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Ecological Study of the Effects of Strip Mining on the Microbiology of Streams

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ECOLOGICAL STUDY OF THE EFFECTS OF STRIP MINING ON THE MICROBIOLOGY OF STREAMS

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ABSTRACT

The microflora of Cane Branch of Beaver Creek in McCreary County, Kentucky, which drains an area that was strip-mined between 1955 and 1959, was studied and compared with that of Helton Branch which drains a comparable area where there has been no mining. Differences include: the establishment of Ferrobacillus ferrooxidans, for which procedures were developed for direct colony isolation from the stream; fewer saprophytic bacteria; more numerous and more diversified filamentous and unicellular fungi; and characteristic differences in algal flora. Representatives of 42 genera of filamentous fungi were identified. Of these, 21 were isolated only from Cane Branch. Representatives of five genera of unicellular fungi were found. One, Rhodotorula, was found consistently in Cane Branch but never in Helton Branch. From 1966 to 1968, Bumilleria appears to have established itself as the dominate alga in Cane Branch at some distance downstream from the strip-mine drainage area. Seasonal differences in the microflora appear to be relatively insignificant, except for the algae.

FCST Category-V-C

Key Words - strip mines, acid streams, acid mine water, aquatic microbiology*, ecology, environmental effects

ECOLOGICAL STUDY OF THE EFFECTS OF STRIP MINING ON THE MICROBIOLOGY OF STREAMS

This study has been conducted on the Cane Branch of Beaver Creek in McCreary County, Kentucky, which drains an area that was strip mined between 1955 and 1959, and Helton Branch which drains a similar area where there has been no strip mining and serves as a control. These streams are within the Daniel Boone National Forest and appeared ideal to study since no domestic waste is discharged into either stream.

The area selected for study is particularly important because of the wealth of information about it that is already available. Between 1955 and 1966 a Work Group Committee made up of representatives of the U.S. Forest Service, the U. S. Army Corps of Engineers, the U. S. Fish and Wildlife Service, the U. S. Soil Conservation Service, the U. S. Bureau of Mines, the U. S. Geological Survey, the Ohio River Valley Sanitation Commission, the Kentucky Department of Economic Development, the Kentucky Department of Fish and Wildlife Resources, the Kentucky Geological Survey, the Kentucky Strip Mining and Reclamation Commission, the Kentucky Division of Flood Control and Water Usage, and the Kentucky Water Pollution Control Commission has been making a study of the hydrologic influences of strip mining on the area.

Different taxonomic groups of microorganisms have been studied with particular attention being paid to microorganisms which may contribute

to or indicate natural recovery of the stream. Molecular filter techniques for isolation and enumeration of specific iron-oxidizing bacterium have been developed. Samples of water were collected from the stream so as to reflect the effect of:

1. Distance from the source of mining pollution,
2. Microenvironment of the stream,
3. Season of year,
4. Quantity of stream flow.

Samples have been collected from six sampling stations in Cane Branch and one in Helton Branch, during the four seasons. The first two stations in the Cane Branch area are located in gullies which receive drainage only from the spoil banks. The third station is at the foot of a 40-foot falls where the water from stations one and two is mixed with water in Cane Branch. Stations 4 and 5 are located in tributaries which flow into Cane Branch and drain different spoil banks in the area. They are approximately 2500 and 3200 feet, respectively, downstream from the falls. The sixth station is located in Cane Branch proper approximately 4000 feet downstream from the falls and the main drainage area. The seventh station, located on Helton Branch, serves as a control. Although there is no strip mining in the Helton Branch area, coal seams are present, which results in a limited amount of seepage. Both surface and bottom samples have been studied and the temperature and pH values of the stream have been recorded at each sampling station during each sampling period.

Temperature and pH

The average pH values at the time of sampling at each station

are given in Table 1. These pH values were obtained with an Instrumentation Laboratory, Inc., "PORTO-matic" pH meter, Model 175. The water temperatures averaged 19°C in the summer; 10°C in the autumn; 3°C in the winter and 14°C in the spring. The average pH values at station 3 through 6 were higher during the winter than at the other seasons. This may be a result of reduced biological activity at lower temperatures and less continuous drainage from the strip-mine spoil during the winter. Since there appears to be no natural buffering in Cane Branch, changes in pH values may be expected to be expressed more readily. Stations 1 and 2 show less change since both are pools formed directly by drainage, in which the water collects before continuing over the falls into Cane Branch proper. The average pH at station 7 was lower in the winter than during the other seasons. This may result from increased carbon dioxide being dissolved at lower temperatures causing a decrease in pH.

TABLE 1

Average pH at Each Sampling Station by Seasons

Station	Summer	Autumn	Winter	Spring
1 (Cane Branch)	3.0	3.0	3.2	3.0
2 (Cane Branch	3.5	3.0	3.2	3.5
3 (Cane Branch)	3.2	3.3	3.6	3.2
4 (Cane Branch)	3.3	3.2	3.8	3.3
5 (Cane Branch)	3.5	3.5	4.1	3.5
6 (Cane Branch)	3.3	3.4	3.9	3.3
7 (Helton Branch)	6.5	6.7	6.3	6.5

Bacteria

Iron oxidizing bacteria appear to be indigenous to bituminous coal regions; therefore, such microorganisms should be present in acid mine water and contribute to the total amount of acid present in such water. A chemosynthetic - iron oxidizing bacterium, Ferrobacillus ferrooxidans, which oxidizes ferrous iron to ferric iron was isolated. This organism grows at a pH value of 3.5 and utilizes ferrous iron as its energy source producing ferric hydroxide and sulfuric acid.

Primary isolation of F. ferrooxidans was accomplished by inoculating 10 ml of stream water into 150 ml of Aleem's modification of Silverman-Lundgren's 9K medium. To prepare this medium a basal salt solution is prepared to contain: $(NH_4)_2SO_4$, 4.50g; KCl, 0.15g; K_2HPO_4, 0.75g; $MgSO_4 \cdot 7H_2O$, 0.75g; and $Ca(NO_3)_2$, 0.015g; in 1050 ml of demineralized water. This basal salt solution is dispensed in 105 ml quantities in 250-ml Erlenmeyer flasks and sterilized by autoclaving for 15 minutes at 121°C. A solution of ferrous sulfate composed of: $FeSO_4 \cdot 7H_2O$, 67.5g; 10N H_2SO_4, 0.4 ml; and 375 ml of demineralized water is filter sterilized. To make the complete medium, 45 ml of this ferrous sulfate solution are added to 105 ml of the basal salt solution. This medium is opalescent green with a pH of 3.5. The inoculated medium was placed on a New Brunswick gyrotory shaker (Model G 10) and incubated at room temperature. Within 5 to 10 days a reddish brown precipitate appeared in the medium, which is characteristic of a viable culture. Growth appeared within two or three days upon subculturing in the same medium.

Ferrobacillus ferrooxidans was isolated during each of the four seasons from Cane Branch. In addition, it was isolated once, during the

summer, from Helton Branch. Although the area drained by Helton Branch contains no mines, evidence for a small amount of drainage from coal seams into the stream was observed following the isolation of the Ferrobacillus. This drainage does not appear to have been sufficient to affect the pH of the stream or to change its flora drastically.

A procedure was developed for producing colonies of F. ferrooxidans on a molecular filter from a culture isolated from the stream. Cells used for culturing on molecular filters were grown on a rotory shaker in 1-liter flasks containing 600-ml amounts of Aleem's modification of the 9K liquid medium. After a 72-hour incubation period, the cells were chilled and then harvested by continuous flow centrifugation. The cell paste and iron precipitate were suspended in 28 ml of cold acidic water, pH 3.0-3.5, shaken, and allowed to stand in the refrigerator over night. The tan supernatant, containing the cells, was removed from the underlying layer of iron precipitate. This cell suspension was centrifuged for five minutes at 270 x g in a Sorvall superspeed automatic refrigerated centrifuge (Model RC2-B) which removed most of the precipitate remaining suspended. The supernatant was decanted and the procedure repeated a minimum of three times. This cell suspension was then centrifuged for 30 minutes at 10,800 x g. The supernatant was decanted and the packed cells were resuspended in 120 ml of cold acidic water. The cell suspension was used within five hours after final dilution. Dilutions of this cell suspension were filtered through Millipore filters (HAWG 047 AO) and the filter membrane placed on absorbent pads which were saturated with liquid medium composed of: $(NH_4)_2SO_4$, 0.114 g; KCl, 0.037 g; K_2HPO_4, 0.37 g; $MgSO_4 \cdot 7H_2O$, 0.375 g; and $Ca(NO_3)_2$, 0.007 g, in 1000 ml of demineralized

water. A solution of 7.5 g $FeSO_4 \cdot 7H_2O$ was made in 100 ml of demineralized water acidified with 0.1 ml 10 N H_2SO_4 and filter sterilized.

The pH value of the basal salt solution was adjusted to 3.5-4.0 with H_2SO_4, dispensed in 100 ml quantities into 250-ml Erlenmeyer flasks and autoclaved for 15 minutes at 121°C. The complete medium was prepared by aseptically adding 1.0 ml of the ferrous sulfate solution to each flask. The pH value of the complete medium was 3.0 to 3.5. These plates were incubated at 28°C.

Each plate contained isolated colonies within 24 hours. The colonies were cream to light tan and approximately 1 mm in diameter. Isolated colonies were inoculated into Aleem's medium and growth occurred within 24 to 36 hours at 28°C. Microscopic examination of the cultures showed the organism to be an actively motile, gram-negative rod measuring 0.6 to 1.0 μ in width and 1.0 to 1.6 μ in length.

F. ferrooxidans was also isolated directly and consistently from the surface and bottom samples on filter membranes in large numbers. The bottom samples were centrifuged at 11,700 x g. The supernatant was decanted and the packed mud treated exactly as the cell paste obtained from the liquid culture of F. ferrooxidans. The only treatment of surface samples was prefiltration using Millipore absorbent pads before filtration through the membrane filter. The same medium and incubation conditions were used as previously described. Again well-isolated colonies appeared within 24 hours. More colonies, 5 to 6 times as many, were obtained from the bottom samples.

Standard plate counts were made at 20 and 35°C to determine the number of saprophytic bacteria in both Cane and Helton Branches. As a

result of sporadic stream flow and turbidity in both streams, consistent counts were not obtained. However, they did show a much larger number of saprophytic bacteria in Helton Branch, 4000 to 50,000, as compared with Cane Branch, 100 to 2000. Attempts to observe periphytic bacteria were made by suspending slides in the streams. However, the distance of the stream from the laboratory made frequent observation impossible and slides were lost due to flooding. Those which were recovered were covered with such a large amount of precipitate as to have little value.

The pH values of Cane Branch in the area which was strip-mined indicates that the increased amount of acid has altered the natural conditions of Cane Branch as compared to Helton Branch. The lowering of the pH, increase of sulfates and almost total elimination of bicarbonate alkalinity have resulted in the alteration of the microflora of Cane Branch. These conditions have resulted in the establishment of F. ferroxidans, which biologically contributes to the acidity of the stream. The isolation of this organism from Helton Branch is not surprising since F. ferrooxidans is believed to be indigenous to bituminous coal regions. However, the density of organisms appears to be so low that no pronounced biological effects are evident. Although counts of saprophytic bacteria fluctuated, the counts of these bacteria in Cane Branch were considerably lower than the counts in Helton Branch which further indicates an alteration of the microflora of Cane Branch.

Fungi

The occurrence of filamentous fungi, yeast, and true aquatic fungi in Cane and Helton Branches was investigated. Filamentous fungi were

isolated by making serial dilutions of the samples and preparing pour plates of neopeptone-dextrose-streptomycin agar. As fungal colonies appeared they were picked and the resultant cultures were checked for purity, and maintained on stock medium until identified. The agar block slide technique was used in identification.

Study of the true aquatic fungi was confined to those easily observed. This was done by covering boiled hemp seeds with approximately 100 ml amounts of sample water in crystallizing dishes. These preparations were allowed to incubate at room conditions of light and temperature for about 14 days. The fungi colonize on the hemp seed and can be identified to genus using the low-power objective of the microscope.

For the isolation of yeasts, two different concentrations of medium were used. The basic ingredient is Wickerham's yeast nitrogen base. A 10X stock solution of this is prepared by adding 6.7 g per 100 ml and filter sterilizing. Two concentrations of glucose solutions, 2% (w/v) and 40% (w/v), are prepared and filter sterilized. The complete medium is prepared by aseptically adding 2.5 ml quantities of the yeast nitrogen base stock solution to 22.5 ml quantities of distilled water previously sterilized by autoclaving for 20 minutes at 121°C in 250-ml Erylenmeyer flasks. Then 25-ml volumes of medium of 2% and 40% glucose solution are added to give final 50-ml volumes of medium of 1% and 20% glucose concentration, respectively. Each concentration of medium was inoculated with 1.0 ml of sample water. The flasks were placed on a rotary shaker and shaken at 120 to 150 oscillations per minute for 64 hours at room conditions of light and temperature. Then the flasks were removed and allowed to stand on the laboratory bench for four hours. The yeast

cells, when present, settle to the bottom, the bacteria remain in suspension, and filamentous fungi remain in suspension or float on the surface. Plates of yeast extract-malt extract-glucose agar were streaked from each flask. These plates were incubated at 20°C for two to three days. If colonies were discrete, transfers were made to neopeptone-glucose agar alants; if not, streaks were made on Diamalt agar plates. Cultures were purified by repeated streaking on neopeptone-glucose agar and maintained on neopeptone-glucose agar slants. The yeasts were identified by microscopic and physiological methods. Carbon replication tests were done to determine the amounts of growth on 30 carbon sources. Carbon and nitrogen auxanograms were made with glucose, sucrose, galactose, lactose, and maltose as carbon sources, and ammonium sulfate, sodium nitrite, and potassium nitrate as nitrogen sources. The abilities of the yeasts to ferment galactose, sucrose, maltose, lactose and raffinose were determined; likewise their abilities to grow in vitamin-free medium and ethanol. The type of pellicle, ring and sediment in liquid wort, and the type of growth on slants of Diamalt agar were observed. The ability to produce pseudomycelium was also determined.

Tables 2 through 8 represent the filamentous fungi isolated and identified during the four seasons. Certain genera were more prevalent in spring, summer and autumn, e.g. Cladosporium, Epicoccum, Mucor, and Phoma. Penicillium was prevalent in all seasons. However, more species were isolated during the winter. One genus, Trichoderma, appeared regardless of season. With the exception of one station, more isolates were obtained from bottom samples during the spring and autumn and from surface samples during the winter. The same station, 2, was the exception

TABLE 2

Fungi isolated at Station 1

Season	Surface	Bottom
Spring	*Cladosporium cladosporioides* *Septonema* sp. *Fusarium* sp. *Phoma* sp. *Aspergillus* sp.	*Rhizopus arrhizus* *Eciococcum purpurascens* *Penicillium* sp. *Mucor* sp. *Trichoderma viride* *Rhinotrichum* sp.
Summer	*Cladosporium cladosporioides* *Epicoccum purpurascens* *Aspergillus* sp. *Alternaria tenuis* *Cephalosporium* sp.	*Trichoderma viride* *Alternaria tenuis* *Aspergillus* sp. *Penicillium* sp. *Mucor angulisporus* *Epicoccum purpurascens* *Phoma* sp. *Absidia* sp.
Autumn	*Cladosporium cladosporioides* *Epicoccum purpurascens* *Alternaria* sp. *Curvularia* sp. *Trichoderma viride*	*Mucor* sp. *Trichoderma viride* *Phoma* sp. *Alternaria* sp. *Penicillium* sp. *Epicoccum purpurascens* *Monosporium* sp.
Winter	*Alternaria* sp. *Fusarium* sp. *Thielaviopsis* sp. *Botrytis* sp. *Trichoderma viride* *Penicillium* (9 species)	*Phialophora fastigiata* *Trichoderma viride* *Penicillium* sp. *Mucor* sp.

TABLE 3

Fungi isolated at Station 2

Season	Surface	Bottom
Spring	*Mucor* sp. *Fusarium* sp. *Beauveria bassiana* *Epicoccum purpurascens* *Phoma* sp. *Trichoderma viride* *Penicillium* sp.	*Phoma* sp. *Mucor* sp. *Trichoderma viride*
Summer	*Fusarium* sp. *Cladosporium cladosporioides* *Alternaria* sp. *Epicoccum purpurascens* *Trichoderma viride*	*Trichoderma viride* *Penicillum* sp. *Mucor* sp. *Alternaria* sp. *Cladosporium cladosporioides*
Autumn	*Mortierella* sp. *Penicillium* sp. *Mucor* sp. *Cladosporium cladosporioides* *Alternaria* sp. *Epicoccum purpurascens* *Trichoderma viride*	*Penicillium* sp. *Mortierella* sp. *Trichoderma viride* *Epicoccum purpurascens* *Pestalotia* sp. *Alternaria* sp. *Mucor* sp.
Winter	*Zygorhynchus moelleri* *Trichoderma viride* *Mucor fragilis* *Penicillium* (3 species)	*Cladosporium cladosporioides* *Trichoderma viride* *Mucor* sp. *Penicillium* sp.

TABLE 4

Fungi isolated at Station 3

Season	Surface	Bottom
Spring	*Penicillium* sp. *Aspergillus* sp.	*Geotrichum candidum* *Rhinotrichum* sp. *Epicoccum purpurascens* *Trichoderma viride* *Penicillium* sp. *Phoma* sp. *Cladosporium cladosporioides* *Fusarium* sp. *Mucor* sp.
Summer	*Calcarisporum* sp. *Oidiodendron* sp. *Trichoderma viride* *Cladosporium cladosporioides* *Alternaria* sp. *Mucor* sp. *Fusarium* sp.	*Alternaria* sp. *Cladosporium cladosporioides* *Trichoderma viride* *Mucor* sp. *Fusarium* sp. *Gongronella butleri*
Autumn	*Trichoderma viride* *Cladosporium cladosporioides* *Mortierella* sp. *Alternaria* sp. *Epicoccum purpurascens* *Penicillium* sp. *Aspergillus* sp.	*Penicillium* sp. *Trichoderma viride* *Zygorhynchus* sp. *Cladosporium cladosporioides* *Phoma* sp. *Alternaria* sp. *Epicoccum purpurascens* *Mortierella* sp. *Fusarium* sp. *Absidia* sp. *Humicola* sp.
Winter	*Phoma* sp. *Fusarium* sp. *Cephalosporium* sp. *Trichoderma viride* *Penicillium canescens* *Mucor* sp.	*Penicillium* sp. *Mucor* sp. *Trichoderma viride* *Gongronella butleri* *Phoma* sp.

TABLE 5

Fungi isolated at Station 4

Season	Surface	Bottom
Spring	*Cladosporium cladosporioides* *Fusarium* sp. *Penicillium* sp.	*Epicoccum purpurascens* *Trichoderma viride* *Phoma* sp. *Alternaria* sp.
Summer	*Trichoderma viride* *Stemphylium botryosum* *Epicoccum purpurascens* *Cladosporium cladosporioides* *Alternaria* sp. *Cephalosporium* sp. *Fusarium* sp.	*Penicillium* sp. *Epicoccum purpurascens* *Trichoderma viride* *Cladosporium cladosporioides* *Aspergillus* sp. *Cephalosporium* sp.
Autumn	*Trichoderma viride* *Penicillium* sp. *Mucor* sp. *Cladosporium cladosporioides* *Fusarium* sp. *Beauveria* sp. *Monosporium* sp. *Epicoccum purpurascens* *Alternaria* sp.	*Trichoderma viride* *Penicillium* sp. *Mucor* sp. *Cladosporium cladosporioides* *Beauveria* sp. *Fusarium* sp. *Phoma* sp. *Gliocladium* sp. *Epicoccum purpurascens* *Alternaria* sp.
Winter	*Humicola* *Cunninghamella japonica* *Trichoderma viride* *Fusarium* sp. *Penicillium* (4 species) *Mucor* sp. *Calcarisporium* sp. *Geotrichum* sp.	*Thysanophora penicilloides* *Trichoderma viride* *Mucor* sp. *Penicillium* sp.

TABLE 6

Fungi isolated at Station 5

Season	Surface	Bottom
Spring	*Cladosporium cladosporioides* *Fusarium* sp. *Trichoderma viride*	*Epicoccum purpurascens* *Fusarium* sp. *Trichoderma viride* *Monilia* sp. *Phoma* sp. *Penicillium* sp. *Pestalotia* sp.
Summer	*Epicoccum purpurascens* *Cladosporium cladosporioides* *Penicillium* sp. *Mucor* sp. *Trichoderma viride* *Alternaria* sp. *Fusarium* sp. *Mortierella* sp. *Phoma* sp. *Nematogonium* sp. *Pestalotia* sp.	*Gongronella butleri* *Cladosporium cladosporioides* *Penicillium* sp. *Mucor* sp. *Trichoderma viride* *Fusarium* sp. *Cephalosporium* sp. *Aspergillus* sp.
Autumn	*Trichoderma viride* *Cladosporium cladosporioides* *Penicillium* sp. *Monosporium* sp. *Chaetomium* sp.	*Trichoderma viride* *Mucor* sp. *Cladosporium cladosporioides* *Penicillium* sp. *Verticillium* sp. *Mortierella* sp. *Fusarium* sp. *Beauveria* sp. *Epicoccum purpurascens*
Winter	*Phoma* sp. *Fusarium* sp. *Absidia coerulea* *Penicillium* (2 species) *Trichoderma* sp. *Mucor* sp.	*Mucor* sp. *Penicillium* sp. *Trichoderma* sp. *Myrothecium* sp.

TABLE 7

Fungi isolated at Station 6

Season	Surface	Bottom
Spring	*Aureobasidium* sp. *Phoma* sp.	*Pestalotia* sp. *Phoma* sp. *Penicillium* sp. *Alternaria* sp. *Cladosporium cladosporioides* *Trichoderma viride* *Mucor* sp. *Fusarium* sp.
Summer	*Cladosporium cladosporioides* *Phoma* sp. *Aspergillus* sp. *Alternaria* sp.	*Cladosporium cladosporioides* *Phoma* sp. *Trichoderma viride* *Penicillium* sp. *Cephalosporium* sp. *Beauveria bassiana* *Fusarium* sp. *Mortierella* sp. *Epicoccum purpurascens*
Autumn	*Trichoderma viride* *Epicoccum purpurascens* *Cladosporium cladosporioides* *Phialopora* sp.	*Trichoderma viride* *Penicillium* sp. *Mucor* sp. *Cladosporium cladosporioides* *Monosporium* sp. *Paecilomyces* sp. *Zygorhynchus* sp. *Cephalosporium* sp.
Winter	*Alternaria* *Cladosporium cladosporioides* *Thysanophora* sp. *Penicillium chrysogenum* *Trichoderma* sp. *Mucor* sp.	*Trichoderma viride* *Penicillium* sp. *Verticillium* sp. *Mucor* sp. *Aspergillus* sp.

TABLE 8

Fungi isolated at Station 7

Season	Surface	Bottom
Spring	*Fusarium* sp. *Monilia* sp. *Cephalosporium* sp.	*Fusarium* sp. *Cladosporium cladosporioides* *Gliocladium roseum* *Penicillium* sp. *Mucor* sp. *Trichoderma viride*
Summer	*Stemphylium* sp. *Penicillium* sp. *Cladosporium cladosporioides* *Epicoccum purpurascens* *Phoma* sp. *Zygorhynchus* sp. *Rhizopus nigricans* *Beauveria bassiana*	*Trichoderma viride* *Fusarium* sp. *Cladosporium cladosporioides* *Pestalotia* sp. *Phoma* sp. *Mucor* sp. *Alternaria* sp. *Peyronelaea* sp.
Autumn	*Trichoderma viride* *Mucor* sp. *Cladosporium cladosporioides* *Fusarium* sp. *Alternaria* sp. *Penicillium* sp. *Aspergillus* sp.	*Trichoderma viride* *Cladosporium cladosporioides* *Mucor* *Alternaria* sp. *Fusarium* sp. *Penicillium* sp. *Monochaetia* sp. *Stachylidium* sp.
Winter	*Mucor fragilis* *Chrysosporium pannorum* *Fusarium* sp. *Gliocladium roseum* *Cephalosporium* sp. *Trichoderma viride* *Penicillium* (3 species)	*Mucor* sp. *Trichoderma viride* *Penicillium* sp. *Gliocladium* sp. *Fusarium* sp.

in all three seasons. Distribution was about equal during the summer.

Table 9 shows the filamentous fungi isolated from Cane and Helton Branches. The fungi were more numerous and diversified in Cane Branch. A total of 42 genera were identified. Of these, 17 were isolated from both areas, 21 only from Cane Branch, 4 only from Helton Branch. Drainage from the strip-mined area appears to have led to an increased fungal flora in Cane Branch.

Representatives of only three genera of true aquatic fungi were found. Achlya, Aphanomyces, and Saprolegnia were identified from Cane Branch, whereas, only Achlya was identified from Helton Branch.

Tables 10 through 16 represent the yeasts isolated and identified during the four seasons. More genera were prevalent during the spring and autumn in Cane Branch whereas more were prevalent during the winter and autumn in Helton Branch.

Table 17 shows the yeasts isolated from Cane and Helton Branches. Representatives of 5 genera were found: one only from Cane Branch, one only from Helton Branch, and three from both. A greater variety of species were found in Cane Branch. Five species of Candida were found only from Cane Branch, one only from Helton Branch, and two from both. Two species of Torulopsis were found only from Cane Branch, one only from Helton Branch, and one from both. Only two species of Cryptococcus were found, one from each branch. Probably significant is the fact that members of Rhodotorula were consistently found in Cane Branch but never in Helton Branch. Members of Trichosporon were found in some samples from Helton Branch, but never in samples from Cane Branch. Representatives of all the genera found in streams containing acid mine waste by other investigators were also found in Cane Branch with the exception of Trichosporon. However, the

TABLE 9

Sources from which Fungi were Identified

Cane and Helton Branches	Cane Branch	Helton Branch
Cladosporium	Septonema	Chrysosporium
Fusarium	Curvularia	Peyronellaea
Phoma	Rhinotrichum	Monochaetia
Rhizopus	Absidia	Stachylidium
Epicoccum	Thielaviopsis	
Penicillium	Phialophora	
Mucor	Botrytis	
Trichoderma	Geotrichum	
Alternaria	Calcarisporium	
Cephalosporium	Oidiodendron	
Beauveria	Gongronella	
Zygorhynchus	Cunninghanella	
Stemphylium	Thysanophora	
Pestalotia	Mortierella	
Monilia	Nematogonium	
Gliocladium	Aureobasidium	
Aspergillus	Monosporium	
	Humicola	
	Chaetomium	
	Verticillium	
	Myrothecium	

TABLE 10

Yeasts isolated at Station 1

Season	Isolate
Spring	*Rhodotorula glutinis* var *rubescens*
	Candida krusei
Summer	*Rhodotorula mucilaginosa*
Winter	*Candida solani*
Autumn	*Torulopsis glabrata*
	Rhodotorula glutinis
	Cryptococcus laurentii
	Candida solani

TABLE 11

Yeasts isolated at Station 2

Season	Isolate
Spring	*Rhodotorula glutinis* var *rubescens* *Torulopsis candida* *Cryptococcus laurentii*
Summer	----------
Winter	*Candida humicola*
Autumn	*Candida solani* *Candida krusei* *Rhodotorula minuta* *Rhodotorula glutinis*

TABLE 12

Yeasts isolated at Station 3

Season	Isolate
Spring	*Candida parapsilosis*
Summer	*Torulopsis glabrata*
Winter	*Candida solani*
Autumn	*Torulopsis glabrata* *Candida solani* *Candida krusei* *Candida parapsilosis* *Cryptococcus laurentii*

TABLE 13

Yeasts Isolated at Station 4

Season	Isolate
Spring	Candida parapsilosis Rhodotorula glutinis var rubescens Torulopsis versatilis Rhodotorula mucilaginosa
Summer	Candida catenulata Candida parapsilosis
Winter	Candida krusei Candida parapsilosis
Autumn	Rhodotorula glutinis Candida solani Candida parapsilosis Candida guilliermondii

TABLE 14

Yeasts Isolated at Station 5

Season	Isolate
Spring	Candida parapsilosis Candida humicola
Summer	Candida melibiosi Torulopsis glabrata
Winter	Candida humicola Candida solani
Autumn	Candida krusei Candida solani Candida humicola

TABLE 15

Yeasts Isolated at Station 6

Season	Isolate
Spring	Candida parapsilosis Rhodotorula glutinis Candida humicola Candida krusei Cryptococcus laurentii
Summer	Rhodotorula glutinis
Winter	Candida krusei Candida melinii Rhodotorula glutinis
Autumn	Candida krusei Cryptococcus laurentii Candida solani Rhodotorula glutinis Candida parapsilosis

TABLE 16

Yeasts Isolated at Station 7

Season	Isolate
Spring	Trichosporon cutaneum var multisporum Candida parapsilosis
Summer	Candida parapsilosis
Winter	Torulopsis glabrata Torulopsis stellata var cambresieri Trichosporon cutaneum var multisporum Candida krusei
Autumn	Candida rugosa Candida krusei Torulopsis stellata var cambresieri Cryptococcus luteolus Trichosporon pullulans

TABLE 17

Sources from which Yeasts were Identified

Cane and Helton Branches	Cane Branch	Helton Branch
Candida parapsilosis	Candida solani	Candida rugosa
Candida krusei	Candida humicola	Torulopsis stellata var cambresieri
Torulopsis glabrata	Candida catenulata	Cryptococcus luteolus
	Candida guilliermondii	Trichosporon cutaneum var multisporum
	Candida melibiosi	Trichosporon pullulans
	Torulopsis candida	
	Torulopsis versatilis	
	Cryptococcus laurentii	
	Rhodotorula glutinis	
	Rhodotorula mucilaginosa	
	Rhodotorula minuta	

species and the number of species in the genera differed. In addition to Candida parapsilosis, six more species of Candida and Rhodotorula minuta, but not Rhodotorula texensis, were found in Cane Branch. Three species of Torulopsis and one species of Cryptococcus were found in Cane Branch whereas representatives of these genera were not reported by other investigators. Although Trichosporon was not found in Cane Branch, two species of this genus were isolated from Helton Branch. However, the species of Trichosporon isolated from acid streams by other investigators was reported as Trichosporon cutaneum. The species isolated from Helton Branch were Trichosporon cutaneum var multisporum and Trichosporum pullulans.

Rhodotorula glutinis has been reported to be associated with accelerated acid formation by Thiobacillus ferrooxidans and T. thiooxidans. Ferrobacillus ferrooxidans was isolated consistently from Cane Branch but only once from Helton Branch. Although Helton Branch does not receive drainage from a strip-mined area, there is evidence of seepage from naturally-occurring coal seams. Therefore, the finding of F. ferrooxidans is not surprising. It appears that the occasional presence of F. ferrooxidans and seepage from coal seams do not influence the pH value of Helton Branch. The occurrence of Rhodotorula only in Cane Branch may be explained by the pH values of the two streams, or the continued association of Rhodotorula and F. ferrooxidans, or both.

Algae

Algae samples were collected from Cane Branch beginning at the

source of the strip-mine drainage and extending downstream. Samples were also collected from Helton Branch. The algae identified during the four seasons in Cane and Helton Branches are shown in Table 18. The algae which were identified during the winter were found only during the winter of 1968. No algae were found in either branch during the winters of 1966-67. The sampling schedule may have been responsible for the failure of observing any algae during these winters. Representatives of 23 genera, Table 19, were found. Of these, four were found in both areas, eleven only in Cane Branch and eight only in Helton Branch. Representatives of the Cyanophyta were found only in Helton Branch. In Cane Branch the amount of algal growth and diversity of types increased from close to the strip-mined area where algal growth was essentially confined to Euglena in pools with direct sunlight, to station 6 where extensive algal growth occurred. Bumilleria sicula was found only in Cane Branch and only at some distance from the strip-mine drainage area. Bumilleria was the predominate alga in Cane Branch proper during the winter of 1968. During this particular season, it was found in Cane Branch at station 6 and upstream too, but not above station 4. Bumilleria has been observed in all seasons except autumn, being the dominate form near station 6 during these seasons. Tribonema, an alga belonging to the same order, Heterothrichales, and family, Tribonemataceae, as Bumilleria was also found at the same locations as Bumilleria during the 1967 summer. The morphology of Bumilleria suggested a close relationship with acid-mine drainage streams. The brown color of the "H-piece" located along the filament, which has been identified as due to iron, suggests that Bumilleria may be utilizing ferrous compounds or that ferric compounds

TABLE 18

Algae Identified from Cane and Helton Branches

Season	Cane Branch	Helton Branch
Spring	Rhizoclonium hieroglyphicum Bumilleria sicula Monocila viridis Euglena polymorpha Stauroneis anceps	Mougeotia parvula Gyrosigma spencerii Fragilaria sp. Microthamnion strictissimum Lyngbya diguetii
	Microthamnion strictissimum Cladophora crispata Cladophora glomerata Euglena sp. Hormidium Klebsii	Micrasterias sp. Meridion circulare Oscillatoria formosa Ulothrix sp.
	Mougeotia parvula Ulothrix aequalis Zygnemopsis decussata	
Summer	Bumilleria sicula Tribonema bombycinum Zygogonium ericetorum Zygnema insigne Euglena sp.	Oedogonium sp. Oscillatoria sp. Lyngbya sp. Stauroneis anceps
	Hormidium subtile Microthamnion strictissimum Stauroneis anceps	
Autumn	Mougeotis sp. Euglena sp. Ulothrix sp. Microthamnion strictissimum Stauroneis sp.	Oedogonium sp. Stauroneis sp.
Winter	Bumilleria sicula Euglena sp. Zygogonium sp. Eunotia sp. Cladophora sp.	Oedogonium sp. Bulbochaete sp.

TABLE 19

Sources from which Algae were Identified

Cane and Helton Branches	Cane Branch	Helton Branch
Mougeotia	Rhizoclonium	Gyrosigma
Microthamnion	Bumilleria	Fragilaria
Ulothrix	Monocila	Lyngbya
Stauroneis	Cladophora	Micrasterias
	Euglena	Oscillatoria
	Hormidium	Oedogonium
	Zygnemopsis	Meridion
	Tribonema	Bulbochaete
	Zygogonium	
	Zygnema	
	Eunotia	

are being precipitated by it in some manner. Therefore similar acid-mine drainage streams other than Cane Branch were investigated to determine if this genus was present. Bumilleria was found in one other stream, pH 2.7, which drains an active strip-mine area. However, it was only observed one time during the summer of 1967. Even though Bumilleria appeared to establish itself as the dominate alga in Cane Branch, at station 6, during the winter of 1968, it was not observed in any other acid-mine drainage stream in the area. However, it has never been found in any stream examined that does not contain acid mine wastes.

The establishment of Bumilleria in Cane Branch some distance from the main drainage area, suggests that this may represent a definite stage in the natural recovery of the stream. If Bumilleria is definitely associated with acid water, the one time observance of this alga in another stream carrying acid mine waste is not surprising. Since the stream drains an area in which there is active mining, the stream is subject to drastic and sporadic physical environmental changes. If these changes predominate for the required length of time, Bumilleria could bloom but not establish itself as a dominate member of the microflora. Unfortunately the literature does not contain information necessary to determine the natural environmental conditions which permit Bumilleria to establish itself as a normal constituent of a microflora.

Discussion:

From the data obtained in this study, it appears that acid-mine drainage waste has altered the microflora of the receiving stream. Specific microorganisms seem to dominate depending upon the degree of acid conditions

existing in the stream over a period of time. Although strip-mining was discontinued on a large scale in the Cane Branch area in August, 1959, the pH of Cane Branch has remained within a range of 3.0 to 4.1 during the two years of this study. This can be attributed to both chemical and biological reactions. Initially, acid was produced by a chemical reaction of the pyritic material exposed as a result of the strip mining. This produced an acid environment which enabled F. ferrooxidans to establish itself as a dominate organism of the soil microflora. As a result of normal rainfall and leaching of the soil, this organism was introduced into Cane Branch and apparently has become a part of the microflora of the stream. This appears to be the case since the organism can be isolated directly from stream water and bottom samples on molecular filters. F. ferrooxidans, having established itself as part of the microflora, continues to contribute to the acidity of the stream producing H_2SO_4 as one of its metabolic end products. The necessity of acid conditions being present in the stream appears to be verified by the fact that F. ferrooxidans has not been isolated repeatedly from Helton Branch. The few times that this organism has been found in Helton Branch there has also been evidence of natural seepage from coal seams. Therefore the continuous isolation of F. ferrooxidans in conjunction with a low pH value, 3.0 to 3.5, of the water appears to indicate well established acid conditions of a stream which results in the alteration of the microflora. Further alteration of the microflora due to low pH is shown by the lower counts of saprophytic bacteria in Cane Branch. Since many protozoa feed on bacteria this affects the population of protozoa present. The alteration of the microflora is further shown by the more numerous and different fungi, filamentous

and unicellular, found in Cane Branch. This is not unexpected since fungi on the ecosystem can not be determined because of insufficient information concerning the physiology and biochemistry of fungi. The isolation of R. glutinis along with F. ferrooxidans may well indicate the continuing influence of F. ferrooxidans on the acidity of Cane Branch. The occurrence of both appears to be correlated with an acid stream and the relationship probably occurs after F. ferrooxidans has become established in the stream. This appears true because F. ferrooxidans has been isolated sporadically from Helton Branch but R. glutinis has never been found.

Acid conditions have had an effect on the algal flora in Cane Branch. Previous investigators reported that representatives of the Cyanophyta have not been observed in acid streams. This has been confirmed by their presence in Helton Branch but not in Cane Branch. The establishment of certain algae in Cane Branch probably indicates natural recovery of the stream. Bumilleria appears to be associated with acid mine drainage streams. When the project began in 1965 this alga was not observed in Cane Branch. During 1966 and 1967 it was observed occasionally and in 1968 it had established itself as the dominate algal form in Cane Branch at some distance, 4000 feet, from the main drainage area. Acid streams in the area of Cane Branch and in Eastern Kentucky were investigated in order to determine some relationship between Bumilleria and acid mine streams. However, Bumilleria was not found in any stream in Eastern Kentucky and only once in an acid stream near the Cane Branch area. There are two reasons why this may have occurred. (1) Strip mining has been discontinued in the Cane Branch area since August 1959. All other streams investigated are located in areas where active strip mining is in progress

or has been discontinued for approximately five years. Therefore it is possible that the establishment of Bumilleria as a dominate form indicates some degree of natural recovery and the streams not yet supporting its growth have not recovered sufficiently. (2) The Cane Branch area is located in a limestone region; the other streams are in a sandstone region. The leaching of water through the different type of rock strata, due to normal rainfall, may contribute either different nutrients or affect the concentrations of the same nutrients.

There appears to be a microflora of acid mine drainage streams which undergoes a gradual change associated with the natural recovery of the stream. As the stream improves the microflora changes and natural recovery is indicated by the dominance of specific organisms or groups of organisms.

Conclusions:

Acid stream conditions began in Cane Branch during 1956 as a result of coal being strip-mined during May 1955 to April 1956. These conditions prevailed through 1958 and were intensified as a result of a second period of strip-mining from August 1958 to August 1959. Concentrations of dissolved solids, sulfate, acidity and the elimination of bicarbonate alkalinity in the water of Cane Branch increased significantly after the mining. The microflora of Cane Branch was altered due to the acid stream conditions as compared to Helton Branch which remained free from acid mine drainage wastes.

Drainage from strip-mined areas appears to have affected the microflora of Cane Branch. Chemical oxidation of pyritic compounds found extensively in the spoil banks has resulted in the formation of ferrous sulfate and sulfuric acid. This appears to have led to the establishment of Ferrobacillus ferrooxidans which contributes to the acid draining into Cane Branch. As a result the pH of the stream has been lowered allowing this organism to be found throughout the stream from the point of initial drainage. This, in turn, causes the filamentous fungi to be more numerous and diversified and the number of saprophytic bacteria to be lower. In agreement with the work of previous investigators, Rhodotorula has been associated with accelerated acid production by T. ferrooxidans. The fact that Bumilleria was found only in Cane Branch and some distance from the strip-mine drainage area may indicate that it is associated in some manner with the natural recovery of the stream.