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# Two-Temperature Membrane Filter Method for Enumerating Fecal Coliform Bacteria from Chlorinated Effluents

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Reports indicate that the standard membrane filter (MF) technique for recovery of fecal coliform bacteria from chlorinated sewage effluents is less effective than the multiple-tube (or most-probable-number [MPN]) procedure. A modified MF method was developed that requires a preincubation period of 5 h at 35°C followed by 18 ± 1 h at 44.5°C. This procedure was evaluated by using both laboratory- and plant-chlorinated primary and secondary effluents. Results obtained by the modified MF method compared favorably with those of the MPN technique for the enumeration of fecal coliforms from chlorinated effluent. Agreement between these two methods was greatest with samples from secondary treatment plants. The average recovery of fecal coliforms by the standard MF procedure was only 14% that of the MPN method, whereas with the modified technique recovery was increased to 68% of the MPN counts. Enhanced recovery resulting from a simple modification in the incubation schedule makes the MF method a valuable adjunct for enumerating fecal coliforms from chlorinated effluents.

In 1958 McKee et al. (5) indicated that the membrane filter (MF) technique for the recovery of total coliforms from chlorinated effluent was less effective than the multiple-tube or most-probable-number (MPN) procedure. Lin (3) in 1973 evaluated methods for enumerating fecal coliforms from chlorinated sewage effluents and likewise concluded that the MF technique, with M-FC medium at 44.5°C, was less efficient than the elevated temperature multiple-tube procedure for fecal coliform recovery. Maxcy (6) suggested that nonlethal injury of coliform bacteria by various treatments, including chlorination, reduced the ability of cells to grow on selective media. Braswell and Hoadley (2) later reported that *Escherichia coli* injured during chlorination of secondary sewage failed to produce colonies on MFs incubated on M-FC medium or to grow and produce gas in lactose broth. In their studies, recovery by the MF method was generally poorer than that by other techniques employed. Because chlorination is currently the most common form of sewage disinfection and is now required year-round in many states, the Environmental Protection Agency has recently suggested that the multiple-tube test be used as the method of choice for fecal coliform determination on effluent containing chlorine (11).

It has been postulated (3) that the broth medium of the presumptive MPN test provides a more favorable environment than the MF surface for the repair of sublethally injured cells. Since damaged cells may be more sensitive to the temperature shock of the immediate 44.5°C incubation required by the MF method, several enrichment procedures have been advanced to increase MF recovery of fecal coliforms. Stevens et al. (8) proposed a 2-day procedure, which included an overnight acclimatizing period for the fecal coliforms on a minimal medium at 25°C. The membrane was then transferred to M-FC agar for an additional 24-h incubation at 44.5°C. Recovery of fecal coliforms from marine waters by this method was equal to about 90% of the MPN recovery when correction was made for the MPN bias.

Rose et al. (7) reported on an improved fecal coliform MF method, using M-FC agar with a lactose agar overlay. After the plates were incubated at 35°C for 2 h, the temperature was increased to 44.5°C for 22 to 24 h. When tested with a variety of water samples, the two-layer agar method yielded almost twice the number of fecal coliform colonies recovered by the standard MF procedure. However, the proposed method was not compared with the multiple-tube test for recovery of fecal coliforms.

Comparisons of fecal coliform recoveries from chlorinated effluents by the standard MF and multiple-tube procedures often show 10-fold-greater MPN counts. Preliminary tests in our

laboratory indicated the importance of temperature acclimation in the recovery of chlorine-injured cells on a selective medium, and this suggested that fecal coliform recovery on M-FC agar could be enhanced by modifying the standard 44.5°C incubation. The purpose of this study was to improve the MF method to the extent that it would be comparable to the MPN test for the recovery of fecal coliforms from chlorinated wastewater.

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## MATERIALS AND METHODS

Preliminary studies with chlorinated effluent were performed to determine the effect of various preincubation times and temperatures on the recovery of fecal coliform bacteria. The standard MF and multiple-tube tests were performed in accordance with the 14th edition of *Standard Methods for the Examination of Water and Wastewater* (1).

**Laboratory-chlorinated samples.** Primary sewage effluent was collected at the Amherst treatment plant and chlorinated in the laboratory. This facilitated standardization of samples and greater predictability in chlorine levels and exposure time. Sodium hypochlorite was added to 1-liter samples to obtain residual chlorine levels (free and combined) up to 2 mg/liter as measured by the orthotolidine test (Fig. 1). Samples were mixed intermittently for contact times varying from 5 to 20 min before being neutralized with an excess of sodium thiosulfate (0.2%). Peptone water (0.1%) was used as a diluent and rinse. Plates of M-FC agar (Difco Laboratories) were usually prepared daily and were always used within 48 h of preparation. Five-tube MPNs were performed by inoculating four serial dilutions into lauryl tryptose broth. Simultaneously, 20 replicate volumes of sample were filtered, and membranes were placed on M-FC agar plates (Millipore Corp. HC membranes were used throughout). Five plates were incubated directly at 44.5°C in a Millipore solid-state heating block. The remaining 15 plates were incubated in a 35°C air incubator. At 3, 4, and 5 h, sets of five plates were transferred to the 44.5°C incubator for the remainder of the incubation period.

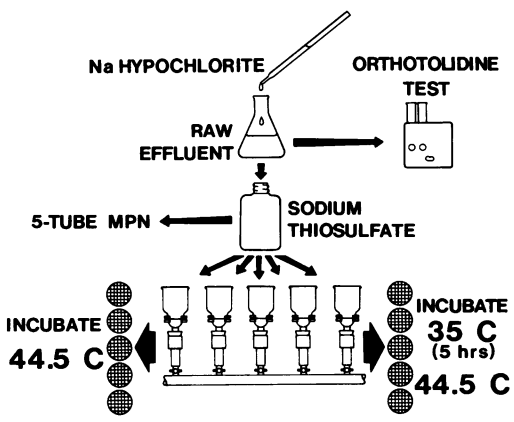


FIG. 1. *Protocol for standard MF-modified MF-MPN comparisons.*

Colonies were counted with the aid of a stereomicroscope and fluorescent light source. Blue colonies recovered by the modified procedure were confirmed as fecal coliforms by gas production in lauryl tryptose broth at 35°C within 24 to 48 h and in EC (elevated coliform) broth at 44.5°C within 24 h. When possible, all blue colonies from a plate were verified to avoid bias.

Incubation at 35°C for 5 h followed by 18 ± 1 h at 44.5°C was determined to be the most productive temperature-time combination, yielding the highest recovery of fecal coliforms without excessive background growth. After the optimum conditions were established, a study was carried out to compare the modified method with the standard MF and multiple-tube methods, using laboratory-chlorinated primary sewage effluent.

**Plant-chlorinated samples.** The study was extended to include an evaluation of the modified method for fecal coliform recovery from plant-chlorinated effluents. Samples were collected from several primary and secondary treatment plants at various times after normal chlorination. Treatment plants cooperating in this project were in the towns of Amherst, Sunderland, Northampton, Greenfield, Millers Falls, and South Deerfield, Mass. Additional samples from Bozeman and Trident, Mont., plants were analyzed at Montana State University to achieve sample diversity. Samples were neutralized immediately or after holding from 4 to 25 min depending upon chlorine residual measurements and the distance they were collected from the chlorinator. After dechlorination, the samples were analyzed as previously described.

Since fecal coliform densities covered many orders of magnitude, all data were expressed and analyzed as logarithms (base 10). For the purpose of comparing methods it was found most illuminating to plot one method against the other as has been done by Lin (3). In this type of graph the line of equality represents perfect agreement between the two methods. The best-fit straight line was computed by the method of least squares, and the correlation coefficient was computed by standard statistical procedures. A measure of productivity or recovery of one method with respect to another was ob-

TABLE 1. *Effect of incubation temperature on recovery of fecal coliform from chlorinated effluent*

| Time (h) at 35°C | Counts/100 ml as % of 5 h[a] | Colonies picked (no.) | Confirmed fecal coliform (%) |
|---|---|---|---|
| 0 | 24.7 | 132 | 93 |
| 3 | 70.8 | 267 | 87 |
| 4 | 76.6 | 278 | 87 |
| 5 | 100.0 | 279 | 89 |

[a] Based on 30 plates per incubation time.

tained by measuring the average distance separating the line of equality and the best-fit line.

## RESULTS AND DISCUSSION

The effects of various preincubation periods at 35°C on the recovery of fecal coliforms from chlorinated effluents are shown in Table 1. Recoveries are expressed as percentages of the 5-h preincubation test, with each figure representing the mean of 30 plates. Preincubation for 5 h at 35°C prior to the 44.5°C incubation appeared to provide the most favorable growth conditions, whereas the standard 44.5°C incubation yielded the lowest recovery of fecal coliforms (24.7%). Preincubation exceeding 5 h resulted in excessive background growth, spreading, and a significantly lower confirmative rate.

Thirty-two laboratory-chlorinated samples

TABLE 2. *Fecal coliform counts by three methods; samples of raw sewage from Amherst Treatment Plant chlorinated in laboratory*

| Date of Run | Chlorine Residual, mg/l | Contact Time, Min | Log Fecal Coliform Count per 100 ml: Standard MF Method | Modified MF Method | 5-Tube MPN |
|---|---|---|---|---|---|
| 11/3/75 | 1.5 | 6 | 3.083[a] | 3.609[a] | 4.041 |
| " | 1.5 | 8 | 2.681 | 3.114 | 3.519 |
| 11/5/75 | 1.5 | 6 | 3.593 | 3.873 | 4.380 |
| " | 1.5 | 8 | 3.029 | 3.477 | 3.690 |
| 11/19/75 | 1.5 | 13 | 2.146 | 2.571 | 2.342 |
| " | 1.5 | 15 | 1.602 | 2.365 | 2.041 |
| 11/20/75 | 1.5 | 15 | 2.215 | 2.617 | 2.690 |
| " | 1.5 | 17 | 2.265 | 2.692 | 2.380 |
| 12/3/75 | 1.5 | 17 | 2.193 | 2.617 | 2.519 |
| " | 1.5 | 19 | 2.146 | 2.683 | 2.663 |
| 12/8/75 | 1.5 | 16 | 1.903 | 2.694 | 3.041;2.845[b] |
| " | 1.5 | 18 | 1.924 | 2.516 | 2.544;2.519 |
| 12/9/75 | 1.5 | 18 | 2.210 | 2.602 | 2.690;2.898 |
| 12/10/75 | 1.5 | 6 | 2.255 | 2.763 | 3.041;2.973 |
| " | 1.5 | 8 | 2.079 | 2.643 | 2.415;2.690 |
| 12/15/75 | 1.4 | 6 | 2.982 | 3.606 | 3.690;3.898 |
| " | 1.4 | 8 | 2.505 | 3.093 | 2.898;2.898 |
| " | 1.5 | 6 | 2.763 | 3.305 | 3.362;3.362 |
| 12/16/75 | 1.5 | 5 | 2.079 | 3.513 | 3.519;4.041 |
| 12/29/75 | 1.75 | 4 | 2.000 | 3.283 | 3.491 |
| " | 2.0 | 7 | 1.301 | 2.623 | 3.230 |
| 12/30/75 | 1.5 | 4 | 2.079 | 3.808 | 4.146 |
| " | 1.5 | 5 | 1.602 | 3.210 | 3.898 |
| " | 1.5 | 7 | 1.602 | 2.441 | 2.845 |
| " | 1.5 | 7 | 1.204 | 2.537 | 2.690 |
| 1/6/76 | 1.25 | 7 | 1.447 | 2.681 | 1.301 |
| 2/17/76 | 1.5 | 5 | 2.422 | 3.459 | 3.898 |
| " | 1.5 | 5 | 2.465 | 3.459 | 4.041 |
| 2/19/76 | 0.8 | 20 | 2.683 | 3.152 | 2.845 |
| " | 0.8 | 15 | 2.757 | 3.391 | 2.898 |
| 3/2/76 | 1.5 | 10 | 1.643 | 2.369 | 3.114;3.230; 3.380 |
| 3/2/76 | 1.0 | 12 | 1.556 | 2.453 | 2.690;2.519; 2.845 |

a. Each figure represents the logarithm of the average of 5 replicate membranes

b. Additional numbers are replicate 5-tube MPN's.

were tested to compare the modified, the standard MF, and the multiple-tube procedures for the enumeration of fecal coliforms (Table 2). After the initial success of the modified MF technique with laboratory-chlorinated effluent, testing proceeded with plant-chlorinated sewage (Table 3). Figure 2 shows the fecal coliform count by the standard MF method plotted against the MPN counts for both laboratory- and plant-chlorinated primary and secondary sewage effluents. Each point represents the average of five M-FC plates compared with the five tubes of the MPN method. The majority of the points fall below the line of equality, indicating consistently greater recovery by the MPN procedure. On the average, the standard MF method yielded only 8% of the fecal coliform recovered by the MPN technique, with a correlation coefficient of 0.84. This low correlation between methods is in accordance with reports of Lin (3) and others. When the modified MF method was compared with the MPN method using the same samples, the points fell closer to the line of equality (Fig. 3). The correlation coefficient was calculated as 0.92, and the mean count by the modified MF method increased to 49% that of the MPN procedure.

Upon closer examination of the data, it was found that agreement between the modified MF method and the MPN method was greatest with samples from secondary treatment plants. Figure 5 shows the standard MF results of 40 samples from plant-chlorinated secondary effluent. The correlation coefficient is high (0.96), but the recovery is only 14% that of the MPN method. The results of the same 40 samples from secondary plants, analyzed by the modified MF methods, are plotted in Fig. 4. The correlation coefficient is 0.97, and the recovery with the modified MF method increased to 68% that of the MPN procedure. This greater agreement between the modified MF method and the MPN method from samples of secondary effluent could be related to the effectiveness of chlorination in effluent containing less organic material.

A total of 3,133 colonies recovered by the modified MF method were verified (Table 4). Of these, 2,910 (93%) were confirmed as fecal coliforms, indicating that the 35°C preincubation does not lower the confirmation rate below that expected when the standard MF technique is used.

In these comparisons the multiple-tube procedure was used as the standard, but it must be noted that there are inherent shortcomings in this technique. The MPN method is based on probability statistics, and estimates of bacterial

TABLE 3. *Fecal coliform counts by three methods; sewage samples chlorinated by the treatment plant*

| Date of Run and Location of Treatment Plant | | Chlorine Residual and Additional Contact Time | | Log Fecal Coliform Count per 100 ml | | |
|---|---|---|---|---|---|---|
| | | mg/l | Min. | Standard MF Method | Modified MF Method | 5-Tube MPN |
| 1/8/76 | Amherst | 1.3 | 7 | 1.079[b] | 2.305[b] | 3.041 |
| 1/21/76 | Amherst | 1.5 | 4 | 2.000 | 2.602 | 3.380 |
| 1/21/76 | Amherst | 1.5 | 7 | 1.301 | 1.602 | 2.447 |
| 1/21/76 | Amherst | 1.5 | 9 | 0.602 | 1.204 | 2.114 |
| 1/22/76 | Amherst | 0.8 | 5 | 0.903 | 2.538 | 3.380 |
| 1/26/76 | Sunderland | 1.0 | 0 | 2.274 | 2.703 | 2.898 |
| 1/27/76 | Northampton | 1.5 | 5 | 2.505 | 3.384 | 3.690 |
| 1/28/76 | Amherst | 0.9 | 10 | 2.158 | 3.759 | 4.732 |
| 2/3/76 | Amherst | 1.0 | 7 | 2.107 | 3.314 | 4.544 |
| 2/11/76 | Northampton | 3.0 | 5 | 0.602 | 2.633 | 3.898 |
| 2/12/76 | Sunderland | 0.9 | 0 | 2.176 | 3.086 | 3.898 |
| 2/12/76 | Sunderland | 0.9 | 0 | 2.086 | 3.025 | 3.041 |
| 2/18/76 | Amherst | 0.8 | 15 | 1.544 | 3.176 | 3.898 |
| 11/24/75 | Bozeman | 0.8 | 0 | 5.415 | 5.987 | 6.204 |
| 11/25/75 | Bozeman | 0.4 | 0 | 5.255 | 5.833 | 5.973 |
| 12/2/75 | Bozeman | 0.4 | 0 | 5.301 | 5.748 | 5.732 |
| 12/3/75 | Bozeman | 0.5 | 0 | 4.991 | 5.681 | 6.041 |
| 12/9/75 | Bozeman | 0.15 | 0 | 5.301 | 5.826 | 5.519 |
| 2/17/76 | Bozeman | 0.4 | 0 | 4.826 | 5.415 | 5.845 |
| 2/17/76 | Bozeman | 0.4 | 5 | 4.556 | 5.301 | 5.544 |
| 2/17/76 | Bozeman | 0.4 | 10 | 4.322 | 5.230 | 5.447 |
| 2/24/76 | Bozeman | 0.6 | 0 | 4.431 | 5.415 | 5.230 |
| 2/24/76 | Bozeman | 0.6 | 5 | 4.204 | 5.255 | 5.204 |
| 2/24/76 | Bozeman | 0.6 | 10 | 4.146 | 5.041 | 5.041 |
| 3/1/76 | Trident | 0.1 | 0 | 4.041 | 4.279 | 4.114 |
| 3/1/76 | Trident | 0.1 | 5 | 3.991 | 4.255 | 4.663 |
| 3/1/76 | Trident | 0.1 | 20 | 3.978 | 4.230 | 4.544 |
| 3/2/76 | Amherst | 1.5 | 10 | 1.342 | 2.580 | 3.898 |
| 3/2/76 | Amherst | 1.0 | 12 | 1.301 | 2.663 | 3.964;3.342; 4.204[c] |
| 3/10/76 | Amherst | 1.25 | 10 | 1.477 | 3.050 | 3.964 |
| 3/10/76 | Amherst | 1.25 | 15 | 1.000 | 1.301 | 2.431 |
| 3/10/76 | Amherst | 1.25 | 20 | 0.602 | 1.380 | 1.301 |
| 3/11/76 | Amherst | 0.75 | 11 | 1.602 | 3.825 | 4.544 |
| 3/11/76 | Amherst | 0.75 | 15 | 1.301 | 2.806 | 3.690 |
| 3/11/76 | Amherst | 0.75 | 20 | 0.778 | 1.477 | 3.380 |
| 3/16/76 | Amherst | 0.5 | 20 | 1.000 | 2.362 | 2.519 |
| 3/18/76 | Sunderland | 1.5 | 0 | 0.301 | 1.892 | 1.519 |
| 3/22/76 | Sunderland | 1.5 | 0 | 2.845 | 3.792 | 3.544 |
| 3/22/76 | Sunderland | 1.5 | 0 | 1.415 | 2.706 | 3.380 |
| 3/23/76 | Northampton | 1.2 | 20 | 1.204 | 2.759 | 3.519 |
| 3/23/76 | Northampton | 1.2 | 25 | 2.021 | 2.593 | 3.732 |
| 3/23/76 | Sunderland | 0.5 | 0 | 1.681 | 2.556 | 3.041 |
| 3/30/76 | Sunderland | 1.6 | 0 | 0.778 | 1.556 | 2.146 |
| 3/30/76 | Sunderland | 1.3 | 0 | <1.000 | 1.820 | 1.322 |
| 4/6/76 | Greenfield | 1.0 | 0 | 0.602 | 0.301 | 1.041 |
| 4/6/76 | Sunderland | 1.2 | 0 | 1.763 | 2.714 | 3.041 |
| 4/6/76 | Sunderland | 1.5 | 0 | | 1.447 | 1.898 |
| 4/6/76 | Millers Falls | 1.5 | 0 | 1.000 | 1.505 | -0.7 |
| 4/5/76 | Bozeman | 0.1 | 0 | 5.519 | 5.944 | 6.342 |
| 4/5/76 | Bozeman | 0.1 | 5 | 5.362 | 5.863 | ≥ 6.380 |
| 4/5/76 | Bozeman | 0.1 | 20 | 5.322 | 5.771 | 5.964 |
| 4/19/76 | Bozeman | 0.15 | 0 | 5.566 | 5.982 | 6.380 |
| 4/19/76 | Bozeman | 0.15 | 10 | 5.431 | 5.892 | 5.447 |
| 4/19/76 | Bozeman | 0.15 | 20 | 5.602 | 5.987 | 5.964 |
| 4/15/76 | Sunderland | 0.8 | 0 | 3.215 | 3.483 | 3.845 |
| 4/15/76 | Sunderland | 1.3 | 0 | 2.164 | 3.024 | 3.732 |
| 4/20/76 | Sunderland | 0.25 | 0 | 2.944 | 3.763 | 3.845 |
| 4/20/76 | Sunderland | 1.5 | 0 | 1.505 | 2.450 | 2.519 |
| 4/26/76 | Sunderland | 2.0 | 0 | 0.505 | 0.857 | 0.903 |
| 4/26/76 | Sunderland | 1.0 | 0 | 3.373 | 3.587 | 2.380 |
| 4/29/76 | Sunderland | - | 0 | 1.000 | 1.663 | 2.362 |
| 4/29/76 | Sunderland | - | 0 | 1.301 | 2.380 | 2.663 |
| 4/29/76 | So. Deerfield | 1.6 | 0 | 1.447 | 1.806 | 2.230 |
| 5/3/76 | Sunderland | 1.5 | 0 | 0.602 | 1.342 | 1.342 |
| 5/4/76 | Sunderland | 0.8 | 0 | 1.602 | 2.000 | 2.690 |
| 5/4/76 | Sunderland | 1.3 | 0 | 1.973 | 2.310 | 2.519 |

a. All samples were chlorinated in the plant for a contact time which depends upon the volume and flow rate in the plant chlorinator; the additional contact time listed here is the time between taking the chlorinated sample and neutralizing it with thiosulfate.

b. Each figure represents the logarithm of the average of 5 replicate membranes.

c. Additional numbers are replicate 5-tube MPN's.

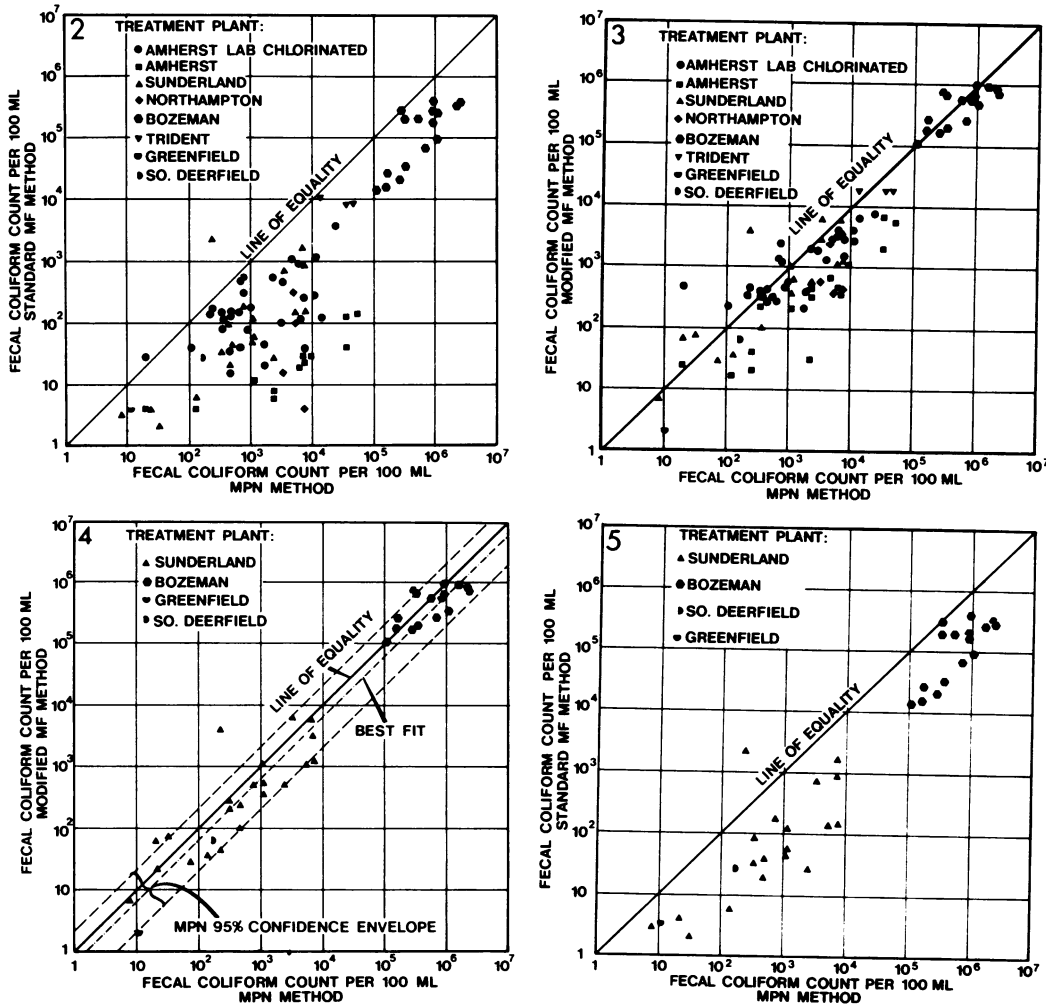


FIG. 2. *Comparison of standard MF method with MPN method. Data from laboratory-chlorinated and plant-chlorinated primary and secondary effluents.*

FIG. 3. *Comparison of modified MF method with MPN method. Data from same samples as for Fig. 2.*

FIG. 4. *Comparison of modified MF method with MPN method. Data from chlorinated effluents from secondary treatment plants only.*

FIG. 5. *Comparison of standard MF method with MPN method. Data from same samples as for Fig. 4.*

TABLE 4. *Verification of fecal coliform recovered by modified MF method*

| Source | Colonies picked (no.) | Colonies confirmed (no.) | Verification rate (%) |
|---|---|---|---|
| Amherst[a] | 1,356 | 1,241 | 92 |
| Amherst[b] | 296 | 296 | 100 |
| Northampton | 160 | 154 | 96 |
| Sunderland | 234 | 219 | 94 |
| Trident | 1,008 | 922 | 92 |
| Bozeman | 79 | 78 | 99 |

[a] Laboratory chlorinated.
[b] Plant chlorinated.

density are known to vary over a 10-fold range on identical samples (9, 10). Membrane filtration with M-FC agar provides a direct bacterial count and requires only 24 h to complete, whereas the MPN test requires 2 to 4 days before final results are obtained.

Referring again to Fig. 4, it is possible that the scatter of points around the best-fit line is due primarily to the inherent variability of the MPN. MPN values are known to follow a normal log distribution, with a standard deviation, $\sigma$ (log), of 0.25 for a five-tube test (10). On Fig. 4 we have represented this predicted variability

as a 95% confidence envelope drawn at $\pm 1.96\ \sigma$, or $\pm 0.48$ log cycle from the best-fit line. Of the 40 points on the graph, five fall outside this envelope, four of these being very close to its boundaries. Thus, the observed scatter does agree with that predicted from MPN theory.

The best-fit line on Fig. 4 lies below the line of equality by 0.17 log unit, indicating an average modified MF recovery of 68% of the MPN. However, it is known both theoretically (9) and experimentally (5) that MPN estimates are positively biased. Thomas (9) gave a factor of 0.851 to correct for the positive bias in five-tube tests, and Lin (3) used this factor in data analyses similar to Fig. 5. Multiplying each MPN by 0.851 has the effect of moving the best-fit line 0.07 log unit closer to the line of equality. The discrepancy is then 0.10 log unit, which corresponds to 79% recovery.

In conclusion, our studies indicate that, whereas the standard MF technique for fecal coliforms from chlorinated effluents compares unfavorably with the MPN method, a simple modification in the incubation procedure greatly enhances recovery. This modification, a 5-h incubation at 35°C, results in fecal coliform counts approximating the MPN in laboratory-chlorinated sewage effluent and plant-chlorinated secondary effluent. Development of a temperature-programmed incubator to make the change from 35 to 44.5°C after the 5-h preincubation period would eliminate any inconvenience to laboratory personnel and provide a practical method for analysis of samples containing injured fecal coliform populations.

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