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Activity of Microorganisms in Acid Mine Water

I. Influence of Acid Water on Aerobic Heterotrophs of a Normal Stream

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Comparison of microbial content of acid-contaminated and nonacid-contaminated streams from the same geographical area indicated that nonacid streams contained relatively low numbers of acid-tolerant heterotrophic microorganisms. The acid-tolerant aerobes survived when acid entered the stream and actually increased in number to about 2×10^3 per ml until the *p*H approached 3.0. The organisms then represented the heterotrophic aerobic microflora of the streams comprised of a mixture of mine drainage and nonacid water. A stream which was entirely acid drainage did not have a similar microflora. Most gram-positive aerobic and anaerobic bacteria died out very rapidly in acidic water, and they comprised a very small percentage of the microbial population of the streams examined. Iron- and sulfur-oxidizing autotrophic bacteria were present wherever mine water entered a stream system. The sulfur-oxidizing bacteria predominated over iron oxidizers. Ecological data from the field were verified by laboratory experiments designed to simulate stream conditions.

High concentrations of iron, sulfate, and hydrogen ions in streams located in coal-mining regions are attributed to oxidation of pyritic minerals associated with coal. The oxidation of reduced iron and sulfur compounds, particularly pyrite and marcasite, was associated with metabolic activities of certain related chemosynthetic autotrophic bacteria (3).

The autotrophic bacteria in the *Thiobacillus-Ferrobacillus* group are responsible for enzymatic oxidation of ferrous ions and reduced sulfur compounds with concomitant production of ferric, sulfate, and hydrogen ions. Aspects of the energy metabolism of iron-oxidizing autotrophs have been reported by Dugan and Lundgren (4). The role of reduced sulfur as an energy source for autotropic bacteria has been reviewed by Peck (8). Silverman and Ehrlich have surveyed and reported microbial interactions with minerals (11).

Sulfuric acid and ferric ions thus produced have a deleterious influence on the heterotrophic biota of streams that receive the mine drainage. Ecological reports (2, 6, 7, 10) have indicated that H_2SO_4 could cause the killing of the normal microflora of affected waters and that aciduric species, notably fungi, appeared to thrive. Ehrlich (5) reported the isolation of large numbers of *Rhodotorula* from acidic copper mine effluents and described an ecological relationship between protozoa and *Thiobacillus* species which he cultured from the water.

This paper describes an investigation of the microbial ecology of acid mine water, in addition to a study of the influences of acid mine water on the microflora of a nonacid-polluted stream. During the investigation we have considered total numbers of organisms without species differentiation based on the thesis that it is total physiological activity which contributes to the succession of events that constitute an ecosystem and that microbial activity is a primary consideration in re-establishing the biota of a "healthy" stream.

We used two separate investigative approaches. The first approach was a field survey, which was conducted for the purpose of correlating bacterial types and numbers with chemical components in the stream. The second approach was to develop an artificial stream system in the laboratory. The laboratory system would approximate stream conditions and allow a measure of control over variables that cannot be controlled in the field. Variables such as temperature, stream flow, aeration, and organic content of water all influence the number of microorganisms per unit volume of water and must be controlled before observations in the natural habitat can be interpreted.

Figure 1 is a schematic diagram of the stream system that was studied in the field. This system was divided into five sampling stations. Sample site B was highly acid water resulting from

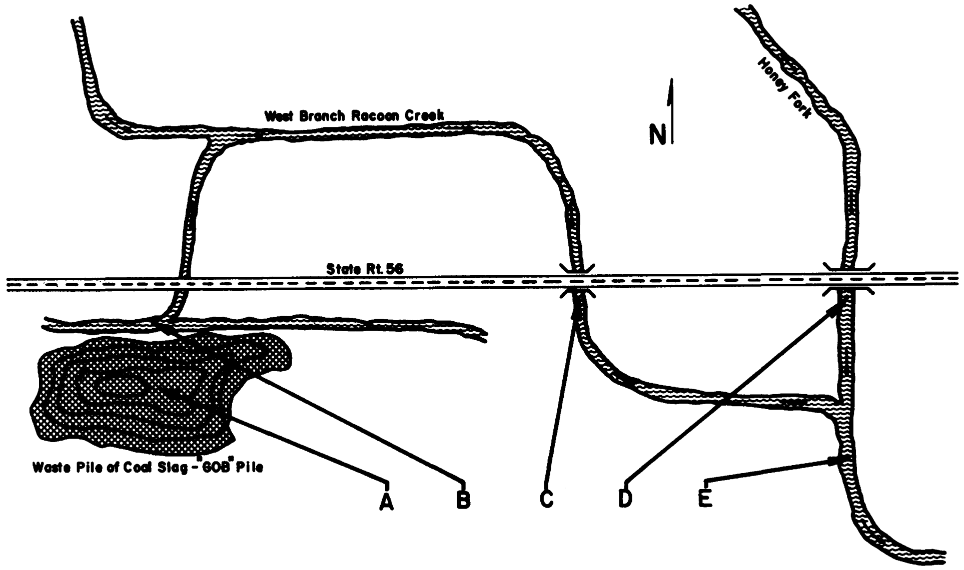

FIG. 1. *Schematic diagram of the stream system. The system is divided into five sampling stations. A represents a waste pyritic coal slag pile. B is a highly acid stream emanating by drainage from A. C is acid water which is diluted by water from other streams. D is a nonacid stream in the same geographical area. E is referred to as a mixed stream resulting from C and D.*

autotrophic bacterial activity present in a large pile of low-grade coal and pyritic minerals located at position A. This mineral waste heap is often referred to as a "gob" pile. The porous mineral deposit is easily permeated with air and rain water which made it suitable for growth of iron- and sulfur-oxidizing bacteria. Water flowing from B became somewhat diluted downstream at point C because it mixed with additional acid and nonacid drainage. An essentially neutral stream (*p*H 6.2 to 8.2) in the same geographical region is shown at D. Stream D flows into the diluted acid stream C and becomes stream E. E is referred to as a mixed system.

MATERIALS AND METHODS

Samples. Water samples were taken in the field in sterile 8-oz (237 ml) bottles and held in a Styrofoam cabinet until they reached the laboratory and were 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 determination. 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 with $BaSO_4$ precipitate as described by the Hach procedure, and *p*H was determined with a Beckman *p*H meter.

Media and growth conditions. Samples (1 ml) of suitable dilutions were pour-plated on Tryptone Glucose Extract (TGE) Agar (Difco), which was supplemented with 0.5 g of yeast extract per liter (TGYE) and on Sabouraud 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 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 (9), with *Desulfovibrio desulfuricans* medium no. 3. Positive tubes were black after incubation at 25 ± 2 C for 6 to 21 days.

Iron-oxidizing chemoautotrophic bacteria were counted by a five tube MPN technique in the salts medium of Silverman and Lundgren (12). Positive tubes were determined after 15 days 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 $FeSO_4 \cdot 7HOH$ 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 2.8). Control tubes and negative growth tubes gave a yellow indicator reaction.

Artificial stream system. The apparatus shown in Fig. 2 was assembled to allow for mixing of acid-contaminated stream water and neutral stream water using controlled flow rates and mixing conditions in an attempt to simulate field conditions. Glass bottles (5 gal) were fitted with 3.0 mm outer diameter glass tubing. A peristaltic pump was calibrated to deliver a reproducible flow of water from the reservoir bottle that contained acidic mine water taken from the field at location C (Fig. 1) into the receiving bottle which was filled to 10-liter capacity with neutral stream water taken from the field at D. The receiving bottle was aerated through a glass-frit dispersion tube to maintain aerobic conditions and to facilitate mixing. The pumping rate was standardized at 180 ml/hr. The receiving bottle was filled after 3 days of elapsed time. The system was maintained at ambient temperature (25 ± 2 C).

A duplicate control apparatus was used simultaneously in which neutral water from station D (Fig. 1) was pumped into neutral water. This control accounted for changes in microbial populations in the receiving bottle which were not attributable to the addition of acidic water.

Subsequent variations in water-mixing procedure were used. Neutral water was pumped into acid water, and, in this case, acid water was added to acid water as a control. This control accounted for changes in microbial populations in acid water which were not attributable to dilution by neutral water or a *p*H rise.

Samples were taken at the time pumping commenced (0 time) and at random intervals over a 3-day period from all receiving bottles (i.e., two sample bottles plus two control bottles) during each experimental run.

Sulfate, iron, and hydrogen ion concentrations were determined on all samples by the techniques previously described.

Portions (1 ml or appropriate dilutions) of each sample were pour plated on SD Agar and TGYE Agar. All plates were incubated at 28 ± 2 C in air for 3 days, and colonies were enumerated.

Fresh water was brought to the laboratory from the field for each experiment. Therefore, control values differed in each experiment (Fig. 3 and 5, and Table 1) because of changing conditions in the field.

Buffering capacity of neutral water in the mixed stream system is demonstrated in Fig. 4. A 100-ml sample of water obtained at site D (Fig. 1) was titrated with 0.02 N H_2SO_4.

Figure 5 illustrates the changes in *p*H and dissolved iron and sulfate in mixed water when neutral water was added to 10 liters of acid water at the rate of 180 ml/hr for a 3-day period.

The survival of heterotrophic aerobic microorganisms recovered on TGYE and SD Agar while either neutral water was added to 10 liters of acid water at the rate of 180 ml/hr or acid water was added to neutral water at the same rate is shown in Fig. 6.

RESULTS

An initial survey of acidic streams over a several square-mile area indicated that bacterial and yeast populations at the various sites examined were similar with respect to types of colonies on the plating media used.

The values (Table 1) represent a compilation of data taken at the locations shown in Fig. 1. The data represent an average of a minimum of

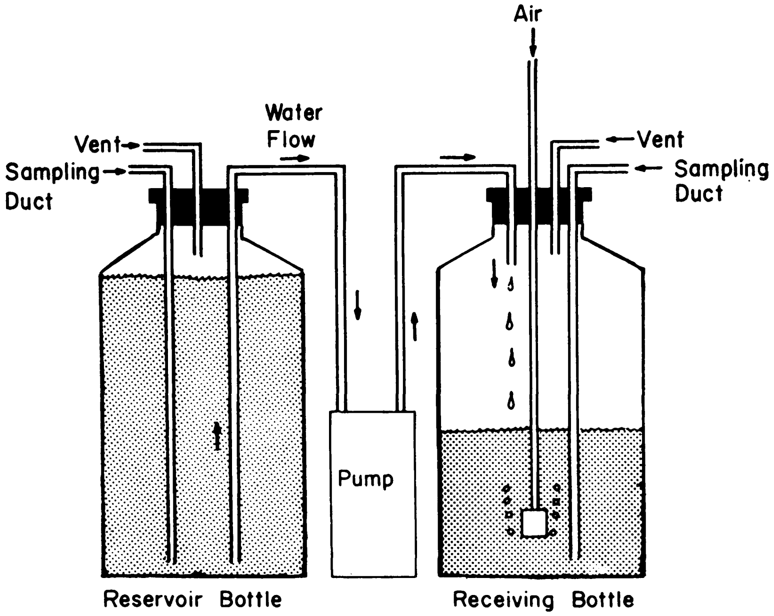

FIG. 2. *Apparatus used as an artificial stream system to simulate the mixing of acidic water with neutral water.*

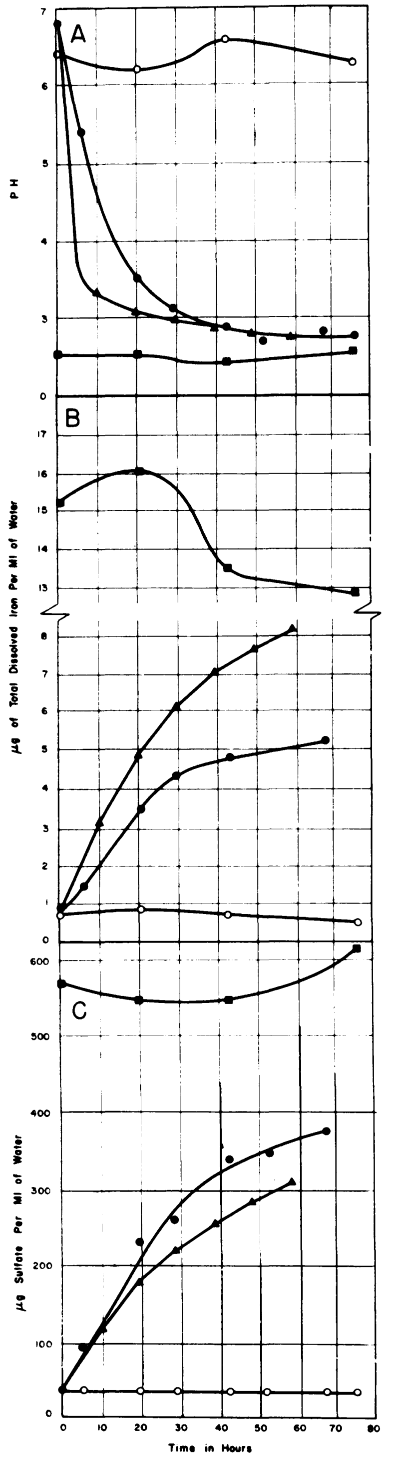

FIG. 3. *Changes in (A) pH, (B) dissolved iron, and (C) sulfate in an artificial stream system when acidic water was added to neutral water. Symbols:* ○, *neutral water control;* ●, *mixture resulting from addition of acid water to neutral water;* ▲, *calculated theoretical curve;* ■, *acid water control.*

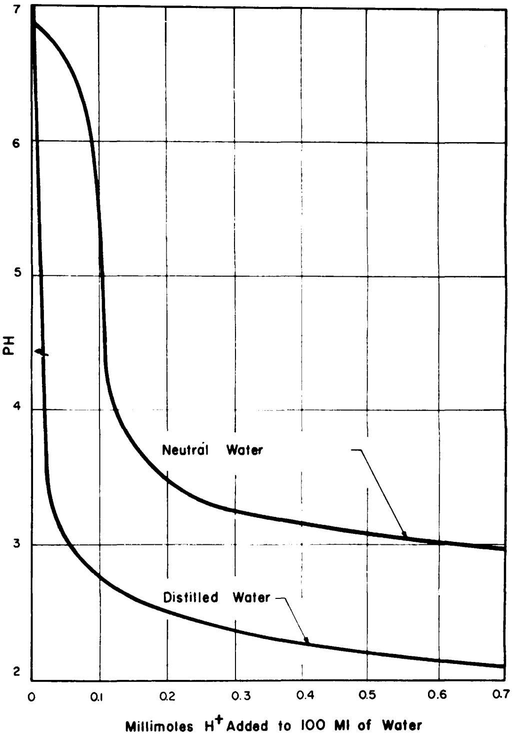

FIG. 4. *Curves showing change of pH in distilled water and neutral stream water when titrated with 0.02* N *H_2SO_4.*

six values. Comparison of numbers of heterotrophic microorganisms counted on either TGYE or SD showed that fewer microbes were present in water as acidity increased from locations D to C to B.

Figure 3 presents data obtained from the artificial stream system and shows that the actual *p*H of the mixture, when acid water (C) was added to neutral water (D), did not decrease as rapidly as predicted by simple dilution (theoretical curve). The theoretical curves plotted in Fig. 3 and 5 show anticipated ion concentrations in the receiving bottles. All theoretical curves were calculated from equation 1. The theoretical ion concentration (equation 1) in receiving bottle is equal to the sum of two determinations: (i) average concentration in reservoir bottle from T-10 hr to T hr times liters of water pumped during 10 hr divided by total liters of water in receiving bottle at T hr, plus (ii) theoretical concentration in the receiving bottle calculated at the previous time interval (T-10 hr) times liters of water in receiving bottle at T-10 hr divided by total liters of water in receiving bottle at T hr.

Discussion

The data (Table 1) suggest that the heterotrophic microflora, which can be recovered from an acid stream on an acid-growth medium (SD Agar), probably represents transient organisms which are not indigenous to the acid stream. For example, an average of 5,776/ml was found in a relatively nonacid stream. When this water was mixed with acid water in approximately equal volumes, the average recoverable heterotrophic population dropped by 78%. The dilution can be approximated by the change in sulfate and iron concentration when stream C mixes with stream D to form E. No significance was attached to differences between values obtained on TGYE Agar as compared to SD Agar. The low number of organisms recovered from locations B and C suggests that a small population of transient heterotrophs was tolerant to the acid environment but did not proliferate there.

Anaerobic bacteria were present in neutral waters, in small numbers (10 or fewer per ml) in diluted acid water (C, E), and absent in highly acid water (B); this suggests that they were not acid tolerant. This may be caused by (i) the high E_h of acidic waters which results from high concentrations of oxidized compounds, (ii) a low concentration of organic material, and (iii) the absence of those microorganisms that utilize oxygen while metabolizing organic materials. On two occasions, the anaerobic species isolated from diluted acid water included sulfate-reducing bacteria. Sulfate-reducing organisms were either lacking or present in very small numbers in acid water. This probably precludes biological sulfate removal from these streams under natural conditions; no evidence of the sulfate removal process was observed.

The predominating aerobic heterotrophic microorganisms in the acid water were yeasts and molds with eight different gram-negative bacilli recovered. The bacilli were generally nonmotile, oxidase-negative, aerobic or facultative species. The bacteria formed white- or cream-colored colonies on the media employed. One aerobic oxidase-positive bacterium was also isolated. The organisms were not thoroughly characterized but

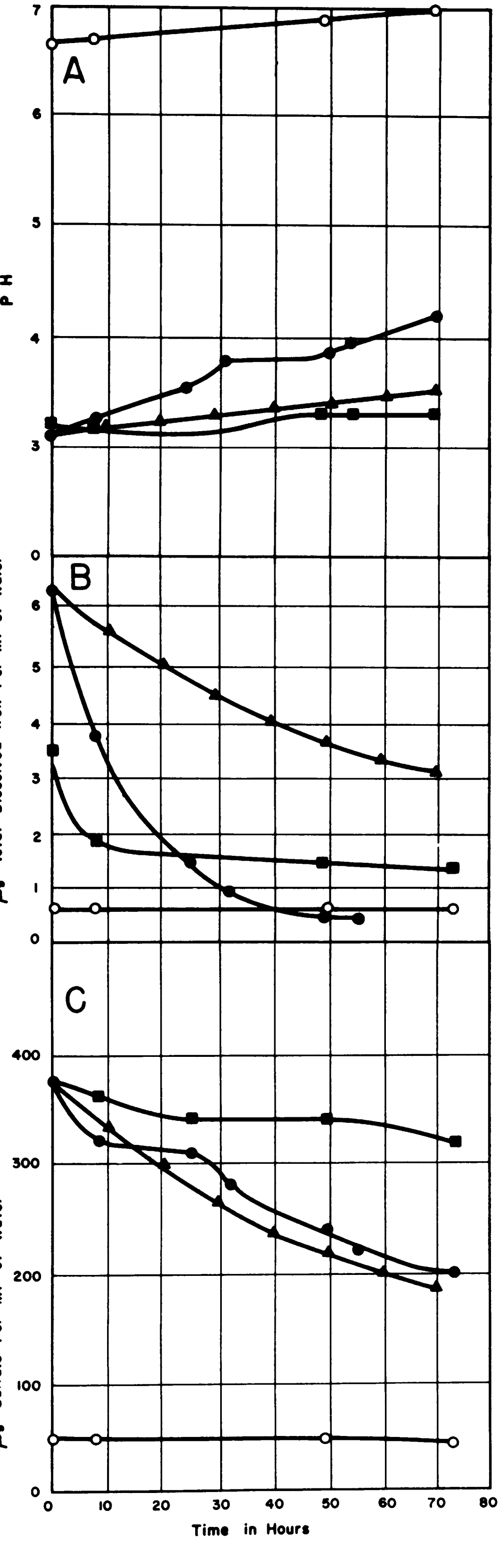

Fig. 5. *Changes in (A) pH, (B) dissolved iron, and (C) sulfate in an artificial stream system when neutral water was added to acidic water. Symbols:* ○, *neutral water control;* ●, *mixture resulting from addition of neutral water to acid water;* ▲, *calculated theoretical curve;* ■, *acid water control.*

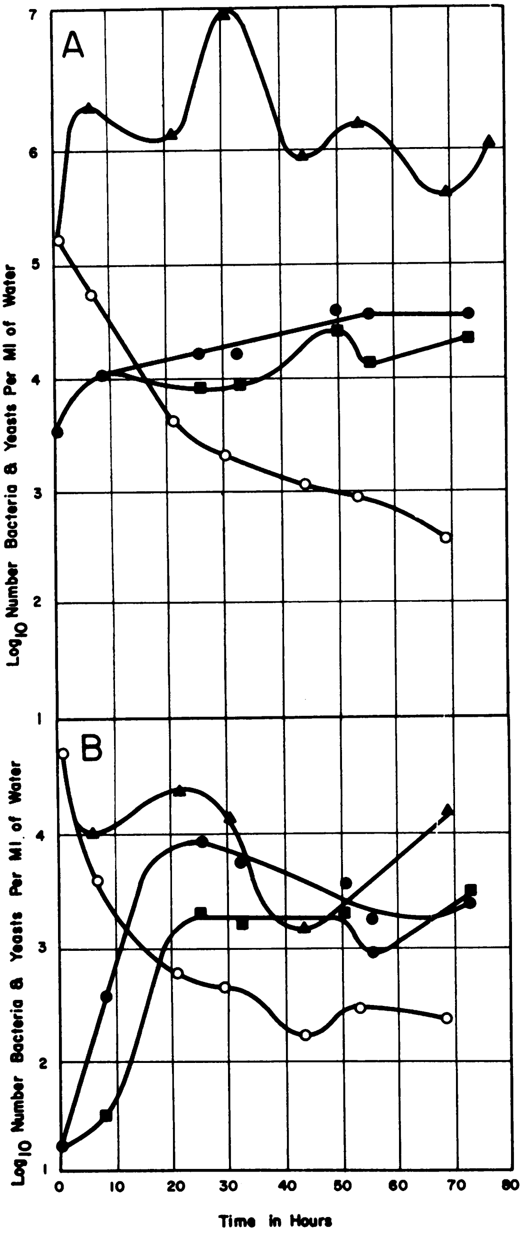

FIG. 6. *Enumeration of heterotrophic aerobic microorganisms in an artificial stream system as determined on (A) Tryptone Glucose Yeast Extract Agar and (B) Sabouraud Dextrose Agar. Symbols:* ○, *in mixture resulting from acid water added to neutral water;* ●, *in mixture resulting from neutral water added to acid water;* ▲, *neutral water control;* ■, *acid water control.*

are tentatively identified as *Achromobacter* and *Pseudomonas*. No gram-positive bacteria were ever isolated from the acid streams with the techniques employed. The organisms that were examined seemed to be transients, entering from a nonacid environment. We must, therefore, presume that the gram-negative bacteria have a greater permeability barrier to hydrogen ions in the environment which gives the gram-negatives survival advantage. The permeability barrier may be related to the higher lipid content of the gram-negative cell envelope as compared to that of the cell wall of gram-positive bacteria.

Comparison of the iron- and sulfur-oxidizing chemosynthetic autotrophic bacteria indicated that the sulfur oxidizers were nearly always present in greater numbers by a factor of 10 than were the iron-oxidizing bacteria. Since the iron oxidizers also oxidize sulfur, the counts of sulfur-oxidizing bacteria may represent both types. The difference can also be attributed to the increased energy available in reduced sulfur compounds over that of ferrous iron. The concentration of sulfate was always greater than 10 times that of iron in the same water. The difference between sulfate and iron concentrations has usually been attributed to removal of iron by precipitation as ferric hydroxides. However, the observed high sulfate concentration may support the views expressed by Silverman and Ehrlich (11), in that oxidation of Fe^{++} will result in formation of SO_4^{-2} from S^{-2}.

Iron and sulfur oxidizers appeared to have a positive correlation with acid concentration. The presence of these bacteria in the neutral stream probably indicates contamination by acid effluents from an unobserved source. The data also suggest that the autotrophic bacteria are tolerant to average *p*H values up to 6.6. Generally, sulfur oxidizers have a wide *p*H range.

Values of the various parameters measured in the field had a wide variance, which can be attributed to daily fluctuations in uncontrolled environmental variables. For example, rainfall can cause a sudden increase in cell numbers in a stream as a result of runoff from surrounding land. The increase in cell numbers would be followed by a sudden decrease in numbers because of dilution. This is particularly true of the autotrophic bacteria, which appear to proliferate in the "gob" pile (Fig. 1A). The organisms, as well as their metabolic by-products, acid, sulfate, and iron ions, are flushed out of the waste pile during a heavy rain causing a sudden surge in concentration in the stream. If the rainfall is prolonged, the reservoir in the waste pile is depleted, and the concentrations of acid, sulfate, and iron ions are diluted markedly in the stream.

A laboratory investigation was conducted for the purpose of controlling environmental variables to assess more accurately the field observations.

TABLE 1. *Organisms, pH, sulfate, and iron concentration at the sample sites*[a]

Sample site	*p*H	SO_4^{-2} concn (M)	Total Fe concn (M)	S oxidizers (MPN/100 ml)	Fe oxidizers (MPN/100 ml)	Anaerobes/ml on Thioglycollate Medium	Heterotrophic aerobes/ml	
							SD Agar	TGYE Agar
B	2.66 (2.5–3.0)	4.0×10^{-2} $(2.3 \times 10^{-2}$–$4.8 \times 10^{-2})$	4.1×10^{-3} $(7.5 \times 10^{-4}$–$9.0 \times 10^{-3})$	1.75×10^{5} $(2.10 \times 10^{2}$–$7.9 \times 10^{5})$	6.89×10^{4} $(2.8 \times 10^{3}$–$3.5 \times 10^{5})$	0	15.1 (1–49)	19 (1–65)
C	3.23 (2.5–4.3)	4.5×10^{-3} $(2.6 \times 10^{-3}$–$6.7 \times 10^{-3})$	2.1×10^{-4} $(7.2 \times 10^{-5}$–$5.2 \times 10^{-4})$	2.20×10^{4} $(2.1 \times 10^{2}$–$1.3 \times 10^{5})$	3.60×10^{2} $(0$–$1.3 \times 10^{3})$	4.4 (1–10)	105 (8–300)	87 (28–150)
D	6.60 (6.2–8.2)	4.3×10^{-4} $(2.2 \times 10^{-4}$–$5.2 \times 10^{-4})$	1.4×10^{-5} $(7.2 \times 10^{-6}$–$1.8 \times 10^{-4})$	5.80×10^{2} $(0$–$3.3 \times 10^{3})$	1.01×10^{2} $(0$–$4.26 \times 10^{2})$	2,800 (100–10,000)	5,776 (86–17,000)	3,379 (762–7,000)
E	3.23[b] (2.6–4.9)	2.1×10^{-3} $(1.5 \times 10^{-3}$–$2.8 \times 10^{-3})$	1.0×10^{-4} $(2.1 \times 10^{-5}$–$1.8 \times 10^{-4})$	7.32×10^{3} $(4.9 \times 10^{2}$–$1.3 \times 10^{4})$	8.03×10^{4}[c] $(8.0 \times 10^{1}$–$3.2 \times 10^{5})$	10 (10–10)	1,371 (63–3,900)	1,257 (220–3,000)

[a] Results given as average values with ranges shown in parentheses.

[b] This average value is probably biased because of a single low result at the lower range limit.

[c] This average value is probably biased because of a single high result at the upper range limit.

The actual iron concentration in the receiving bottle did not increase as rapidly as predicted (Fig. 3). Sulfate concentration increased more rapidly than expected, to a maximum of about 65 μg/ml greater than theoretical. An explanation of this phenomenon may be that (i) neutral water acts as a buffer in the mixed water system, (ii) iron precipitates from solution, and (iii) sulfate is formed in the mixed water probably as the result of oxidation of reduced sulfur compounds contributed by the neutral water. Figure 4 demonstrates a buffering effect by neutral water over the entire *p*H range of the titration. However, buffering capacity probably varied with environmental conditions in the field. The second possibility mentioned above is not unexpected because iron was observed to precipitate as yellow-orange material on the bottom of the receiving bottle. As indicated in Fig. 3B, iron also precipitated in the acid water control (reservoir). This factor influenced the actual iron concentration in the mixture because, as time progressed, less iron per hr was added to the receiving bottle. The calculations involved in the determination of the theoretical curve (see equation 1) allow for this factor. The difference between the actual and theoretical iron concentrations can then be attributed to precipitation of iron in the receiving bottle. The unexpected increase in the sulfate concentration in the mixture (Fig. 3C) is attributable to changes in the mixture and not in the acid water contained in the reservoir bottle. The sulfate concentration in the neutral water control remained constant throughout the experiment. The discrepancy between actual and calculated curves may be due to biological, as well as chemical and physical factors. Production of sulfate via microbial oxidation of reduced sulfur compounds in the neutral water is a possibility. Sulfate may also be released from precipitated ferric iron resulting from variation in *p*H.

When neutral water (D) was added to acid water (C), the actual *p*H in the mixture increased more rapidly than the theoretical *p*H (Fig. 5). This is consistent with the observed buffering capacity of the neutral water. The flat portion of the actual *p*H curve between 32 and 45 hr probably indicates oxidative production of hydrogen ion, which compensates for an anticipated rise in *p*H caused by dilution by neutral water.

The iron concentration (Fig. 5B) in the mixed water (receiving bottle) decreased more rapidly than predicted by dilution of the acid water with neutral water. Virtually all of the iron in the mixture precipitated as the *p*H increased. This is probably due to oxidation of ferrous to ferric iron and the decreased solubility of ferric iron with increasing *p*H. The iron concentration of the neutral water control remained constant throughout the experiment. Precipitation occurred rapidly during the first 8 hr in the acid water control and at a slower rate after 8 hr.

After 15 hr, the actual sulfate concentration in the mixture (Fig. 5C) was greater than could be accounted for on the basis of dilution. No additional sulfate was produced in the acid water alone, and the data obtained in this mixed system are in accordance with the data obtained when acid water was added to neutral water (Fig. 3C).

In the artificial stream system, the numbers of microorganisms in the neutral water controls fluctuated with time, although they were generally present at 10^6/ml or greater. Comparison of the neutral control curve shown in Fig. 6A (approximately 10^6/ml) to that in Fig. 6B (approximately 10^4/ml) suggests that less than 10% of the organisms in neutral water were tolerant to the acidity of SD Agar (*p*H 5.5). This observation was verified by comparing the sharp drop in numbers of organisms over the first 20 hr when acid was added to neutral water to the change in *p*H (Fig. 3A). A plot of the total counts of organisms versus time appears quite similar to the *p*H versus time plot.

The numbers of organisms per ml of acid water control increased during the first 20 hr (Fig. 6B), which shows that the acid water (*p*H 2.5) was able to support growth of organisms up to a level of about 2×10^3/ml. Addition of neutral water to acid water resulted in organism counts stabilizing at about 5×10^4/ml (Fig. 6A), whereas the counts were approximately 10^3 when acid water was added to neutral water. This again indicates that the acid water contained an acid-tolerant microflora which was maintained when neutral water was added and that relatively few acid-tolerant microbes entered the system with the neutral water. Acid water added to neutral water resulted in a lower stabilization value, which is related to the relative number of acid-tolerant microbes present.

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