Optimum Membrane Structures for Growth of Coliform and Fecal Coliform Organisms

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The purpose of this study was to determine the optimum membrane filter structure and characteristics for recovery of coliform organisms. Additionally, other factors such as sterilization method and membrane composition were examined. Fecal coliform growth tests with varied samples indicated that the most critical factor in recovery was surface pore morphology and not other factors previously suspected. Fecal coliform counts showed a dramatic increase, with increasing surface opening sizes. Membrane structures with surface openings large enough to surround the entrapped bacteria are required for optimum growth of fecal coliform organisms. Maximum fecal coliform recoveries are obtained using membranes composed of mixed esters of cellulose exhibiting a surface opening diameter of 2.4 μm and a retention pore size of 0.7 μm.

Since its introduction as a tentative method for coliform enumeration in the 10th edition of Standard Methods in 1955 (1), the membrane filter has gained wide usage not only for total coliform, but also for fecal coliform, total bacteria, and a variety of other bacterial tests. The unique advantage of the membrane over other test methods is its ability to concentrate and localize bacteria from large sample volumes. Hence, the membrane increases the sensitivity of quantitative bacteriology into the range well below one organism/ml. Once the bacteria are localized, the membrane provides a structure for counterdiffusion of nutrients and metabolic products as well as a " hospitable" growth environment. In these functions, the membrane differs little from the earlier pour- and streak-plate methods.

The earliest techniques for bacteriological analysis with membrane filters involved direct microscopic examination of bacteria trapped on the membrane surface. Here, the optimum structure required pores smaller than the organisms being trapped for examination, so that they would lie in a single microscopic plane. This surface planar retention facilitated finding the organisms under high-power microscopy. The above requirements evolved naturally to the practice of retaining organisms on the membrane surface for various culture techniques. At that time, not much thought was given to developing an optimal membrane structure for colony growth.

The ideal characteristics of a membrane for quantitative bacteriology would appear to be pores small enough to retain bacteria but open enough to provide optimal diffusion of media and a hospitable surface for growth. However, upon examining the variety of bacterial methods utilizing membranes, one finds a considerable range of bacteria sizes, types, and metabolic requirements. These considerations led us to wonder if it was possible to develop membranes which would be especially favorable for the growth of particular types of organisms, such as coliform group.

The critical step in development of a colony from a single bacterium is the onset of cellular division, and it is not unreasonable to postulate that this process could be affected by the extent and nature of the contact of the organism with the solid portion of the membrane filter and the extent and thickness of the nutrient film surrounding the organism. Further, nutrient supply by diffusion of medium and removal of subsequent metabolic waste products must be a function of membrane structure and pore morphology.

With these factors in mind, we began this study with the objective of defining the optimum membrane structure for growth of coliform bacteria.

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MATERIALS AND METHODS

Several types of membranes were used in this study. There were type MF mixed esters of cellulose (Millipore Corp.), type GA cellulose acetate (Gelman Instrument Co.), and a noncellulosic polyaryl...
ester membrane which is not available commercially. The surface structures of these were characterized using a Coates & Welter CWISSCAN 100-4 scanning electron microscope. Before observation, the membranes were coated with 1- to 2-nm layer of gold.

Fecal coliform and total coliform determinations were performed in accordance with Standard Methods (2), sections 408 A and B, with the following modifications. To achieve the closest possible similarity between membrane tests and streak plate controls, the membranes were plated on a 0.34-cm thickness of agar medium in 47-mm petri dishes; each streak plate was prepared by spreading a 0.1-ml aliquot of sample onto a 0.34-cm thickness of agar in a 90-mm dish. The reason for using a controlled thickness of agar is that we had found, in earlier experiments, that fecal coliform recovery is a function of agar thickness.

M-FC broth and M-Coliform broth were obtained from the BioQuest Division of Becton, Dickinson, and Co. Agar (15 g/liter) was added when the media were prepared. Agar plates were stored at 5 C and were used within 48 h of preparation. Fecal coliform plates were incubated at 44.5 ± 0.2 C in Blue M water baths equipped with calibrated recording thermistors. Total coliform plates were incubated at 35 ± 0.5 C in circulating air incubators.

Most of the water samples were untreated sewage, obtained from the masher section of the Billerica, Mass., Sewage Treatment Plant. River samples were also used. Samples were stored at 5 C and were used within 30 h of collection.

Some refinements of technique were needed to make it possible to run experiments involving large numbers of samples. Initially, it was found that noticeable die-off occurred in 15 min when the source water was diluted with phosphate buffer. The use of 0.1% buffered peptone, however, stabilized the count for a period of 1 h. It was also found to be important to restrict the time between plating and incubation to 15 min or less. The complete procedure was then as follows. A preliminary count was obtained when the sample was taken. The following day, a dilution was prepared to give a count of 200 to 1,000 bacteria/ml, using buffered peptone diluent. The diluted sample was mixed for 30 min on a mechanical shaker. Then groups of about 18 membranes and nine streak plates were prepared from 0.1-ml aliquots, plated, and incubated. This was repeated throughout the experiment. Using this method, up to 100 membranes plus associated streak plate controls could be run within the 1-h limit. To confirm fecal coliforms, typical blue colonies were transferred into lauryl tryptose broth and then into EC broth.

Surface pore morphology. Membrane filter structure can be characterized by several parameters. The retention pore size is a measure of the smallest particle which is retained by the structure and is best measured by direct determination of passage of particles (or microbes) of known size. This technique is described by Rogers and Rossmoore (6).

In the present investigation, we were interested not only in bacterial retention but also in how the bacteria are situated on the membrane. It is reasonable to expect that the environment of retained bacteria depends on the retention pore size as well as the structure of the surface layer in which they are retained.

Figure 1 gives scanning electron photomicrographs of a series of eight membranes made from mixed esters of cellulose. The photomicrographs show similar structures which differ only in the size of the openings. In each photomicrograph relatively large surface openings can be seen overlying a system of finer pores. The large surface openings were characterized by the surface opening diameters reported on the figure. These were determined by direct measurements on each photomicrograph or, in the case of the smaller size openings, by measuring enlargements of the photomicrographs. The retention characteristics of these membranes for coliform organisms were determined by passage tests, as described in the following section.

In summary, the way in which bacteria are situated on a membrane is determined by a new parameter, the surface opening diameter, which is observable from scanning electron photomicrographs. The retention of bacteria is determined by the more familiar retention pore size, which is found from passage tests.

RESULTS AND DISCUSSION

Figure 2 shows fecal coliform counts on the series of membranes described above. There is a remarkable increase in counts at surface opening diameters between 1.0 and 2.0 μm. The decrease in counts at the largest opening size is evidently due to passage of organisms through this very coarse structure. The dotted line labeled "passage" was obtained by refiltering the effluent through a 0.45-μm retention pore size membrane and plating this membrane on M-FC agar in the usual way. On the basis of both growth and passage tests, the optimum membrane structure was determined to have a 2.4-μm surface opening diameter with smaller (fecal coliform retentive) voids of approximately 0.7 μm internally. Results of this plus three other fecal coliform runs are given Fig. 3. In all four runs, the abrupt increase in recovery at a surface opening diameter of 1.0 to 2.0 μm is evident, with the optimum structure, i.e., zero passage and optimum growth, occurring with 2.4-μm surface openings.

Typical blue colonies were picked for confirmation from the 0.7-, 1.4-, and 2.4-μm surface opening membranes. The ratios of confirmed/picked were 18/20 for the 0.7-, 19/20 for the 1.4-, and 17/20 for the 2.4-μm surface opening membrane.

Figure 4 presents the results of two total coliform experiments on the same series of filters. Here, only a slight effect may be observed occurring only at the smallest and largest sur-
Fig. 1. Scanning electron micrographs of a series of mixed esters of cellulose membranes. Numbers shown are surface opening diameters.
Fig. 2. Fecal coliform count versus surface opening diameter. Each count was obtained from a 0.1-ml aliquot of a single sewage sample.

Fig. 3. Normalized fecal coliform counts for four different water samples. To put results of four water samples onto a single graph, the count on each membrane was divided by a scale factor. For a given water sample, the scale factor was the mean count on the 2.4-µm surface opening membrane.

face opening sizes. Evidently, whereas the fecal coliform test requires a surface opening diameter of 2.4 µm of optimum growth, the total coliform test is less demanding and works well on membranes of surface opening diameter in the range of 1 to 3 µm.

At this point, it appeared that surface opening diameter was definitely a primary determinant of fecal coliform recovery. However, other factors, such as chemical composition and methods of sterilization, remained to be investigated.

Effect of chemical composition. In the foregoing set of tests, membranes employed were composed of mixed esters of cellulose. A second series of experiments was designed employing cellulose acetate membranes. Cellulose acetate has a much smaller affinity for proteins, and presumably bacteria, than does the mixed esters material used in the previous test. Thus, if surface adhesion affects growth, a difference between the acetate and mixed esters results should be evident.

In the next experiment, recovery on the 2.4-µm (surface opening) mixed cellulose esters membrane was compared with that of a 3.8-µm (surface opening) cellulose acetate membrane. In addition, an experimental polyaryl ester (noncellulosic) membrane composition having 3.0-µm-diameter surface openings was included. Results are given in Fig. 5. The actual counts on each five replicates are plotted here, as well as passage counts obtained by refiltering the effluents from each. The results show no significant difference in count between the three membrane compositions, suggesting that membrane composition is not an important factor in fecal coliform recovery.

Effect of sterilization method. Several authors (3, 5) have suggested that bacterial recov-
eries may be affected by the method of sterilization. They did not, however, present data derived from comparing identical membranes, where the only variable was the method of sterilization. To test for possible sterilization effects, membranes were selected from the group exhibiting optimum growth characteristics (2.4-μm surface openings). These membranes were then divided into four groups using random sampling techniques. One group was left unsterilized, one was autoclaved at 121 C for 15 min, one was exposed to ethylene oxide using a standard sterilization cycle (the cycle used a 2-h exposure to 12% ethylene oxide at 130 F [54.5 C] and 60% relative humidity [4]) and was aerated 3 days, and the fourth group was sterilized by irradiation at a dose of 1.0 megarads using gamma rays from a cobalt 60 source. Mean counts and 95% confidence limits on the means are given in Table 1. There are no significant differences between counts on the unsterilized membranes and counts on the membranes sterilized by the three methods used.

The data collected to this point strongly suggest that neither chemical composition nor method of sterilization have any significant effect, but that the primary determinant of fecal coliform growth on a membrane filter is that of the surface pore morphology (specifically with respect to the size of upper surface openings).

We speculated that since surface effects are strongest at surface void sizes which are close to coliform dimensions, some sort of fit of the organism into the pore might be required for optimum growth. To visualize the fit of the organism into the surface structure, electron photomicrographs of an Escherichia coli isolated from sewage on the 2.4-μm surface opening cellulose ester membrane were obtained. Figure 6 shows a field in which it appears that an organism has penetrated into a large surface void.

**Effect of nutrient supply.** Concerning fecal coliform recovery, the mechanism of the effect could be that organisms which are deposited on very fine surface structures are incompletely surrounded by nutrient, whereas ones that fit into surface openings can be cradled below the level of nutrient that is drawn up by capillary forces. Because of evaporation, an incompletely surrounded bacterium might be subjected to a locally hypertonic solution, with resulting plasmolysis and death. This effect would be particularly evident at the elevated temperature (44.5 C) of the fecal coliform test.

To test this hypothesis, three methods of supplying nutrient were compared. The 0.7-μm and the optimum 2.4-μm surface opening cellulose ester membranes were used. One set was plated in the standard manner, one set was plated face down on the M-FC agar, and the third set was plated right side up with 2.0 ml of M-FC agar overlayed onto each membrane. Results are summarized in Table 2.

Due to the confluence of colonies, accurate counts could not be obtained from the membranes plated face down. However, it was clear that the number of colonies on membranes having the smaller surface openings (0.7 μm) was substantially increased by plating face down. Overlaying these membranes gave a dramatic increase in counts. The increase in growth thus seen from inverting the filter, plus the close agreement in counts of the two membrane groups when the lower yield filters were overlayed with nutrient, give strong evidence that complete nutrient coverage of the organisms is required and that this is achieved only with larger surface opening sizes.

During the comparison testing of the membranes for the 0.7- and 2.4-μm surface opening groups, some additional benefits were noted relative to the latter. These predictable, but nonetheless important, phenomena were an increase in the flow rate through the membrane, an increased diffusion rate of media to the membrane surface, and, significantly, an increase capacity to filter larger volumes of water, particularly those where algae or other colloidal turbidity would otherwise limit the sample size.

In summary, the factors expected to have an effect on fecal coliform recovery were investigated. The only one showing a significant effect was that of surface pore morphology. The evidence suggests that fecal coliforms must be cradled slightly below the membrane surface for optimum recovery at 44.5 C. This suggests an optimum membrane structure, with surface pores slightly larger than the fecal coliform organisms but with internal bacterial retentive pores.

### Table 1. Effect of sterilization on mixed cellulose ester membranes having 2.4-μm surface opening diameter

| Method        | Total coliform count* | Fecal coliform count* |
|---------------|-----------------------|-----------------------|
| Unsterilized  | 42 ± 6                | 99 ± 9                |
| Ethylene oxide sterilized | 44 ± 6                | 103 ± 9               |
| Autoclaved    | 38 ± 6                | 108 ± 9               |
| Irradiated    | 40 ± 6                | 94 ± 8                |
| Streak plate  | 50 ± 6                | 82 ± 6                |

* Two different sewage samples were used. Each mean is an average of five replicates.
Until now, membranes recommended for bacterial testing have been specified by a retention pore size of 0.45 μm. Typical 0.45-μm retention membranes have surface opening diameters of 1 to 2 μm. A slight shift of position on the curve in the range of 1- to 2-μm surface openings can have a large and significant effect on recovery (Fig. 2 and 3).

Since membranes of different manufacture, all having 0.45-μm retention size, may exhibit differences in surface morphology (i.e., in relative surface opening diameters), they may also exhibit considerable differences in fecal coliform recovery.

A change to the optimum 2.4-μm surface

**Fig. 6.** Scanning electron micrograph of E. coli on 2.4-μm surface opening diameter cellulose ester membrane.

**Table 2. Effect of plating method and pore size on fecal coliform test using sewage sample**

| Method of plating                  | Mean counts and 95% confidence limits |
|-----------------------------------|----------------------------------------|
|                                   | 0.7-μm surface openings  | 2.4-μm surface openings |
| Membrane plated in standard manner | 14 ± 3                        | 44 ± 10                  |
| Membrane inverted on agar         | 30±                          | 45±                      |
| Membrane plated and overlayed     | 46 ± 7                        | 53 ± 8                   |
| Streak plate                      | 35 ± 5                        |                          |

* Approximation.
opening size will not only provide higher fecal coliform counts, but will also lead to a smaller sensitivity to small differences in surface morphology.

For the total coliform test (Fig. 4), however, membrane performance is not sensitive to surface morphology (except in the range below 1-μm surface opening size). The new 2.4-μm surface opening/0.7-μm retention pore size membrane developed in this work should be regarded as an improvement for fecal coliform tests and may also be used for total coliform, with results equivalent to 0.45-μm retention membranes.

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LITERATURE CITED
1. American Public Health Association. 1955. Standard Methods for the examination of water and wastewater, 10th ed. American Public Health Association, Inc., New York.
2. American Public Health Association. 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Inc., New York.
3. Dutka, B. J., M. J. Jackson, and J. B. Bell. 1974. Comparison of autoclave and ethylene oxide-sterilized membrane filters used in water quality studies. Appl. Microbiol. 28:474-480.
4. Kereuk, K., and R. S. Lloyd. 1969. Ethylene oxide sterilization. J. Hosp. Res. 7:7-75.
5. Presswood, W. C., and L. R. Brown. 1973. Comparison of Gelman and Millipore membrane filters for enumerating fecal coliform bacteria. Appl. Microbiol. 26:332-336.
6. Rogers, B. G., and H. W. Rossmoore. 1970. Determination of membrane filter porosity by microbiological methods. Dev. Ind. Microbiol. 11:453-459.