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# Evaluation of Recovery Methods to Detect Coliforms in Water

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Various recovery methods used to detect coliforms in water were evaluated by applying the membrane filter chamber technique. The membrane filter chambers, containing pure-culture suspensions of *Escherichia coli* or natural suspensions of raw sewage, were immersed in the stream environment. Samples were withdrawn from the chamber at regular time intervals and enumerated by several detection methods. In general, multiple-tube fermentation techniques gave better recovery than plating or membrane filtration procedures. The least efficient method of recovery resulted when using membrane filtration procedures, especially as the exposure period of the organisms to the stream environment increased. A 2-h enrichment on a rich, nonselective medium before exposure to selective media improved the recovery of fecal coliforms with membrane filtration techniques. Substantially enhanced recoveries of *E. coli* from pure-culture suspensions and of fecal coliforms from raw-sewage suspensions were observed when compared with recoveries obtained by direct primary exposure to selective media. Such an enrichment period appears to provide a nontoxic environment for the gradual adjustment and repair of injured cells.

Much work has been directed toward improving the sensitivity and selectivity of specific bacterial detection methods in an attempt to maximize the recovery of indicator organisms from aquatic environments. Bissonnette et al. (2) reported that prolonged exposure of pure cultures of *Escherichia coli* or *Streptococcus faecalis* to certain aquatic environments frequently resulted in a substantial loss in recovery of these organisms upon application of selective enumeration media when compared with detection with rich, nonselective media. Problems with the recovery of *E. coli* from chlorinated secondary sewage have recently been reported by Braswell and Hoadley (3). Unchlorinated secondary sewage effluent was autoclaved, inoculated with a pure culture of *E. coli* ATCC 27622, and then chlorinated with NaOCl. The researchers observed a substantial loss in recovery of *E. coli* ATCC 27622 when using the fecal coliform standard-methods membrane filtration procedure (1) as opposed to the total viable count obtained on Trypticase soy agar spread plates. Apparently the discrepancies in colony-forming units between nonselective and selective media indicate that a significant portion of bacterial cells become physiologically injured due to the environmental stress imposed by the aquatic environment.

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It has also been reported that a nonlethally injured population of *E. coli* was capable of repairing such injury received in the aquatic environment if exposed to a rich, nonselective broth before application of specific selective media (2). This capability of repair lends support to those workers (4, 8, 9, 11, 15) who have suggested enrichment techniques to improve recovery of indicator organisms from various aquatic environments. In the present research, membrane filter chambers of McFeters and Stuart (12) were used to examine the recovery efficiency of several enumeration methods used to detect coliforms in water as a function of exposure time in the aquatic environment. Pure-culture suspensions of *E. coli* and natural populations of bacteria found in raw sewage were exposed to the natural stream environment for timed intervals, and their recovery was compared by using conventional membrane filtration, pour plate, and most-probable-number (MPN) techniques as well as an enrichment membrane filtration method.

## MATERIALS AND METHODS

**Source of test microorganism and raw sewage.** The pure culture used in this study was *E. coli* C32OMP25. The source and physiological identification of this organism have been presented elsewhere (2). Raw sewage was obtained at the Bozeman Wastewater Treatment Plant.

**Preparation of cell suspensions.** Preparation of pure-culture suspensions of *E. coli* C32OMP25 fol-

lowed previously outlined procedures (2). Description, sterilization, and aseptic assembly of the membrane filter chambers followed the procedures of McFeters and Stuart (12). For those experiments using natural samples, a 20-ml sample of raw sewage was loaded directly into the chamber without being pretreated. The filled chambers were suspended in stream water at a site located on the East Gallatin River, designated as site EG6 in a previous publication (2). A description of this site and sampling procedures has been presented (2).

**Enumeration methods for *E. coli* C32OMP25.** The three general methods simultaneously used to enumerate *E. coli* C32OMP25 were pour plate, MI'N, and membrane filtration. In the pour plate procedure, enumeration with a nonselective medium was accomplished by using Trypticase soy agar supplemented with 0.3% yeast extract and 0.5% glucose (TSY agar). Violet red bile (VRB) agar and deoxycholate lactose agar (DLA) were used as the selective solid media. Duplicate sets of plates were made for each dilution and medium used. All plates were overlayed 15 min after plating with an additional 5 ml of the same medium. The plates were inverted and incubated at 35°C for 24 h before counting.

Liquid-media enumeration procedures using the MPN method involved using three different broths. The rich, nonselective broth used was Trypticase soy broth supplemented with 0.3% yeast extract and sufficient glucose to make the final concentration 0.5% glucose (TSY broth). Other liquid media used were lactose (LAC) broth and brilliant green lactose bile (BGB) broth. A total of five tubes in each of five successive decimal dilutions were used in the MPN procedure. A 1-ml amount of the appropriately diluted sample was inoculated into a tube containing 9 ml of the medium. The tubes were incubated at 35°C for 48 h. The MPN determinations were estimated based on the tubes showing gas production for LAC and BGB broth and those showing growth in the nonselective TSY broth.

Membrane filtration procedures for the enumeration of *E. coli* C32OMP25 used three different media: TSY agar, m-endo MF agar, and m-FC medium. The procedure used for membrane filtration with these media followed *Standard Methods* (1). Membrane filters used were Millipore filters, type HAWG 047SO, with a mean pore size of 0.45 μm. Duplicate plates of all samples were run in each experiment. TSY and m-endo MF plates were incubated at 35°C for 24 h, whereas m-FC plates were sealed with waterproof tape, placed in waterproof plastic bags, and incubated in a water bath for 24 h at 44.5°C.

**Enumeration of coliforms and fecal coliforms in raw sewage.** The total coliform population in raw sewage was determined by using LAC MPN and m-endo MF membrane filtration procedures as described above. The presence of probable coliform colonies was confirmed by transferring isolated colonies from membrane filters to BGB broth. Plates were divided into quarters, and all suspect coliform colonies from a single quarter were picked into BGB broth. Also, BGB broth was inoculated from those presumptive tubes of LAC broth showing gas after 48 h. All BGB tubes containing gas within 48 h at 35°C were considered evidence that coliform organisms were present. Additionally, all positive BGB tubes were further analyzed for the presence of fecal coliforms by transferring the culture to EC medium followed by incubation at 44.5°C. Production of gas within 24 h in EC medium indicated the probable presence of coliforms of fecal origin and was used to calculate fecal coliform densities. The number of fecal coliforms was also determined by the membrane filtration method using m-FC medium as previously described.

**Enrichment procedures.** An enrichment procedure was used to determine whether improved recovery of fecal coliforms could be attained by exposing cells to a rich, nonselective medium before transfer of the organisms to a specific selective medium. The enrichment technique was used with both a pure culture of *E. coli* and a natural suspension of raw sewage.

After immersion of a membrane filter chamber containing a suspension of *E. coli* in the stream environment, 1-ml samples were withdrawn at the start of the experiment and after 1, 2, and 3 days of exposure to the stream water. Enumeration was done by membrane filtration methods using both the conventional direct technique and an enrichment technique. Duplicate filtrations were performed with all dilutions such that one set could be enumerated by the direct method and the other by an enrichment technique. The direct technique was done by transferring one set of the membrane filters to TSY agar, m-endo MF agar, and m-FC broth. The enrichment technique consisted of first placing the duplicate set of membrane filters in petri dishes containing pads soaked with 2 ml of TSY broth. These enrichment plates were then incubated upright for 2 h at 35°C. After this 2-h enrichment period, the filters were transferred from the TSY broth pads to the respective media used in the direct techniques, i.e., TSY agar, m-endo MF agar, and m-FC broth. Incubation temperatures and times have been previously described.

A suspension of raw sewage was analyzed in exactly the same manner as the pure culture of *E. coli*. In addition, probable coliform colonies from one quarter of the membrane filters were picked into BGB broth for confirmatory purposes. Those tubes showing the presence of gas within 48 h at 35°C were then further analyzed by transfer to EC medium and incubation at 44.5°C for 24 h. The results were used to calculate the fecal coliform densities obtained by the respective media and methods.

## RESULTS AND DISCUSSION

**Recovery of *E. coli* C32OMP25.** A summary of *E. coli* C32OMP25 recovery by various enumeration methods is presented in Table 1 as a function of the percent recovery on TSY pour plates. In this experiment, a chamber containing a suspension of *E. coli* at an original concentration of approximately $10^6$ cells/ml was immersed in the stream water at site EG6.

TABLE 1. *Comparison of recovery efficiencies of different enumeration methods for E. coli C32OMP25 with selective and nonselective media over a 3-day exposure period in the stream environment*

| Exposure time (days) | %[a] cells detected by: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pour plate | | | MPN | | | Membrane filtration | | |
| | TSY | DLA | VRB | TSY | LAC | BGB | TSY | m-Endo MF | m-FC |
| 0 | 100.0 | 93.8 | 93.8 | 137.5 | 137.5 | 106.3 | 106.3 | 106.3 | 87.5 |
| 1 | 100.0 | 96.8 | 87.3 | 174.6 | 125.4 | 125.4 | 93.7 | 42.9 | 22.2 |
| 2 | 100.0 | 3.5 | 1.5 | 65.0 | 85.0 | 16.5 | 95.0 | 0.3 | 0.3 |
| 3 | 100.0 | 0.1 | 0.1 | 159.1 | 159.1 | 5.9 | 131.8 | 0.1 | 0.0 |

[a] As a percentage of counts obtained on TSY pour plates.

Periodically, samples were withdrawn and analyzed simultaneously by MPN, plating, and membrane filtration methods. The most efficient recovery was obtained with the rich, nonselective TSY medium for all the methods followed by the LAC MPN procedure. The more highly selective BGB broth MPN procedure reflected an extremely rapid loss in recovery efficiency after the first day of exposure to the water. Membrane filtration using m-FC medium in conjunction with the elevated incubation temperature was least effective in recovering the viable population, since only 0.3% of the cells were detected by this method after 2 days of exposure in the stream. m-Endo MF medium was slightly more efficient in recovery than was m-FC medium. Pour plate procedures using either DLA or VRB agar reflected a rapid loss in recovery efficiency after the first day of exposure. It should be noted, however, that the use of 45°C agar in the pour plate overlay procedure may have contributed to the loss in recovery efficiency of this technique. Overall, MPN procedures gave the greatest recovery efficiency, membrane filtration procedures the least, and pour plate procedures intermediate. Caution should be observed, however, when comparing MPN values with results obtained by pour plate or membrane filtration techniques. Due to the built-in positive bias of the MPN index, the 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 on the same sample.

These results support previous data (2) indicating that upon exposure to the aquatic environment, a significant proportion of *E. coli* cells lose their ability to produce colonies on a selective medium, yet retain this ability on a nutritionally rich, nonselective medium when using plating procedures. Apparently, a substantial portion of the bacteria become nonlethally injured due to the environmental stress imposed by the aquatic environment, resulting in discrepancies in colony-forming units between nonselective and selective media. The present research indicates that even greater injury to *E. coli* is reflected by membrane filtration procedures, especially when applying the currently accepted elevated incubation temperature of 44.5°C (1). The commonly used LAC MPN procedure exhibited efficient recovery of the total population over the entire exposure period.

**Recovery of coliforms and fecal coliforms in raw sewage.** Comparison of recovery efficiencies of various enumeration methods was also conducted with natural suspensions of raw sewage. In one such experiment, a membrane filter chamber containing a suspension of raw sewage was immersed in the stream water and samples were withdrawn at 24-h intervals over a 2-day exposure period. A comparison of the recovery efficiencies of the various methods of enumerating total coliforms and fecal coliforms from this natural suspension of bacteria is presented in Table 2. The membrane filtration method using m-endo MF medium was less effective in detecting the total coliform population than was the MPN procedure.

Detection of fecal coliforms from raw sewage was also less efficient when using membrane filtration methods as opposed to MPN procedures. The direct enumeration of fecal coliforms by elevated temperature was the least efficient recovery method. A two-step membrane filtration method using m-endo MF medium followed by identification of fecal coliforms with EC medium somewhat improved recoverability of fecal coliforms.

Other workers have observed significantly poorer recoveries of indicator organisms by membrane filter techniques than by MPN methods, especially when examining toxic wastes or chlorinated effluents. McKee et al. (13) demonstrated substantially lower recovery of coliforms from chlorinated settled sewage

TABLE 2. *Comparison of recovery efficiencies of different enumeration methods for coliforms and fecal coliforms from a suspension of raw sewage over a 2-day exposure period in the stream environment*

| Exposure time (days) | % cells detected by given method[a] | | | | |
|---|---|---|---|---|---|
| | Coliforms[b] | | Fecal coliforms[c] | | |
| | m-Endo MF MF[d] | LAC MPN | m-Endo MF MF | LAC MPN | m-FC MF |
| 0 | 100.0 | 150.3 | 100.0 | 165.4 | 75.7 |
| 1 | 100.0 | 118.6 | 100.0 | 135.9 | 53.4 |
| 2 | 100.0 | 307.4 | 100.0 | 537.5 | 48.9 |

[a] As a percentage of counts obtained by m-endo MF.
[b] Counts based on confirmation in BGB broth.
[c] Counts based on confirmation in BGB broth and EC medium.
[d] Membrane filtration.

when using membrane filter techniques as compared with accepted MPN procedures. Lin (10) also observed superiority of the MPN procedure over the membrane filter technique when attempting to detect fecal coliforms from chlorinated secondary effluents. Similarly, Shipe and Cameron (18) demonstrated superior recovery of coliforms by MPN procedures as opposed to membrane filter techniques when examining river water containing toxic wastes. The authors reasoned that the poorer recovery on membrane filters could be due to an accumulation of toxic substances on the filter, or that "weakened cells" might recover more readily in the environment of broth than under the conditions found on a membrane filter. The data in the present research corroborate these earlier findings and further indicate that the discrepancies in recovery on some selective media become more pronounced as the exposure time in the aquatic environment increases.

**Enrichment experiments.** Due to the low precision, prolonged analysis time, and other inherent limitations of the MPN technique, attempts have been made to improve the recovery efficiency of membrane filtration techniques. Observations by Bissonnette et al. (2) have shown that the injury acquired by a population of *E. coli* when exposed to the aquatic environment can be rapidly repaired in a rich, nonselective broth medium. This resuscitation period allowed the injured subpopulation of cells to repair themselves such that they became insensitive to the later application of selective media containing various inhibitory agents. Application of enrichment techniques to improve the recovery of coliforms, fecal coliforms, and fecal streptococci has been advocated by several workers (4, 8, 9, 11, 15, 16). Recently, Lin (10) demonstrated that an enrichment LES two-step membrane filtration procedure significantly improved recovery of total coliforms from chlorinated secondary effluents as compared with a one-step method.

In the present research, an enrichment procedure was compared with the conventional membrane filtration direct procedure for the recovery of *E. coli* C32OMP25 while exposed to the stream environment (Fig. 1). The application of direct primary exposure to the elevated temperature of 44.5°C in the presence of m-FC medium was found to severely limit the recoverability of *E. coli* as evidenced by the fact that the recovery of the total viable population was approximately 23% after 1 day, 32% after 2 days, and less than 1% after 3 days of exposure to the stream environment. Enrichment of bacteria on membrane filters with TSY medium for 2 h before transfer to m-FC medium at 44.5°C improved the recovery of *E. coli* by more than twofold after all exposure periods in the

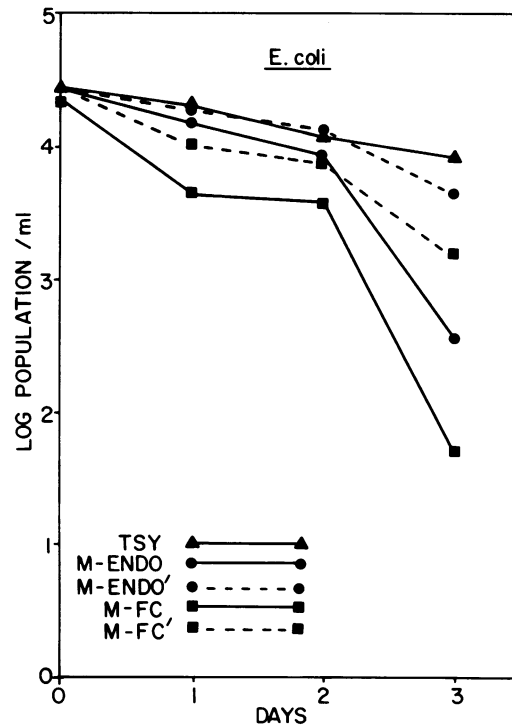


FIG. 1. *Comparison of direct and enrichment membrane filtration techniques for the recovery of a suspension of E. coli C32OMP25 during a 3-day exposure period to the stream environment. Direct techniques (——) consisted of the transfer of membrane filters to TSY, m-endo MF, and m-FC media. Enrichment techniques (-----) consisted of a 2-h incubation on TSY medium before transfer of filters to m-endo MF and m-FC media.*

stream water. The direct use of m-endo MF medium also inhibited the recovery of *E. coli* as compared with TSY medium; yet primary exposure to m-endo MF medium was found to be less harmful than direct use of m-FC medium. A 2-h enrichment period before the transfer of filters containing bacteria to m-endo MF medium substantially improved recovery of stressed cells of *E. coli*. Sufficient repair of the injured cells occurred during the enrichment period such that essentially equal recoverability was observed on m-endo MF medium as compared with TSY medium during the first 2 days of exposure to the aquatic environment. For all exposure periods, enrichment on TSY medium followed by transfer to m-endo MF medium was the most efficient selective method studied to recover *E. coli*.

Recovery efficiency of direct and enrichment methods for fecal coliforms from raw sewage is depicted in Fig. 2. A 2-h enrichment on TSY medium before application of selective m-endo MF medium substantially enhanced the recovery of fecal coliforms over that observed by direct enumeration using m-endo MF medium. Similarly, enrichment before transfer of filters to m-FC medium incubated at the elevated temperature resulted in improved recoverability of fecal coliforms in comparison to recovery efficiency using direct exposure to m-FC medium at 44.5°C. Throughout the entire 3-day exposure period of the raw sewage to the stressful environment of the stream water, the most efficient method of recovering fecal coliforms was a 2-h enrichment on TSY medium followed by transfer to m-endo MF medium. The least efficient method was observed when using the membrane filtration procedure with m-FC medium incubated at 44.5°C.

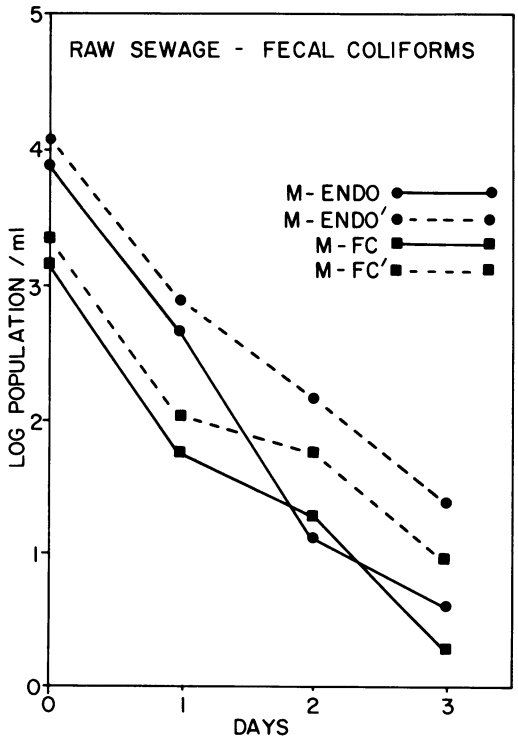


FIG. 2. *Comparison of direct and enrichment membrane filtration techniques for the recovery of fecal coliforms from a suspension of raw sewage during a 3-day exposure period to the stream environment. Direct techniques (——) consisted of the transfer of membrane filters to m-endo MF and m-FC media. Enrichment techniques (-----) consisted of a 2-h incubation on TSY medium before transfer of filters to m-endo MF and m-FC media.*

A distinct limitation of enrichment techniques such as those used here is the fact that considerably more time, equipment, and manpower are required for analysis as compared with direct techniques. Recently, however, Rose et al. (15) have minimized these limitations by proposing a two-layer agar membrane filter procedure that allows for repair and subsequent reproduction of those fecal coliforms that have been debilitated by exposure to natural or toxic waters. Use of this two-layer medium procedure automatically shifts the culture contact from an enrichment growth substrate to the differential medium phase. Although direct plating procedures are not currently accepted standard methods to detect coliforms in water, Hartman et al. (6) have reported substantially improved recovery of stressed coliforms from water by using a modified direct-plating procedure whereby plates containing inocula are poured with a basal medium that contains all the ingredients of VRB agar except bile salts and dyes. The sterile overlay consisted of VRB agar containing double the usual concentrations of bile salts no. 3, neutral red, and crystal violet.

An important factor to be considered when using membrane filtration methods concerns the reported discrepancies in recovery of coliforms and fecal coliforms when using different brands of membrane filters (5, 7, 14, 17). Recently, Sladek et al. (19) presented evidence that the primary determinant affecting fecal coliform recovery was the surface pore morphology of the membrane filter rather than the chemical composition or method of sterilizing the membrane filter. These workers observed that fecal coliform counts showed a dramatic increase with increasing surface opening sizes, resulting in the development of specially designed membrane filters exhibiting a surface

opening diameter of 2.4 μm and a retention pore size of 0.7 μm.

The data in the present research demonstrate the need for an awareness of the limitations of currently accepted methods used to detect indicator organisms in water. Through the use of membrane diffusion chambers it was possible to follow the recovery phenomena of several methods and media used to detect indicator organisms as a function of exposure time in the aquatic environment. Since significant proportions of cells may become physiologically injured or "weakened" during exposure to certain aquatic environments, it is evident that special precautions are required to ensure maximal recovery. The recent investigations into membrane filter structure and composition as well as the development of more economical and rapid enrichment techniques should considerably enhance the recovery of these organisms from aquatic environments.

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