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Influence of coal source and treatment upon indigenous microbial communities

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SUMMARY

The leaching of six Eastern coals was investigated using experimental coal columns subjected to simulated leaching events. Measurements of CO_2 assimilation and specific enrichment cultures indicated that the microbial communities of all leachates were dominated by iron- and sulfur-oxidizing chemoautotrophic bacteria. Comparison of CO_2 assimilation rates in leachates and core samples of leached coal indicated that most chemoautotrophs remained within coal columns during leaching. Mean numbers of chemoautotrophic bacteria in leachate samples were correlated with concentrations of dissolved iron and sulfate. Leachates from unwashed, run-of-mine coals contained more chemoautotrophs and more iron and sulfate than did leachates from washed, final product coals. After several leachings, the ratio of sulfur oxidizers to iron oxidizers tended to increase. These data suggest that the chemoautotrophic community of final product coals may be pyrite-limited. Aerobic heterotrophs constituted a minor component of the microbial community in leachates from the six coals and their abundance and metabolic activity were apparently not influenced by the beneficiation history of the coal. Changes in rates of acetate metabolism may have been related to microbial succession within the heterotrophic community of coal columns. In all leachates, rates of tritiated methylthymidine assimilation were correlated with rates of acetate incorporation but not with CO_2 assimilation, even though autotrophs dominated the microflora. Thus, thymidine assimilation rates appear to reflect activities or growth of mainly heterotrophic microorganisms in leachate.

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INTRODUCTION

Coal is likely to become increasingly important as a fuel for electric power generation during the next two decades, as the demand for petroleum be-

gins to exceed its availability. The consequent increase in outdoor storage of coal will present an environmental hazard insofar as highly acid solutions containing potentially toxic organic and inorganic materials result from the leaching of coal during precipitation events. These substances, if not contained, may then enter surface and subsurface drainage systems. Although it is known that acid production and leaching of inorganics from pyritic minerals in coal materials are catalyzed by chemoautotrophic bacteria, particularly *Thiobacillus ferrooxidans*, the role of chemoautotrophs in leaching of fuel-grade coals under typical storage conditions has received little attention. Likewise, the role of naturally occurring heterotrophic bacteria in fuel-grade coals has been virtually unknown.

In attempting to determine the influence of bacteria upon coal leaching, we have investigated bacterial abundance and metabolic activities associated with several Eastern coals stored under various conditions. Our initial investigation showed that large numbers (up to $>10^7$ cells/ml) of chemoautotrophic bacteria were present in leachates from columns containing a low-sulfur fuel-grade Eastern coal [19]. Autotrophic bacterial activity in the coal itself was much higher than in leachates, suggesting that those bacteria appearing in leachates represented only a fraction of the total autotrophic community in the coal columns. Autotrophs were responsible to a large extent for acid production and for leaching of iron and sulfur as a result of pyrite oxidation. As the autotrophic population grew, the consequent decline in pH led to a suppression of the heterotrophic community in leachates and probably in the coal itself.

Although the above scenario describes microbial leaching of one coal type under laboratory conditions, actual coal storage piles often represent a range of coal types. Coal is derived from a wide variety of plant materials and consequently varies in composition. In addition to regional differences, coal of different strata in the same mine can also vary considerably. Sulfur, ash, and BTU content of the mined coal may vary daily or even more frequently as different parts of a mine pit are exploited. Although it is common practice to blend coals to attain the desired sulfur, ash, and BTU levels, the resulting product may still vary with regard to other characteristics. An additional source of variability is introduced by the practice of washing some coals to reduce pyrite levels.

Such differences in coal characteristics might be expected to result in considerable variation in the natural microbial assemblages which mediate the leaching process and thereby to affect the rate of leaching or the composition of leachates. A wide range of leachate chemical characteristics has been reported for different coals [2,6,20,21]. To our knowledge, however, the study reported here is the first to consider the influence of coal type and mode of preparation on naturally occurring microbial communities during storage and leaching of coal. In particular, we focus on the effects of coal composition and level of processing (beneficiation) on bacterial growth, metabolic activities, and succession in leachates from six Eastern coals.

MATERIALS AND METHODS

Coal sources and characteristics

Six different coals were selected for study. The coals comprised two major groups according to the level of beneficiation (processing) they had received before shipment. Final product (FP) coals represented a blend of coals which had been ground and washed to reduce pyrite levels, while run-of-mine (ROM) coal had been ground but not washed or blended. Coals A, B, and H were FP bituminous coals from the Leslie mine in western Pennsylvania (Power Operating Co., Phillipsburg, PA) and were used 3–22 days after collection. Coal H was collected before train shipment and within 1 week of being mined, while A and B were collected at the Potomac Electric Power Company's Chalk Point generating facility (Aquasco, MD) within 1 week of arrival. A detailed examination of Coal A leachates has been reported elsewhere [19]; these data are summarized herein to facilitate comparison with other coals.

Coals D, E, and F were ROM coals which had undergone size reduction but not washing or blending. Coal D was provided by the Power Operating

Company (Phillipsburg, PA) and was used 43 days after collection. Coals E and F were supplied by the Delta Mining Company (Ocean, MD) and Flango Brothers, Inc. (Phillipsburg, PA) respectively, and were used 19–27 days after collection. After collection, coal samples were stored in an unheated shed. Although some moisture was present in all coal samples, no additional water was introduced until experimental leaching commenced.

Before the coals were used for leaching experiments, particles of greater than 13 mm diameter were removed and discarded. Of the remaining particles, 50–61% fell into the size range $>0.5-<5.0$ mm. Particle sizes were estimated by sieve analysis [10], and surface areas were estimated with the assumptions that particle sizes were normally distributed within each size range, that the particles were spherical, and that coal density was 0.8 g/cm^3 [22].

Leaching columns and conditions

Leaching columns containing 6.8 kg of coal were prepared as described previously [19]. Coal A was leached in triplicate columns, while duplicate columns were used for other coals. All columns were incubated in a room maintained at 25 ± 2°C. Air-saturated distilled water (1.5 liters, pH 5.6) was added by means of a perforated plastic bucket to each Coal A column at 10-day intervals, and 2.0 liters was added to other columns at 14-day intervals. Within 5 h after each leaching event, coal core samples were taken from Coal H and F columns. The cores (mean wet weight, 8.82 g; mean water content, 14.8%) were collected to a depth of about 8 cm with modified 10-cm^3 plastic syringes. Leachate volumes (ca. 1.4 liters) were measured at each leaching event. Each coal was subjected to six to eight leaching events.

Chemicals and chemical analyses

The total carbon and nitrogen contents of the six coals were determined using a Perkin-Elmer model 240B elemental analyzer. Ash, sulfur, and BTU content were determined by Penniman and Brown, Inc. (Baltimore, MD) using ASTM methods D3174, D4239-C, and D-2015 respectively [1].

Total extractable aliphatic and aromatic contents of the six coals were measured as described by Fendinger (Chemical characterization of organic components in leachates from coal, Ph.D. dissertation, University of Maryland, 1987). A 2.0 g sample of each coal was extracted by sonication in 10–20 ml of pesticide grade dichloromethane for 10 min. Aliphatic and aromatic fractions were then isolated using silica gel chromatography. The aliphatic fraction was eluted with 2 column volumes (10 ml) of hexane, while the aromatic fraction was eluted with two column volumes of 3:1 dichloromethane/hexane. The extracts were then analyzed by means of a Hewlett Packard Model 5840 gas chromatograph equipped with a flame ionization detector (GC/FID). Chromatographic separation of components in the extracts was accomplished using a 30 m × 0.25 mm fused silica SPB-5 (5% diphenyl/94% dimethyl/1% vinyl polysil-oxane) bonded phase capillary column (Supelco, Inc.). The chromatographic conditions were: injection, splitless; injection temperature, 250°C; initial column temperature, 50°C for 4 min; initial ramp, 10°C/min to 100°C; secondary ramp, 5°C/min to 270°C; linear velocity, 43 cm/s, He; detector temperature, 270°C. Total aliphatic and aromatic concentrations were determined by summing the areas of peaks from the GC/FID chromatograms of each fraction.

Leachate pH was determined electrometrically immediately after collection of leachates. Dissolved iron and sulfate were measured by ion chromatography and atomic absorption spectroscopy, respectively; detailed results have been described elsewhere [15].

Choline *O*-sulfate was prepared as described previously [19]. The compound was used for screening of organosulfur-utilizing aerobic heterotrophs.

Microbial enumeration and metabolic activity measurements

Enumerations of iron-oxidizing chemoautotrophs, sulfur-oxidizing chemoautotrophs, and choline *O*-sulfate utilizers were made at each leaching event using a three-tube MPN (most probable number) procedure. Media and scoring procedures have been described previously [19]. Total recoverable aerobic heterotrophs in Coal A leachates were

enumerated by colony counts [14,19], while a three-tube MPN procedure was used for other leachates. These latter measurements were made using peptone-glucose medium [14] at pH 3.5 and pH 7, modified by the omission of agar and the addition of 100 mg cycloheximide/l (Sigma Chemical Co.) to retard fungal growth. Bacterial numbers are expressed as the mean number of cells washed out of one column (6.8 kg coal) at one leaching event (calculated for the first six leaching events carried out with duplicate or triplicate columns; $n = 12$ or 18). Expressing the data in this manner permits comparisons among leaching columns having different leachate volumes. For Coals H and F, bacterial numbers are also shown for each leaching event.

Dark assimilation of CO_2 in all leachates and [^{14}C]acetate assimilation and respiration in Coal A leachates were measured as described previously [19]. In leaching experiments with Coals B, H, D, E, and F, measurements of acetate metabolism were modified to permit simultaneous determination of [^{3}H]thymidine assimilation [16]. Four replicate samples (10 ml of leachate) were placed in capped 50 ml centrifuge tubes. One sample was treated with 0.5 ml of 37% formaldehyde and served as a control. A mixture of sodium [2-^{14}C]acetate (51 μCi/μmol, ICN) and [^{3}H]methylthymidine (6.7 Ci/mmol, ICN) was injected through a silicone rubber sealed port in the cap to give a final radioactivity of 0.053 μCi/ml of [^{3}H]methylthymidine and 0.01 μCi/ml of sodium [2-^{14}C]acetate. After a 1 h incubation period (25 ± 2°C), the reaction was terminated by injection of 1.0 ml of ice-cold 55% (w/v) trichloroacetic acid. Injection ports were immediately resealed and the tubes were shaken (120 rpm, 1 h) to facilitate the release of unincorporated carbonates. Contents of CO_2 trapping cups containing a filter paper wick and 0.3 ml phenethylamine were placed into scintillation vials containing 10 ml Instagel (United Technologies Packard) and the mixtures counted using a Packard Tri-Carb liquid scintillation spectrometer operated in the DPM mode. Tube contents were filtered (0.2 μm membrane), after which filters and suspended material were rinsed (2 × 5 ml) with 25% (v/v) ethanol and then counted as described above. During each experiment, CO_2 trapping efficiency was determined by measuring the recovery of $^{14}CO_2$ from duplicate formalin-killed samples injected with 0.1 μCi of NaH $^{14}CO_3$. Metabolic rates for leachate samples are expressed as μmol acetate or nmol thymidine metabolized per day, and represent mean rates (calculated over six leachings of duplicate or triplicate columns; $n = 12$ or 18) for the entire leachate output (ca. 1.4 liters) of one column (6.8 kg coal) at one leaching event. Acetate metabolism rates for Coals H and F and CO_2 assimilation rates for Coal H are also plotted for individual leaching events.

Levels of dark assimilation of CO_2 and of acetate respiration in coal core samples were measured as described previously [19]. Values are plotted for individual leaching events and represent μmol acetate respired or μmol CO_2 incorporated per coal column per day. Bacterial abundances and rates of acetate and thymidine incorporation could not be reliably determined in core samples.

Statistical analysis

Large confidence intervals, frequently spanning more than an order of magnitude, are associated with individual estimates of bacterial numbers obtained by the three-tube MPN method. However, the use of duplicate or triplicate coal columns and six leaching events allowed differences between coals to be tested for significance by two-way analysis of variance with coal type and leaching event as classification variables. A logarithmic transformation was used to normalize data for all coal columns and leaching events. 95% LSD intervals were used to compare mean bacterial numbers for the six coals, or to compare mean heterotroph numbers obtained at two pH values.

Relationships between bacterial numbers or metabolic activities and physical or chemical characteristics of the coals were tested by linear regression analyses. These analyses were based on mean values over the six leaching events for bacterial numbers and activities in each individual coal column, and physical and chemical characteristics determined for each of the six coal types.

RESULTS

Autotrophic microbial community

Depending on the source coal, mean numbers of iron oxidizers in leachates ranged from 2.7×10^9 cells per column per leaching event to 5.6×10^{10} cells per column per leaching event, while sulfur oxidizers ranged from 1.8×10^8 cells per column per leaching event to 2.8×10^9 cells per column per leasing event (Fig. 1A). In leachates from all six coals, more bacteria were recovered when iron rather than sulfur was the energy source in the enumeration medium.

ROM coals D and F contained significantly more iron oxidizers than any of the FP coals ($P \leq 0.05$), while the difference between ROM Coal E and FP coals A and H was not significant. Sulfur oxidizers were significantly more numerous in the three ROM coals than in FP Coals B and H ($P \leq 0.05$), but data for ROM Coals D and E did not differ significantly from those for FP Coal A. Although not conclusive, these results suggest that beneficiation influences numbers of iron and sulfur oxidizers. On the other hand, major chemical characteristics of the six coals (N, S, C, ash, or BTU content) bore no obvious relationship to coal beneficiation (Table 1). Removal of large numbers of autotrophs during washing of FP coals at the mine or a lower inorganic sulfur/total sulfur ratio might cause energy-limited growth of autotrophs and contribute to the lower numbers of iron and sulfur oxidizers in FP coals.

When the three ROM coals were considered separately, the mean abundance of autotrophs in leachates appeared correlated with coal nitrogen content ($r^2 = 0.77$ for sulfur oxidizers, 0.79 for iron oxidizers; $n = 6$, $P \leq 0.05$) (Fig. 1A, Table 1). Although these correlations may not have been meaningful due to the small variation in N content (0.96–1.3%) among the coals, they suggest that nitrogen limitation plays a role in determining autotroph abundance in ROM coal leachates. Among the three ROM coals, mean numbers of autotrophic bacteria in leachates also varied with coal particle surface area ($r^2 = 0.73$ for iron oxidizers, 0.87 for sulfur oxidizers with $n = 6$; $P \leq 0.05$ and 0.01 for iron and sulfur oxidizers, respectively). These findings are not unexpected in view of the greater accessibility of pyrite to bacterial attack afforded by more finely ground coal.

Whether because of energy limitation, nutrient limitation, or physical removal of autotrophs, lea-

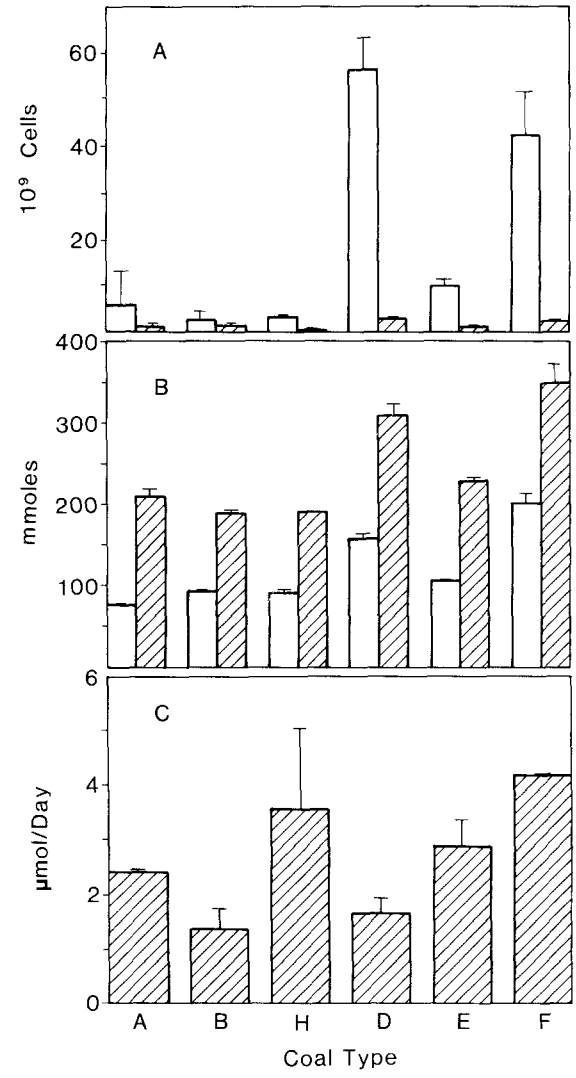

Fig. 1. Autotrophic bacterial community of leachates. (A) Mean number of iron-oxidizing bacteria (unhatched bars) and sulfur-oxidizing bacteria (hatched bars) in leachates from six coals. (B) Mean iron content (unhatched bars) and sulfur content (hatched bars) of leachates. (C) Mean rates of dark CO_2 assimilation in leachates. Values are calculated over six consecutive leaching events and represent mean output per column (6.8 kg coal) per leaching event. Error bars show the upper range of mean outputs for triplicate columns (Coal A) or duplicate columns (Coals B, H, D, E, and F).

Table 1

Chemical characteristics of coal used in leaching experiments

Values for all coal parameters except surface area are reported on a dry weight basis.

Coal	Beneficiation	%C	%N	%S	%Ash	BTU/lb	Surface area (m^2/6.8 kg)	Mean leachate volume (ml)
A	FP	69.0	1.16	3.38	14.8	12833	15.6	1404
B	FP	74.5	1.30	2.70	14.7	13090	21.7	1266
H	FP	71.4	1.00	1.99	13.8	13085	35.4	1422
D	ROM	83.1	1.34	2.54	9.8	13908	41.5	1305
E	ROM	47.2	0.96	3.75	18.2	11976	30.6	1258
F	ROM	75.8	1.36	5.22	18.5	12422	34	1563

chates from washed coals contained less dissolved iron and sulfur (Fig. 1B) than did leachates from ROM coals. These data suggest that metabolic activity, as well as the abundance of autotrophs, should have been lower in FP coal than in ROM coal. However, dark CO_2 assimilation rates in leachates (Fig. 1C) showed no apparent correlation with washing, but these rates may not reflect autotrophic activity within the columns themselves to the same degree in all coals. Dark CO_2 assimilation (Fig. 1C) and mean concentrations of iron and sulfur in leachates (and hence, rates of Fe and SO_4 leaching) were also not correlated with total S content of the parent coal (Fig. 1B).

In interpreting results obtained with leachate samples, we have assumed that events occurring in leachates reflect those within coal columns. In order to elucidate the relationship between changes in leachate samples and events within the coal itself, we compared microbial activities in core samples and leachates from Coal H. In leachates from a column containing FP Coal H, dark CO_2 assimilation increased with time to a maximum value of 4.47 μmol/day at 28 days (Fig. 2A) and then declined to 1.5–2.1 μmol/day for the remainder of the experiment. A qualitatively similar increase occurred in Coal H cores (Fig. 2A) during 0–28 days, although mean CO_2 assimilation rates in the coal peaked at 70 days. CO_2 assimilation in cores was about 2–5.6 times greater than in leachates at 0–56 days, and was 8 times higher than in leachate at 70 days. These data suggest that autotrophs tended to remain, and, in fact, to increase within Coal H during leaching.

Bacteria capable of iron oxidation significantly ($P \leq 0.01$) outnumbered those capable of sulfur oxidation by more than an order of magnitude in Coal H leachates during the 0–56 day period (Fig. 2B). During this time, changes in CO_2 assimilation rates in leachates (Fig. 2A) were paralleled by changes in abundance of iron oxidizers. After 56 days, the number of iron oxidizers began to decline, while organisms capable of using sulfur comprised an increasingly large percentage of the community (Fig. 2B). At 70 days, numbers of iron oxidizers and sulfur oxidizers no longer differed significantly. Since the iron content of leachates began to decline after day 0 (Fig. 2C), this change may have been due to energy limitation of that portion of the community which preferentially used iron as an energy source. Sulfate levels in leachates also declined after day 0 (Fig. 2C), but were about twice as high as iron levels, reflecting the stoichiometry of iron pyrite (FeS_2) oxidation.

In leachates from the ROM coal F, shown for comparison with FP Coal H, significantly ($P \leq 0.01$) more bacteria also were recoverable in iron-containing medium than in elemental sulfur medium (Fig. 2B) at 0–56 days. As with Coal H, an increased proportion of the autotrophic community was recovered in sulfur medium at 70 days. At this time, sulfur oxidizers significantly outnumbered

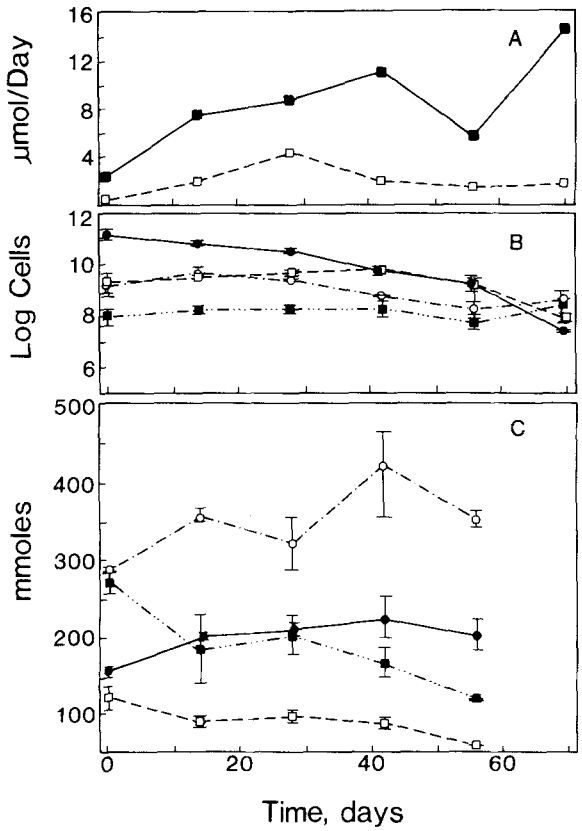

Fig. 2. Temporal changes in autotrophic bacterial activity associated with FP Coal H and ROM Coal F. Data points represent mean values for an entire coal column (6.8 kg coal) or the entire leachate output (ca. 1.4 liters) at each leaching event. Error bars show the range of mean outputs for duplicate columns.(A) Dark CO_2 assimilation in leachates and core samples from a single column containing Coal H. Symbols: □, Coal H leachate; ■, Coal H cores. (B) Iron- and sulfur-oxidizing bacteria in leachates from FP Coal H and ROM Coal F. Symbols: □, iron oxidizers, Coal H; ●, iron oxidizers, Coal F; ■, sulfur oxidizers, Coal H; ○, sulfur oxidizers, Coal F. (C) Iron and sulfate content of leachates from Coals H and F. Symbols: □, iron content, Coal H leachate; ■, sulfur content, Coal H leachate; ●, iron content, Coal F leachates; ○, sulfur content, Coal F leachates.

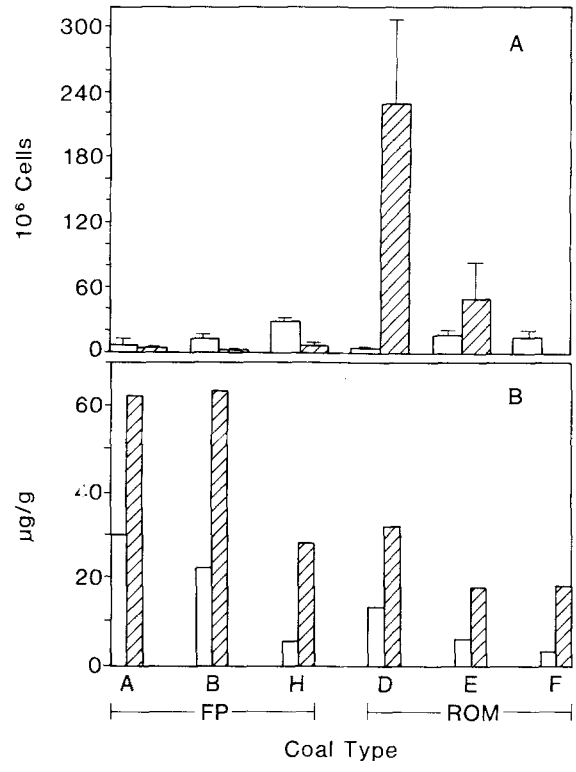

Fig. 3. Aerobic heterotrophic bacterial community of leachates and organic content of six coals. (A) Mean number of aerobic heterotrophs recovered from leachate in peptone-glucose medium at pH 7 (unhatched bars) and pH 3.5 (hatched bars). Values are calculated over six consecutive leaching events and represent mean output per coal column (6.8 kg coal) per leaching event. Error bars show the upper range of mean outputs for triplicate columns (Coal A) or duplicate columns (Coals B, H, D, E, and F). (B) Total extractable aliphatic (unhatched bars) and aromatic (hatched bars) hydrocarbon concentrations (µg/g dry wt. coal) in FP coals (A, B, H) and ROM coals (D, E, F).

iron oxidizers ($P \leq 0.05$). After undergoing an overall increase during the 0–42 day period, leaching of iron and sulfur decreased slightly after 42 days, again suggesting that energy limitation may have been involved in the changing composition of the autotrophic community.

Heterotrophic microbial community

The abundance of aerobic heterotrophs in leachates, unlike that of autotrophs, was not related to coal preparation (Fig. 3A). Mean heterotroph numbers were also unrelated to concentrations of aromatic or aliphatic hydrocarbons within the coal (Fig. 3B), to coal carbon content (Table 1), or to coal nitrogen content (Table 1). Although our work with limed coal columns [19] indicated that pH was a major influence on the heterotrophic community, no connection was seen between pH and hetero-

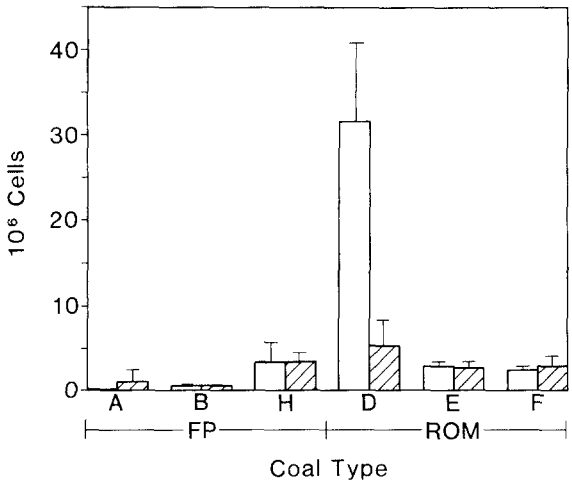

Fig. 4. Mean numbers of organosulfur-utilizing aerobic heterotrophs recoverable in leachates using choline *O*-sulfate medium at pH 7 (unhatched bars) and pH 3.5 (hatched bars). Values are calculated over six consecutive leaching events and represent mean output per coal column (6.8 kg coal) per leaching event. Error bars show the upper range of mean outputs for triplicate coal columns (Coal A) or duplicate columns (Coals B, H, D, E, and F).

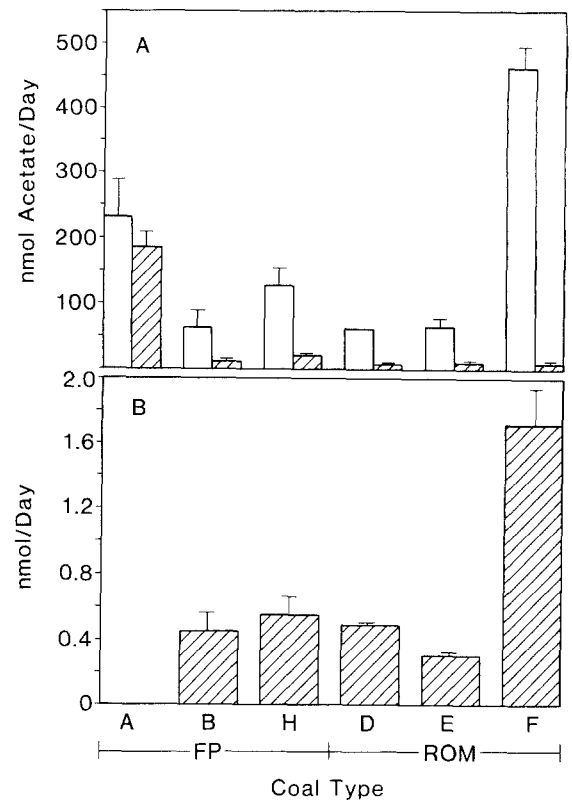

Fig. 5. Heterotrophic activity in leachates from six coals. (A) Incorporation (unhatched bars) and respiration (hatched bars) of sodium [2-^{14}C]acetate in leachates. (B) Incorporation of [^{3}H]methylthymidine in leachates. Values are calculated over six consecutive leaching events and represent mean rates over the entire leachate output of one coal column (6.8 kg coal) at one leaching event. Error bars show the upper range of mean values for triplicate columns (Coal A) or duplicate columns (Coals B, H, D, E, and F). Thymidine incorporation was not measured in Coal A leachates.

troph abundance in leachates from the six coals. This suggests that the six coals, whose mean leachate pH values were 1.74–2.11, had pH values that were already below the threshold at which growth of most heterotrophs could occur. Fewer than 2.9 $\times$ 10^7 cells per column per leaching event and 2.4 $\times$ 10^8 cells per column per leaching event were recovered from any leachate with peptone-glucose medium at pH 7 and pH 3.5, respectively.

Leachates from the six coals differed with regard to the relative abundance of heterotrophs culturable at pH 7 and at pH 3.5 (Fig. 3A). Heterotrophs capable of growth at pH 7 predominated in leachates from FP Coals B and H as well as ROM Coal F ($P \leq 0.05$), while acidophilic or acidotolerant forms dominated the heterotrophic microflora in leachates from ROM Coal D ($P \leq 0.05$). In FP Coal A, numbers of aerobic heterotrophs did not differ significantly at the two pH values. Furthermore, the portion of the heterotroph population capable of growth on choline *O*-sulfate varied significantly ($P \leq 0.01$) in the different coals (Fig. 4). Mean values for the recovery of heterotrophs in choline *O*-sulfate medium ranged from about 2 orders of magnitude lower to about one order of magnitude higher than those in peptone-glucose medium at either pH (Figs. 3A, 4). However, their abundance never exceeded 1.2 $\times$ 10^8 cells/column/leaching event at pH 7 or 3.7 $\times$ 10^7 at pH 3.5 during any one leaching event. In Coal D leachates, significantly more choline *O*-sulfate utilizers were culturable at pH 7 than at pH 3.5 ($P \leq 0.05$) while significantly fewer were culturable at pH 7 than at

pH 3.5 in Coal A leachates ($P \leq 0.05$). Approximately equal numbers of the two types were found in leachates from Coals B, E, F, and H. Abundance of choline *O*-sulfate utilizers did not follow any major inorganic chemical parameter and was unrelated to carbon content or to total extractable aliphatic or aromatic hydrocarbon content of the parent coal (Table 1, Fig. 3B).

As well as being less abundant than autotrophs, heterotrophs accounted for a smaller proportion of microbial community metabolism and productivity in leachates. In all six leachate types, rates of acetate metabolism (Fig. 5A) were an order of magnitude or more lower than rates of dark CO_2 assimilation (Fig. 1C). Mean acetate incorporation varied from 60 to 465 nmol · day^{-1} and appeared unrelated to either coal preparation or heterotroph numbers. Mean acetate respiration rates were lowest in leachates from the ROM coals (7–185 nmol · day^{-1}), but the differences among the six coals were small, except for Coal A. No relationship was observed between acetate metabolism and pH (which differed only slightly between the six coals). Acetate metabolism was also unrelated to carbon content, nitrogen content, total aromatic content, or total aliphatic hydrocarbon content of the coals.

Leachates with higher acetate incorporation rates (Fig. 5A) tended to exhibit higher thymidine assimilation rates (Fig. 5B). The regression of mean thymidine incorporation on mean acetate incorporation was highly significant ($r^2 = 0.97$, $n = 10$, $P \leq 0.01$). Thymidine assimilation rates did not correspond to relative rates of dark CO_2 assimilation (Fig. 1) among the six leachate types ($r^2 = 0.36$ for the regression of mean thymidine incorporation on mean CO_2 assimilation with $n = 10$), even though autotrophy was the dominant metabolic activity in all leachates. Thus, thymidine incorporation rates measure primarily heterotrophic bacterial activity in coal leachates.

To examine the extent to which leachate data reflected events occurring within coal samples, we measured acetate respiration rates in cores as well as in leachates from Coals H and F at each leaching event. In Coal H, peak acetate respiration rates occurred at 14 days in leachates but at 28 days in cores

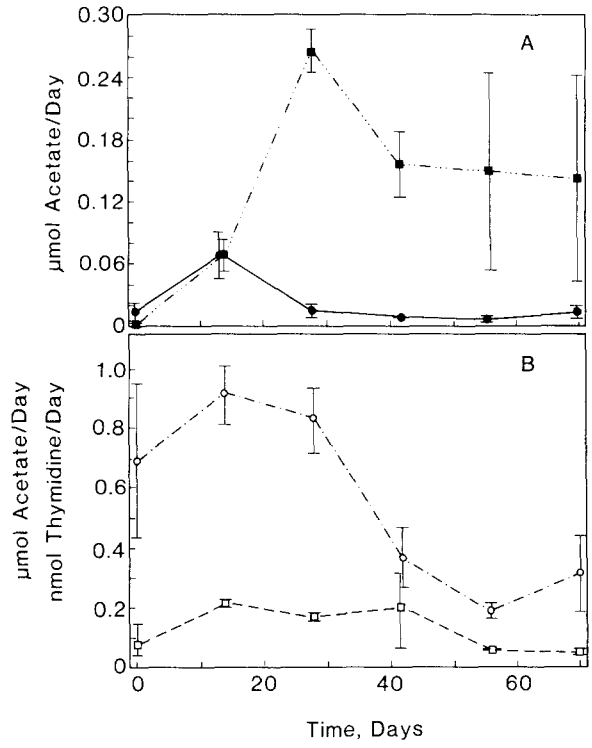

Fig. 6. Temporal changes in heterotrophic activity in cores and leachates from Coal H. (A) Acetate respiration in cores (■) and leachates (●). (B) Incorporation of acetate (□) and thymidine (○) in leachates. Data points represent estimated mean values for an entire coal column (6.8 kg coal) or the entire leachate output (ca. 1.4 liters) at each leaching event. Error bars show the range of mean outputs for duplicate columns.

(Fig. 6A). Two other measures of heterotrophic activity, thymidine assimilation and acetate incorporation (Fig. 6B), also reached maximum mean values at 14 days in leachates. Thymidine incorporation thus appeared to follow acetate incorporation rather than dark CO_2 assimilation, which peaked at 28 days (Fig. 2A).

As was the case with other coals we have studied, the microbial community of Coal H leachate assimilated carbon as acetate much less rapidly than as CO_2 (Figs. 2A, 6A). In leachate, the ratio of CO_2-carbon assimilated to acetate respired ranged from 14.5 to 154. In core samples, this ratio ranged from 16.5 to 492.4 (Figs. 2A, 6A). Assuming that the leachate-associated and core-associated ratios between

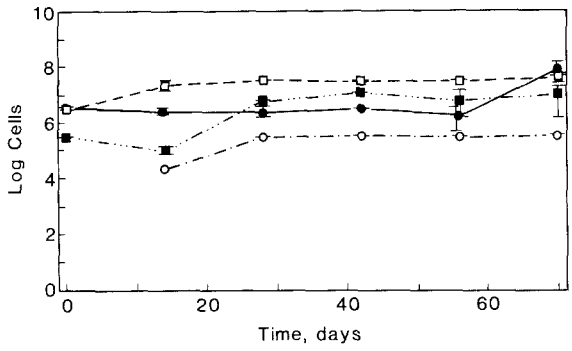

Fig. 7. Temporal changes in abundance of aerobic heterotrophs in leachates from FP Coal H and ROM Coal F. Data points represent estimated mean values for the entire leachate output (ca. 1.4 liters) at each leaching event. Error bars show the range of mean outputs for duplicate columns. Symbols: □, aerobic heterotrophs recovered at pH 7 from Coal H leachates; ■, aerobic heterotrophs recovered at pH 3.5 from Coal H leachates; ●, aerobic heterotrophs recovered at pH 7 from Coal F leachates; ○, aerobic heterotrophs recovered at pH 3.5 from Coal F leachates.

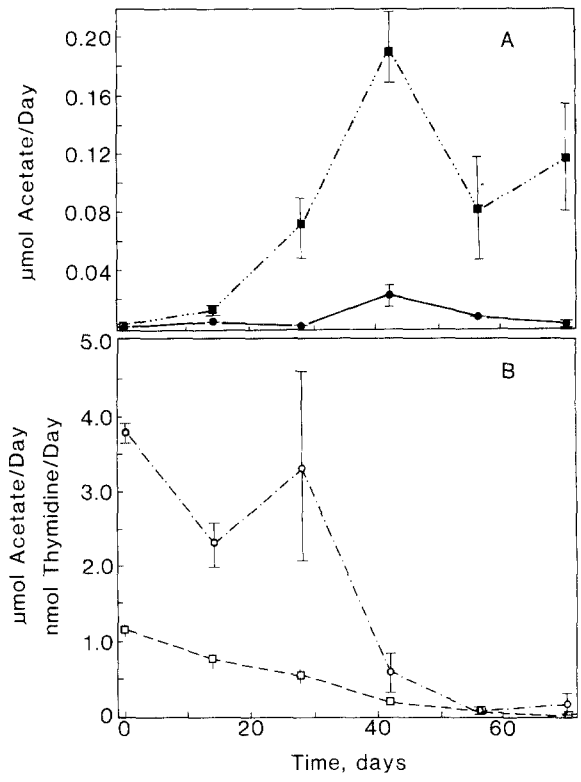

Fig. 8. Temporal changes in heterotrophic bacterial activity in cores and leachates from Coal F. (A) Acetate respiration in cores (■) and leachates (●). (B) Incorporation of acetate (□) and thymidine (○) in leachates. Data points represent estimated mean values for an entire coal column (6.8 kg coal) or the entire leachate output (ca. 1.4 liters) at each leaching event. Error bars show the range of mean outputs for duplicate columns.

acetate incorporation and respiration were similar at the same leaching events, this implies that autotrophic activity dominated Coal H cores throughout the experiment, as it did in the leachates.

The heterotrophic community of Coal H leachates was dominated by species or strains culturable at pH 7, but the number of heterotrophs recoverable at pH 3.5 increased about 70-fold between 14 and 28 days (Fig. 7). During the same time period, acetate respiration rates in coal cores began to greatly exceed those in leachates (Fig. 6A). These data suggest that changes in heterotrophic activity in cores are related to microbial succession, and that heterotrophic bacteria of later successional stages (compared to the heterotrophic community initially present in the coal) show a greater tendency to remain within coal columns rather than to be lost during leaching.

In Coal F, as in Coal H, acetate respiration rates in cores and leachates were initially similar, but became considerably higher in cores than in leachates as leaching continued (Fig. 8A). In both cores and leachates, peak respiration rates occurred at 42 days. However, acetate incorporation rates in leachates, and consequently the ratio of incorporation to respiration, decreased markedly throughout the experiment (Figs. 8A, B). Measurements of thymidine assimilation supported the idea that heterotrophic growth was much slower in later leaching events than in the 0–28 day period (Fig. 8B). Thus, in Coal F leachates and probably in cores as well, the heterotrophic community became less productive with time, although the number of heterotrophs recovered from leachates either remained stable or increased with time (Fig. 7). This decrease in productivity with higher bacterial abundance can be explained by a shift in species or strain composition of the heterotrophic community.

In Coal F leachates, as in Coal H leachates, fewer heterotrophs were culturable at pH 3.5 than at pH 7 at any given leaching event (Fig. 7). The apparent failure of acidophiles to dominate the heterotrophic population might indicate that some factor other than pH was involved in limiting heterotrophic growth. However, it is also possible that the extremely low pH (as low as 1.6) of Coal F leachates may not have favored growth of organisms culturable at pH 3.5 over that of strains culturable at pH 7.

DISCUSSION

Although the chemistry of leachates and slurries have been compared for a number of coal types [2,6,13,20,21 and M.C. Reid, Characterization of organic contaminants present in coal slurry wastewaters, Ph.D. dissertation, University of Tennessee, 1984], previous investigations have not compared the naturally occurring microbial populations that influence the leaching process. Most work in this area has involved the use of only a single coal material and/or has used artificially introduced microorganisms in an effort to manipulate desulfurization processes [5,7,8,9,14,17,18]. Our study, therefore, represents the first attempt to determine the influence of beneficiation processes upon coal microbiology.

In general, our results for several coals agree with those reported previously for Coal A [19]. Most notably, iron- and sulfur-oxidizing bacteria dominated the microflora of leachates from all six coals studied, and the size of the autotrophic community was related to mean iron and sulfur levels in leachates ($r^2 = 0.71$ for the regression of iron oxidizers on iron content, 0.70 for the regression of sulfur oxidizers on sulfur content with $n = 13$, $P \leq 0.01$). It is, therefore, likely that these features are characteristic of Eastern coals in general. Furthermore, our results with Coal H, together with previous data on Coal A [19], indicate that autotrophs are dominant within coal columns as well as in leachates.

Although the leaching process follows a generally similar pattern in the six Eastern coals we have studied, the method of coal preparation (grinding alone versus grinding, washing, and blending) has a major influence on autotroph abundance and leaching of iron and sulfur. Beneficiation tends to decrease the abundance of autotrophic bacteria as well as the iron and sulfur content of leachates. Although detachment of bacteria during washing might have resulted in low initial abundances of autotrophs, the low average abundances in FP leachates compared to ROM leachates as leaching continues suggest nutrient or energy limitation as well. Although little or no relationship between autotroph abundance and coal chemical parameters was observed, pyrite or nutrients may have been selectively removed from coal particle surfaces during washing, leaving the remaining energy sources or nutrients less accessible to bacteria. The shift in the ratio of sulfur oxidizers to iron oxidizers in leachates from Coals H and F suggests that iron in particular may become limiting after prolonged leaching of some coals. Even in coals with a high pyrite content, particle surfaces might eventually become coated with elemental sulfur, decreasing the susceptibility of pyrite to bacterial attack. Coating of particle surfaces with elemental sulfur is known to occur during bacterial leaching of copper ores [4].

In leachates from ROM coals, autotroph abundance varied according to the amount of coal surface area exposed by the grinding process, again suggesting that energy limitation influences autotroph abundance. Thus, the microbial leaching process in ROM coals might be slowed by storing the coal in a more coarsely ground form, thereby minimizing the amount of pyrite exposed to microbial attack.

Mean levels of autotrophic activity in the six coals were not consistently related to autotroph numbers or to leaching of iron or sulfur. From our detailed study of Coal A [19] and H, it is apparent that cell-specific CO_2 assimilation rates vary with time, probably due to changes in the nutritional status of the autotrophic community. Apparently, considerable variability also exists between mean CO_2 assimilation rates in leachates from different coals. One cause of such variability might be a dif-

ference between strains of autotrophs in the six coals. In addition, the ratio between CO_2 assimilation in Coal H cores to that in leachates also changes with time. This could indicate a difference in physiological condition between particle-associated and unattached cells. The tendency of the core/leachate CO_2 assimilation ratio to increase with time also suggests that progressively smaller portions of the autotrophic community are washed out with successive leachings.

Our results with Coal A [19] indicated that the size of the heterotroph population in coal columns was limited by acid production resulting from autotrophic metabolic activity. The results reported here are, in general, consistent with this idea. However, differences in heterotroph abundances among the six coals could not be predicted on the basis of the minor pH differences seen in leachates, indicating that other factors are probably also involved. Unlike autotrophs, heterotrophs were not dramatically affected by washing of coal, suggesting that they are not readily removed during washing and that they are not growth-limited by substances removed during washing.

As well as varying in abundance, the heterotrophic microflora of the six coals also varied in composition and in cell-specific rates of acetate metabolism. Differences in heterotrophic populations of the six coals could not be attributed to any one chemical parameter of the coals or their leachates. Although there are slight indications of a relationship between acidophilic or acidotolerant organosulfur utilizers and nitrogen content or types of organics present in the coal, factors (other than pH) controlling the remainder of the heterotroph population remain unclear. This may be due to differences in the original species composition of the six coals, the complexity of the coal habitat, and possibly to the presence of microenvironments of varying chemical characteristics within the coal columns. It seems probable that heterotrophic activity in coal columns is influenced by microbial succession during the course of leaching experiments, and that proportionately fewer heterotrophs are washed out of the columns in later leaching events (compared to early leaching events). We can, however, conclude that when pH is not artificially controlled, heterotrophs represent only a minor component of the microflora of Eastern coals subjected to leaching in laboratory columns. It is therefore likely that the development of processes for biodegradation of organic sulfur in coal will depend either on artificially maintaining a high pH or on optimizing conditions for the growth of acidophilic heterotrophs.

The close correspondence between acetate metabolism and [^{3}H]methylthymidine assimilation in leachates suggests that a substantial portion of the heterotrophic community is capable of acetate uptake, thus tending to validate the use of acetate as a substrate for measuring heterotrophic activity in coals and coal leachates. The fact that dark CO_2 assimilation measurements fail to follow thymidine incorporation suggests that iron- and sulfur-oxidizing autotrophs (e.g. *Thiobacillus* spp.) assimilate thymidine much more slowly than do heterotrophs in the leachate environment. Photoautotrophic microorganisms also assimilate radiolabeled nucleotides slowly compared to heterotrophs [3,11,12], as does an exponential phase pure culture of a chemoautotrophic hydrothermal vent sulfur-oxidizing bacterium (Radway, J.C., C.L. Divan and J.H. Tuttle. Comparison of adenine and thymidine incorporation by selected microbial cultures. American Society for Microbiology, 1987 Abstracts, p. 254). However, an estuarine chemoautotrophic strain assimilates thymidine at rates comparable to those of heterotrophic isolates, although the proportion of labeled thymidine incorporated into DNA is not yet known. Uptake rates may also vary with the culture's growth phase (Radway, J.C., C.L. Divan and J.H. Tuttle. Comparison of adenine and thymidine incorporation by selected microbial cultures. American Society for Microbiology, 1987 Abstracts, p. 254). Thus, measurements of short-term thymidine uptake rates probably do not reflect heterotrophic production alone in all environments, although it seems possible that the technique might provide such an estimate in coal leachates.

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