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Microbial Dissimilatory Sulfur Cycle in Acid Mine Water

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Ferric, sulfate, and hydrogen ions are produced from pyritic minerals associated with coal as a result of autotrophic bacterial metabolism. Water carrying these ions accumulated behind a porous dam composed of wood dust originating at a log-cutting mill. As water seeped through the porous dam, it was enriched in organic nutrients which then supported growth and metabolism of heterotrophic bacteria in the water downstream from the dam. The heterotrophic microflora within and below the sawdust dam included dissimilatory sulfate-reducing anaerobic bacteria which reduce sulfate to sulfide. The sulfide produced caused the chemical reduction of ferric to ferrous ion, and black FeS precipitate was deposited on the pond bottom. A net increase in the *p*H of the lower pond water was observed when compared to the upper pond water. Microbial activity in the wood dust was demonstrated, and a sequence of cellulose degradation processes was inferred on the basis of sugar accumulation in mixed cultures in the laboratory, ultimately yielding fermentation products which serve as nutrients for sulfate-reducing bacteria. Some of the microorganisms were isolated and characterized. The biochemical and growth characteristics of pure culture isolates were generally consistent with observed reactions in the acidic environment, with the exception of sulfate-reducing bacteria. Mixed cultures which contained sulfate-reducing bacteria reduced sulfate at *p*H 3.0 in the laboratory with sawdust as the only nutrient. Pure cultures of sulfate-reducing bacteria isolated from the mixed cultures did not reduce sulfate below *p*H 5.5.

Autotrophic bacteria in the *Thiobacillus-Ferrobacillus* group are responsible for the enzymatic oxidation of ferrous sulfide minerals (e.g., pyrite and marcasite) which are often found associated with coal in nature (4). The net result of microbial oxidation of pyritic minerals is an accumulation of ferric, sulfate, and hydrogen ions. Drainage water in regions where such minerals are mined become highly acidic and contain relatively high concentrations of iron, sulfate, and other ions. A marked alteration in the microbial ecology can be observed under these conditions, and certain aspects of this ecosystem have been described (20). The inhibitory effects of acid mine water on heterotrophic bacteria of neutral streams were reported to result from hydrogen ions and, to a lesser extent, sulfate ions, but not from iron ions (10).

In this report, we consider the metabolic activities of microorganisms involved in the reduction of iron and sulfate ions and in the neutralization of acid in naturally occurring acidic mine water. To our knowledge, the only report of sulfate-reducing bacteria in acidic mine drainage is that of Leathan (8), and this report attributes no significance to the presence of sulfate reducers in acid mine water.

Figure 1 is a schematic representation of the acidic stream studied. The stream flow was impeded by a dam composed primarily of wood dust, which was a waste product from a small log-cutting mill in the hills of southeastern Ohio. The retarded flow of water resulted in a pond behind the dam which is hereafter referred to as the upper pond. Uneven terrain downstream from the dam resulted in the formation of the lower pond. The porous quality of wood dust allowed the acidic water to permeate through the wood at a low rate and to enter the lower pond. The flow of water from the system at location 7 was slightly less than the flow entering the upper pond. Presumably, some water was lost via leaching and evaporation. Wood dust was sampled at location 3, whereas water was examined at the other six locations. Although samples from all locations

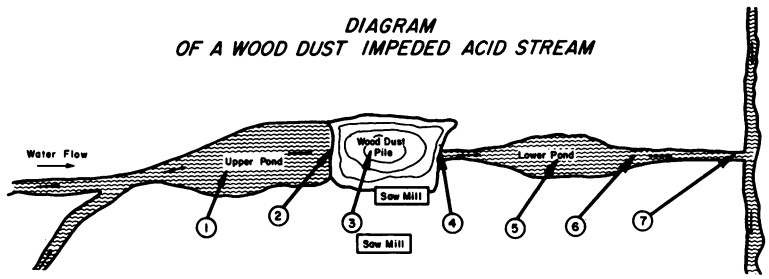

FIG. 1. *Schematic outline of an acid stream which is impeded by a dam composed of wood dust, showing the upper and lower ponds and sample locations.*

were required for the evaluation, the greatest emphasis was given to samples from the upper and lower ponds.

MATERIALS AND METHODS

Samples. Water samples taken in the field were placed in sterile 8-oz (0.236 liter) bottles, held on ice in a Styrofoam cabinet until reaching the laboratory, and then refrigerated at 8 C. All water samples were plated on bacteriological culture media within 24 hr after they were taken from the stream.

Chemical determinations. Total dissolved iron was measured colorimetrically by the phenanthroline method, according to the procedure described for a Hach Field Kit (Hach Chemical Co., Des Moines, Iowa).

Sulfate was determined turbidimetrically as $BaSO_4$ precipitate, as described by the Hach procedure, and the *p*H was determined with a Beckman *p*H meter.

H_2S was semiquantitatively determined by the Hach procedure, which involved comparison of the amount of black PbS precipitate formed on a lead acetate-impregnated filter paper with papers blackened by a known concentration of H_2S. This procedure was sensitive to 0.1 μg of H_2S per ml of water.

Pond water was analyzed directly for aldehydes by the Tollens method (15) and after 20-fold and 40-fold concentration by evaporation at 100 C.

Chromatography of soluble sugars in wood dust extracts. Extracts were prepared by adding 200 ml of distilled water to 100 g of wood dust which had been obtained from either the top 6 inches (15.24 cm; fresh wood dust) or from a depth of 3 ft (91.44 cm) into the pile (partially decomposed wood dust). The mixtures were allowed to stand at 25 ± 2 C for 2 hr with frequent shaking. Wood dust particulates were then removed by filtration through Whatman no. 1 paper. The resulting filtrates were filtered through sterile membranes (0.45 μm pore size; Millipore Corp., Bedford, Mass.) to remove small suspended particles and to sterilize the solution.

A 100-ml amount of each extract was evaporated under vacuum to dryness over KOH pellets at 22 ± 2 C. The dried residues were prepared for chromatography by resuspending in 1.0 ml of distilled water. Each solution (0.01 ml) was applied separately to Whatman no. 1 paper which had been previously spotted with glucose, xylose, cellobiose, and galactose marker solutions. Descending chromatograms were developed in isopropanol-water (1:4) at 25 ± 2 C. After development for 24 to 27 hr, sugars were detected by the Trevelyan silver nitrate method (21). A 40-fold concentration of lower pond water was also chromatographed in *n*-butyl alcohol-acetic acid-water (40:10:22). The concentrated sample was dialyzed against distilled water (8 C) for 12 hr in an attempt to remove inorganic ions which interfere with chromatographic separation and then were chromatographed in the same solvent system.

Mixed culture flask systems. Wood dust (400 g) and water (1 liter) were added to 2-liter Erlenmeyer flasks in the following combinations of wood dust and water: acid mine water plus partially decomposed wood dust, acid mine water plus fresh wood dust, distilled water plus partially decomposed wood dust, distilled water plus fresh wood dust. Separate flasks containing each of the four combinations were incubated at 22 ± 2 C, 37 C, and 50 C, respectively. Portions of liquid from flasks containing acid mine water were routinely assayed for iron, sulfate, and *p*H as described above. All flasks were assayed for carbohydrates by the anthrone method of Steinecher and Rheins (18) and by the Nelson reducing sugar test (3).

Media and growth conditions. Portions (1 ml or appropriate dilutions) of each water sample were plated on Tryptone Glucose Extract (TGE) Agar (Difco), which was supplemented with 0.5 g of yeast extract per liter (TGYE), and on Sabouraud's Dextrose (SD) Agar (Difco). The cultures were incubated at 25 ± 2 C in the air for 3 days.

Anaerobic microorganisms, other than *Desulfovibrio*, were determined by adding 1.0-ml portions of a single tube dilution series to 9.0 ml of Thioglycollate Medium (Difco). The reciprocal of the highest dilution that showed growth after 7 days at 25 ± 2 C, as determined by the appearance of turbidity in the anaerobic zone of the tubes, was taken as the number of organisms.

Sulfate-reducing bacteria were enumerated with a standard three-tube most probable number (MPN) method (1), according to the tube culture technique described by Postgate (13) with *Desulfovibrio desul-*

furicans medium no. 3. Positive tubes were black after incubation at 25 ± 2 C for 6 to 21 days.

Cellulose-digesting microorganisms were isolated and cultured in the cellulose enrichment medium of McBee (9). Cellulose was obtained from rope cotton which was hydrolyzed in 11.8 N HCl for 48 hr at 25 ± 2 C, washed seven times with distilled water, and ground to a paste with a mortar and pestle. Plates were incubated at 25 ± 2 C for 14 days. Cellulose digesters were identified by the appearance of a clear zone around the colony.

Iron-oxidizing chemoautotrophic bacteria were counted by a five-tube MPN technique (1) in the salts medium of Silverman and Lundgren (16). Positive tubes were determined after 15 days of incubation at 25 ± 2 C by the presence of a dark red-brown precipitate. Uninoculated control tubes and those in which growth did not occur contained a light yellow-brown precipitate at the end of the incubation period due to autooxidation of ferrous iron.

Sulfur-oxidizing chemoautotrophic bacteria were enumerated in the same manner as were the iron oxidizers. Elemental sulfur (0.1 g per 10 ml) replaced the $FeSO_4 \cdot 7H_2O$ in the culture medium, and positive tubes were taken as those in which sufficient acid was produced to cause five drops of a 1% thymol blue solution to turn red (red, *p*H 1.2, to yellow, *p*H 2.8). Control tubes and negative growth tubes gave a yellow indicator reaction.

Aerobic heterotrophic and facultatively aerobic heterotrophic isolates other than *Streptomyces* were examined for the ability to ferment or assimilate glucose, lactose, adonitol, arabinose, dulcitol, galactose, inositol, inulin, levulose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, trehalose, and xylose. Glucose and lactose utilization was determined in tubes of Purple Broth Base (PBB; Difco) which contained 1.0% (w/v) sugar. All other carbohydrates were added as sterile differential discs (Difco) to plates of PBB containing 1.5% agar, which had been spread with 0.1 ml of a 24-hr Nutrient Broth culture of the isolate. All cultures were incubated at 25 ± 2 C for 48 hr. A color change of purple to yellow (bromocresol purple, yellow, *p*H 5.2, to purple, *p*H 6.8) was taken as an indication of fermentation. Enhancement of growth around the disc was considered as an indication of oxidative utilization. All other sugar fermentation determinations were conducted with standard microbiological methods (18).

Oxidase production was determined by the method of Kovacs (7).

Nitrate reduction was determined according to the method described in the *Difco Manual* (9th ed., Difco Labs., Inc., 1953). Cultures were incubated at 25 ± 2 C for 18 hr. An 0.1-g amount of zinc dust was added to each negative tube, and the presence of a pink color assured that the nitrate had been reduced only to nitrite and not to N_2 gas, which may have resulted in a false negative observation.

Gelatin liquefaction was determined by the method described in the *Difco Manual* after incubation at 25 ± 2 C for 5 days.

Streptomyces species isolated from wood dust were tested for their ability to ferment cellulose, glucose, and xylose at 22 ± 2 C, 37 C, and 50 C. Cells were inoculated into PBB containing 1% (w/v) glucose, xylose, or cellulose (Whatman no. 1 paper powder) and were incubated for 48 hr at the respective temperatures.

RESULTS

Table 1 is a compilation of the average chemical and biological conditions (data) obtained from samples taken from the upper and lower ponds over a 1-year period. Each average represents a minimum of four determinations. The sulfate-reducing bacteria from the lower pond represent two different types. These have been tentatively identified as a *Desulfovibrio* and a *Desulfotomaculum* species. The *Desulfotomaculum* isolate was a peritrichously flagellated sporeforming anaerobic rod when observed under the electron microscope. It resisted heat (80 C for 15 min). The *Desulfovibrio* isolate was a polarly flagellated, slightly curved or straight anaerobic rod and produced considerably more sulfide than the sporeforming isolate.

Data obtained from samples taken on the same day are summarized in Table 2. The heterotrophic aerobes listed in Table 1 consist of 10 different yeasts and 12 different bacteria.

Ten different yeasts were isolated from the upper and lower ponds, and were examined for sugar-fermenting capacity because of their potential production of alcohols and organic acids which could serve as nutrients for the dissimilatory sulfate-reducing bacteria found in both the wood dust pile and the lower pond. Three of the isolates have tentatively been characterized as strains of *Rhodotorula glutinis*. None of the isolates fermented or utilized the following compounds: dulcitol, inositol, inulin, melibiose, rhamnose, salicin, sorbitol, and trehalose.

Four gram-positive isolates, tentatively classified as *Bacillus* species on the basis of aerobic spore formation, were obtained from the upper and lower ponds.

Seven physiological types or groups of gram-negative rod-shaped aerobic bacteria were isolated from the stream system. The bacteria were partially characterized with standard biochemical reactions (17). One type was isolated from both upper and lower ponds. This organism was tentatively identified as an *Aerobacter* or closely related genus on the basis of glucose and lactose fermentation, facultative aerobic growth on Thioglycollate Medium (Difco), and colony characteristics in Eosin Methylene Blue Agar (EMB; Difco).

The remaining gram-negative rods were considered to be pseudomonads on the basis of lack of fermentation of adonitol, arabinose, dulcitol, fructose, galactose, glucose, inositol, inulin, lac-

TABLE 1. *Chemical and biological changes which occurred in acid mine water upon passage through wood dust*[a]

Determination	Upper pond (1)[b]	Lower pond (5)
pH	2.84 (3.90–2.40)	3.38 (4.85–2.70)
SO_4 concn (μmoles/ml)	8.765 (5.205–12.492)	6.100 (3.277–10.306)
Total Fe concn (μmoles/ml)	1.067 (0.788–1.325)	0.313 (0.064–0.681)
Sulfur-oxidizing bacteria (MPN/100 ml)	9,580 (130–33,000)	1,820,000 (23,000–7,000,000)
Fe-oxidizing bacteria (MPN/100 ml)	9,520 (490–33,000)	426,000 (33,000–1,400,000)
Anaerobes/ml on Thioglycollate Medium	2.5 (0–10)	528 (10–1,000)
So_4^{2-} reducers (MPN/100 ml)	0	876 (0–2,400)
Heterotrophic aerobes/ml on		
SD Agar	15.4 (2–44)	82,1000 (49–290,000)
TGYE Agar	47.4 (2.5–110)	350,000 (470–1,700,000)

[a] Values are averages calculated from a minimum of four readings. Ranges of values are given in parentheses beneath the average values.

[b] The numbers in parentheses after upper pond and lower pond refer to the schematic outline shown in Fig. 1.

TABLE 2. *Chemical, biological, and temperature differences in samples collected on the same date*

Sample location[a]	Air temp (C)	Sample temp (C)	pH	SO_4^{2-} conc (μmoles/ml)	Total Fe concn (μmoles/ml)	Sulfur oxidizers (MPN/100 ml)	Fe oxidizers (MPN/100 ml)	SO_4^{2-} reducers (MPN/100 ml)
1	−6	6	3.1	10.098	1.325	1.3×10^2	4.9×10^2	0
2	−6	6	2.8	9.890	1.468	1.1×10^2	8.0×10^1	0
3	−6	33						
4	−6	13	4.3	3.825	0.681	10^7	4.9×10^5	7.0×10^2
5	−6	6	3.4	3.825	0.663	7.0×10^6	1.4×10^6	1.4×10^3
			4.0[b]	4.268[b]	0.734[b]	7.9×10^{6b}	1.0×10^{7b}	1.9×10^{3b}
6	−6	6	3.35	4.268	0.573	2.2×10^4	2.1×10^5	2.0×10^2
7	−6	6	3.2	5.309	0.645	4.9×10^4	4.6×10^5	0

[a] As shown in Fig. 1.

[b] Samples taken at bottom-water interface.

tose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Six different pseudomonad types were differentiated on the basis of the presence or absence of motility, ability to reduce nitrate to nitrite, gelatin hydrolysis, presence or absence of oxidase (7), and pigmentation. No attempt was made to study flagellation or to classify these organisms as to genus. The organisms were presumed to be *Pseudomonas*, *Xanthomonas*, *Acetomonas*, or closely related bacteria.

Microbiological examination of the wood dust yielded seven different isolates which were tentatively identified as *Streptomyces* species on the basis of filamentous growth habit and colony characteristics. A summary of cellulose, glucose, and xylose utilization by these isolates after 48 hr of incubation is shown in Table 3. In general, the isolates grew much better at 37 C than at either 50 or 22 C. Isolates 6 and 7 had little activity on the substrates tested at any of the incubation temperatures employed.

One white-rot type of *Basidiomycete* was observed to have extensive mycelial growth in the top several inches of the wood dust pile. In addition, one *Clostridium* strain was isolated. Gram-negative rod-shaped bacteria were also present in the wood dust. No attempt was made to isolate

and identify these species, except one, which was similar to the *Aerobacter* species isolated from the upper pond.

Aldehydes were present in both upper and lower ponds (Tollens method; 15), but the intensity of the reaction indicated semiquantitatively that a greater concentration of aldehydes was present in the lower pond.

Chromatographic examination of concentrated lower pond water indicated the presence of sugars. The components could not be resolved by the techniques employed, and much tailing occurred because of the high salt content of the water. Dialyzed concentrates did not contain sugars, and the procedure could not be used to remove the tailing due to salts. Fresh wood dust extracts also contained sugars. Two components co-chromatographed with glucose and xylose markers. Partially degraded wood dust extracts did not contain these components. Both extracts contained inseparable components which did not migrate as far as the glucose, xylose, cellobiose, and galactose markers.

Figures 2 and 3 show the effect of temperature and wood dust condition on sulfate reduction. Maximal sulfate reduction occurred in flasks containing partially degraded wood dust.

Figures 4 and 5 show the changes in *p*H in the same flask cultures. Note that the rise in *p*H correlates with the removal of sulfate.

Table 4 is a compilation of the anthrone and reducing sugar concentrations in culture supernatant fluids which contained a mixed microflora taken from wood dust. Data from flask cultures in which distilled water was substituted for acid mine water are also included. The anthrone procedure measures total soluble sugars, providing that the monomeric units are able to form furfurals in the presence of concentrated sulfuric acid. In contrast to the anthrone procedure, Nelson's test does not hydrolyze glucosidic linkages. Therefore, an increase in reducing power over the amount of anthrone measurable sugar is interpreted as a decrease in the average chain length of soluble cellulose polymers.

TABLE 3. *Utilization of glucose, cellulose, and xylose by Streptomyces isolates at three different temperatures*[a]

Isolate no.	Glucose			Cellulose			Xylose		
	22 C	37 C	50 C	22 C	37 C	50 C	22 C	37 C	50 C
1	−	+	−	±	+	−	±	±	−
2	+	++	−	+	++	−	+	++	−
3	A	A	−	++	++	−	++	++	−
4	+	+	−	+	+	−	++	+	−
5	−	+	−	−	+	−	−	++	−
6	−	−	−	−	−	−	±	−	−
7	−	−	−	−	−	−	−	−	−

[a] Reactions are indicated as follows: −, negative response; ±, slight utilization; +, utilization; ++, greater utilization than +; A, acid produced.

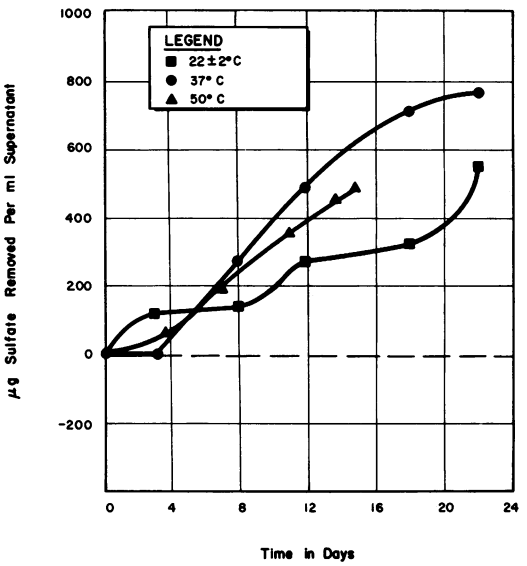

FIG. 2. *Influence of temperature on sulfate reduction in flasks containing a mixed microflora and partially decomposed wood dust.*

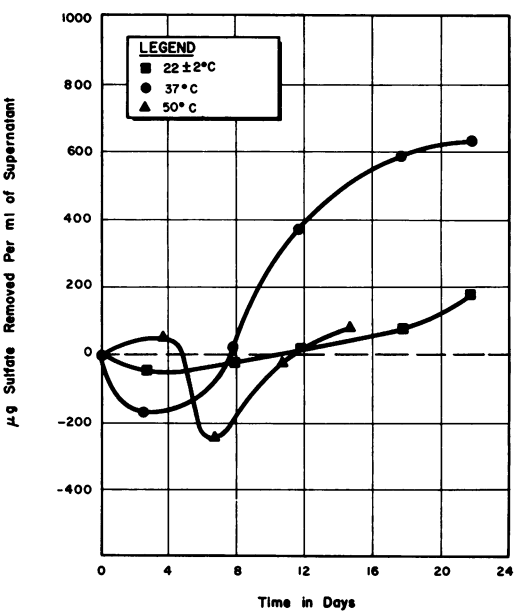

FIG. 3. *Influence of temperature on sulfate reduction in flasks containing a mixed microflora and fresh wood dust.*

TABLE 4. *Values showing change (μg/ml) in concentration of total and reducing sugars as result of mixed fermentation of wood dust at three different temperatures*

Determination	Sugar	22 C			37 C			50 C		
		0 Time	14 Days	Net gain	0 Time	14 Days	Net gain	0 Time	14 Days	Net gain
Distilled water + fresh wood dust	A[a]	275	360	+85	275	215	−60	185	440	+225
	R[b]	70	250	+180	60	200	+140	175	350	+175
Acid mine water + fresh wood dust	A	190	240	+50	170	235	+65	130	460	+330
	R	45	130	+85	15	350	+335	95	275	+180
Distilled water + partially degraded wood dust	A	65	55	−10	65	55	−10	30	85	+55
	R	50	45	−5	40	50	+10	40	245	+205
Acid mine water + partially degraded wood dust	A	45	50	+5	40	45	+5	5	95	+90
	R	0	5	+5	20	40	+20	10	235	+225

[a] Anthrone total soluble sugars.
[b] Nelson's reducing sugars.

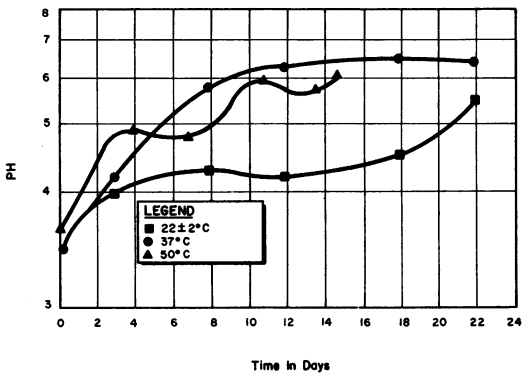

FIG. 4. *Influence of temperature on pH change in flasks containing a mixed microflora and partially decomposed wood dust.*

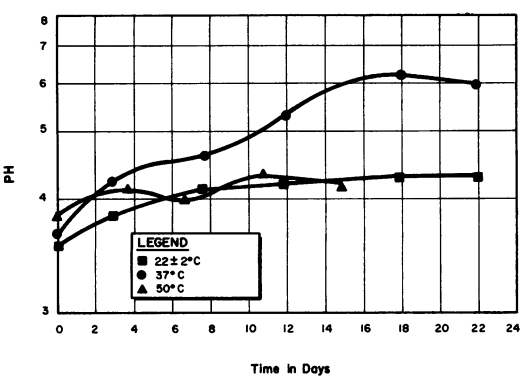

FIG. 5. *Influence of temperature on pH change in flasks containing a mixed microflora and fresh wood dust.*

DISCUSSION

Water entering the upper pond was typical of acidic mine drainage as regards low *p*H, high iron and sulfate ion concentrations, numbers of iron- and sulfur-oxidizing autotrophic bacteria, and low numbers of heterotrophic bacteria, particularly anaerobes. No sulfate-reducing bacteria could be found in the upper pond. Four gram-positive species were isolated from the upper pond and were tentatively identified as *Bacillus* species. This is of interest because we had previously reported that gram-positive bacteria of neutral streams were extremely susceptible to acid mine water (20).

In contrast, the lower pond contained a vastly different microflora. Aerobic heterotrophic microorganisms were present in markedly higher numbers. The predominant aerobes or facultative aerobes were yeasts and higher fungi and 12 different physiological types of bacteria. Most were tentatively classified as pseudomonads. Higher plate counts on TGYE agar than on the more acid SD agar indicate that, although many of the organisms in the lower pond were acid-tolerant and viable, they grew more favorably on the neutral TGYE growth medium. This suggests that they were entering the lower pond from the wood dust pile and were probably not growing in the lower pond.

A 200-fold increase in numbers of anaerobic bacteria was observed in the lower pond as compared to the upper pond. This is undoubtedly due to organic materials from the wood dust which serve as nutrient sources, and the effect of these nutrients on lowering the oxidation-reduction (O/R) potential.

The increase in *p*H and the decrease in iron and sulfate concentrations (Table 1) in the lower pond, in comparison to the upper pond, can be attributed to the influence of the wood dust dam. This can be seen by comparing the chemical parameters at station 4, where the water leaves the wood dust, with the same parameters at station 5 (Table 2). Although some activity with respect to sulfate reduction occurs in the lower pond, most of the sulfate reduction process undoubtedly occurs in the wood dust. The discrepancy between chemical parameters at the surface and at the bottom of the lower pond probably results from the ability of the chemical assays used to detect ions present in insoluble form as precipitates in the pond bottom samples [e.g., $Fe_2(SO_4)_3$].

Stream flow rates which are influenced by environmental conditions caused fluctuations in measured parameters in the field. The parameters appeared to coincide with observed water conditions. For example, when the lower pond water had a reddish color, the sulfate concentration and *p*H were nearly equal to the upper pond water. Under these conditions, low numbers of sulfate reducers were recovered. When the lower pond was greenish-black, the acidity and sulfate concentration were lower than in the upper pond and sulfate-reducers were always recovered in higher numbers. Free H_2S was detected in the water only when the color was greenish-black. The color was undoubtedly due to precipitation of FeS, which is in agreement with the lower iron concentration found in the lower pond.

It is interesting to note that dissimilatory sulfate reducers are active in a highly acidic environment. The two cultures which were isolated did not reduce sulfate in artificial media in the laboratory when the *p*H was below 5.5. However, sulfate was reduced in the laboratory when sawdust was used as the substrate in a mixed culture system at a *p*H of 2.8. It is possible that a microenvironment of higher *p*H is set up around wood or other suspended particulates in a system having a low *p*H.

It must be emphasized that sulfate reduction cannot occur in the absence of organic materials, since sulfate-reducing bacteria are generally regarded as heterotrophic (11, 12, 14). The substrate range for these bacteria consists chiefly of fermentation products and not sugars or polysaccharides. Recalling that the lower pond contains soluble sugar and that sugars believed to be glucose and xylose disappeared in the depths of the sawdust, it seems clear that a cellulose degradation process involving several physiological types of microorganisms is required to provide substrate for the sulfate-reducing bacteria. The data in Table 4 give some insight into this process.

In the 22 or 37 C cultures containing fresh wood dust and either distilled water or acid mine water, reducing power (Nelson's method) increased more than anthrone measurable sugar. This suggests that sugars in relatively long chain lengths are degraded to smaller fragments more rapidly than insoluble wood dust is converted to sugars, otherwise one would expect a greater increase in nonreducing as compared to reducing sugar. This is especially true of the 37 C culture which contained acid mine water. At 14 days, the ratio of reducing sugar to anthrone sugar is greater than 1. It also suggests activity of mesophilic fermenting microorganisms at 37 C in fresh wood dust. These organisms are probably anaerobic, since aerobic bacteria would be expected to utilize reducing sugars either fermentatively or oxidatively. It must be emphasized that acid had no effect upon cellulose degradation in this system, and that traces of H_2S present had no effect upon the determination of reducing sugars. Then, the difference in reducing power between the distilled water and acid mine water, fresh wood dust cultures can be attributed to a lowering of the O/R potential by H_2S and to the effect of the lowered O/R potential on the bacteria responsible for the fermentation process. The ratio of reducing sugars to anthrone sugars (greater than 1:1) indicates the presence of aliphatic aldehydes and ketones in units of four carbons or less as opposed to compounds of five carbons or more, which are required to react with anthrone reagent.

In contrast to fresh wood dust cultures at 37 C, the supernatant fluids of fresh wood dust cultures at 50 C show a greater buildup of anthrone sugars than of reducing sugars. This suggests the activity of thermophilic cellulose-splitting microorganisms upon the insoluble wood dust cellulose. The greater buildup of anthrone sugars in the acid mine water culture, in which sulfate reduction was occurring, also indicates that this may be an anaerobic process.

The lack of change in anthrone or reducing sugars in cultures at 22 or 37 C, which contained partially degraded wood dust and either distilled water or acid mine water, suggests a steady degradation sequence: insoluble wood dust → anthrone sugar → reducing sugar → fermentation products which were utilized by sulfate-reducing bacteria. Comparison to cultures in fresh wood dust suggests that substrates available to the microflora have been depleted in the degraded wood dust.

At 50 C, in both cultures which contained partially degraded wood dust, reducing sugars increased over anthrone sugars. These data suggest that the primary activity of thermophilic cellulose-splitting microbes in partially degraded wood dust was the cleavage of soluble degrada-

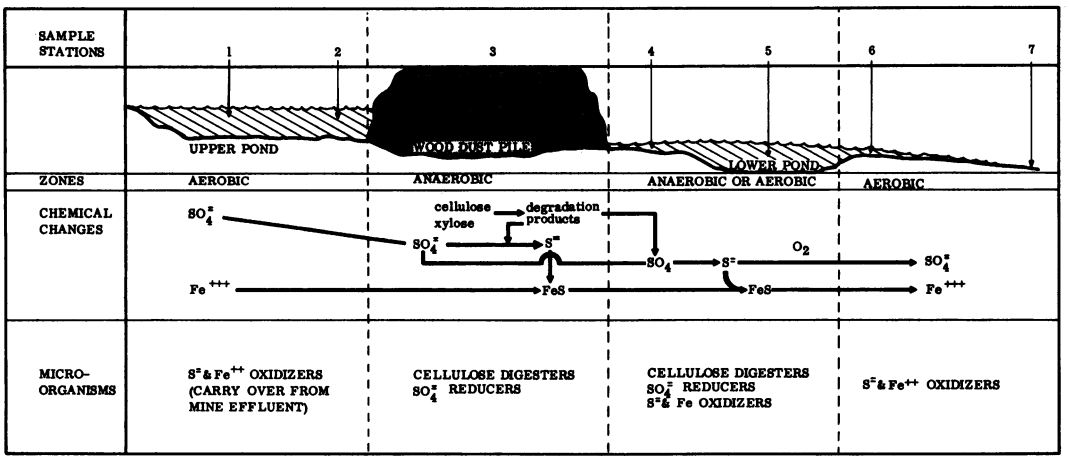

FIG. 6. *Schematic outline of the acid stream system depicting the distribution of chemical changes and microorganisms along the seven sample locations.*

tion products to smaller units. The buildup of reducing materials at 50 C but not at 37 or at 22 C suggests that mesophilic bacteria were responsible for their further degradation.

It is clear that the microorganisms isolated from the wood dust do not represent the overall activity. Any or all of these species, particularly the *Streptomyces* species which degraded cellulose, may be important to the sulfate reducers, not only as a source of nutrients but also as metabolic agents for lowering the O/R potential. This is also true of the heterotrophic lower pond isolates.

It is quite surprising to find higher numbers of aerobic iron- and sulfur-oxidizing bacteria present in the lower pond under generally anaerobic conditions when compared to the upper pond. We must assume they are increasing in numbers in the lower pond. This may reflect the availability of ferrous and sulfide ions as energy sources. Growth of these bacteria in the lower pond probably results in reacidification of the water. We have not examined the isolates for the possibility of facultative autotrophic growth on sulfur and organic compounds, although reports indicate (2, 5; C. Remsen and D. Lundgren, Bacteriol. Proc., p. 83, 1963) that heterotrophic growth of these organisms might be possible. Origin of the oxygen requirement for autotrophic iron and sulfide oxidation is a puzzling question in this environment. We do not understand how O_2 exchange at the air water interface can satisfy the O_2 demand of the organisms in the presence of detectable H_2S. One possibility is a cyclic presence and absence of O_2 which could be related to photosynthetic cycling. Numerous *Euglena* cells were observed microscopically in wet mounts prepared from the lower pond water. These organisms may liberate O_2 during daylight and the system would be anaerobic during dark periods, allowing sulfate reduction to preceed. Our data, however, do not suggest such fluctuations since H_2S is detected in the light. Another possibility is the simultaneous growth resulting from a symbiotic association of sulfate reducers, iron and sulfur oxidizers, and photosynthetic *Euglena*. Ehrlich reported on the balance of ecology with reference to carbon metabolism in an acidic environment (6) and discussed the role of algae, protozoa, yeasts, and chemoautotrophic bacteria.

Figure 6 is a schematic summary of the activities of microorganisms in the upper and lower pond system as influenced by metabolic activity in the wood dust pile. This represents a complete dissimilatory sulfur cycle as well as an $Fe^2 \rightarrow Fe^3 \rightarrow Fe^2$ cycle in a rather unusual ecosystem.

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