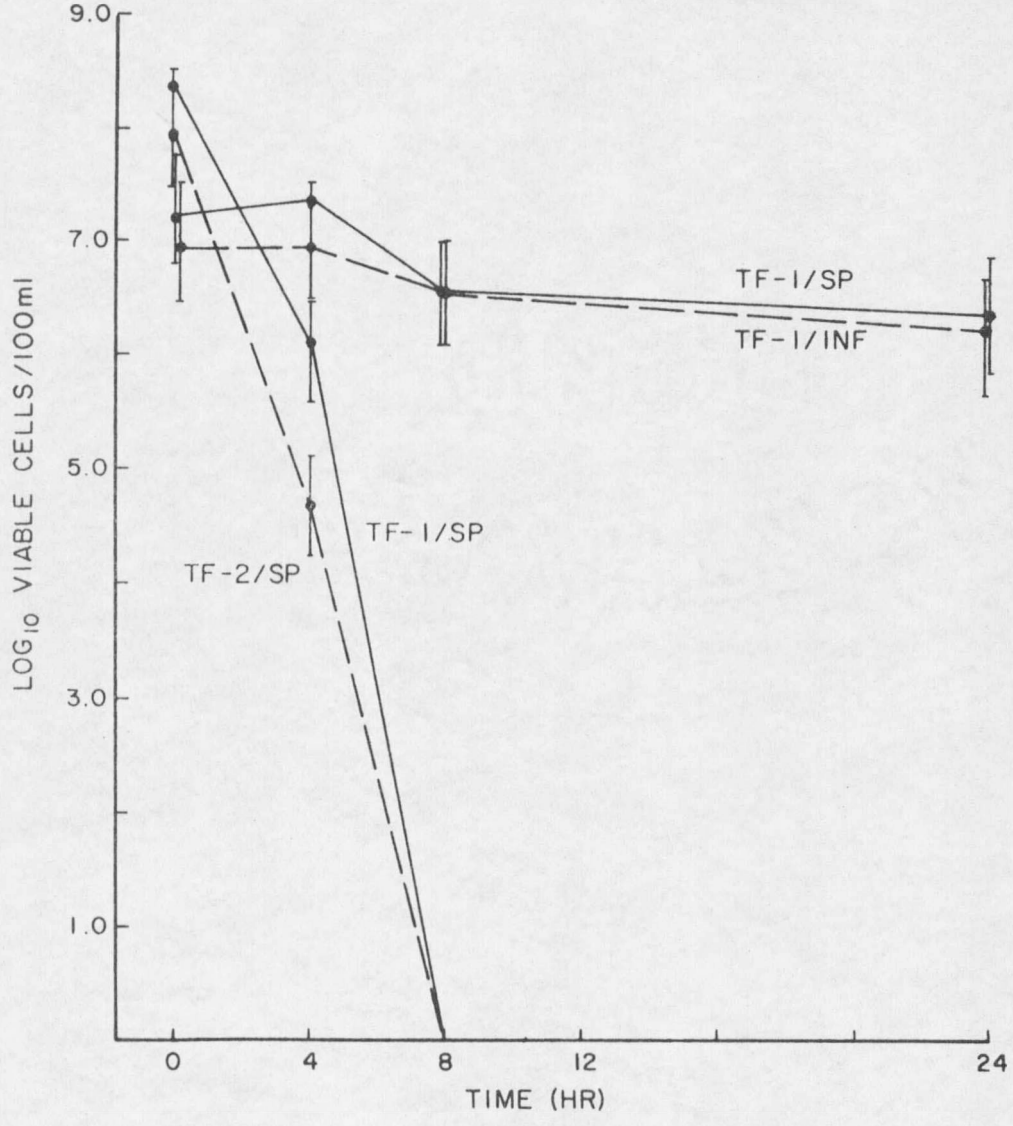
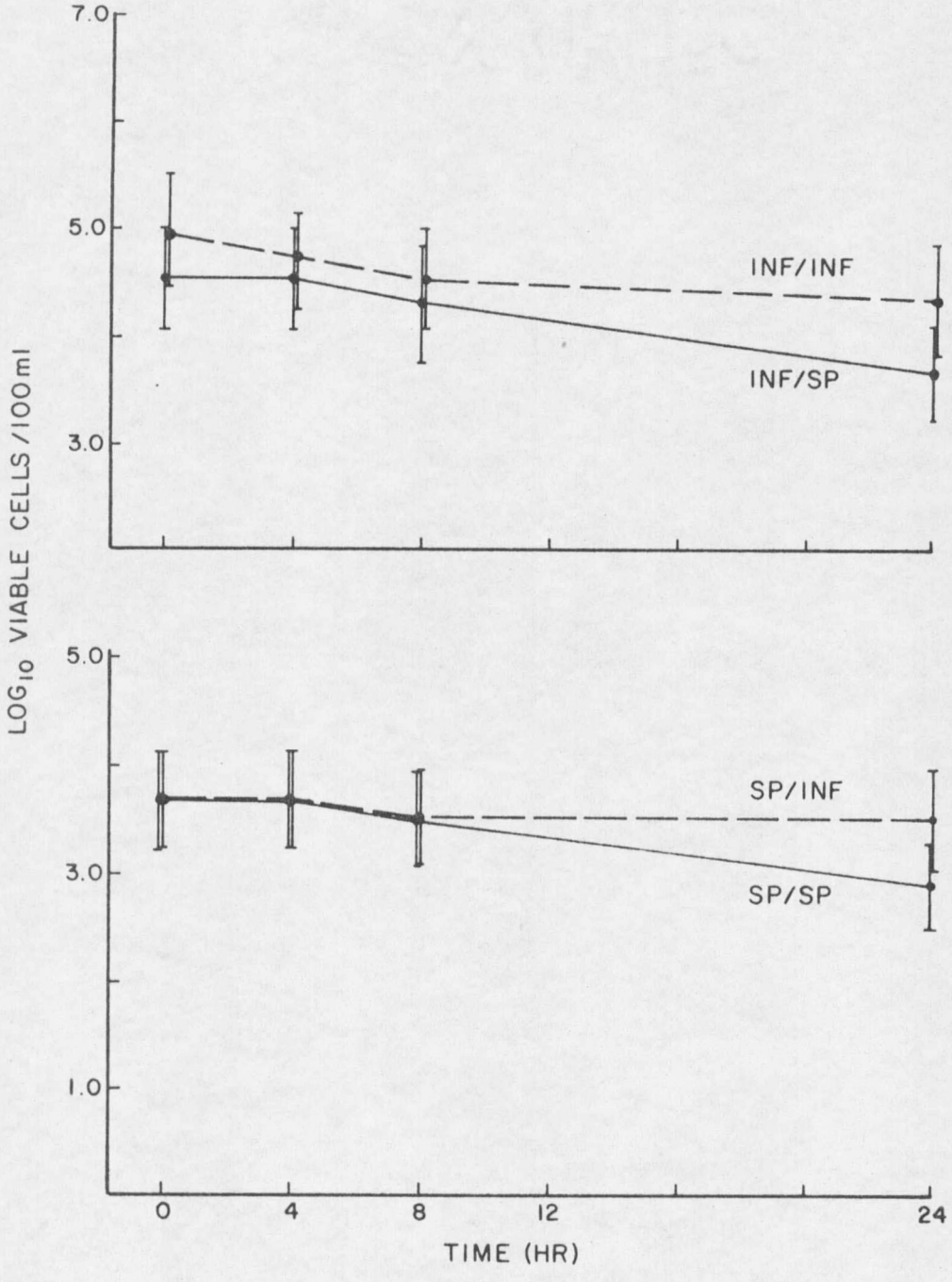

9.0
7.0
5.0
3.0
1.0
LOG10 VIABLE CELLS/100ml
TF-1/SP
TF-1/INF
TF-1/SP
TF-2/SP
0
4
8
12
24
TIME (HR)

Figure 5. Survival of indigenous populations (influent and settling pond) of iron oxidizing bacteria in settling pond and influent waters in membrane chambers. Bar indicates 95% confidence limits. INF/SP indicates influent organisms incubated in the settling pond.


7.0
5.0
3.0
INF/INF
INF/SP
5.0
3.0
1.0
SP/INF
SP/SP
0
4
8
12
24
TIME (HR)
LOG10 VIABLE CELLS/100 ml

off a little more slowly when incubated in the influent compared to the settling pond, however, the large confidence intervals inherent in the MPN procedure make a more definite statement difficult to justify. It was thought that incubation temperature may have had an important effect on the observed die-off rates, since June temperatures were much higher than October temperatures (approximately 25 C vs. 10 C), however an experiment performed in the laboratory showed no significant difference in die-off between a suspension of isolate TF-1 in settling pond water incubated at 10 C with one incubated at 25 C in membrane chambers.

SULFATE REDUCTION RATES

The radioisotope technique of Ivanov (20) was employed to assess the activity of sulfate reducing bacteria in the sediments of the Decker mine settling pond. Results (Table 10) from September, October, and December, 1976, showed an active rate of sulfate reduction in these sediments, ranging from 1.27 to 10.50 mg H_2S per l of sediment per day. A spike of lactate in the October experiment (300 mg/l) stimulated the rate by more than three-fold, indicating that organic matter was limiting the process. Lack of stimulation in the December experiment indicated something other than organic matter was limiting the sulfate reduction process. The June, 1977, experiment was run to determine how accurately the rate of sulfate reduction could be measured in

TABLE 10. RATES OF SULFATE REDUCTION IN THE DECKER MINE SETTLING POND. SULFATES ARE GIVEN IN MG/1. THE RATE OF SULFATE REDUCTION IS GIVEN IN MG H_2S PRODUCED PER 1 OF SEDIMENT PER DAY. LACTATE SPIKES WERE ADDED TO THE INDICATED TUBES AT 300 MG/1 (OCTOBER, DECEMBER 1976) OR 100 MG/1 (OCTOBER, 1977)

DATE	LOCATION/DEPTH(CM)		TREATMENT	SO_4	TEMP	RATE
9/76	NORTH END	0-10	NONE	150	16	1.27
	SOUTH END	0-10	NONE	150	17	3.98
10/76	SOUTH END	0-10	NONE	180	6	10.50
			LACTATE			34.85
12/76	SOUTH END	0-10	NONE	44	1	1.83
			LACTATE			1.54
6/77	SOUTH END	20-40	NONE	63		0.54
						0.62
						0.71
						0.97
9/77	SOUTH END	0-20	NONE	36	16	2.83
		20-40	NONE	21		0.27
		40-60	NONE	109		0.60
	NORTH END	0-20	NONE	276		0.00
		20-40	NONE	294		1.03
		40-60	NONE	44		0.95
10/77	SOUTH END	0-20	NONE	488	11	0.00
			LACTATE			0.95
		20-40	NONE	93		3.13
			LACTATE			6.64
		40-60	NONE	41		1.80
			LACTATE			2.44
	NORTH END	0-20	NONE	175		0.73
			LACTATE			9.74
		20-40	NONE	157		0.71
			LACTATE			10.53
		40-60	NONE	173		1.14
			LACTATE			11.76

replicate samples from the same homogenized sediment core. Values compared reasonably well, ranging from 0.54 to 0.97 mg H_2S per 1 of sediment per day. During June, 1977, an unusually heavy rainstorm hit the mine area, and large volumes of silty water were pumped out of the mine pit and through the pond system. This resulted in an accumulation of sandy silt about 20 to 30 cm thick on top of the old surface layers of the settling pond sediment. The September and October, 1977, measurements of sulfate reduction rates indicated there were areas in the new surface sediment layer that recovered quickly in the sulfate reduction process, and others that were slower in recovery. The addition of lactate (100 mg/1) greatly stimulated sulfate reduction at all levels in the northern end of the pond, and in the upper level in the southern end of the pond in October, 1977.

METAL BOUND SULFIDES

The content of metal bound sulfides in the settling pond sediments seemed to parallel the activity of the sulfate reducing bacteria. A drastic drop in the content of metal bound sulfides in the surface layers of the settling pond occurred after the heavy June, 1977, rainstorm because of the sediment washed into the pond (Table 11). Recovery of the content of metal bound sulfides, presumably linked to the generation of hydrogen sulfide by the sulfate reducing bacteria, was sporadic in the surface layers of the settling pond sediment.

TABLE 11. CONTENT OF METAL BOUND SULFIDES IN THE DECKER SETTLING POND SEDIMENTS.

DATE	LOCATION/DEPTH(CM)		WEIGHT 1 ML SEDIMENT (G)	METAL BOUND SULFIDES MG/1	MG/G DRY WEIGHT
2/77	SOUTH END	0-20	0.2450	608	2.26
7/77	SOUTH END	0-20	-	0	0.00
		20-40	0.6507	1320	2.03
	NORTH END	0-20	-	0	0.00
		20-40	0.9401	401	0.43
9/77	SOUTH END	0-20	0.4328	795	1.83
		20-40	0.3082	443	1.42
		40-60	0.5081	394	0.79
	NORTH END	0-20	0.3980	112	0.28
		20-40	0.7055	103	0.15
		40-60	0.6433	613	0.98
10/77	SOUTH END	0-20	0.5048	54	0.11
		20-40	0.7530	1121	1.49
		40-60	0.3637	521	1.43
	NORTH END	0-20	0.3033	239	0.79
		20-40	0.3987	336	0.84
		40-60	0.9893	822	0.83

On one occasion some of the heavy metals solubilized by hydriodic acid treatment of settling pond surface sediment were quantified. Iron and manganese were the predominant metals found, and cadmium, copper, nickel, lead, and zinc were also detected (Table 12).

SULFATE REDUCTION IN WELL WATERS

Sulfate reducing bacteria were recovered from many wells in southeastern Montana (Table 13), however it was not possible to demonstrate activity of these organisms using radiolabeled sulfate. In a few well water samples sulfates and sulfides were removed and trapped for the purpose of sulfur isotope ratio determinations. The values obtained in the isotope analyses demonstrate a clear fractionation between sulfates and sulfides in the same water sample, indicating the sulfides arose as a result of bacterial dissimilatory sulfate reduction.

OTHER SULFUR BACTERIA

Thiothrix

Through the use of the key and descriptions in Bergey's Manual (3), the cream colored filaments lining the mine sump rivulets were determined to be predominantly Thiothrix. Laboratory experiments on agar plates showed no evidence of gliding motility. Using bright field optics, the filaments were seen to contain numerous refractile

TABLE 12. HEAVY METAL CONTENT OF A HYDRIODIC ACID EXTRACT OF SETTLING POND SURFACE SEDIMENT. DATA FROM THE SETTLING POND WATER IS TAKEN FROM THE REPORT OF GREGORY (13).

	SETTLING POND SEDIMENT		SETTLING POND WATER
	MG/1	MG/G DRY WT	RANGE (MG/1)
CADMIUM	0.38	0.0016	<0.001-0.002
COPPER	3.0	0.0122	<0.01 -0.02
IRON	142	0.5794	0.08 -1.91
MANGANESE	67	0.2734	<0.01 -0.42
NICKEL	5.5	0.0224	<0.01 -<0.05
LEAD	2.0	0.0082	<0.05 -0.08
ZINC	12.0	0.0490	<0.01 -0.03
MERCURY	-	-	<0.0002-0.0089

TABLE 13. RESULTS OF WELL WATER SAMPLING NEAR MINING AREAS IN SOUTHEASTERN MONTANA. SULFUR ISOTOPE DATA ARE EXPRESSED AS $\delta^{34}S$ ‰.

WELL	LOCATION	DEPTH (M)	H_2S ODOR	MPN (100 ML)	$^{35}SO_4$ REDUCED	SULFUR ISOTOPE RATIOS Ag_2S	$BaSO_4$
1	10 KM NE OF DECKER	15	+	$1.7x10^3$	-	+6.68	+45.17
2	15 KM W OF COLSTRIP	?	-	$2.0x10^1$	ND		
3	10 KM S OF COLSTRIP	50	-	$1.3x10^3$	-	-36.82	+3.08
4	10 KM S OF COLSTRIP	?	+	$1.4x10^2$	ND		
5	15 KM E OF COLSTRIP	15	+	$2.4x10^3$	-		
6	60 KM NE OF DECKER	15	+	$>2.4x10^4$	-		
7	60 KM NE OF DECKER	80	-	0	ND		
8	55 KM NE OF DECKER	110	-	$7.9x10^2$	ND		
9	50 KM NE OF DECKER	50	+	$7.0x10^2$	-		
10	30 KM NE OF DECKER	250	+	$>2.4x10^4$	-	-11.43	+6.04
11	20 KM N OF DECKER	55	-	$1.1x10^2$	ND		
12	DECKER MINE SUMP	5	+	$3.5x10^3$	-	-32.90	-0.20

TABLE 13. CONTINUED.

WELL	LOCATION	DEPTH (M)	H_2S ODOR	MPN (100 ML)	$^{35}SO_4$ REDUCED	SULFUR IROTOPE RATIOS Ag_2S	$BaSO_4$
13	95 KM NE DECKER	20	-	$1.4x10^3$	-		
14	105 KM NE DECKER	50	+	$>2.4x10^4$	ND	-38.50	+9.63
15	105 KM NE DECKER	?	+	$>2.4x10^4$	-	-34.65	+2.68
16	130 KM NE DECKER	100	+	$>2.4x10^4$	-	-36.43	+4.11
17	120 KM NE DECKER	50	+	$3.5x10^3$	ND	-27.68	+3.33
18	125 KM NE DECKER	250	-	$1.4x10^2$	ND		
19	15 KM S COLSTRIP	?	+	$2.8x10^3$	-	-35.11	+1.85

granules. These granules did not stain with acidic methylene blue or Sudan black, but rapidly disappeared when a drop of warm acetone or ethanol was drawn under the coverslip. An acetone extract of a tuft of filaments gave a strong positive reaction for elemental sulfur. The extract was found to contain about 15 mg/l elemental sulfur. The filaments were about 1.5 µm wide with rounded ends, and their length varied greatly, ranging up to several hundred µm long. Occasionally, rosettes could be seen, however, holdfasts could not be clearly distinguished. Occasional branching of the filaments occurred, at right angles to the filament. These branches were sometimes of considerably smaller diameter than the main filament. After the original source of the organisms was buried (the sump pit), slides were placed in the influent channel, attached to rocks, to see if recolonization would occur. After six weeks the slides had not become colonized, however, the rocks to which the slides were attached had developed areas of cream-colored filamentous growths which were intimately associated with an algal community consisting of diatoms and other types of algae. Microscopically, these filaments resembled the ones collected from the sump rivulets.

Thiobacilli

Isolates from the thiosulfate oxidation MPN tubes (from settling pond waters) were identified as Thiobacillus neapolitanus (two isolates)

and Thiobacillus denitrificans (one isolate) using the procedures suggested by Hutchinson, et al. (17) for the identification of thiobacilli. The results of the testing are shown in Table 14. Attempts were also made to isolate low pH thiosulfate oxidizers (pH 4.5), as well as low pH heterotrophic bacteria (pH 3.0). Turbidity was occasionally observed in enrichment flasks of the thiosulfate medium, however growth on agar was not successful. The only growth obtained at low pH on heterotrophic media were a few surface fungal colonies in broth.

TABLE 14. RESULTS OF HUTCHINSON, ET AL. TESTING SCHEME FOR IDENTIFICATION OF THIOBACILLI. (+) INDICATES GROWTH.

	ISOLATES			
	TD-1	S6A	SZ	CONTROL
FINAL pH (S6 MEDIUM)	5.6	3.0	3.0	6.5
% THIOSULFATE OXIDIZED (S6)	3	91	92	0
SULFUR DEPOSITED ON THIOSULFATE AGAR (S6)	+	+	+	-
KOSER CITRATE	-	-	-	-
NUTRIENT AGAR	-	-	-	-
S3 (ANAEROBIC) MEDIUM	+	-	-	-
GAS PRODUCTION (S8 MEDIUM)	+/-	-	-	-
S6 BROTH PLUS 5% NaCl		+	+	-
IDENTIFICATION	T. DENITRIFICANS	T. NEAPOLITANUS	T. NEAPOLITANUS	

DISCUSSION

Microorganisms indigenous to alkaline mine waters have received little attention and consequently the microbiology of this type of mine drainage is poorly understood (32,55). This is particularly true of the functional role of thiobacilli in this setting. Adverse impacts of coal mining on water quality in the eastern United States are well known and studied, but much less is known concerning the potential for water quality degradation in the West (40). This study attempts to answer some of the questions that exist regarding occurrence and activity of some of the important sulfur cycle bacteria (some of which are known to contribute to water pollution problems) found in alkaline waters of a western coal strip mine.

Water chemistry data indicate that the waters in the Decker mine had none of the attributes which characterize acid mine drainage. In contrast, No Name Creek, which drains abandoned bituminous coal mines near Great Falls, illustrated a typical acid mine drainage stream possessing low pH values, high sulfate and iron concentrations, and a virtual absence of higher forms of life.

The extreme environments, such as acid mine drainage, inhabited by T. ferrooxidans are well known, however, virtually nothing is known of its distribution elsewhere (62). This organism may actually exist in far more soil and water environments than has previously been supposed (63). Fjerdingstad (11) was able to isolate T. ferrooxidans

from lignite pit sludge of pH values as high as 6.35. Tuttle, et al. (64) detected acidophilic iron and sulfur oxidizing bacteria in a neutral stream and attributed their presence to an unobserved acidic input. Lyalikova (33) was able to detect T. ferrooxidans in small numbers in alkaline waters associated with copper-nickel deposits and attributed its presence to creation of acidic microzones in the ore, and Karavaiko (24) reached a similar conclusion in a study of oxidation of neutral to alkaline sulfur bearing limestone by T. thiooxidans. Sokolova and Karavaiko (51) state that T. ferrooxidans is frequently detected in neutral water sources in mines in which focal oxidation of ores was observed.

The present study indicated that T. ferrooxidans had a widespread distribution in mine waters and sediments and in associated sedimentary environments outside the mines in eastern Montana and Wyoming. The numbers of this organism were surprisingly high in the neutral to alkaline mine settling pond influent waters, comparable to values reported by Tuttle, et al. (64,65) for acidic mine drainage streams. Dugan (8) has stated that the very presence of T. ferrooxidans in mine water indicates that the organism has already been responsible for the formation of acid from pyrite, the numbers of bacteria correlating with the amount of pyrite oxidized. Tabita, et al. (55) feel that alkaline mine waters favor the activity of organisms oxidizing reduced sulfur

compounds whereas the predominance of reduced iron and pyrite in acidic drainage favors T. ferrooxidans. Numbers of T. ferrooxidans were at least equal to, and often greater than numbers of bacteria oxidizing thiosulfate at neutral pH in the settling pond influent waters. These statements (8,55) and the results of this study suggest an appreciable amount of activity of T. ferrooxidans occurred in the coal bearing strata of the Decker mine. However, since the content of bicarbonate in the groundwaters of the Decker area was so high (69), any acid formed was quickly neutralized. This concept is supported by the data of VanVoast and Hedges (69) who found high sulfate concentrations in the mine settling pond waters relative to those found in groundwater samples (they attributed this increase in sulfate to dissolution from freshly crushed coal during the mining process). It seems likely that the high sulfate concentrations resulted from the microbial oxidation of sulfuritic materials found in association with the coal bearing strata. The sulfuric acid formed would be quickly neutralized by bicarbonate in the water, but the sulfates would remain to be eventually passed into the settling pond. It also seems likely that any activity of T. ferrooxidans is limited to small areas or microzones within the coal bearing strata since 1) wells sampled in the area show no evidence of acidity (69) and acidity has never been noted in water samples taken in the mine, and 2) the organisms are not active at

neutral pH values in laboratory culture, and die-off in mine waters. In addition, Chadwick, et al., in an analysis of the Rosebud and McKay coal seams (part of the Fort Union formation) near Colstrip (5), found pyrite and other trace metals to have significant vertical variations over short distances. Pyrite and other heavy metals were concentrated near the base of the coal seams. A similar phenomenon could also occur at Decker. Since the overall sulfur content of the Decker coal is only 0.3 to 0.4%, and the pyritic sulfur content is half or less of this value (36), it is also possible that overburden materials and/or the rider coal seam contribute a large portion of the potential oxidizable sulfuritic materials, and this could be where most of the activity of T. ferrooxidans is taking place. The inability to culture the iron oxidizers from core samples of coal and to show acid production from crushed coal samples tends to support this point. In addition, Temple and Kimble (59) found chemoautotrophic bacteria to be widely distributed in overburden cores from eastern Montana coal fields. A few of these core samples developed low pH values upon leaching (<pH 4). They concluded that activity of these organisms in core samples was likely, but that in most cases there was only a small amount of oxidizable iron or sulfur compared to the carbonate buffering capacity. Once acidic microzones are established, it is also possible that sulfide in the groundwater could serve as an energy source for T. ferrooxidans.

The isolates of T. ferrooxidans which were obtained appeared to be physiologically similar to those previously described in the literature. This is further evidence that acid production occurred in the mine environment. Generation times of 7.7 to 15.9 hours when the isolates are grown on iron compare closely to the figures of 6.5 to 15.0 hours quoted by Tuovinen and Kelly (62) in their review on this organism. Respiration on iron resulted in QO_2 (protein) values of 240 to 984, equivalent to QO_2 (N) values of 1500 to 6150. Silverman and Lundgren (48) reported a QO_2 (N) range of 2027 to 4516 in different cell harvests of Ferrobacillus ferrooxidans strain TM grown under optimal conditions on 250 µmoles of ferrous iron. They obtained a maximum value of 5130 using 500 µmoles of ferrous iron. Beck (2) found a maximum QO_2 (N) of 4200 with his isolate. In a comprehensive study on factors affecting bacterial respiration using iron, Landesman, et al. (28) found they could obtain QO_2 (N) values as high as 22,500.

The difficulties encountered in getting strains of T. ferrooxidans to grow at the expense of thiosulfate have been described by Tuovinen and Kelly (63) who had to use large inocula to successfully adapt the organism to growth on this compound. Difficulties of this sort had for years caused confusion in the nomenclature of the acidophilic iron oxidizing bacteria Ferrobacillus ferrooxidans, Ferrobacillus sulfooxidans, and Thiobacillus ferrooxidans. Kelly and Tuovinen (25) have

only recently clarified this situation by finally cultivating all three organisms on elemental sulfur and thiosulfate, eliminating the previously utilized criteria for separating the organisms. The earliest named organism, T. ferrooxidans (57) is the correct name to be used. The isolates obtained in the present study were also difficult to cultivate on thiosulfate until large inocula (10^9 cells) were used. Smaller numbers of iron or sulfur grown cells could not be successfully transferred to thiosulfate.

Growth of the isolates on elemental sulfur was not difficult if the technique of Dugan and Tuttle (9) was followed, involving cultivation in the presence of both iron and sulfur before growth on sulfur alone was attempted.

To summarize, the isolates of T. ferrooxidans obtained from various non-acidic environments appear to be physiologically and morphologically similar to those previously described from acidic mine drainage. These organisms were not able to grow at neutral pH or survive well in mine waters indicating they must be active in low pH areas within the coal bearing strata.

The present study indicates that an active rate of sulfate reduction occurred in the sediments of the Decker mine settling pond, up to 10.5 ml H_2S per 1 of sediment per day. Although reports of direct measurements of sulfate reduction are rare in the literature (22,61), especially outside the Soviet Union, some comparison of the

data can be made. Chebotarev (6) termed rates of sulfate reduction of 6.2 and 13.3 mg H_2S per kg sediment per day "very vigorous". Kuznetsov (27) listed "intensive" rates of sulfate reduction ranging from 4.2 to 40 mg H_2S per 1 of sediment per day. The rates determined for the Decker settling pond sediments (supporting a substantial population of these organisms) therefore seemed to be significant.

Whenever sulfate reducing bacteria are active, metals are precipitated as metal sulfides (42). This indicated that the Decker settling pond could have acted as a trap for heavy metals which entered it. Ilyaletdinov, et al. (18) encouraged the growth of sulfate reducing bacteria by addition of a natural source of organic matter (chopped reeds) to a metallurgical plant settling pond. The bacteria produced hydrogen sulfide which precipitated copper in solution, reducing the concentration of this element to acceptable levels in the effluent from the pond. Tuttle, et al, (65) showed a wood dust dam to be an effective method of encouragement of sulfate reducing bacteria, which resulted in a pH rise and precipitation of iron from acid mine waters. Mercury, a particularly dangerous heavy metal, is effectively removed from contaminated waters by precipitation as a sulfide (21,45,54,71) and in this form it is unavailable for methylation (45). Sulfate reduction could easily be encouraged in the settling pond sediments as evidenced by the rapid recovery in activity after the June, 1977 rainstorm. The Decker mine settling pond, therefore, could function

as an effective trap for heavy metals. Metal bound sulfides in the sediments of the pond were found to comprise, at times, over 0.2% of the dry weight. Sorokin (52) felt that sulfides in concentrations of 410 to 4520 mg per 1 of sediment (making up as much as 1% of the dry matter of the sediment) were "enormous" quantities.

At the present time, the Decker mine water discharged into the Tongue River is generally within United States Environmental Protection Agency standards for non-hazardous public water supplies or the protection of aquatic life (1). Concentrations of mercury, however, exceeded the recommended levels by occasionally greater than an order of magnitude (up to 0.87 µg/1) (66). Discharge from the mine did not present a source of mercury pollution to the aquatic environment outside of the mine because of negligible loading to the Tongue River (13). Data obtained by Gregory (13) indicate that concentrations of certain other heavy metals, including lead and selenium occasionally exceeded U.S. Environmental Protection Agency recommendations. In that study, mercury concentrations of up to 8.9 µg/1 were measured in the settling pond waters. This value exceeds the recommended levels by greater than 100 times. Encouragement of sulfate reduction in the settling pond sediments could decrease the concentrations of mercury of other heavy metals in the pond effluent by a significant margin. In other mining areas where heavy metal loading poses a greater threat to the the surrounding aquatic environment, metal sulfide precipitation in

mine settling ponds could prove to be a valuable process.

In many instances, sulfate reducing bacteria have been implicated in the contamination of ground waters with hydrogen sulfide (15,20,30). However, with the exception of work by Russian scientists (especially Ivanov), there appears to be almost no quantitative data available regarding the activity of these organisms in groundwater. This study demonstrated the occurrence of sulfate reducing bacteria in the groundwaters of the coal deposit regions in southeastern Montana. Attempts to show activity of these organisms in this environment have, however, failed. Ivanov (19) suggested that sulfate reducing bacteria in the adsorbed state may contribute much more to sulfate reduction than those found free in the water. Perhaps this was the case in the waters investigated in this project in that areas of intensive sulfate reduction could be localized in the aquifers. The widespread occurrence of the sulfate reducers in the groundwater samples indicated they were likely native to the habitat and that there are probably areas of sulfate reduction in the aquifers (39). With the exception of geothermal areas, hydrogen sulfide occurring in nature nearly always results from microbiological processes, usually dissimilatory sulfate reduction. Additional support for this explanation in the groundwaters studied in this project comes from the sulfur isotope ratios which revealed an enrichment of the lighter isotope of sulfur (^{32}S) in

groundwater sulfides relative to sulfates. A major fractionation of sulfur isotopes (^{32}S and ^{34}S are the two major stable isotopes) occurs when sulfate reducing bacteria reduce sulfate to sulfide, resulting in an enrichment of ^{32}S in the product sulfide (23). A comparison of the sulfur isotope composition (^{32}S and ^{34}S) of sulfates and sulfides in the groundwater will indicate if the sulfides have arisen as a result of biological sulfate reduction. In the present study, sulfides were enriched in ^{32}S (depleted in ^{34}S) relative to sulfates, indicating microbial sulfate reduction was responsible for the production of sulfide. The isotope data are, therefore, consistent with the idea that the sulfides arise from microbial sulfate reduction.

Other types of sulfur bacteria occurring in mine waters

Thiothrix was identified in the channels leading to the sump pit. Of interest is the fact that there were occasional smaller branches off of the main filaments, a characteristic which this genus supposedly does not possess (3). It is possible that other filamentous, sulfur depositing bacteria were mixed in with the population of Thiothrix. Other species of sulfur bacteria identified included Thiobacillus neapolitanus and Thiobacillus denitrificans. It is likely that other species of Thiobacillus occurred in the mine. Sulfur oxidation positive MPN tubes may have contained T. thiooxidans however it was not possible to grow these cultures on low pH colloidal sulfur or thiosulfate agar

plates for isolation.

SUMMARY

In order to better understand the possible adverse effects of surface coal mining in southeastern Montana on the scant water resources of that region, a study of microbial sulfur cycle organisms, some of which are known to cause serious acid water pollution problems in connection with mining, was undertaken at the Decker coal mine in southeastern Montana.

There was no evidence of acidity in waters of the Decker mine, however, Thiobacillus ferrooxidans, a major contributor to acid mine drainage, was consistently detected in surprisingly high numbers in the Decker mine waters and sediments. The organism was also found at other mines in the region and in some environments outside of the mines; all of which were non-acidic. After some isolates of the organism were obtained, physiological studies were performed to determine if these iron and sulfur oxidizing bacteria were indeed similar to those previously described from acidic environments. The isolates resembled typical T. ferrooxidans in their oxidation of iron, sulfur, thiosulfate, and pyrite at low pH, and would not grow above pH 5.7 on sulfur or thiosulfate. Two of the isolates were used in survival experiments and it was found they died off in mine waters. Experiments with crushed rider seam coal were unsuccessful in showing acid production. Based on these experiments it is believed that these organisms were active in microzones within the coal bearing

strata, and were continuously released into the associated waters. The acid that was produced by these bacteria was no doubt quickly neutralized by the high concentrations of bicarbonate present in the groundwaters.

The Decker settling pond sediments supported a large and active population of sulfate reducing bacteria, producing up to 10.5 mg H_2S per liter of sediment per day. Since the content of metal bound sulfides in the settling pond sediments was so high it seemed likely that sulfate reducing bacteria were precipitating heavy metals in the settling pond waters. This process could be more efficient if these organisms were encouraged by manipulating the pond design characteristics.

Several wells in southeastern Montana were sampled. Hydrogen sulfide commonly occurs in the groundwaters of that region and sulfate reducing bacteria were present in all wells except one. The bacterial production of radioactive H_2S from $^{35}SO_4$ could not be demonstrated in these wells. A number of sulfur isotope analyses, however, showed the sulfides in a given sample were enriched in ^{32}S relative to the sulfates, indicating the sulfides were likely formed by sulfate reducing bacteria which preferentially utilize ^{32}S in the sulfate reduction process. It seems likely that there are areas of active sulfate reduction in the aquifers.

Other sulfur bacteria were found in the Decker mine environment including Thiothrix, Thiobacillus neapolitanus, and Thiobacillus denitrificans.

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Aspects of microbial sulfur cycle activity
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