Microbial effect on the quality of leach water from eastern Montana coal mine spoils
by Patrick Ferol Kimble

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE i n Microbiology
Montana State University

Abstract:
Eight test hole cores of overburden grab samples from the Bear Creek Study Site of the West Moorhead coal deposit in eastern Montana were received from the U.S. Geological Survey. The samples were visually inspected for evidence of mineralization, which was aided by an accompanying geological log for each core. Each sample was ground prior to its analysis for lead content, pH value, conductivity value, and chemoautotrophic bacteria presence. The mineralization of the core samples was quantitatively different for the strata, with no consistent relation between physical and chemical descriptions.

Chemoautotrophic bacteria, both sulfur and iron oxidizing bacteria, were isolated from a number of core samples. Difficulty was encountered in obtaining pure cultures of these bacteria. Growth was not enhanced when yeast extract, cysteine, glutathione or IM-MF additives were included. The fastidiousness of these cultures does not typify sulfur or iron oxidizers in general, and therefore is characteristic of these isolates. A culture of iron oxidizing bacteria was isolated from a revegetation study site from the Colstrip coal deposit in eastern Montana. The growth of this culture was similar to the typical iron oxidizing bacterium, Thiobacillus ferrooxidans.

Leaching studies were performed on samples which exhibited a wide range of lead concentrations, pH values and conductivities. A <80 mesh ground sample was used in the leaching studies. The study included the comparison of static and shaking conditions, with and without glucose conditions, and autotrophic, soil, and no inoculum conditions. The fact that lead values in the leachates were roughly similar and not proportional to the lead content of the core sample shows that autotrophic oxidation did not proceed at a rate associated with unbuffered high pyrite ores. Most strata did not develop low pH values on leaching but a few strata did, with pH values seen as low as pH 1.61.

An algal bioassay procedure was developed to determine the possible toxic effect of the leachates. The bioassay utilized an inoculum of Selenastrum capricornutum PRINTZ, and included a 1:10 dilution of the leachate. Growth of the algae was monitored by fluorescence spectrophotometry on daily intervals. Some leachates produced inhibition , of algal growth, that was explained by low pH values while other toxic leachates could not be explained by either pH values or lead content of the leachates. For the majority of leachates no toxicity was observed, instead some leachates produced stimulatory effects.

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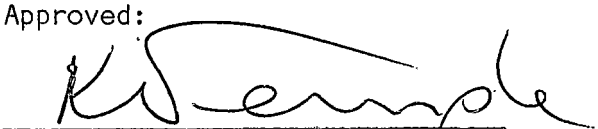

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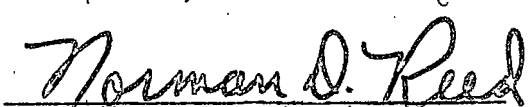

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ABSTRACT

Eight test hole cores of overburden grab samples from the Bear Creek Study Site of the West Moorhead coal deposit in eastern Montana were received from the U.S. Geological Survey. The samples were visually inspected for evidence of mineralization, which was aided by an accompanying geological log for each core. Each sample was ground prior to its analysis for lead content, pH value, conductivity value, and chemoautotrophic bacteria presence. The mineralization of the core samples was quantitatively different for the strata, with no consistent relation between physical and chemical descriptions.

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INTRODUCTION

The solubilization of metals from mining spoils, including coal mine spoils, has been well documented by Galbraith et al. (13), Silverman and Ehrlich (36), Tuovinen and Kelly (49), and Fjerdingstad et al. (12). The metals, as sulfides, will oxidize upon exposure to oxygen resulting in the formation of the corresponding metal sulfates, by the following reaction (3,12,33,36,45).

$$MeS + 2O_2 = MeSO_4$$

The presence of pyrite (iron disulfide, FeS_2) in spoils will cause the most vigorous solubilization of metals, as a consequence of bacterial production of ferrous sulfate, ferric sulfate, and sulfuric acid from the pyritic material (39,45,46). The ferric iron ion, produced as ferric sulfate through bacterial action, is a potent chemical oxidant, resulting in the solubilization of minerals (39,45). Members of the bacterial genus Thiobacillus catalyze the solubilization of metal sulfides due to the general ability of the genus to oxidize reduced sulfur compounds and to the specific ability of the species, Thiobacillus ferrooxidans, to also oxidize the reduced ferrous iron ion (36,39,45, 46). According to Temple and Koehler (46), the overall reaction sequence may be expressed as follows:

Initial chemical reaction:

$$FeS_2 + 3\text{-}1/2O_2 + H_2O = FeSO_4 + H_2SO_4$$

Bacterial reaction carried out by T. ferrooxidans:

$$2FeSO_4 + 1/2O_2 + H_2SO_4 = Fe_2(SO_4)_3 + H_2O$$

Subsequent chemical reactions:

$$Fe_2(SO_4)_3 + FeS_2 = 3FeSO_4 + 2S$$

Bacterial reaction carried out by T. thiooxidans:

$$S + 1\text{-}1/2O_2 + H_2O = 2H^+ + SO_4^{=}$$

According to Silverman (37) and Beck and Brown (2), the biological oxidation of pyrite is thought to proceed by two mechanisms, concurrently: a direct contact mechanism which requires physical contact between bacteria and pyrite particles and an indirect contact mechanism according to which the bacteria oxidize ferrous ions to the ferric state, thereby regenerating the ferric ions required for chemical oxidation of pyrite. Singer and Stumm (39) and Bosecker et al. (3) postulate that the oxidation of metal sulfides in the presence of pyritic material is mainly restricted to the chemical oxidation of sulfides via ferric iron. They also postulate that the activity of chemoautotrophic bacteria is mainly restricted to the reoxidation of chemically reduced ferric iron. Bryner and Anderson (6) did observe the bacterial oxidation of a metal sulfide, molybdenite (MoS_2), in the absence of pyrite, but the rate of oxidation was much slower than the rate of oxidation observed when pyrite was included. From these references, it could be assumed that oxidation of metal sulfides by chemoautotrophic bacteria will occur with or without pyrite, but that the rate of oxidation will be more rapid when associated with pyrite. Other bacteria and fungi, such as

Bacillus species and *Penicillium simplicissimum*, respectively, have been shown to aid in the solubilization of metals, through the production of organic acids and some unidentified compounds (45,49). However, the contribution of heterotrophic microorganisms in metal solubilization may be limited, due to low leaching yields, whereas the participation of *Thiobacillus* species in metal solubilization has been widely accepted and exploited (49).

Water which has been in contact with a particular stratum is influenced by the chemical composition of that stratum and is known as the leachate of that stratum. The toxicity of the leachate depends upon the minerals present and their rate of oxidation in relation to water flow. The identity of toxic elements, such as copper, arsenic, boron, zinc, and lead, is highly variable depending upon the composition of the ore body or the overburden. In the most extreme cases with high pyrite and low carbonate conditions, the leachate is strongly acid (45). The acid-forming chemoautotrophic bacteria, *Thiobacillus* species, are active in acid production of these high pyrite and low carbonate ores (22,23,45), as well as being active under neutral or alkaline conditions which are due to carbonate minerals (21). In the latter circumstance, visible halos may often be seen surrounding metal deposits. These halos consist of dissolved and reprecipitated minerals which may be acid salts and are often formed by the chemical reaction of sulfuric acid resulting from microbiological leaching and with

carbonates found in the ore or overburden (36). In the case of the low carbonate ores, the sulfuric acid and the metal sulfates formed through bacterial leaching would be found in the receiving waters of the ore.

Deleterious influence on the flora and fauna in receiving waters is often associated with high concentrations of heavy metals and low pH (10). One method of assessing this influence is through algal bioassays, using the green alga, Selenastrum capricornutum, in accordance with the Environmental Protection Agency methods (50,51). This algal bioassay procedure has also been utilized in the management of water quality, in the evaluation of water fertility, and in the determination of inorganic or organic compound toxicity (14,30,51). Selenastrum belongs to the group of ubiquitous algae which include Chlorella, Scenedesmus, and Ankistrodesmus, which have a wide tolerance towards environmental conditions (51). The parameter used to describe growth of the test alga is maximum standing crop, which is defined as the maximum biomass achieved during incubation, but for practical purposes is less than 5% per day (50,51). Biomass can be monitored by several methods, which include dry weight (gravimetric), dry weight (indirect electronic particle counting), chlorophyll a (in vivo fluorescence, extracted fluorescence, and extracted absorbance), direct microscopic enumeration, and turbidity (absorbance at 750 nm)(50,51).

Statement of Purpose

As coal mining expands with the current and future energy demand a more complete understanding of alkaline and acid mine drainage from coal mine spoils is desired. This project studied coal mine spoils from eastern Montana which was characterized by alkaline mine drainage. The purpose of the project was to determine the microbial effects on leaching of undesirable substances from coal mine spoils by ground water, to identify bacteria naturally present in the spoils which may be involved in mobilization or immobilization of these substances, and to determine the effect of coal mine spoil leachates on the organisms in receiving waters.

Site Description

The major study area was the Bear Creek Coal Study in the West Moorhead coal deposit of the Fort Union coal formation in eastern Montana (Fig. 1). Overburden cores were obtained from an area which, to date, is not being mined. One overburden sample was taken from a revegetation study site, 1969-11, at Colstrip, Montana, which is also part of the Fort Union coal formation (Fig. 1). The sample was obtained from an exposed chunk of coal in the overburden which was surrounded by an acid salt halo.

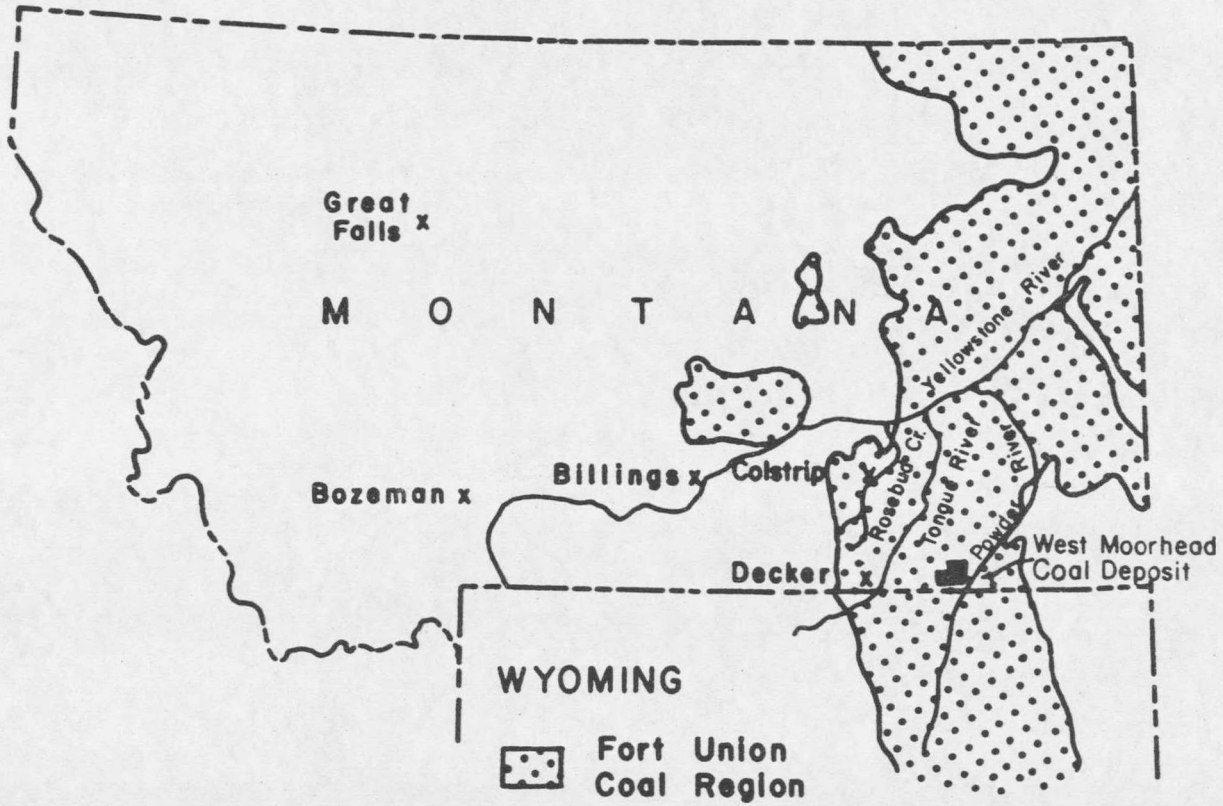

Fig. 1. Map showing the West Moorhead area, as well as the Decker area and the Colstrip area, and the Fort Union coal region. Adapted from VanVoast and Hedges (52).

MATERIALS AND METHODS

Media

Water utilized. The water utilized was either double-distilled water or reagent grade water that had been processed by a Milli-Q water system (Millipore Corporation, Bedford, Massachusetts) following single distillation and was stored in Pyrex glass.

Autotrophic media. The autotrophic media were described by Hutchinson et al. (16,17,18), Silverman and Lundgren (35), and Manning (25). These media utilized thiosulfate or ferrous-iron as their energy sources, and are listed in Table I.

ISP medium was utilized only as a solid medium, while the other media were utilized as both liquid and solid media. To solidify the medium, "Ionagar" no. 2 (Colab Laboratories, Chicago Heights, Illinois) was added at a concentration of 1.5% to permit plating of bacterial specimens on the often acid medium.

Leachate medium. The basal salt medium of Brierley and Brierley (4) was used in the leaching experiments. This medium was used with and without glucose added to a final concentration of 0.1% (w/v). The medium pH was adjusted to the measured pH of the core sample being leached.

Algal Bioassay Medium. The medium (AAP) utilized in the algal bioassays was prepared as described by U.S. Environmental Protection Agency (50).

Table 1. Autotrophic growth media used in these studies.

Reference	Media acid thiosulfate	neutral thiosulfate	ferrous-iron
Hutchinson	S6	S5	Fe
Silverman and Lundgren			9K
Manning			ISP

Preparation of Glassware

All glassware, except pipettes, was machine washed and air dried. Items that were acid washed were soaked in 3 N HCl for a minimum of thirty minutes, rinsed six times with tap water followed by six rinses with double distilled water or Milli-Q reagent grade water, and air dried. Glassware sterilized by autoclaving was either covered with aluminum foil or stoppered with gauzed cotton plugs and processed for 15 minutes at 15 pounds pressure.

Pipettes were soaked in chromic acid for 30 minutes, rinsed at least fifteen times with tap water and air dried. Pipettes and glass petri dishes were placed in metal cans or boxes and oven sterilized at 350°F for 3 hours.

Sampling

Overburden grab samples were obtained from eight test holes of the Bear Creek Coal Study of the West Moorhead coal field of the Fort Union coal deposit in eastern Montana by the U.S. Geological Survey. Each of the eight test hole cores was accompanied by a geological log. Each sample consisted of a portion of the test hole core, between known depths, and was placed in plastic bags for transport. The samples were stored in plastic bags at room temperature in the dark.

A sample was obtained from a revegetation study site (1969-11) at Colstrip, Montana. The sample was taken from an exposed area which

exhibited evidence of iron oxidation in close association with coal distributed in the overburden. This sample was stored in a plastic bag at room temperature.

Sample Analysis

Each sample was visually inspected for evidence of mineralization. Core samples were then ground in a mortar and pestle, analyzed for pH, conductivity, and lead, and cultured for chemoautotrophic bacteria.

pH measurement. For pH measurement, 10 g of ground sample were mixed with 20 ml of double distilled water and allowed to stand for four hours before making the pH reading with a Radiometer model 25 pH meter.

Conductivity measurement. Conductivity was measured by adding ten g of ground sample to 20 ml of double distilled water of known conductivity, and allowing the suspension to stand for 4 hr prior to making conductivity readings. The conductivity was obtained using a Lab-Line Lecto model MC-1 Mark IV ohm meter. The listed values are therefore relative figures representing the conductivity of 20 ml of water which has been exposed to contact with 10 g of crushed sample. These values should not be considered to represent the specific values of leach water. The conductivity was recorded as μmhos/cm at 25°C.

Lead measurement. Each sample was extracted by adding 3 ml of concentrated HCl and 1 ml of concentrated HNO_3 to 1 g of ground sample in an acid washed screw capped tube. The mixture was boiled for 1-1/2 minutes prior to the addition of 3 ml of double distilled water, followed by boiling for another 1-1/2 minutes. The extract was cooled and filtered through Whatman no. 4 filter paper into an acid washed 10 ml volumetric flask. The flasks were filled to volume with double distilled water. The extracts were mixed and then stored in acid washed screw capped tubes at 4°C until analyzed. Lead was measured by atomic absorption spectrophotometry, using an Instrumentation Laboratory, Inc. model 151 atomic absorption/emission spectrophotometer. The lead concentration was reported as µg per g core sample.

Bacterial enrichment. Enrichment cultures for the chemoautotrophic bacteria, thiobacilli, were attempted with each sample. One gram of ground sample was added to each of three 125 ml erlenmeyer flasks, each flask contained one to 3 media, S6, S5, or Fe. These flasks were incubated statically at 28°C in the dark. After two weeks incubation, 0.5 ml from each flask was subcultured into 5 ml of fresh medium in capped tubes. After one month incubation, pH and thiosulfate or ferrous-iron concentration were measured on each of the original enrichment flasks (40,42). The amount of both thiosulfate and ferrous-iron oxidized was reported as a percent of the uninoculated control incubated for the same one month period. The presence of

chemoautotrophic bacteria was determined from the resulting percentages and from elemental sulfur flocculation and from turbidity of the medium or from visible iron oxidation. The liquid cultures showing growth were transferred to fresh liquid medium and also plated on solid medium. When growth in the liquid medium transfers did not result, transfers were made to liquid medium (S6, S5, or Fe) which had been supplemented with yeast extract (0.05%, w/v), cysteine (10 mg/100 ml; J. A. Brierley, private communication), glutathione (10 mg/100 ml), or IM-MF additives (0.1%; 43). Liquid cultures were subsequently transferred weekly. Representative colonies were selected from the plates at weekly intervals and restreaked for purification of the culture. Strains were purified by at least three single colony isolations (16). Pure cultures were characterized to species according to the scheme of Hutchinson et al. (19) for thiobacilli taxonomy. The resulting pH and percent thiosulfate oxidized values for thiobacilli according to the diagnostic tests of Hutchinson et al. (19) are illustrated in Table 2.

The sample from the revegetation study site was cultured for ferrous-iron oxidizing bacteria in Fe medium. The culture was subsequently transferred to 9K medium and 9K medium plus yeast extract (0.05%, w/v), cysteine (10 mg/100 ml), glutathione (10 mg/100 ml), or IM-MF additives (0.1%). Iron oxidation and final pH were measured after incubation for 18 days.

Table 2. Percent of thiosulfate oxidized and resulting pH values for thiobacilli according to the diagnostic tests of Hutchinson et al. (19)[a].

Thiobacillus species	Percent thiosulfate oxidized	Resulting pH
Thiobacillus novellus	<30%	6.6-5.0
Thiobacillus denitrificans	<90%	6.6-5.0
Thiobacillus thioparus	>90%	6.6-3.5
Thiobacillus neapolitanus	>90%	3.5-2.8
Thiobacillus thiooxidans	>90%	<2.0
Thiobacillus ferrooxidans	>90%	<2.0
Thiobacillus intermedius	>90%	<2.8->2.0

[a]Values determined from cultures grown in S6 or S5 medium for a period of 28 days.

Leaching Studies

Core sample selection. Samples were selected which exhibited a wide range of pH values, conductivity values, and lead contents.

Core sample preparation. Samples to be leached were ground and sized to <80 mesh (less than 117 µm).

Autotrophic inoculum. Autotrophic bacteria, both sulfur and iron oxidizing bacteria, were obtained from various sources. Sulfur oxidizing bacteria were isolated from the Artist's Paint Pot area and Geyser Springs, Yellowstone National Park. Iron oxidizing bacteria, isolated from the settling pond of the Decker Coal Mine, Decker, Montana, were graciously supplied by Dr. Greg Olson. These cultures were maintained separately, and mixed prior to inoculation of the leach flasks.

Soil inoculum. A general soil inoculum was obtained from greenhouse pots immediately prior to the inoculation of the leach flasks.

Experimental leaching design. Leaching was performed in erlenmeyer flasks, under either static or shaking conditions. Shaking was on a New Brunswick model VS gyrotory shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) at a speed of 180 revolutions per minute. The flask size, the medium volume, core sample weight, and inoculum volume or weight were different for the two conditions, as shown in Table 3. Six flasks were used for each condition for each

Table 3. Content of the leach flasks for both static and shaking conditions.

Condition	erlenmeyer flask volume ml	core sample weight g	medium volume ml	inoculum size autotrophic ml	soil g
Static	250	5	150	1.0	5
Shaking	125	2.5	75	0.5	2.5

core sample. Each of the six flasks contained core sample. Three of the six flasks contained the leach medium without glucose added, while the others contained the leach medium with glucose added. The inoculum was the same for both sets of three flasks; one flask was an uninoculated sample control, one flask was inoculated with the mixture of autotrophic bacteria, and one flask was inoculated with soil from the greenhouse pots. The experiment was incubated for 30 days at room temperature in the dark.

Handling and analyses of leachates. Following the leaching period, a 15 to 20 ml portion of each leachate was removed to obtain the leachate's pH reading and lead content. Prior to lead analysis the samples were filtered through Whatman no. 4 filter paper into acid washed screw capped tubes and stored at 4°C.

The remaining leachate was used in the algal bioassays. This portion of the leachate was prefiltered (Millipore, AP25) followed by filtering through 0.45 µm membrane filter (Millipore, type HAWP) into sterile glassware. The leachate was stored in acid washed containers, either plastic bottles or glass tubes, at 4°C until the bioassay was performed.

The concentration of lead in a leachate is reported as parts per million lead. The reported values of µg lead per g of core were determined by using the following relationships.

parts per million = mg per liter = µg/g

Y µg/g = (X mg/l) (150 ml/5 g) (1000 µg/mg)

Y µg/g = 30 (X µg/g).

X = the ppm value of the leachate

Y = the recorded µg/g of the leachate

Algal Bioassays

Organism utilized. A culture of Selenastrum capricornutum PRINTZ was obtained from the Environmental Protection Agency, Corvallis, Oregon.

Culture maintenance. The culture of Selenastrum capricornutum PRINTZ was maintained on AAP medium (50) by transfer of 5 ml of culture to 100 ml of fresh medium in 250 ml erlenmeyer flasks. The recommended routine stock culture transfer schedule of weekly transfers (50) was initially decreased to 6 days, but after further experiments transfer every 2 days was found necessary to maintain a "healthy" culture for experimental work.

Test conditions. All flasks, either for maintenance or bioassay, were incubated at room temperature under continuous cool-white fluorescent lighting at 400 ft-c (50). The flasks were continuously shaken at approximately 100 oscillations per minute on a G10 gyrotory shaker (New Brunswick Scientific Company, Inc.). Growth curve studies were performed with 10 replicate 500 ml erlenmeyer flasks containing

200 ml of AAP medium. All other bioassays were performed in 250 ml erlenmeyer flasks containing 100 ml of AAP medium.

Preparation of inoculum. Initially, cells from the stock culture were prepared by washing as described by the U.S. Environmental Protection Agency (50). Centrifugation was performed at 10,000 rpm for 10 minutes using a Sorval superspeed RC2-B automatic refrigerated centrifuge. After some experimentation the inoculum was taken directly from the flasks, without washing and centrifuging.

Amount of inoculum. An inoculum giving a starting cell concentration in the test flasks of 10^3 cells per ml was used (50). The cell concentration (X) was determined from the fluorometer reading (Y) by the following equation (11).

$$\frac{X}{10^3 \text{ cells/ml}} = \frac{X}{15.4 \text{ relative fluorescence units}}$$

Preparation of glassware. Glassware was acid washed and sterilized as previously described. All test flasks were stoppered with gauzed cotton plugs.

Biomass monitoring for growth curve studies. Several methods were used to determine the biomass during growth curve studies; these methods are subsequently described.

Turbidity. In vivo optical density was determined using a Varian Techtron model 635 spectrophotometer at 750 nm (50) with a one centimeter path length in the cuvette.

Fluorescence. Fluorescence was used as one means of measuring chlorophyll, with both in vivo and extracted suspensions (50). The extraction methods described by Yentsch and Menzel (54) were followed with the following substitutions: 0.45 µm cellulose acetate membrane filter (Millipore) for glass fiber filter, sodium bicarbonate (15 mg/l) for magnesium carbonate (1 g/100 ml), 5 ml of 90% acetone for 2 ml of 90% acetone added to the grind tube, and after grinding for 1 to 2 minutes, samples were frozen overnight prior to centrifuging instead of allowing centrifuged samples to stand for 1 to 2 hours. The tissue grinder was a Lightnin model L mixer (Mixer Equipment Company, Inc., Rochester, New York). Fluorescence was determined using a G. K. Turner Associates model no. 111 fluorometer (Palo Alto, California). Chlorophyll and phaeophytin were measured by reading the initial fluorescence (F_o), and the fluorescence (F_a) after the addition of 2 drops of 2 N HCl. Chlorophyll a (F_{chl}) was calculated from these readings using the formula of Yentsch and Menzel (54).

$$F_{chl} = 1.77 (F_o - F_a)$$

Chlorophyll a was used in the plotting of the extracted chlorophyll data.

Absorbance. Chlorophyll was determined by absorbance using the Varian Techtron spectrophotometer at 665 nm and 750 nm (50,54). Extraction and acidification methods were performed as for chlorophyll a

fluorescence. Chlorophyll *a* and phaeopigment *a* were calculated according to the formula in Weber (53) and Standard Methods (1).

$$\text{chlorophyll } a = \frac{26.7\ (665_b - 665_a)\ E}{VL}$$

$$\text{phaeopigment } a = \frac{26.7\ (1.7\ (665_b) - 665_a)\ E}{VL}$$

665_a = absorbance after acidification
665_b = absorbance prior to acidification
E = volume (ml of 90% acetone added
V = volume (ml) of extract filtered
L = path length (cm) of cuvette, 1 cm

Cell count. Direct cell count was determined using a Petroff-Hausser bacterial counter (C. A. Hausser and Son, Philadelphia, Pennsylvania).

Dry weight. Dry weight and ash-free weight were determined following the methods of Weber (53). Five ml of sample were pipetted into the tared porcelain crucible.

Biomass monitoring for bioassays. *In vivo* chlorophyll *a* fluorescence was used to monitor the biomass in the bioassay studies. The data was reported as percent inhibition, indicated by a negative value, or stimulation, indicated by a positive value, as compared to the maximum standing crop of algal control flasks. The values were calculated by the following formula.

$$X = \frac{100\ (A - B)}{B}$$

X = tabulated value, percent
A = leachate bioassay fluorescence reading
B = algal control fluorescence reading at maximum standing crop

Maximum standing crop is defined as the maximum algal biomass reached during incubation (50).

Algal growth studies. The growth of Selenastrum capricornutum PRINTZ as influenced by either pH, glassware cleanliness, inoculum size, inoculum age, or inoculum washing was followed by fluorescence readings.

Medium pH. The pH of the AAP medium was adjusted with a sodium acetate-acetic acid buffer (27) to 5 pH values, 3.6, 4.2, 4.6, 5.0, and 5.4.

Glassware acid washing. Glassware was washed with boiling chromic acid which was swirled to coat the glassware surface, rinsed 3 times with tap water, rinsed 3 times with a 3:1 concentrated HCl:concentrated HNO_3 solution, and rinsed 6 times with both tap water and double distilled water.

Inoculum size. The glassware acid washing study was run in conjunction with a study in which the inoculum size was doubled from 10^3 cells per ml to $2x10^3$ cells per ml.

Inoculum age. The inoculum age was varied from the usual six-day-old inoculum to two- and four-day-old inocula.

Inoculum washing. Inoculum washing was examined by inoculating flasks with cells washed in sodium bicarbonate (15 mg/l) and with cells unwashed.

Algal bioassays of leachates. Leachate bioassays were performed using 10 ml of filtered leachate, 90 ml of AAP medium, and a volume of cells as inoculum so that the test flasks contained 10^3 cells per ml. The pH of some leachates which produced inhibition in the algal bioassays was raised to pH 8.0 with 1 N NaOH.

RESULTS

Sample Analysis

The diversity of the core samples can be observed from the analyses presented in Table 4-11. The core samples were composed of some combination of shale, sandstone, siltstone, coal, or clay. There was visual evidence of mineralization in some samples, which was exemplified by the presence of iron oxidized portions and salt crystals. Some layers of the overburden cores were composed entirely of coal, as seen in Tables 4, 5, 7, and 10, while each of the cores had coal as a part of some of their samples. The coal was often associated with samples of lower pH than most of the other samples. The pH of most samples was above pH 6.0, and often as high as pH 8.8, with the highest pH value being pH 9.65 (Table 10). The majority of the coal samples had pH values of less than pH 5.54, although not all of the samples containing coal were of these low pH values. The sample with the lowest pH value, pH 2.31 (Table 11), contained coal along with carbonaceous shale. While the pH values encompassed a wide range, the lead content of the samples covered a much narrower span of values, specifically 0.0 to 57.0 µg per g of sample. Although lead was measured as high as 49.1 and 57.0 µg/g (Tables 5 and 11), the majority of samples had less than 20 µg/g of sample. The amount of lead in the sample did not appear to be related to any of the other analyses. Conductivity values ranged from the low of 162 µmhos/cm at 25C to the high value of 9232 µmhos/cm

Table 4. Analysis of core sample DH75-102.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm,25 C)
0- 10	Sandy clay; yellow, Fe-oxidized spots	8.15	6.8	3449
10- 20	Sandy clay	8.64	4.3	3359
20- 30	Clay; yellow, Fe-oxidized pebbles	8.43	7.5	1001
30- 40	Sandy clay, carboniferous shale; yellow, Fe-oxidized pebbles	8.49	8.2	829
40- 50	Coal	5.47	2.7	4929
50- 60	Shale and sandstone; carboniferous specks	8.46	14.4	3059
60- 70	Siltstone (light grey)	8.24	5.6	849
70- 80	Shale and siltstone	8.63	4.8	1358
80- 90	Shale; carboniferous fragments	7.29	2.8	1429
90-100	Siltstone; carbonaceous spots	7.41	4.8	1441
100-110	Siltstone; carboniferous specks	7.48	12.4	2206

Table 5. Analysis of core sample DH75-103.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0- 10	Sandy shale; Fe-oxidized spots	8.43	19.3	3133
10- 20	Sandstone; carbonaceous streaks	8.32	23.8	3703
20- 30	Shale; orange (Fe-oxidized) spots	8.14	13.2	1714
30- 40	Carbonaceous clay; coal	7.69	15.1	1803
40- 50	Layered coal	6.51	1.9	1032
50- 60	Coal	5.54	6.3	3922
60- 70	Shale	8.65	15.6	678
70- 80	Shale and sandy siltstone	7.42	7.6	1210
80- 90	Shale	8.51	10.6	986
90-100	Sandstone	8.67	4.7	772
100-110	Shaley sandstone; carbonaceous streaks	8.49	49.1	1060
110-120	Shale with carbonaceous specks and coal	6.67	21.8	1830
120-130	Sandy shale	6.23	24.3	2826
130-140	Sandstone and shale	8.59	4.7	1062
140-150	Coal and sandstone	8.92	10.3	892
150-160	Shale; carbonaceous layers	8.65	14.5	1485
160-170	Sandstone with Fe-oxidized spots; shale with carbonaceous streaks	7.91	8.6	1775
170-180	Silty clay	8.71	7.3	1191
180-188	Silty clay; carbonaceous spots	8.99	6.9	918

Table 6. Analysis of core sample DH75-104.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 17	Layered, Fe layers (HCl positive), soft shale layers, sandy shale	7.44	24.2	2279
17.5- 32.8	Sandy shale; hard carbonaceous shale; clay shale	6.21	17.2	2638
37.9- 53.1	Sandy and clay shale	9.01	17.2	679
53.1- 70.9	Silty-compact shale	8.84	16.3	825
70.9- 85.2	Sandy shale, hard layered carboniferous shale	6.24	15.9	2352
85.2-100.2	Nonlayered silty and clay shale	8.84	21.2	1098
100.2-114.4	Mixed layers of clay, carboniferous shale, Fe spots	8.36	16.2	1354
114.4-131.0	Layered clay, carboniferous shale	5.73	20.0	3485
131.0-153.1	Coal and compact clay	7.61	16.4	754
153.1-169.7	Silty sandstone, crumbly coal	6.72	20.7	3065
169.7-187.1	Sandstone	8.91	17.5	1078
187.1-200.1	Sandy clay	7.06	22.8	2929
200.1-219.5	Clay, shale, and sandstone	7.15	18.9	4139
219.5-234.2	Sandy shale	8.44	13.6	1609
234.2-254.9	Compact clay, Fe spots	8.78	14.0	1482
254.9-284.8	Silt, coal	8.68	9.0	963
284.8-304.5	Siltstone, coal	6.69	6.0	3239

Table 7. Analysis of core sample DH75-106(A).

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
5-10	Yellow sand, spots of iron, dark sand in core middle	8.32	11.7	3881
10-11	Black specks (coal), may be extraneous from pulling core out; soft	8.25	11.3	3518
17-25	Natural fracture, carboniferous	8.62	7.6	2302
30-31		8.60	6.6	2215
31-32	Carboniferous material-fracture; sedimentary material with organics	8.65	7.9	1514
40	Sandstone, soft-moist	8.80	0.0	990
40-41	Carbonaceous material, soft	8.73	12.0	1372
59-60	Hard shale	8.98	7.5	874
72-73	Heavy, dense sandstone, little nonuniformity	8.99	7.6	833
78		8.89	5.8	1064
94	Coal, moldy, supporting more life than usual	8.41	5.8	1506

Table 8. Analysis of core sample DH75-106(B).

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 15	Silty shale, siltstone; Fe-oxide chunks, calcareous spots	7.2	11.6	3292
15 - 30	Silty, moist shale; Fe-oxidized silt	7.4	8.0	2170
30 - 41	Silty sandstone; Fe-oxidized silt	8.3	6.2	997
41 - 53.5	Carbonaceous shale, coal; Fe-oxidized spots	7.1	14.0	1434
53.5- 71	Shale, some carbonaceous; coal	6.7	10.0	2058
71 - 88	Shale, siltstone	8.8	17.0	720
88 -110.5	Shale, sandstone, gray claystone	9.1	15.5	858
110.5-115	Shale with Fe spots and carbonaceous spots: coal	7.9	16.5	2058
115 -133.5	Sandstone and shale; carbonaceous spots	9.1	14.2	825
133 - 146	Sandstone; carbonaceous streaks, mold	9.3	9.0	481
146 - 163	Light and gray shale, coal	7.3	14.0	2463
163 -178	Silty and coal shale, gray clay with carbonaceous spot	8.6	11.6	1341
178 -201.8	Carbonaceous shale	8.9	20.0	743
201.8-233.4	Anderson coal; salt crystals	4.2	13.0	5678
242.6-270.5	Shale with sandstone; carbonaceous streaks	8.7	11.0	582
270.5-280	Carbonaceous shale and sandstone	5.3	9.9	2463

Table 9. Analysis of core sample DH76-108.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 18	Sandy clay; Fe-oxide spots (active HCl reaction), gypsum precipitate	8.46	11.3	3082
18 - 20.3	Soft sandstone	8.59	4.9	774
20.3- 43.8	Shale; Fe-oxidized, calcareous and carbonaceous spots	8.09	15.0	2122
75.8- 79.2	Very carbonaceous shale	3.43	11.5	3422
79.2- 90	Shale and sandstone	8.53	7.2	507
90 - 94.8	Shale in layers	8.07	13.3	1150
94.8-105.5	Clayey sandstone	8.27	2.8	357
105.5-138.6	Shale, carbonaceous shale, little coal	8.72	12.5	595

Table 10. Analysis of core sample DH75-109.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 10	Sandy clay; tan, black carbonaceous spots, salt spots	8.59	10.1	4512
10 - 15.5	Sandy clay; black spots, salt spots, orange (Fe) deposits	8.59	12.2	3397
17 - 22.8	Sandy clay and sand; fine black, shite, and orange granules	8.54	9.5	2679
22.8- 38.9	Clay; black specks, Fe-oxide deposits, gypsum (separate bag)	7.52	15.7	3190
38.9- 60.0	Clay, coal, clinker	8.10	8.2	3014
60 -114.6	Siltstone, light shale; carboniferous spots, Fe-oxide, gypsum	6.06	8.5	2937
114.6-122.5	Coal	7.16	3.5	850
122.5-123.1	Shale; coal specks	8.59	14.4	451
123.1-142.3	Sandy siltstone	9.40	6.9	558
142.3-147.5	Shale; carbonaceous streaks	7.83	10.4	1361
147.5-149	Siltstone	9.51	8.2	472
149 -159	Shale, dark siltstone with coal deposits	8.72	10.4	840
159 -167.9	Siltstone, clay	9.46	11.1	524
170	Black shale; calcareous streaks	9.04	13.2	782
167.9-177.5	Silty siltstone and shale; black deposits	9.65	9.2	458
177.5-200	Silty siltstone and clayey siltstone	8.85	9.2	590
200 -222.2	Coal, siltstone with coal deposits	7.63	6.8	1129
222.2-230	Sandy siltstone; black deposits	8.35	6.2	784
230 -243.4	Shale; carbonaceous	8.59	8.8	709

Table 11. Analysis of core sample DH76-111.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 0.5	Sandy clay; roots	8.50	57.0	162
0.5- 13.4	Silty sandstone; few Fe-oxidized spots, few calcareous spots	9.24	5.5	1383
13.4- 30.5	Coal, shale; Fe-oxidized chunks and streaks	6.93	15.7	3962
30.5- 50	Clayey sandstone	8.89	9.8	1057
50 - 71.7	Carbonaceous shale	8.96	15.4	587
102 -105	Coal and carbonaceous shale	2.31	20.2	9232
111.5-118	Shale	9.03	12.5	932
126.5-131.7	Carbonaceous silty shale	8.48	4.8	478
131.7-156.5	Shale	8.19	17.8	1001

at 25C (Table 11), but most of the samples had conductivity values below 3000 µmhos/cm at 25C. As was the case with lead content, the conductivity values did not appear to be consistently related to either the physical composition or to the other chemical components which were measured. The samples with pH values less than pH 5.73, except for DH75-106(B) sample depth 270.5-280 ft (Table 8), also had higher conductivities than most of the samples. These high conductivities ranged from 3422 µmhos/cm at 25C to the highest conductivity of 9232 µmhos/cm at 25C. Conductivities, as great as these, also occurred with samples which had pH values higher than pH 5.73.

Enrichment Cultures

Enrichment cultures, enriching for thiosulfate oxidizing bacteria in S6 and S5 media and for ferrous-iron oxidizing bacteria in Fe medium, were incubated for one month. The amount of thiosulfate or iron oxidation and the resulting pH were assayed (Tables 12-19). The presence of bacteria was substantiated in many cases by microscopic examination of wet mounts, and also by observation of sulfur flocculation on the surface of the medium or by medium turbidity. As Table 3 exemplified, the different species of thiosulfate oxidizing bacteria can exist over a wide range of pH and oxidize variable percentages of thiosulfate. The S6 medium produced more cultures which oxidized 90% or more of the thiosulfate than did the S5 medium. The final pH values

Table 12. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-102.

Depth (ft)	S6 Percent thiosulfate oxidized	pH	S5 Percent thiosulfate oxidized	pH	Fe Percent ferrous-iron oxidized	pH
0- 10	33	6.24	90	6.59	46	3.00
10- 20	62	6.14	92	5.59	34	2.96
20- 30	96	4.84	93	5.42	38	2.75
30- 40	90	5.06	95	5.50	35	2.90
40- 50	94	4.06	97	2.57	26	2.29
50- 60	94	4.57	73	4.63	16	2.74
60- 70	91	2.97	42	4.54	10	2.35
70- 80	95	2.90	26	4.49	7	2.38
80- 90	92	5.36	97	2.58	18	2.50
90-100	97	2.46	95	3.42	0	2.45
100-110	100	2.78	95	4.10	0	2.37