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Comparison of Autoclave and Ethylene Oxide-Sterilized Membrane Filters Used in Water Quality Studies

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Autoclave and ethylene oxide-sterilized membrane filters manufactured by Gelman, Millipore, and Sartorius were field tested for their recovery of total coliforms, fecal coliforms, fecal streptococci, and heterotrophs. The data were analyzed by using split-plot analysis of variance and significance tests. Membranes were also tested for pH and toxicity using *Escherichia coli*. The mean data summaries indicated that Gelman membrane filters generally produced the highest counts during the field studies. Statistical analyses of the March data showed that there were significant differences between membrane filters at 1% level; however, statistical analyses of June data revealed no significant differences except in total coliform recoveries. Toxicity tests at 35 C indicated that Gelman and Millipore autoclaved membrane filters were able to recover 92% of the test organisms. Toxicity tests performed at 44.5 C revealed that no membranes were able to recover more than 40% of the test organisms. Since differences were found in the ability of the three brands of membrane filters to recover bacteria from natural and controlled sources, membrane filters from different manufacturers cannot be readily interchanged. There is a need for a standardized procedure for testing bacterial recovery by membrane filters.

With the acceptance of the membrane filter technique as an official method of evaluating water quality (1, 3, 4, 7), the microbiologist is faced with the dilemma of selecting a membrane filter from a variety of commercially available filters, each with its own brand name and characteristics. Production processes and quality control test procedures for determining pore characteristics, such as air flow rates, water flow rates, and bubble point, vary from manufacturer to manufacturer. In addition, there are no standardized procedures to ensure retention and propagation of microorganisms.

In comparative studies of membrane filters by Levin et al. (5) and Presswood and Brown (6), differences were found in the relative efficiency of membrane filters in supporting the growth of coliforms from pure culture and river water. If these differences are routine, then the validity of some water quality assessments is in doubt, and serious consideration must be given to the failure of some manufacturers to comply with standard membrane filter specifications (1).

Since doubts have been raised on the recovery of microorganisms by the various commercially produced membrane filters, a field study was initiated to compare and assess the performance of some commonly used membrane filters sterilized by autoclave and ethylene oxide procedures.

MATERIALS AND METHODS

Sampling. Water samples were collected from three sites: Paris and Brantford on the Grand River, and the Burlington Canal in Southern Ontario. These samples were iced and returned to the laboratory, and processing was completed within 5 h of collection. During the study periods, 12-16 March and 4-9 June 1973, samples were collected daily between 9:00 and 10:00 a.m. from the above sites.

Procedure and culture media. Using procedures and media as outlined in American Public Health Association *Standard Methods* (1971), membrane filtration tests were performed as follows: (i) total coliform (1) using m-Endo agar LES (Difco), control no. 56423 in both studies; (ii) fecal coliform (1) using m-FC agar (Difco), control no. 277057 in the March studies and no. 277057 in the June studies; (iii) fecal streptococcus (1) using m-Enterococcus agar (Difco), control no. 271855 in both studies; (iv) heterotrophic counts, using a specially formulated MF agar (Difco), control no. 178440, containing peptone (3.0 g); K_2HPO_4, (0.2 g); $MgSO_4$ (0.05 g); $FeCl_3$ (0.001 g); soluble casein (0.5 g); agar (15 g); and distilled water (1 liter); pH 7.2.

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Membrane filters. Since various lots of membrane filters from manufacturers were available, it was decided to use these membranes interchangeably on the assumption that all membranes from each manufacturer had similar characteristics. Nonsterile membrane filters were processed in an autoclave used only for freshly prepared media and membrane filters to eliminate variability in autoclaving procedures. The following 47-mm, 0.45-μm gridded membrane filters were used in these studies: (i) Gelman GN-6, 64194, lot no. 80578 (Gelman Instrument Co., Ann Arbor, Mich.). Sterilized in the laboratory autoclave, referred to as GNA in the March study; (ii) Gelman GN-6, 64194, lot no. 80630. Sterilized in the laboratory autoclave, referred to as GNA in the June study; (iii) Millipore HAWGO47AO, lot no. 9893-13 (Millipore Corp., Bedford, Mass.). Sterilized in the laboratory autoclave, referred to as MA in the March and June studies; (iv) Millipore HAWGO47SO, lot no. 08619-4. Ethylene oxide sterilized by the manufacturer, referred to as ME in the March study; (v) Millipore HAWGO47SO, lot no. 08619-4, 48542-4, 2989-1, and 48542-3. Ethylene oxide sterilized by the manufacturer, referred to as ME in the June study; (vi) Sartorius 11406, lot no. 304382709 (Sartorius Membrane Filter Products, British Drug Houses, Toronto, Ontario). Sterilized in the laboratory autoclave, referred to as SA in the March study; (vii) Sartorius 11406, lot no. 308181734, 308201734, and 308181774. Sterilized in the laboratory autoclave, referred to as SA in the June study; (viii) Sartorius 13706, lot no. 87917417. Ethylene oxide sterilized by the manufacturer, referred to as SE in the March study; (ix) Sartorius 13706, lot no. 301813186. Ethylene oxide sterilized by the manufacturer, referred to as SE in the June study.

pH of aqueous extracts of membranes. Membrane filter pH was examined to assess the importance of this factor in explaining membrane filter variations. The pH values of the membrane filter tested were as follows: Gelman GN-6, lot no. 80578, autoclaved, pH 4.150; Gelman GN-6, lot no. 80630, autoclaved, pH 5.600; Millipore HA, lot no. 08619-4, ethylene oxide treated, pH 5.350; Millipore HA, lot no. 9893-13, autoclaved, pH 5.425; Sartorius lot no. 304382709, ethylene oxide treated, pH 4.850; and Sartorius lot no. 308181734, autoclaved, pH 5.850. Three samples were used for all determinations.

It was assumed that microorganisms do not interact with the inert porous part of the membrane but do interact with the residuals or byproducts left over from the manufacturing process. The residuals were extracted in an aqueous phase from the membrane, and their pH was tested to evaluate the relevance of this factor to membrane recovery of bacteria.

Clean and contamination-free equipment and techniques were required because of the small differences which were measured. Extraction procedures were carried out in an Erlenmeyer flask with ground glass, attached to a heat exchanger-condenser. The condenser was used to prevent the escape of possible volatile components and to keep the extracting phase constant. Extraction was completed by moderately boiling the membranes for 2 h. The extracting solution was 200 ml of glass-distilled, deionized water, conductivity 4 to 7 μohms/cm, resistivity of 0.159 to 0.250 Mohms/cm, and pH 6.1 to 6.4, into which three membrane filters were placed. Violent boiling was not used because degradation of the polymer might occur.

Toxicity studies. To determine whether the various membranes exhibited a toxic effect on *Escherichia coli* growing at 35 and 44.5 C, pure culture studies were initiated by using random samples of the different lots of membrane filters (Table 1 and 2).

E. coli ATCC 25922 was grown in Trypticase soy broth (BBL) for 24 h at 35 C. The culture was serially diluted 10^{-6} with sterile phosphate buffer (1). Throughout the test, 5.0 ml of this suspension was used, alternating the membrane filter technique with the pour-plate technique. Five membrane filters, randomly selected from different lots representing the different manufacturers, were used. For the membranes to be considered nontoxic according to military specifications MI-L-D-37005 (DSA-DM), the arithmetic mean of the count on five membrane filters on m-Plate Count Broth (Difco) at 35 ± 0.5 C was to be at least 90% of the arithmetic mean of the count on five agar (m-Plate Count Broth plus 1.5% agar) plates. As yet, there have been no criteria set for testing at 44.5 C.

Statistical analyses. Counts of each bacterial type were tabulated separately by two time periods, three locations, five filter treatments, and five days. The plan was to collect three replicates in March and five replicates in June. However, excess sediment and algae in some samples resulted in several missing values. This negated the use of analysis of variance (ANOVA) of multiple classification, with days a cross-classification, for testing the effectiveness of the filter-treatment combinations.

To keep the loss of information minimal, the means of the replicate counts were analyzed. Any missing mean counts were estimated to the closest value by the standard statistical procedure for split-plot ANOVA, losing one degree of freedom for each estimate (2, 8). It was assumed that mean counts were unbiased and random. For each bacterial type and period the standard deviation and mean count were first plotted for the five filter-treatment combinations. In nine of these ten preliminary graphs, standard deviation markedly increased with mean count, a sign of heterogeneous variance. To help equalize variance and provide a more normally distributed variable, mean counts were transformed to logarithms ($\log_{10}$). The graphs of standard deviation (in log units) on mean count confirmed that $\log_{10}$ mean count was a preferable metameter for ANOVA.

In the split-plot design used, locations were considered random or model II. Filter-treatment combinations were handled as major plots and days as subplots. Both were considered fixed or model I. Major plot and subplot error terms were pooled only if the ratio of their variances (calculated F) was less than tabular F at a probability of 0.20. When mean counts for filter treatment were significantly different by F test, two methods were used to isolate the differences. One method used t tests to compare the effectiveness of the following sets chosen a priori:

TABLE 1. *Recovery of E. coli at 35 C by the membrane filters used in the study*[a]

Replicate no.	No. of colonies per 5.0 ml											
	GNA lot no. 80630	Pour plate control	GNA lot no. 80578	Pour plate control	MA lot no. 9893-13	Pour plate control	ME lot no. 9891-1	Pour plate control	SA lot no. 308181734	Pour plate control	SE lot no. 301813186	Pour plate control
1	88	130	120	130	100	110	92	140	100	140	110	120
2	110	120	120	130	100	120	110	120	100	120	66	96
3	100	110	120	130	120	120	96	110	99	100	52	110
4	120	110	120	110	100	100	110	120	110	110	47	140
5	120	120	110	140	110	130	99	130	93	120	94	120
Total count	540	590	590	640	530	580	510	620	500	590	370	590
Mean count	110	120	120	130	110	120	100	120	100	120	74	120
Mean filter/ mean pour × 100 (%)	92		92		92		83		83		62	

[a] Control plate counts were obtained from pour plates of mPCB plus 1.5% agar at 35 C.

TABLE 2. *Recovery of E. coli at 44.5 C by the membrane filters used in this study*[a]

Replicate no.	No. of colonies per 5.0 ml											
	GNA lot no. 80630	Pour plate control	GNA lot no. 80578	Pour plate control	MA lot no. 9893-13	Pour plate control	ME lot no. 9891-1	Pour plate control	SA lot no. 308181734	Pour plate control	SE lot no. 301813186	Pour plate control
1	48	140	43	110	35	130	4	120	52	140	56	140
2	55	120	28	130	25	130	6	120	48	130	57	120
3	51	120	48	120	40	120	6	120	48	120	46	130
4	34	120	50	110	45	130	5	110	37	120	44	120
5	47	140	37	130	46	130	10	130	42	120	44	120
Total count	240	640	210	600	190	640	31	600	230	630	250	630
Mean count	48	130	42	120	38	130	6	120	46	130	50	130
Mean filter/ mean pour × 100 (%)	37		35		29		5		35		38	

[a] Control plate counts were obtained from pour plates of mPCB plus 1.5% agar at 44.5 C.

GNA versus SA; GNA versus MA; SA versus SE; and MA versus ME. In the second method, mean counts from all possible pairs of filter-treatment combinations were compared, using the Student-Newman-Keuls (SNK) multiple-range test at $P = 0.05$.

RESULTS AND DISCUSSION

Problems typical of environmental water quality studies such as sediment- and detritus-laden waters and algae blooms caused the loss of some replicates and samples. Other problems were also encountered with some of the membranes. In several instances Sartorius membranes were found to have hydrophobic areas which limited the true filtering area (Fig. 1). Both Millipore and Sartorius membranes, upon autoclaving, became distorted and somewhat fragile (Fig. 2). This distortion was probably caused by shrinkage. It was not established whether these changes affected recovery of bacteria on the membranes.

When testing for fecal coliform densities, the Millipore membrane filter often produced a beige-yellow background, which, in some instances, made counting somewhat difficult. This finding was also noted by Presswood and Brown (6).

Paris and Brantford samples varied greatly in their bacterial content during the survey pe-

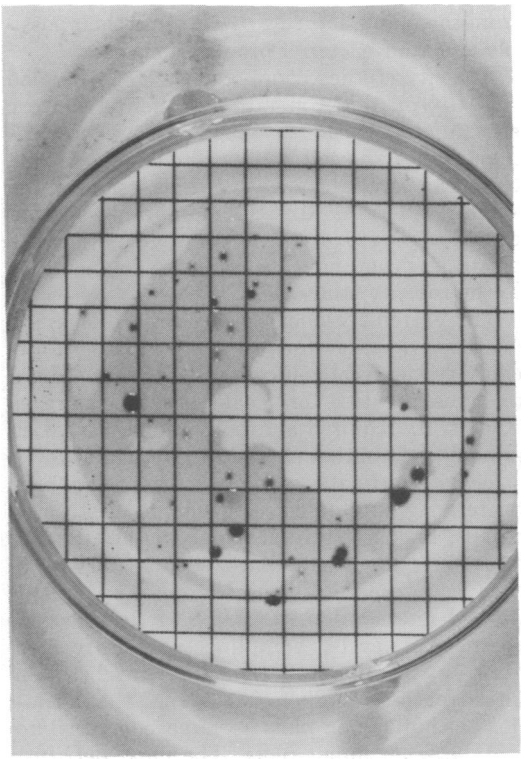

FIG. 1. *Typical hydrophobic areas found on some membranes.*

riods. Depending on the bacterial type examined, March densities were 3 to over 100 times those found in June. However, Burlington bacterial populations tended to be slightly lower in March.

The summarized mean data (Tables 3 and 4) indicate that GNA membranes generally produced the highest mean counts during the two studies. This was especially noticeable in the coliform, fecal coliform, and heterotrophic density tests.

Results of the split-plot ANOVA and significance tests are summarized in Table 5. The a priori *t* test and the less sensitive and efficient SNK test generally support the same statistical conclusions. For example, the *t* test indicates that significantly fewer total coliform bacteria were trapped and grown on the SA and MA filters in comparison to those trapped and grown on the GNA filters in the March studies. Furthermore, there was no significant difference in the effectiveness of the SA and SE filters and of the MA and ME filters. Since SA versus SE and MA versus ME results are similar, GNA were more efficient statistically than all other membrane filters in recovering total coliforms in March. Corresponding SNK tests of total coliforms support this conclusion.

The *t* test analysis of March fecal coliform tests show that significantly fewer bacteria were able to grow on SA and SE filters than on GNA filters. However, the GNA, MA, and ME filters did not differ significantly in their ability to trap and propagate these bacteria. The SNK test shows similar, though not identical, overlapping.

The separation of filter treatment is not as clear for fecal streptococcus during March. For both experimental designs, the *t* tests were identical (Table 5). These indicate that even though the SE and MA filters were less efficient than the GNA, SA, and ME filters, the latter three did not differ significantly in their recovering ability. Both SNK tests show overlapping of mean counts.

The *t* test analysis of March heterotrophic densities showed that SA and MA filters were less effective than the GNA filters. However, it would not be safe to draw the same conclusions between SE, ME, and GNA filters. Even though pairs SA and SE and MA and ME did not differ significantly, the mean for SE and ME are closer to the mean for GNA.

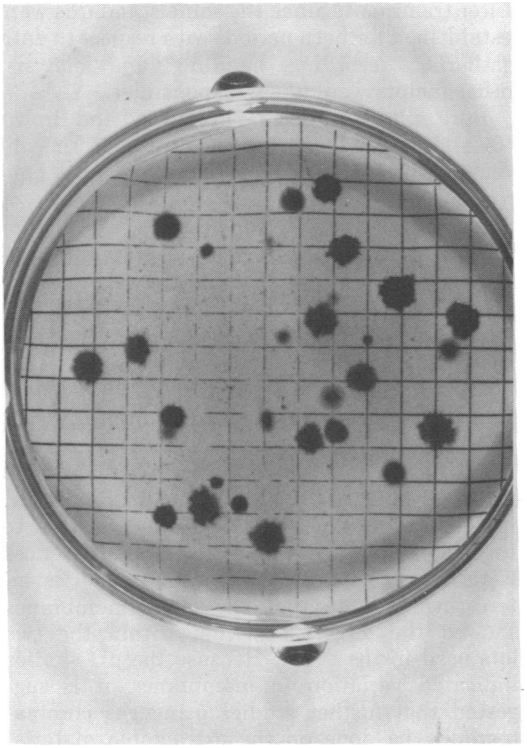

FIG. 2. *Distortion of membrane after routine autoclaving.*

TABLE 3. *Summary of mean data, March study*

Membrane tested	No. of times specific membranes recovered the highest mean and lowest mean counts							
	Coliform		Fecal coliform		Fecal strepto-coccus		Hetero-troph	
	High	Low	High	Low	High	Low	High	Low
GNA	11	0	9	0	5	0	9	1
SA	1	7	3	5	5	1	2	4
SE	0	4	1	7	1	3	2	3
MA	3	1	1	2	0	5	1	5
ME	1	3	2	3	1	4	2	4

With respect to June total coliform counts, *t* tests indicate that the GNA filter retained and propagated more coliforms than the MA filters and probably more than the SE filters, as there was a significant difference between SA and SE recoveries. However, the GNA, SA, and ME filters were about equally effective (Table 5). The SNK test shows no clear distinction.

Counts made in June for fecal coliforms, fecal streptococci, and heterotrophic bacteria did not show a significant difference by filter treatment. This is in marked contrast with all of the above data sets where F was highly significant for filter treatment. Since the same standards were established for both periods with respect to data gathering, recording, handling, and analysis, other factors were likely responsible.

Pure culture studies by Presswood and Brown (6) and Levin et al. (5) on fecal coliforms indicated that GNA recovered more fecal coliforms than ME filters. Perhaps the lack of agreement with Presswood and Brown's (6) pure culture studies were due to the evaporation of ethylene oxide residuals since the ethylene oxide-sterilized membrane filters used in this study were at least 6 months old. Presswood and Brown (6) suspected that this difference might be due to pH, whereas Levin et al. (5) believed these differences to be due to "inhibiting toxic effects of the filter membranes."

Studies on aqueous extracts of the membrane filters revealed that the ethylene oxide-treated membranes had a lower pH value relative to their autoclaved counterpart. The differences were between 0.075 to 1.45 pH units. It is interesting to note that the GNA membranes showed the largest variation within the two lots used in the study. Because the pH studies indicated variation in membranes, it is suggested that further studies using gas chromatography be done on the extractable material of the membranes to identify the components from the manufacturing process and their possible effect on bacterial propagation.

Toxicity studies conducted at 35 C revealed that GNA and MA membranes showed no toxic effects on *E. coli* (Table 1). However, ME membranes showed a 9% reduction in colony counts in comparison with MA. SA showed the same recovery as ME. SE membranes showed even lower recovery. GNA and MA were the only filters that met U.S. military specifications in the toxicity study.

The extremely poor recovery rate at 44.5 C (Table 2) requires further investigations, especially as the fecal coliform test is gaining more favor as the test for fecal pollution. Further studies are required and nongridded as well as gridded membranes should be compared.

Density estimates made in June for the three bacterial groups (fecal coliform, fecal streptococcus, and heterotrophs) at Paris and Brantford were about one-tenth the magnitude of their counterparts during March. Apparently, when bacterial densities drop, reflecting low population levels, sampling variability can mask any possible statistical difference between filter treatments. There is evidence that variation within major plots is large compared to that between major plots. The clay, detritus, and algae encountered during the June study, combined with the lower bacterial densities, were likely contributors to the lack of variation between membrane filter recoveries (Table 5).

Since the results of this study and those of Levin et al. (5) and Presswood and Brown (6) indicate that there are differences between membrane filters in their ability to recover and grow bacteria, then what choice does the practicing water microbiologist have? If one uses membrane A, one may, in testing a specific water body, find 100 fecal coliforms per 100 ml. If one uses membrane B, one may find 300 fecal coliforms per 100 ml. Thus, by using membrane

TABLE 4. *Summary of mean data, June study*

Membrane tested	No. of times specific membranes recovered the highest mean and lowest mean counts							
	Coliform		Fecal coliform		Fecal strepto-coccus		Hetero-troph	
	High	Low	High	Low	High	Low	High	Low
GNA	10	0	7	0	5	3	10	2
SA	1	1	1	3	3	1	1	1
SE	2	6	5	2	2	7	4	2
MA	0	5	2	1	4	4	1	7
ME	1	2	1	5	5	3	0	4

TABLE 5. *Summary of split-plot ANOVA and significance tests of all data, March and June studies*[a]

Time period (1973)	Test	No. of locations	No. of filter treatments	No. of days	df[b] lost via missing plot technique	Filter treatment		Ranked filter treatment and results of SNK multiple range tests	A priori *t* test of filter treatment mean differences
						df in mean square	significance of *F*		
12-16 March	Total coliform	3	5	5	0	40	**[c]	GNA 3.3534 MA 3.2043 [d] ME 3.1282 SE 3.0845 SA 3.0514	GNA vs. SA ** GNA vs. MA * SA vs. SE NS MA vs. ME NS
12-16 March	Fecal coliform	3	5	5	1	39	**	GNA 2.8527 ME 2.7930 MA 2.7389 SA 2.7178 SE 2.6291	GNA vs. SA * GNA vs. MA NS SA vs. SE NS MA vs. ME NS
12-16 March	Fecal streptococcus	3	5	3	0	24	**	GNA 2.7400 SA 2.7385 ME 2.5523 SE 2.5464 MA 2.3627	GNA vs. SA NS GNA vs. MA ** SA vs. SE * MA vs. ME *
12-16 March	Fecal streptococcus	3	5	4	6	26	**	GNA 2.7815 SA 2.7790 ME 2.6190 SE 2.6124 MA 2.4655	GNA vs. SA NS GNA vs. MA ** SA vs. SE * MA vs. ME *
12-16 March	Heterotrophic	3	5	5	1	39	**	GNA 4.6569 ME 4.5258 SE 4.4662 MA 4.4404 SA 4.4032	GNA vs. SA ** GNA vs. MA ** SA vs. SE NS MA vs. ME NS
4-8 June	Total coliform	3	5	4	0	32	**	GNA 3.5608 SA 3.4411 ME 3.3420 SE 3.2469 MA 3.1876	GNA vs. SA NS GNA vs. MA ** SA vs. SE * MA vs. ME NS
4-8 June	Fecal coliform	3	5	2	0	8	NS	SE 1.9596 GNA 1.9594 MA 1.8959 SA 1.8761 ME 1.8714	
4-8 June	Fecal coliform	2	5	5	5	4	NS	GNA 1.8488 SE 1.8029 MA 1.7963 SA 1.7871 ME 1.7692	
4-8 June	Fecal streptococcus	3	5	2	2	38	NS	SA 1.6452 SE 1.6100 GNA 1.5674 ME 1.5437 MA 1.5356	
4-8 June	Heterotrophic	3	5	5	0	8	NS	GNA 3.3790 SE 3.3337 SA 3.2986 MA 3.2722 ME 3.2585	

[a] Metameter is $\log_{10}$ bacterial counts.
[b] df, Degrees of freedom.
[c] Difference between means is: not significant (NS); significant at 5% level (*); significant at 1% level (**).
[d] Brackets enclose means not significantly different at 5% level.

A, the water body meets the recreational water quality standards. However, by using membrane B, the water fails to meet the requirements as a recreational facility.

How many times have microbiologists compared data from a specific water body and found widely fluctuating counts and blamed faulty media, techniques, or seasonal variations, and assumed all membranes to be equal? Perhaps these fluctuations were due to membranes of different batch numbers, different manufacturers' membranes, or different sterilization procedures. Since these and other studies have shown that there are differences in the abilities of membranes to grow bacteria, there should be standardized methods for bacteriological testing of membrane filters. The establishment of such recovery and propagation standards is a critical current need.

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