Ecology of Iron-Oxidizing Bacteria in Pyritic Materials Associated with Coal

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A technique was developed for measuring $^{14}\text{CO}_2$ uptake by chemolithotrophic bacteria directly in pyritic materials associated with coal and coal refuse. There was good correlation between $^{14}\text{CO}_2$ uptake, as determined by this technique, and the most probable number of iron-oxidizing bacteria. Maximal $^{14}\text{CO}_2$ uptake occurred in coal refuse material 2 to 3 years old, and only slight incorporation was demonstrated in fresh material or material 40 years old. Samples taken from the surface of the coal refuse pile always demonstrated maximal $^{14}\text{CO}_2$ uptake, and in most samples, only slight activity was demonstrated at depths below 8 to 10 cm. Optimal uptake of $^{14}\text{CO}_2$ by natural samples occurred at 20 to 30 °C and at a moisture content of between 23 and 35%. In addition to chemolithotrophic bacteria, heterotrophic fungi and yeasts were also routinely isolated in high numbers from acidic coal refuse. In contrast, acidophilic, heterotrophic bacteria were either absent or present in low numbers in such acidic samples.

Although various metabolic activities of the iron- and sulfur-oxidizing bacteria have been investigated under controlled laboratory conditions (for review, see reference 10), little is known about the activities of these chemolithotrophs directly in nature. At least part of this dearth of knowledge can be explained by the lack of suitable techniques for studying metabolic activities directly in nature. Recently, Smith et al. (17) published a technique for measuring the in situ uptake of $^{14}\text{CO}_2$ by soil microorganisms. This technique has been successfully applied to the study of the activities of soil algae (D. W. Smith, Ph.D. thesis, University of Wisconsin, Madison, 1972) and chemolithothrophic bacteria (6) present in hot acid soils.

In the present paper, we would like to report a modification of the technique of Smith et al. (17) which is suitable for the in situ study of $^{14}\text{CO}_2$ fixation by chemolithothrophic bacteria in pyritic materials associated with coal and coal refuse. By using this procedure, the present study was undertaken in an attempt to define the various environmental factors affecting the in situ activity of chemolithothropic bacteria in coal and coal refuse materials.

MATERIALS AND METHODS

Habitats. Three different coal mining regions were used in this study: (i) an abandoned 40-year-old coal refuse pile located at Friar Tuck Mine at Dugger, Ind.; (ii) an active mining operation located at
Blackfoot Mine no. 5, Winslow, Ind.; and (iii) an active mining operation located at Peabody Coal Co., S. Wilmington, Ill. These regions provided a variety of coal refuse piles of different ages.

**Habitat measurement and sampling.** Coal refuse samples were collected at various depth intervals with a calibrated stainless-steel hand corer. Each sample was homogeneously mixed, and its moisture content was determined by the procedure of Fliermans and Brock (6). The pH of the sample was determined by using a Corning model 12 research pH meter after making a 1:1 mixture of coal refuse and deionized water. Temperatures were measured with a telethermometer (Yellow Springs Instrument Co., model no. 425 C) and a "banjo" probe (model no. 408). The probe and telethermometer were periodically checked with a standard mercury thermometer.

**14CO2 generation.** For the generation of 14CO2, 2 ml of 10 N H2SO4 was added to a 70-ml glass serum bottle. The bottle was capped with a rubber stopper which had been previously coated with clear silicone adhesive sealant (Sears, Roebuck and Co., Chicago, Ill.). A 3.3 ml amount of air was removed from the bottle with a syringe fitted with a 26-gauge needle, and an equal volume of NaH14CO3 (20 μCi/ml) (New England Nuclear Corp., Boston, Mass.) was added. The contents of the bottle were mixed by gentle shaking to avoid contact of acid with the rubber stopper.

Five ml of air was injected into the 14CO2 generation serum bottle with a 5-ml gas-tight syringe (Hamilton Co., Whittier, Calif.) fitted with a 26-gauge stainless-steel needle and connected to a repeating dispenser (model PB 600-10, Hamilton Co., Whittier, Calif.). The syringe was filled with 5 ml of a 14CO2-air mixture, and 0.3 ml was injected into each sample serum bottle. After all injections were performed, the activity of the generated 14CO2 was determined by injecting 0.1 ml into each of two or three vials containing 10 ml of toluene-fluor mixture with 0.375 g of 2,5 diphenyloxazole (POPOP, Beckman Instruments Inc., Fullerton, Calif.) and 0.1 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)benzene (POPOP, Packard Instrument Co., Inc.) per 1,000 ml of toluene. In addition, each vial contained 2.5 ml of a mixture of one part Nuclear-Chicago solubilizer (NCS) and two parts of phenethylamine (Fisher Scientific Co.), to trap the 14CO2, plus two parts of analytical grade methanol (0.05% water, Fisher Scientific Co.).

Samples were counted with Beckman LS-100, or β Mate II liquid scintillation systems and corrected for background radiation.

**Incubations.** A 1- or 2-g amount of sample was placed in a tared 5-ml stoppered serum bottle. The bottles were weighed on a Mettler balance, type H5 (Mettler Instrument Corp., Hightstown, N.J.), and the exact weight of the added coal refuse material was determined. Bottles were preincubated in the dark at the indicated temperature for 15 min to reduce any possible pressure build-up. The samples were aspirated with a 26-gauge needle, and 0.3 ml of air was removed prior to injection of 0.3 ml of 14CO2-air mixture. Incubations were stopped by removing the rubber stoppers from the serum bottles and by the addition of 2 ml of 1 N perchloric acid (PCA).

**Oxidation of samples.** After incubation, samples were removed from the serum bottle by using approximately 8 ml of carbonate flush solution (57 ml of concentrated H2SO4 with 92 g of FeSO4.7H2O in 600 ml of distilled water) (2) and placed in a 500-ml round-bottom flask. The samples were rapidly brought to a boil and kept at a slow boil for exactly 20 s to remove any radioactive carbonate formed nonbiologically during the incubation period. Any further heating resulted in partial oxidation of the sample. The flask was then flushed with air for about 20 s to ensure the total removal of 14CO2 from the air phase.

Because of the large amount of gas generated from the oxidation of coal and coal refuse materials, the oxidation technique of Smith et al. (17), using sealed ampoules, was unsatisfactory. After testing several other possible methods, a modified procedure of Allison et al. (2) was found to be most satisfactory for the oxidation of coal and coal refuse materials.

The apparatus used in the oxidation of the samples is shown in Fig. 1. Two or 3 g of K2Cr2O7 was added to the samples after cooling to room temperature, and the flask was attached to the condenser. Scintillation vials containing the toluene-based fluor with added phenethylamine and NCS, as described above, were screwed into the caps. Twenty-five milliliters of digestive acid mix (concentrated H2SO4 and 85% H3PO4 in a 6:4 ratio) was added to the funnel. The stopcock was opened, and after the addition of the acid to the flask, it was immediately closed. Cold water was circulated through the condenser, and the air flow was adjusted to a rate of 2 bubbles/s. Heat was applied with a flask heater (model H 1540, Scientific Products, Evanston, Ill.), and the sample was rapidly brought to a boil. The heat intensity was reduced to a rheostat setting of 6, which permitted a slow boiling of the sample. If a white precipitate developed in the scintillation vial, heating was fur-

![Fig. 1. Apparatus for processing samples.](http://jb.asm.org/)
ther reduced. After 10 min, the source of heat was removed, and the system was aerated for 10 min with a flow of 6 to 8 bubbles/s. The scintillation vials were removed from the apparatus and counted as described above.

Smith et al. (17) have demonstrated that there is no interference in the phenethylamine-containing fluor by high concentrations of chloride ions (up to 20%, wt/vol) and by water concentrations higher than 0.15 ml/vial. Therefore, these possible variables are not a problem when using coal refuse materials with the present technique. To test the efficiency of recovery of 14CO2 in the present system, 50,000 counts/min of 14C-glucose (U) (New England Nuclear Corp., Boston, Mass.) was added to 1 g of coal refuse material, and the sample oxidized. Results of this experiment demonstrated 100% recovery of the initial radioactivity as 14CO2 when three vials were used in the apparatus. Additional data on recovery efficiency are given by Smith et al. (17).

Most-probable-number determinations. The numbers of chemolithotrophic bacteria, heterotrophic bacteria, and fungi were determined by the most-probable-number technique. Numbers of iron-oxidizing bacteria were estimated using the 9-K medium of Silverman and Lundgren (15) at pH 2.5. Fungal numbers were estimated by using the basal salts medium of Allen (1) with added 0.1% yeast extract and 0.1% glucose and adjusted to pH 2.5 with 10 N H2SO4. Heterotrophic bacteria were estimated by using a similar medium as for the fungi, except that cycloheximide was added at a final concentration of 0.1 mg/ml. One gram of sample was added to a bottle containing 100 ml of the appropriate medium. Serial 10-fold dilutions were prepared in triplicate for each sample. Tubes were incubated in the dark for 14 days at 30 C. Growth of the iron-oxidizing bacteria was initially determined by the formation of a red-orange color of Fe(OH)3 in the tube. Fungal growth was determined by the presence of mycelia, and bacterial growth was initially determined by the presence of visible turbidity in the tube. All initial determinations of microbial growth were confirmed by microscopy examination.

The most-probable-number of organisms per gram of soil were determined by using the tables of Collins (3).

RESULTS

14CO2 incorporation as a function of time. Surface samples (0 to 4 cm) from a 5-year-old coal refuse pile located near Wilmington, Ill., were injected with gaseous 14CO2 (about 100,000 counts/min) and incubated in the dark at 30 C. After various time intervals, the incubations were stopped by the addition of 1 N PCA, and the samples were processed. The results are shown in Fig. 2 and indicate that 14CO2 uptake is approximately linear during the first 24 h of incubation. Approximately 11% of the 14CO2 was incorporated after this period of time. As a control, a sample of coal refuse material which had been previously subjected to autoclaving for 4 h was also included. This control sample demonstrated only slight 14CO2 uptake during the time period tested.

Evidence that 14CO2 uptake is due to chemolithotrophs. To provide evidence that the 14CO2 uptake is due to the activity of the iron-oxidizing bacteria, 1 g of FeSO4·7H2O was added to a 10-g sample of homogenized material taken from a 5-year-old pile. Gaseous 14CO2 (about 100,000 counts/min) was injected, and the samples were incubated for 18 h prior to processing. Results demonstrated approximately a ninefold stimulation of 14CO2 uptake in samples with added FeSO4 (11,973 counts per min per g) as compared with control samples without FeSO4 (1,333 counts per min per g).

Additional evidence that 14CO2 uptake is due to chemolithothrophic bacteria was obtained in experiments by J. L. Mosser from our laboratory. In these experiments, measurement was made of oxidation of 35S-labeled elemental sulfur to 35SO42− (using the method of Shively and Brock) (12) by samples of coal refuse material. The reactions were stopped by the addition of 2 ml of formaldehyde solution (40%, wt/vol), and the amount of 35SO4 production was determined by extraction according to the method of Fiererans and Brock (7) and was assayed according to the procedure of Shively and Brock (12). Results of these experiments demonstrated a close correlation between 14CO2 and 35SO4 production. For example, one particular set of samples taken from various depths in a coal refuse pile demonstrated 14CO2 uptake of 7,194 counts per min per g (0 to 2 cm), 3,080 counts per min per g (2 to 4 cm) and 1,520 counts per min per g (4 to 6 cm), and 35SO4,

![Fig. 2. 14CO2 uptake as a function of time. The average of triplicate determinations are shown. Solid line represents active coal refuse material. Dotted line represents control samples which were autoclaved prior to injection.](http://jb.asm.org/Downloaded from http://jb.asm.org/)
production of 2,331, 1,096, and 659 counts per min per g, respectively.

\(^{14}\)CO\(_2\) uptake as a function of depth of coal refuse pile. To study the distribution of chemolithotrophic bacteria in coal refuse piles, samples were taken from various depths in the pile, and \(^{14}\)CO\(_2\) uptake and numbers of iron-oxidizing bacteria were determined. Results demonstrate that most of the \(^{14}\)CO\(_2\) incorporation and the highest numbers of iron-oxidizing bacteria are concentrated at or near the surface of the pile and that in most cases only slight activity is detected at depths below 8 or 10 cm (Table 1). A degree of variation occurred from site to site; and this variation is probably due to the heterogeneous nature of the coal refuse pile.

In addition to \(^{14}\)CO\(_2\) uptake and numbers of iron-oxidizing bacteria, the numbers of heterotrophic bacteria and fungi in the samples were also determined with the most-probable-number technique. Results of these studies (Table 1) indicate that heterotrophic bacteria growing at pH 2.5 in a basal salts medium supplemented with glucose and yeast extract were quite low in number (10\(^7\) or 10\(^8\) organisms/g). In contrast to bacterial numbers, most-probable-number counts of fungi in many of the samples were much higher (10\(^7\) or 10\(^8\) organisms/g).

The predominant fungal isolate obtained from the acidic coal refuse piles used in our experiments has been identified as *Aureobasidium pullulans* (M. R. Tansey, Indiana University, personal communication). In addition to *A. pullulans*, other fungi tentatively identified as members of the genus *Penicillium* and several as yet unidentified yeasts were also isolated from these samples. In the absence of cycloheximide, acidophilic, heterotrophic bacteria were never obtained in these experiments. However, in the presence of this antibiotic, the predominant bacterium isolated in low numbers was a gram-negative, nonmotile, non-spore-forming rod which formed a light yellow pigment when grown on solid medium.

Incorporation of \(^{14}\)CO\(_2\) as a function of age of coal refuse material. Samples were obtained from coal refuse material of various ages, and \(^{14}\)CO\(_2\) incorporation and the number of iron-oxidizing bacteria were determined. Results of this experiment (Table 2) also demonstrate a strong

### Table 1. \(^{14}\)CO\(_2\) incorporation and numbers of iron-oxidizing bacteria, heterotrophic bacteria, and fungi at various depths in a coal refuse pile

<table>
<thead>
<tr>
<th>Site*</th>
<th>Depth (cm)</th>
<th>pH</th>
<th>(^{14})CO(_2) incorporation(^{a})</th>
<th>No. of iron-oxidizing bacteria per gram(^{a})</th>
<th>No. of heterotrophic bacteria per gram(^{a})</th>
<th>No. of fungi per gram(^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0-4</td>
<td>1.4</td>
<td>&gt;1.1 x 10(^{9})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td></td>
<td>4-8</td>
<td>1.5</td>
<td>3,659</td>
<td>4.6 x 10(^{7})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td></td>
<td>8-12</td>
<td>1.6</td>
<td>507</td>
<td>1.5 x 10(^{8})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td></td>
<td>12-16</td>
<td>1.2</td>
<td>446</td>
<td>1.1 x 10(^{8})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td></td>
<td>16-20</td>
<td>1.1</td>
<td>305</td>
<td>2.1 x 10(^{8})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td>II</td>
<td>0-2</td>
<td>1.6</td>
<td>4,533</td>
<td>4.3 x 10(^{8})</td>
<td>2.4 x 10(^{8})</td>
<td>2.1 x 10(^{8})</td>
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<td></td>
<td>2-4</td>
<td>1.6</td>
<td>5,388</td>
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<td>9.3 x 10(^{8})</td>
<td>2.9 x 10(^{8})</td>
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<td></td>
<td>4-6</td>
<td>1.5</td>
<td>3,114</td>
<td>7.5 x 10(^{8})</td>
<td>4.3 x 10(^{8})</td>
<td>1.2 x 10(^{8})</td>
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<td></td>
<td>6-8</td>
<td>1.5</td>
<td>1,310</td>
<td>2.4 x 10(^{8})</td>
<td>2.4 x 10(^{8})</td>
<td>2.0 x 10(^{8})</td>
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<td></td>
<td>8-10</td>
<td>1.5</td>
<td>1,350</td>
<td>4.3 x 10(^{8})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td></td>
<td>10-12</td>
<td>1.5</td>
<td>897</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td>III</td>
<td>0-2</td>
<td>2.4</td>
<td>7,194</td>
<td>3.1 x 10(^{9})</td>
<td>6.2 x 10(^{9})</td>
<td>1.1 x 10(^{10})</td>
</tr>
<tr>
<td></td>
<td>2-4</td>
<td>2.2</td>
<td>3,080</td>
<td>6.2 x 10(^{9})</td>
<td>9.1 x 10(^{9})</td>
<td>6.2 x 10(^{9})</td>
</tr>
<tr>
<td></td>
<td>4-6</td>
<td>2.2</td>
<td>3,086</td>
<td>9.0 x 10(^{9})</td>
<td>9.1 x 10(^{9})</td>
<td>3.1 x 10(^{10})</td>
</tr>
<tr>
<td></td>
<td>6-8</td>
<td>2.2</td>
<td>1,520</td>
<td>ND(^{d})</td>
<td>6.2 x 10(^{9})</td>
<td>6.2 x 10(^{9})</td>
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<tr>
<td>IV</td>
<td>0-4</td>
<td>2.1</td>
<td>8,581</td>
<td>&gt;1.1 x 10(^{9})</td>
<td>4.3 x 10(^{9})</td>
<td>&gt;1.1 x 10(^{9})</td>
</tr>
<tr>
<td></td>
<td>4-8</td>
<td>1.8</td>
<td>1,956</td>
<td>7.5 x 10(^{9})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td></td>
<td>8-12</td>
<td>1.9</td>
<td>2,767</td>
<td>2.4 x 10(^{9})</td>
<td>4.3 x 10(^{9})</td>
<td>&gt;1.1 x 10(^{9})</td>
</tr>
<tr>
<td></td>
<td>12-16</td>
<td>1.6</td>
<td>2,112</td>
<td>4.6 x 10(^{9})</td>
<td>ND(^{d})</td>
<td>ND(^{d})</td>
</tr>
<tr>
<td></td>
<td>16-20</td>
<td>1.7</td>
<td>3,720</td>
<td>4.6 x 10(^{9})</td>
<td>2.4 x 10(^{9})</td>
<td>&gt;1.1 x 10(^{9})</td>
</tr>
</tbody>
</table>

\(^{a}\) Samples were obtained from different sites on a 5-year-old coal refuse pile located at Wilmington, III.

\(^{b}\) \(^{14}\)CO\(_2\) (100,000 counts/min) was added to each sample prior to incubation at 30 C for 24 h. Each value represents the average of triplicate determinations and represents counts per minute per gram.

\(^{c}\) Determined by the most-probable-number technique.

\(^{d}\) Value not determined.
correlation between $^{14}$CO$_2$ incorporation and the number of iron-oxidizing bacteria. Maximal incorporation occurred in samples 3 to 5 years old, and only slight incorporation was demonstrated in fresh samples or in coal refuse material 40 years old. The pH of fresh coal refuse was routinely near neutrality, as compared with 2-year-old samples which were usually pH 2.5 or lower. The acidic pH in 2-year-old material can be correlated with an increase in the activity and number of iron-oxidizing bacteria.

**Effect of temperature on $^{14}$CO$_2$ uptake.** To determine the effect of varying the temperature of incubation on $^{14}$CO$_2$ uptake, samples taken from a 5-year-old coal refuse pile located at Wilmington, Ill., were incubated for 18 h at the various temperatures indicated in Fig. 3. Results of this study demonstrated slight $^{14}$CO$_2$ uptake at 10 or 70 C, with an optimum occurring between 20 and 30 C.

**Effect of soil moisture on $^{14}$CO$_2$ uptake.** To determine the effect of varying the moisture content of the sample on $^{14}$CO$_2$ uptake, a sample of coal refuse material was dried with a vacuum desiccator. At various time intervals, samples were removed from the desiccator, and $^{14}$CO$_2$ uptake and moisture content were determined (Fig. 4). $^{14}$CO$_2$ incorporation was detected at all moisture contents tested between 12 and 35%, with an optimal moisture content between 23 and 35%.

**DISCUSSION**

The present technique makes possible for the first time the measurement of the in situ activity of chemolithotrophic iron-oxidizing bacterial directly in coal refuse piles and provides a valuable tool for studying the effect of various environmental parameters on the activity of these acid-producing microorganisms.

Considerable evidence supports the conclusion that $^{14}$CO$_2$ uptake in the present study is due to the activity of chemolithothrophic iron-oxidizing bacteria rather than heterotrophic populations present in coal refuse materials. (i)

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**TABLE 2. Effect of age of coal refuse material on $^{14}$CO$_2$ uptake**

<table>
<thead>
<tr>
<th>Site*</th>
<th>Ageb</th>
<th>Age</th>
<th>pH</th>
<th>Time of incubation (h)*</th>
<th>Number of iron-oxidizing bacteria per gram*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1 day</td>
<td>6.2</td>
<td>156.0</td>
<td>1154.0</td>
<td>4073.0</td>
</tr>
<tr>
<td>I</td>
<td>1 month</td>
<td>5.1</td>
<td>665.0</td>
<td>2817.0</td>
<td>8333.0</td>
</tr>
<tr>
<td>II</td>
<td>2 months</td>
<td>6.5</td>
<td>ND</td>
<td>ND</td>
<td>3054.0</td>
</tr>
<tr>
<td>II</td>
<td>2 years</td>
<td>2.2</td>
<td>ND</td>
<td>ND</td>
<td>3054.0</td>
</tr>
<tr>
<td>II</td>
<td>3 years</td>
<td>2.1</td>
<td>ND</td>
<td>ND</td>
<td>3054.0</td>
</tr>
<tr>
<td>I</td>
<td>5 years</td>
<td>2.0</td>
<td>337.0</td>
<td>3919.0</td>
<td>10607.0</td>
</tr>
<tr>
<td>I</td>
<td>40 years</td>
<td>2.6</td>
<td>ND</td>
<td>ND</td>
<td>2782.0</td>
</tr>
</tbody>
</table>

*Sampling sites were located at Wilmington, Ill. (site I); Winslow, Ind. (site II) and Dugger, Ind. (site III).

bEstimated age of sample.

cpH of a mixture of one part sample and one part distilled water.

dHomogenized surface samples (0 to 4 cm) were incubated with gaseous $^{14}$CO$_2$ (100,000 counts per min per g) and incubated at 30 C for the time periods shown. Values are expressed as counts per minute per gram.

* Determined by the most-probable-number technique.

† Value was not determined.
14CO2 uptake always correlated with the number of iron-oxidizing bacteria, but not with the number of heterotrophic bacteria and fungi present in the sample. (ii) After the addition of FeSO4 to a sample of coal refuse material considerable stimulation of 14CO2 uptake was observed. Presumably, this stimulation is due to the utilization of ferrous ions as a source of energy by the iron-oxidizing bacteria. (iii) 14CO2 uptake was maximal in acidic samples of coal refuse in which chemolithotrophic iron-oxidizing bacteria were most active. (iv) There is a close correlation between the oxidation of 35S-labeled elemental sulfur by natural samples of coal refuse and 14CO2 uptake as determined by the present technique (J. L. Mosser and T. D. Brock, unpublished data).

From the aspect of possibly controlling microbial acid formation, it may be important to determine where in the coal refuse pile maximal activity and numbers of the iron-oxidizing bacteria are localized. The data presented in this study indicate that maximal 14CO2 uptake occurs at or near the surface of the coal refuse pile and that in most samples there is little incorporation at depths below 8 to 10 cm. This observation is in agreement with determinations of the most probable number of iron-oxidizing bacteria, and it suggests that chemolithotrophic bacteria are localized on the surface of the pile and that there is little activity of these acid-producing organisms at depths below 10 cm. Recently, Shumate and Bryant (13) have discussed evidence indicating that oxygen permeates to a depth of only 6 to 14 in. in a coal refuse pile of claylike nature and that no weathering of pyrite can be observed below this depth. These results would suggest that most of the acid is produced at or near the surface of the coal refuse pile and that such acid is leached from the surface during periods of precipitation.

The exact reason for the concentration of chemolithotrophs at the surface of the coal refuse pile is not known. However, these organisms are strict aerobes and require CO2 when growing autotrophically. Presumably, at lower depths, exchange of O2 or CO2 through the pile may be limiting for the growth of these bacteria.

In addition to localizing microbial activity at or near the surface of the coal refuse pile, several experiments were also performed to determine possible effects of the age of the coal refuse pile on chemolithotrophic activity. Results of these studies indicate that maximal activity and numbers of iron-oxidizing bacteria occur in coal refuse piles 3 to 5 years old. Very little 14CO2 uptake was demonstrated in fresh samples of coal refuse, and our results indicate that a period of 2 to 3 years is required before iron-oxidizing bacteria have developed in high numbers and before significant 14CO2 uptake can be demonstrated. The presence of increasing numbers of iron-oxidizing bacteria in coal refuse piles 2 to 3 years old is well correlated with a drop in the pH of the coal refuse material from near neutrality to pH 2 during this time period.

The absence of significant 14CO2 uptake in samples of coal refuse piles with neutral or slightly acidic pH values suggests an absence of large numbers of chemolithotrophic bacteria in these samples. Reports of thiobacilli (e.g., Thiobacillus thiopeanus, T. neapolitanus, T. novellus, T. intermedius, and T. prometabolis) growing at near or neutral pH values have been well documented in the literature (8, 11). Although no attempts were made in our study to quantify numbers of these "neutral pH thiobacilli," results of in situ 14CO2 uptake studies suggest that these bacteria are either absent or present in low numbers in such coal refuse materials. Additional investigations are warranted to determine whether "neutral pH thiobacilli" play a significant role in microbial acid formation in coal refuse piles or whether most of the acid in coal refuse piles above pH 4.0 is produced by the auto-oxidation of iron, as has been proposed by Singer and Stumm (13).

The exact reason for the decrease in numbers and activity of the iron-oxidizing bacteria in the 40-year-old coal refuse pile sampled is unclear. However, it seems feasible that the bacteria in such older coal refuse piles may have exhausted the supply of readily available energy sources or that certain toxic products may have accumulated over the years. Our observation of low microbial 14CO2 uptake in older coal refuse piles suggests that bacterial acid production is minimal in these piles and that most of the acid present in older piles was produced at an earlier time. This observation of minimal activity in older coal refuse piles may have significance in any attempts to control microbial acid leaching from such older piles.

By using the present technique it has been possible to define, for the first time, the environmental factors affecting the activity of chemolithothrophic bacteria in nature. Results of our studies indicated that the temperature optimum for maximal 14CO2 uptake is between 20 and 30 C. To our knowledge, no laboratory studies of the effect of temperature on 14CO2 incorporation by T. ferrooxidans have been reported in the literature. However, the optimal temperature for growth of T. ferrooxidans has been reported to be 28 C (15).

The results of the present study suggest that
the chemolithotrophic bacteria present in coal refuse material are metabolically active over a wide range of moisture contents (12 to 35%) and have an optimal moisture content for $^{14}$CO$_2$ uptake of 23 to 35%. Because the moisture contents of the coal refuse samples used in our study were rarely below 20 or above 35%, we believe that moisture is not an important factor determining the activity of chemolithotrophic bacteria in the coal refuse pile.

In addition to the chemolithotrophs, our studies demonstrate that acidic coal refuse piles also have a population of heterotrophic bacteria and fungi. The predominant bacterium growing at pH 2.5 is a light-yellow pigmented, gram-negative nonspore-forming rod which is present in rather low numbers ($10^3$ or $10^4$ organisms/g). In contrast to bacterial numbers, most probable numbers of fungi were extremely high ($10^7$ to $10^8$ organisms/g). Although several different fungi have been isolated in our experiments, the predominant one has been identified as A. pullans (M. R. Tansey, unpublished data). Whether this fungus is present in coal refuse primarily as a spore or as a metabolically active filament remains to be determined. However, its wide distribution in coal refuse material and its ability to grow at low pH would suggest that it may be growing in such habitats.

Several questions remain concerning the activity and ecology of chemolithotrophs in coal mine drainage areas. Of particular significance to an understanding of microbial acid production may be the effect of addition of organic compounds on chemolithotrophic activity (8, 11), as well as various interactions between these bacteria and heterotrophic populations. Hopefully, the present technique for determining in situ $^{14}$CO$_2$ uptake by chemolithotrophic bacteria can be applied to the study of these remaining problems, as well as to the study of the ecology of other habitats where chemolithotrophs are present in high numbers.

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