Enumeration of coliforms from streams containing acid mine water

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A major pollution problem in many Appalachian coal mining regions in the U. S. involves the introduction of acid mine drainage into freshwater streams. In addition, these same acid mine streams frequently serve as a repository for domestic wastewater, and hence, any associated waterborne microbial pathogens. Because detection systems for pathogenic microorganisms lack sensitivity and are at best semiquantitative, so-called indicator organisms are used to determine the bacteriological quality of such water. Most sanitary-indicator organisms, as well as enteric waterborne pathogens, are bacteria whose natural habitat is the intestine of man and other warm-blooded animals. Thus, their presence in water suggests the possibility of contamination by pathogens of fecal origin.

Many investigators have concluded that polluted streams receiving acid mine drainage are rapidly self-purified in regard to most bacteria of public health significance.[1, 2] The lack of recovery of indicator organisms from these aquatic environments may indicate these organisms are dead or destroyed as a result of the extreme stress of the acid mine drainage. However, mineral recovery of sanitary-indicator organisms from such extreme environments may reflect the inadequacy of the recovery procedures used to detect and enumerate these organisms.

Recently, Hackney and Bissonnette [3] demonstrated that following exposure of pure cultures of indicator organisms of the coliform group to acid mine water, a substantial proportion of the survivors failed to form colonies on a selective medium, yet retained their ability to form colonies on a relatively rich, nonselective medium. These findings suggested that coliform enumeration procedures using selective media failed to detect sublethally injured survivors from acid mine water. It seems quite likely that previous studies undertaken to examine the sanitary significance of bacteria in acidic waters have been in error. Improved methods need to be developed for the detection of coliforms that have been subjected to acid mine water.

The purpose of this research report is to present results of studies that critically examine the effectiveness of presently available recovery techniques for the enumeration of the total

TABLE I. Description and location of sampling sites.

Site	Description and Location
1	Monongahela River at Walnut Street, approximately 4.0 km upstream from the outfall of the Morgantown wastewater plant.
2	Monongahela River at Sixth Street, approximately 2.7 km upstream from the outfall of the Morgantown wastewater plant.
3	Monongahela River at the Star City bridge, approximately 500 m downstream from the outfall of the Morgantown wastewater plant.
4	Dent's Run, approximately 1.0 km from its junction with the Monongahela River.
5	Scott's Run, approximately 100 m from its junction with the Monongahela River.
6	Robinson Run, approximately 3.2 km from its junction with the Monongahela River.
7	Unnamed stream of acid pH draining a sanitary landfill area, approximately 70 m from its junction with Hartman Run.
8	Hartman Run, approximately 25 m downstream from its junction with the unnamed stream draining the sanitary landfill area.
9	Hartman Run, approximately 5 m upstream from a sewage pipe effluent outfall.
10	Wastewater pipe effluent outfall (unchlorinated) discharging into Hartman Run.
11	Hartman Run, approximately 50 m downstream from the wastewater pipe effluent outfall.

coliform group from acid mine water environments.

MATERIALS AND METHODS

Sampling. Samples were collected from selected stream sites (Table I) using sterile, 1-liter bottles. The sampling sites were located close to the laboratory, enabling collection and processing within 5 hours. Samples not immediately processed were stored at 5°C. Water temperature was recorded at each site, and pH determinations were performed in the laboratory.

Standard coliform analysis. When necessary, appropriate sample dilutions were prepared using sterile 99-ml, 0.1% peptone blanks.[4] Samples were analyzed for total coliforms using two enumeration procedures simultaneously, the "Standard Methods" [4] most probable number (MPN) and membrane filtration (MF) techniques.

The presumptive coliform five-tube MPN test was performed using single-strength lactose broth. Positive tubes were then subjected to the confirmed MPN test using brilliant-green lactose bile (BGLB) broth.[4] A confirmed MPN was then calculated for each sample, and a completed MPN was routinely performed on samples collected during the early portion of the study. False-positive total coliforms were rarely detected by the completed MPN; therefore, the completed MPN was not regularly performed during future analyses.

The MF procedure used for total coliform analysis also conformed to "Standard Methods." [4] All filtrations were done using Gelman GN-6 Metrical filters (Gelman Instrument Co., Ann Arbor, Mich.). Following filtration of the water sample or its appropriate dilution, filters were transferred to petri dishes containing M-coliform agar (M-coliform broth containing 1.5% agar). Duplicate filtrations were done for each dilution of all samples analyzed. After incubation at 35°C for 24 hours, colonies were counted on a colony counter. A representative number of randomly selected colonies from those plates containing from 20 to 80 coliform colonies were subjected to confirmatory procedures using BGLB broth.

Total coliform enrichment procedure. In some experiments, recovery of total coliforms by enrichment methods was simultaneously compared to recovery obtained by the standard one-step MF coliform procedure previously described. Preliminary experiments compared the recovery efficiency of resuscitation broth [5] with the lauryl tryptose broth enrichment medium of "Standard Methods" [4] as well as Brain Heart Infusion broth. Results from these experiments reflected slightly greater recovery of total coliforms when using resuscitation broth; therefore, resuscitation broth was selected for future work.

When the enrichment procedure was used, absorbent pads were soaked with 1.8 to 2.2 ml of resuscitation broth (40 g peptone, 6 g yeast extract, 30 g lactose, 1 000 ml distilled water, and pH 7.4). Duplicate filtrations were performed for each dilution of all samples. Membrane filters were aseptically placed on the absorbent pads directly from the filtration unit, and the filters were then incubated in an upright position at 25°C for 2 hours. After the 2-hour enrichment period, the filters were aseptically transferred to M-coliform agar. The plates were incubated, counted, and confirmed for coliform colonies as previously described in the standard MF technique.

RESULTS

MPN versus MF recovery. Recovery problems were encountered when attempting to detect and enumerate coliforms from streams containing acid mine water. As indicated by the recovery ratios in Table II, the MPN technique was the superior recovery method when compared with the MF technique. In general, the highest recovery ratios were found to be associated with those streams exhibiting the lowest pH values. Recovery ratios could not be calculated for three of the stream sites adversely affected by acid mine drainage (sites 6 to 8) because coliforms could not be detected in 100 ml of sample when using the MF technique. However, coliforms could be detected in each case using the MPN procedure. In contrast, the discrepancies between the two techniques were minimal with the less acidic samples collected from the Monongahela River (sites 1 to 3).

Enrichment effects on MF total coliform recovery. In an attempt to increase the recovery efficiency of the MF technique when examining acid mine water environments, a coliform enrichment MF procedure was used. Recovery obtained by the enrichment MF method was compared with coliform recovery detected by the direct MF and standard MPN procedures. As evidenced by the recovery ratios in Table III, the MPN method proved, in most cases, to be the most effective method for the recovery of coliform bacteria, while the one-step MF method was the least efficient.

TABLE II. A comparison of total coliform recovery (per 100 ml of sample) by MPN and direct MF procedures on three different days.

Site	Day	Water[a] Temp.	pH	MF	MPN	Recovery[b] Ratio
1	1	1	6.3	2.3×10^3	7.9×10^4	34.3
	2	3	6.4	1.2×10^4	2.4×10^5	20.0
	3	3	6.5	1.0×10^4	4.0×10^4	40.0
2	1	1	6.6	8.8×10^2	1.1×10^5	125.0
	2	3	6.5	3.9×10^3	2.0×10^4	5.1
	3	3	6.5	4.9×10^2	1.2×10^4	24.1
3	1	2	6.7	1.6×10^4	5.4×10^6	337.5
	2	3	6.4	3.2×10^4	4.3×10^5	13.4
	3	3	6.7	7.9×10^3	4.9×10^5	60.0
4	1	0	6.5	1.0×10^1	4.9×10^3	490.0
	2	0	5.3	2.5×10^1	4.0×10^4	1600.0
	3	0	6.0	3.7×10^1	3.7×10^3	100.0
5	1	1	4.8	2.6×10^2	1.1×10^4	42.3
	2	5	4.5	3.0×10^0	2.7×10^3	900.0
	3	5	4.3	1.0×10^0	2.2×10^3	2200.0
6	1	1	3.1	<1	7.9×10^1	—[c]
	2	7	3.2	<1	4.0×10^0	—
	3	8	3.1	<1	2.6×10^3	—
7	1	1	3.2	<1	1.7×10^2	—
	2	5	3.4	<1	1.1×10^1	—
	3	5	3.2	<1	1.6×10^1	—
8	1	1	3.0	<1	1.3×10^3	—
	2	5	3.4	<1	1.1×10^1	—
	3	5	3.2	<1	1.6×10^1	—

[a] Degrees centigrade.
[b] MPN index/MF density.
[c] Not calculable.

The data also demonstrate that the enrichment MF procedure resulted in an appreciable improvement of coliform recovery when compared to the direct MF method (Table III). On close examination of water samples of acidic pH (sites 7, 9, and 11), the value of the enrichment technique is clearly evident. The recovery of coliforms from these acidic water samples with the two-step enrichment technique more closely approximates the MPN values than do the direct MF recoveries. In contrast, comparison of coliform detection from a domestic wastewater effluent by the two MF methods suggests little value in using enrichment techniques. In these cases, recoveries obtained by direct MF methods were quite comparable with those obtained by two-step enrichment procedures.

DISCUSSION

Currently, there are two accepted standard methods to detect and enumerate coliforms in water, the MPN and MF techniques.[4] Ideally, the two procedures should give relatively similar results. Severe limitations of the MF technique have been recognized, especially when examining wastewaters containing toxic chemicals, particularly wastewater effluents containing high concentrations of chlorine.[6-9] These findings support the recommendation [4] that the coliform MF technique is not applicable to the examination of waters receiving chlorinated effluents or wastewaters containing toxic metals or phenols. Apparently, the concentration of toxic materials on the surface of membrane filters may prevent growth of some microorganisms, which is a problem not encountered in the liquid environment used in the multiple-tube fermentation technique.

Similarly, it is evident that problems arise when applying conventional MF recovery methods to detect and enumerate coliforms from streams containing acid mine water (Tables II and III). The MPN values for all samples collected were consistently higher than the corresponding coliform density obtained

TABLE III. A comparison of total coliform recovery (per 100 ml of sample) from various streams by MPN, direct MF, and enrichment MF procedures.

Site	pH	MF[a]		Recovery Ratio	MPN	Recovery Ratio
		Direct	Enriched[b]	Enriched/Direct		MPN/Direct
4	6.0	3.9×10^4	6.3×10^4	1.6	7.9×10^4	2.0
7	2.9	6.0×10^1	3.4×10^3	56.7	1.3×10^3	21.7
9[c]	2.9	<1	2.5×10^0	>2.5	7.9×10^2	>790.0
9	3.0	1.5×10^1	7.5×10^2	50.0	9.3×10^2	62.0
9	3.0	1.1×10^1	6.2×10^1	5.6	4.5×10^1	4.1
10[c]	6.9	3.6×10^7	3.9×10^7	1.1	4.9×10^7	1.4
10	6.3	1.2×10^7	1.4×10^7	1.2	2.5×10^7	2.1
10	6.9	2.9×10^7	3.1×10^7	1.1	1.3×10^7	0.5
11[c]	3.3	2.1×10^5	2.9×10^5	1.4	1.3×10^6	6.2
11	3.2	8.9×10^4	5.3×10^5	5.9	6.8×10^5	7.6
11	3.6	4.3×10^3	3.2×10^5	74.4	4.9×10^5	113.9

[a] Average of two replicates.
[b] Enriched with resuscitation broth.
[c] Data for sites 9 to 11 represent three different sampling days.

by the direct MF method. Furthermore, the greatest discrepancies between the two enumeration methods were usually associated with those samples collected from streams with extremely depressed pH values. In some instances, the MF procedure failed to detect any coliforms in 100 ml of sample, while the corresponding MPN analysis gave evidence of greater than 1 000 coliforms/100 ml. Caution should be observed, however, when comparing MPN values with results obtained by MF procedures. Bissonnette *et al.*[10] have emphasized that statistically based MPN values are not always representative of the absolute density of a given water sample and will always be difficult to interpret in comparison with results of direct count procedures because of the built-in positive bias of the MPN index. Nevertheless, the large differences in coliform recovery obtained between MF and MPN techniques in streams containing acid mine water support the use of the MPN technique and cast doubt on the reliability of the MF technique.

Although the liquid environment of the MPN method may augment bacterial growth, this method has certain limitations, including prolonged analysis time and low precision. Therefore, in an attempt to increase the recovery efficiency of the MF technique when examining water containing acid mine drainage, a two-step enrichment MF technique was compared to the direct MF and standard MPN techniques (Table III). Enrichment procedures were examined because considerable evidence has accumulated indicating that after exposure to environmental stress in aquatic environments, some microorganisms are metabolically or structurally damaged to such an extent that significant problems arise on attempts of detection and enumeration.[3, 10-12] The detection of such nonlethally injured cells becomes more complicated by the use of selective media. Thus, an important consequence of this physiological alteration is that such cells are commonly unable to produce colonies on selective media that are regularly recommended for their enumeration.

Therefore, the combination of environmental stress and subsequent utilization of selective media may result in significantly lowered recoveries of nonlethally injured cells, leading to erroneous conclusions regarding the true density of bacterial populations in an environmental sample. With regard to the likelihood of acidic environments contributing to sublethal debilitation of sanitary-indicator organisms, Roth and Keenan[13] demonstrated that strains of *Escherichia coli* sublethally damaged in acidic environments showed an increased sensitivity to selective violet red bile agar. More recently, Hackney and Bissonnette[3] observed that strains of *E. coli* and *Enterobacter aerogenes* exposed to acid mine water demonstrated increased sensitivity to selective deoxycholate lactose agar.

The enrichment technique used in this study consisted of a two-step procedure whereby bacterial cells were initially exposed to a rich, nonselective medium where appreciable repair of the injured cells could be obtained prior to application of the standard selective medium.

Other workers [8, 10, 14-18] have observed improved recovery of indicator organisms from various aquatic environments on application of similar resuscitation or enrichment procedures. As evidenced by Table III, an enrichment MF procedure resulted in a minimum of 2.5-fold to a maximum of 74.4-fold increase in recovery of total coliforms from streams of low pH (pH less than 4.0). Resuscitation proved to be of little value for coliform enumeration from a domestic wastewater effluent as reflected by nearly equal recovery by either the one-step or two-step MF procedure. Thus, it appears that enrichment techniques are particularly valuable when attempting to recover coliforms from the stressed environments of streams containing acid mine water.

Apparently, the application of a two-step enrichment procedure when using the MF method promoted repair of some of the injured cells, allowing for a substantial increase in detection of viable coliform organisms. Such resuscitation procedures are not necessary when examining unchlorinated wastewater discharge of the type examined in this study.

CONCLUSIONS

Valid sanitary evaluations of waters receiving acid mine water and organic wastes are strongly dependent on maximizing the sensitivity of recovery procedures for bacterial indicator organisms. It has been shown that improvements of the MF technique are possible, namely the incorporation of an enrichment step utilizing resuscitation broth. Although in most cases the MPN method produced greater coliform densities in comparison with the MF method, the disadvantages inherent in the multiple-tube technique suggest that this method should not be the sole criterion used in determining bacteriological water quality. However, the MPN method remains a valuable and powerful tool, in that it may detect bacterial pollutants that may otherwise go undetected by the MF procedure.

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