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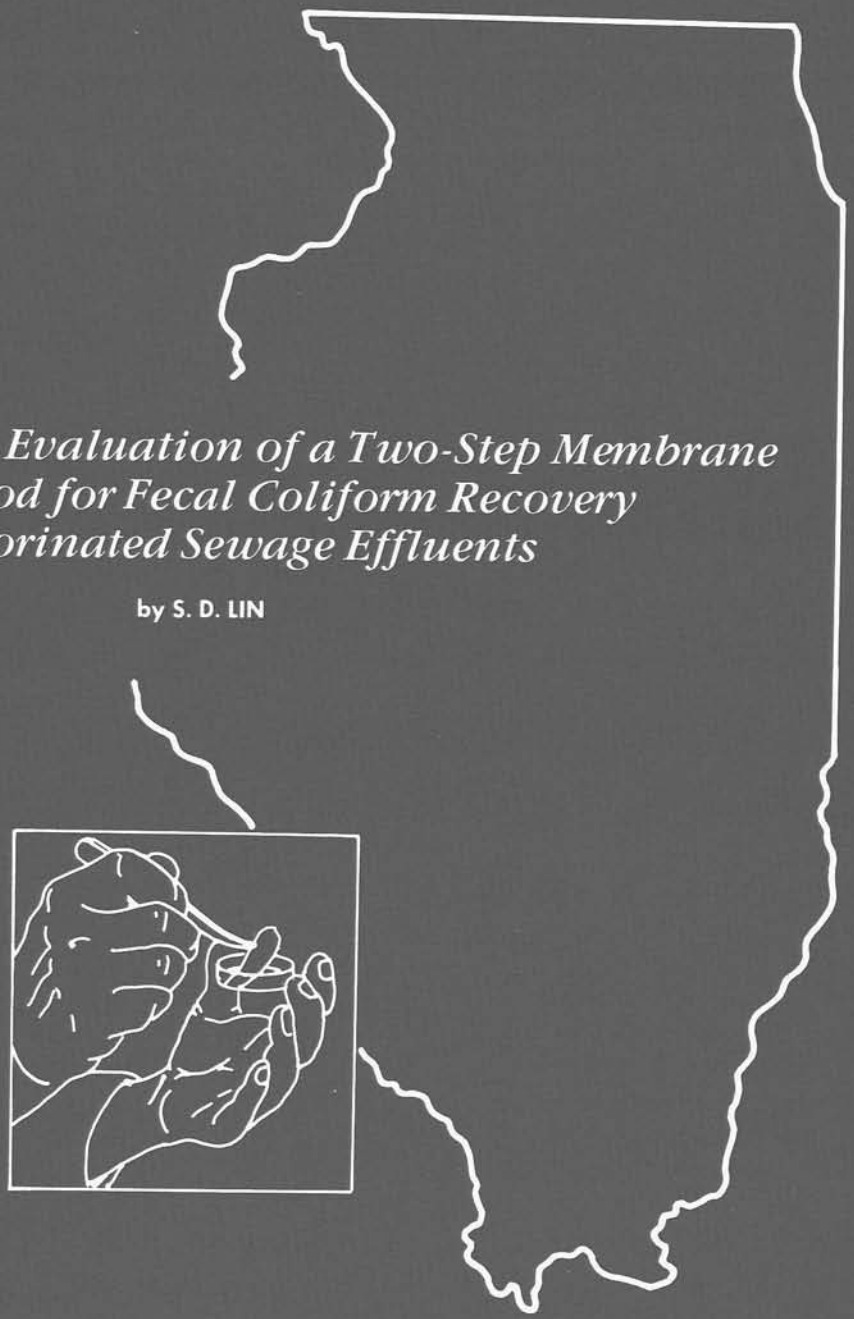

Development and Evaluation of a Two-Step Membrane Filter Method for Fecal Coliform Recovery in Chlorinated Sewage Effluents

by S. D. LIN

ILLINOIS STATE WATER SURVEY
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REPORT OF INVESTIGATION 87

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by S. D. LIN

Title: Development and Evaluation of a Two-Step Membrane Filter Method for Fecal Coliform Recovery in Chlorinated Sewage Effluents.

Abstract: A membrane filter (MF) method for fecal coliform (FC) in chlorinated wastewater effluents was developed and evaluated with 10 different types of MFs. A membrane is transferred onto M-FC agar for incubation at 44.5°C for 18±2 hours after pre-enrichment with phenol red lactose broth and incubation at 35°C for 4 hours. Results of 126 comparisons indicated that the FC recoveries by this two-step MF method were comparable to those obtained by the multiple tube (MPN) procedure. The results of comparisons of 10 MF types for FC recovery in chlorinated effluents from five plants with the two-step MF procedure were analyzed by Duncan's multiple range test. The FC recovery rates of the MFs tested can be ranked as follows: Millipore HC ~ Gelman GN-6 ~ Sartorius green > Helena Titan GH ~ Nuclepore N040 ~ Sartorius SM-137 56 ~ Schleicher & Schuell BC 07 > Johns-Manville Sterilized ~ S & S B-9 > J-M Radiation.

Reference: Lin, S. D. Development and Evaluation of a Two-Step Membrane Filter Method for Fecal Coliform Recovery in Chlorinated Sewage Effluents. Illinois State Water Survey, Urbana, Report of Investigation 87, 1978.

Indexing Terms: bacteria analysis, chlorinated wastewater effluents, Duncan's multiple range test, fecal coliform, membrane comparison, multiple-tube (MPN) method, two-step membrane filter method.

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SUMMARY AND CONCLUSION

The standard one-step M-FC broth membrane filter (MF) procedure for recovery of fecal coliform (FC) in chlorinated wastewater effluents is much less effective than the multiple-tube (most probable number, MPN) technique. A two-step MF method was developed. Using a pre-enrichment technique with phenol red lactose broth and incubation at 35 C for 4 hours, followed by plating on M-FC agar and incubation at 44.5 C for another 18±2 hours, enhanced FC recovery in chlorinated effluents. The results of 126 comparisons using chlorinated effluents from five wastewater plants showed that FC recovery with the two-step MF method is comparable to that with the MPN procedure.

In the development of the two-step MF method for FC recovery in chlorinated effluents, only the Millipore HC filters were used. Literature shows that different bacteria recovery capabilities are reported for various membranes. Therefore, nine other types of membranes were compared with Millipore HC for their fecal coliform recovery in chlorinated effluents from the five wastewater treatment plants.

The results of 61 comparisons were analyzed by Duncan's multiple range test. The 10 membranes can be divided into four groups. Millipore HC, Gelman GN-6, and Sartorius SM 138 06 (green) are equivalent and give the highest FC recovery in chlorinated effluents. The next group is Helena Titan GH, Nuclepore N 040, Sartorius SM-137 56, and Schleicher & Schuell (S & S) BC 07. Johns-Manville (J-M) sterilized and S & S B-9 belong to the other group and have an FC recovery better than J-M radiation. The J-M radiation gives the lowest FC recovery rate in chlorinated effluents.

In all, 1002 blue colonies from all types of filters were isolated for fecal coliform verification. The average percentage of verification was 93.2.

INTRODUCTION

In Illinois and other states year-round disinfection (generally chlorination) of wastewater effluents is mandatory. Illinois requirements specify a maximum permissible fecal coliform (FC) density in treated effluents. This requires FC enumeration in chlorinated effluents. For FC determination, both the M-FC broth membrane filter (MF) method and the most probable number (MPN, or multiple tubes) procedure are being used. The U. S. Environmental Protection Agency recently permits MF analysis of chlorinated wastewater effluents in monitoring provided some parallel testing with the MPN test has been done.

The MF method, using M-FC broth at 44.5°C for FC recovery, was proposed by Geldreich et al.[1] in 1965. The M-FC broth MF method was incorporated into the 13th edition (1971) of *Standard Methods*[2] as an approved procedure for determining FC concentrations in waters. The problems with this one-step MF method have been reported by many investigators in the past five years. The coliform recoveries by the MF method are influenced by the brand of membrane used (table l),[3-12] the sterilization procedure for the membrane,[7] the medium,[5] the temperature of incubation,[5] the sources of the coliform bacteria,[10] and the surface pore morphology of the membrane filter.[11-13] The conclusions of these investigations might be influenced by

Table 1. Summary of Published Comparison Studies on Membrane Filters

Reference	*Membrane type*	*Source of test organism*	*Medium*	*Results and conclusion*
Levin *et al.*[3]	Schleicher & Schuell, Millipore, Oxoid, & Gelman 27A	*E coli* ATCC 8739	MF MacConkey broth	No difference for S & S, Millipore, & Oxoid. Gelman gave much less adverse
Presswood and Brown[4]	Gelman GN-6 & Millipore HA	Mixed 25 strains of *E. coll* type I isolated from river waters & sewages	M-FC broth	Gelman agreed with control plate counts. Gelman recovered FC 2.3 times more than Millipore
Hufham[5]	Gelman GA-6 & Millipore HA	*E. coli (ATCC* 11775) *E. aerogenes* (ATCC 13048) isolated from lake waters	M-coliform broth, M-FC broth	No difference between 2 brands for TC recovery. Gelman was better for FC recovery. Neither brand gave adequate results.
Harris[6]	Gelman (GN-6) (assumed type), Millipore (HA)	Unchlorinated aquatic sources		Gelman averaged 2.5 times greater FC recovery than Millipore. TC recoveries are similar with 2 brands.
Dutka *et al.*[1]	Gelman GN-6 (G), Millipore HA(M), & Sartorius 11406 & 13706 (S). (A:autoclaved E:ethylene oxide)	River waters	M-Endo agar LES, & M-FC agar	For TC recovery: March: GA > MA, SA; MA ~ ME, SA ~ SE June: GA > MA, SE; SA ≠ SE; GA ~ ME ~ SA. For FC recovery: March: GA > SA, SE; GA ~ MA ~ ME June: No significant difference by filter treatments.
Schaeffer *et al.*[8]	Gelman (GN-6) (assumed typed), Millipore (HA)	Natural waters	(M-Endo medium*) & (M-FC broth)	Gelman was superior to Millipore for TC recovery, but they were equivalent for FC recovery
Green *et al.*[9]	Millipore HA & HC, Gelman GN-6, S & S B-9, Sartorius & Johns-Manville	Unchlorinated waters	M-FC agar	For FC recovery. Millipore HC > Gelman > Johns-Manville ~ Sartorius > Millipore HA > S & S
Brodsky and Schiemann[10]	Millipore HA, Sartorius S-M 11456, J-M 045M047SG, & 045M047LG, & Gelman GN-6	Diluted EC broth cultured waters, and river waters	M-Endo agar & M-FC agar	EC cultured waters: TC: J-M ~ S ~ M FC: J-M > S; J-M ~ M; & M ~ S River waters: TC: J-M > S; J-M ~ M, & M ~ S FC J-M > S; G ~ M
Standridge[11]	Millipore HA & HC, & Gelman GN-6	None (Scanning electron microscope used)	None	Surface pore morphology of Gelman is similar to that of M-HC, but smaller than M-HC & larger than M-HA.
Lin[12]	MUlipore HA & HC	Natural waters & chlorinated effluents	M-Endo agar LES, MFC broth & agar, & Entrococcus agar	HC was superior to HA for FC recovery. HC can be used for TC & FS detection

the experimental design[5] and by statistical analyses used.[8] Hufham[5] suggested that the M-FC broth one-step MF method should not be recommended as an analytical tool for *E. coli* enumeration. These studies all used unchlorinated effluents or natural waters.

For the recoveries of indicator organisms from chlorinated secondary and tertiary effluents, previous studies[14,15] show that total coliform (TC), FC, and fecal streptococcus (FS) recoveries by the one-step MF method were significantly less than those by the MPN procedure. The enriched two-step MF method for the TC and FS recoveries from chlorinated effluents were subsequently proposed. Similar study by Greene et al.[16] confirmed the work of Lin.[12] The two-step MF method for TC recovery from chlorinated effluents is adopted by the new (14th ed.) issue of *Standard Methods.*[11]

Improvement in the MF method for FC recovery in chlorinated effluents is needed. Among the methods proposed to improve the recovery of nonlethally injured bacterial cells have been pre-enrichment,[14,15,18,19] temperature acclimation,[19, 20] and alternate media usage,[21 -23] or a combination of these.[24] In 1975, Rose et al.[24] proposed a two-layer agar MF method which increased sensitivity to FC detection in natural waters and chlorinated effluents. Comparison of the FC recovery on the two-layer agar MF with the MPN method was undertaken.

There are many commercially available membranes for bacterial detections. It is reasonable to assume that the different brands of membrane have different capabilities for FC recovery in chlorinated effluents. The question arises, are all brands of filter applicable to the method developed and here reported.

The requirement for indicator bacteria enumeration in

treated effluents necessitates the development and evaluation of an adequate economical procedure for determining FC densities in chlorinated effluents. The information presented in this report should be useful to wastewater works operators, sanitary microbiologists, and regulatory agencies.

Objective and Report Plan

During this series of study, two separate investigations were performed. One dealt with the methodology development, the other with evaluation of the procedure developed. The purposes of the study were:

1) To develop an MF procedure for FC recovery in chlorinated wastewater effluents
2) To examine the efficiencies of many brands of MF with the developed procedure

This report describes the procedure used in the study. It also presents the results related to the two objectives and includes discussions on the development and evaluation of the proposed method.

Acknowledgments

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MATERIALS AND METHODS

Grab samples of secondary and tertiary effluents from five wastewater treatment plants serving the Illinois cities of Peoria, East Peoria, Morton, Pekin, and Washington were used. The Peoria plant employs the high-rate activated sludge process with tertiary stabilization ponds, treating a combination of domestic and industrial wastewaters. Contact stabilization comparable to the standard-rate activated sludge process is used at East Peoria, Morton, and Pekin. These plants principally treat domestic wastewaters. Washington is served by an aerated lagoon, trickling filter, and tertiary filters. Samples were collected from the trickling-filter effluent. This plant also principally treats domestic wastewater.

One-liter portions of effluent from each plant were dosed with calcium hypochlorite (HTH, 70 percent available chlorine) at concentrations up through 7 mg of chlorine per liter. The samples were stirred gently but intermittently, and after varying periods of contact time (up to 30 minutes) they were dechlorinated with an excess of sodium thiosulfate. The dechlorinated samples were assayed immediately for FC densities by parallel MPN and MF methods during the first phase of the study. Generally, all media used were freshly prepared, and no medium was more than 4 days old.

The MPN procedures were performed by using a series of four-decimal dilutions per sample, with five tubes for each dilution. Lauryl tryptose broth was used for the presumptive tests. For FC confirmation, EC medium at 44.5±0.2 C (water bath) was used.

For MF procedures, three to six replications for each sample were filtered through 0.45 µrn membrane filters (Millipore Corp., types HCWG 04753 and HAWG 04750) for each test run. Parallel tests using the one-step MF method with M-FC broth or M-FC agar at 44.5±0.2°C were performed. A 5-ml portion of M-FC agar was dispersed into each 50X12 mm tight-fitting petri dish. Pre-enrichment media consisting of EC medium, M-Endo broth, M-FC broth, and phenol red lactose broth (PRLB) were used as part of a two-step MF method with M-FC agar. Various temperatures and incubation periods were tried.

During the course of the second (last) phase of this study, FC assay was performed for dechlorinated samples with 10 types of membranes by the 2-step MF method which was developed during the first phase of this study. Table 2 gives the detailed description of the 10 membrane filters used. Membranes B through H are conventionally used for coliform detection. The other three membranes (A, I, and J) are relatively new ones. Membrane J is claimed by the manufacturer as comparable to membrane A with a retention pore size of 0.7 µm. Membranes are gridded except Johns-Manville 045M047SP (lot 422J286, B), Johns-Manville M045PR47C (lot 429J393, C), and membrane F. With the exception of membrane F, all membrane filters are made of cellulose. Pre-autoclaved and autoclaving mean that the membranes are sterilized at the factory and the laboratory, respectively. Millipore's absorbent pads were used with membranes F and I which had no pads.

Table 2. Description of 10 Membranes Used in the Study

Symbol	Manufacturer	Filter type	Pore size, μm	Material	Sterilized by	Lot No	Absorbent pad used
A	Millipore Corp.	HC	0.70	Mixed cellulose acetate and cellulose nitrate	Ethylene oxide	37158 6, 37158 7, 37158 8,8c 14028	Millipore
B	Johns-Manville	Membra-Fill 045 M047SP, 045 M047SG	0.45	Mixed esters of cellulose diacetate & nitrocellulose	Ethylene oxide	422 J 286	Johns-Manville
C	Johns-Manville	Membra-Fil M045P R47C, M045G R47C	0.45	Mixed esters of cellulose diacetate & nitrocellulose	Radiation	429 J393, & 422 J290	Johns-Manville
D	Helena Lab.	Titan GH	0.45	Mixed cellulose esters	Ethylene oxide or Pre-autoclaved	280, 296, & 311	Helena
E	Schleicher & Schuell	B-9 (BA 85/21)	0.45	Nitrocellulose	Autoclaving	11775, & 8055	S8cS
F	Nuclepore Corp.	N 040	0.40	Polycarbonate plastic	Autoclaving	81B1B10A	Millipore
G	Gelman Inst. Co.	GN-6	0.45	Mixed esters of cellulose	Pre-autoclaved	80935,8c 8211 81317	Gelman
H	Sartorius-Membran-filter GmbH	SM-137 56	0.45	Cellulose nitrate	Pre-autoclaved	30798 3734	Sartorius
1	Sartorius-Membran-filter GmbH	SM-138 06 (Green)	0.45	Cellulose nitrate	Autoclaving	50145 5788	Millipore
J	Schleicher & Schuell	BC 07	0.70	Nitrocellulose	Autoclaving	50/6	S8cS

Three replicates of each membrane type were tested for each sample. No dilution was needed for any of the samples. Filtration order on the six manifolds used was randomized to prevent bias. The filtered membrane was placed on the absorbent pad which contained 1.8 to 2.0 ml of phenol red lactose broth (Difco) on the top of the culture dish. Dishes were incubated in an inverted position at 35±0.5 C for 4 hours. After the pre-enrichment period, the membrane filter was transferred onto the M-FC agar (Difco) and was incubated in either a water bath (sealed in plastic bags) or a Millipore MF incubator (solid-state air heating block) at 44.5±0.2 C for additional 18±2 hours. Blue colonies were counted with the aid of a binocular widefield dissecting microscope at 10 to 15 magnifications.

For FC verification purposes, representative colonies (3 to 6 blue colonies per filter) were inoculated into PRLB and incubated for 24 to 48 hours at 35 C. All colonies showing gas production within the incubation period were confirmed by subculturing in EC medium in a water bath at 44.5±0.2°C for 24 hours.

RESULTS

Methodology Development

Membrane Filters. Recently, the Millipore Corporation introduced a type HC membrane filter to replace the conventional type HA filter for cohform analyses. According to Sladek et al.,[13] FC recoveries on M-FC agar with type HC filters were equal to or greater than those with corresponding spread-plate controls. Their results indicated that the optimum membrane structure was a 2.4 *μm* surface opening size and a retention pore size of 0.7 *μm*. A comparison study by Green et al.[9] indicated that among the six types of filters tested, type HC filters gave the highest FC recoveries in unchlorinated waters.

Type HC and type HA filters were evaluated for FC recoveries from three types of chlorinated wastewater effluents. The chlorine residuals before dechlorination were 0.05 to 0.30 mg/1. The results are shown in table 3. It appears that the type HC filter is only slightly better than the type HA for FC recoveries, but results were not statistically different. This is presumably due to the one-step MF method. Type HC filters were used in this study.

M-FC Broth versus M-FC Agar. M-FC broth is widely used for FC detection with the standard one-step MF method. Recently, M-FC agar was reported to be more satisfactory for FC recoveries,[13,14] especially for those from chlorinated wastewater.[13] The difference in constituents between M-FC broth and M-FC agar is an additional 1.5 percent of agar in the latter. Volumes of 5.0 and 1.7 ml were used for M-FC agar and M-FC broth, respectively. It was assumed that the FC recoveries from chlorinated effluents on M-FC agar would be greater than those on M-FC broth because of possible residual toxicities in the absorbent pad used as a substrate. The results shown in table 4 support the view that the FC

Table 3. Comparison of Type HA and Type HC Membrane Filters for Recovery of Fecal Coliform from Three Types of Chlorinated Effluents on M-FC agar at 44.5°C

Number of organisms (counts)			*Number of organisms (counts)*	
Type HA	*Type HC*		*Type HA*	*Type HC*
36	36		23	38
46	76		35	39
107	105		47	65
23	36		49	53
60	51		42	51
103	95		33	29
71	86		33	38
49	79		28	28
52	99		11	6
6	7		10	14
12	7		10	7
18	18		33	37
47	50		34	35
78	60		36	48
86	71	Mean	41	46
24	21	SD	26	28

Table 4. Comparison of the M-FC Broth and M-FC Agar Membrane Filter Counts at 44.5°C for Fecal Coliform Recoveries from Chlorinated Secondary Sewage Effluents

Effluent	*M-FC broth count*	*M-FC agar count*
Contact stabilization	2	7
(Pekin)	2	7
	5	18
	13	65
	9	53
	5	51
Contact stabilization	16	21
(Morton)	23	38
	12	39
	15	29
	6	38
	13	28
Trickling filter	23	50
(Washington)	32	60
	52	71
	0	6
	1	14
	1	7
High rate activated sludge	53	86
(Peoria S.D.)	45	79
	77	99
Mean	20	41

Table 5. Comparison of Fecal Coliform Counts from Chlorinated Sewage Effluents by the MF Method on M-FC Agar with and without Enrichment

	Pre-enriched at 35°C then on M-FC agar at 44.5°C									
	M-FC agar	*M-FC broth*		*Phenol red lactose broth*		*EC broth*		*M-Endo broth*	
	at 44.5°C	*2 br*	*4 br*	*2 br*	*4 br*	*2 br*	*4 br*	*2 br*	*4 br*
	36	40	68	59	74	47	61	32	
	76	93	128	128	173	123	117	66	
	105	140	223	121	390	243	159	97	
	36	25	32	30	29	23	11	21	21
	51	56	72	60	96	54	25	40	46
	95	114	117	108	203	82	39	104	98
	7	11	17	17	23	11	21	12	13
	7	23	38	25	36	17	19	14	15
	18	28	60	43	57	38	49	30	47
	50	71	137	62	142	57	88	72	91
	60	75	138	99	231	73	94	93	86
	71	144	186	126	246	115	104	131	216
	21	43	36	21	36	23	15	16	48
	38	51	36	47	59	19	17	33	52
	39	47	52	40	50	28	28	41	23
Mean	47	64	80	66	123	64	56	53	60

counts on M-FC agar are significantly higher than those obtained on M-FC broth. Therefore, M-FC agar was used in this study as the culturing medium when using pre-enrichment procedures.

Enrichment Broth. Four media were tested for pre-enrichment purposes. The results in table 5 show that FC counts on membrane filters were generally higher with the pre-enrichment technique. Of the four media, M-FC broth and PRLB produced the best FC recoveries. Overall, PRLB gave the highest FC recoveries from chlorinated effluents and was therefore selected as the pre-enrichment broth for the two-step MF method.

Pre-Enrichment Temperature. The effect of temperature on PRLB pre-enrichment is shown in table 6. The enriched membrane filters were transferred to M-FC agar and incubated at 44.5±0.2°C, after pre-enrichment incubation at 35 and 44.5°C for periods of 2 and 4 hours. FC counts significantly increased with PRLB pre-enrichment at 35°C. At 44.5 C not only was there no increase in FC recoveries, but there was deterioration in recovery compared with the non-enrichment step. On the basis of data shown in table 6, a temperature of 35 C was selected for pre-enrichment incubation with PRLB.

Pre-Enrichment Period. FC counts generally increased with pre-enrichment time (tables 5 and 6). To determine the optimum period of incubation for PRLB pre-enrichment, 140 sample runs were performed for periods of 1 through 10 hours at 35±0.5 C. Most of the runs were performed at 2, 4, and 6 hours.

Figure 1 shows three typical curves of FC counts as a function of pre-enrichment incubation time. FC recovery is enhanced by time of incubation up to a point, after which counts level off. This generally occurs around 4 hours (figure 1a). In figures lb and 1c, the solid lines represent typical-

Table 6. Comparison of Temperature Effects of FC Count Recoveries from Chlorinated Effluents on M-FC Agar with Phenol Red Lactose Broth Pre-Enrichment

	M-FC agar at 44.5°C	*Transferred to M-FC agar after enriching with PRLB at 35° C*		*44.5°C*	
		2br	*4 br*	*2 br*	*4 br*
	20	62	112	20	15
	21	59	112	19	19
	20	71	102	21	15
	37	68	107	18	17
	35	57	98	12	28
	48	60	127	22	8
Mean	30	63	110	19	17

sized colonies; the dashed lines include both atypical and typical colonies. Atypical colonies (very tiny ones) usually appeared after 5 hours of incubation but occasionally after 4 hours. These minute atypical colonies increased in numbers with time of pre-incubation. For some samples, typical-sized FC colonies decreased in numbers after 4 hours of incubation (figure 1c).

The verification of typical and atypical tiny blue colonies from PRLB-pre-enriched M-FC agar cultures is shown in table 7. Of the 354 colonies of typical size subcultured from incubation periods of 4, 5, and 6 hours, 327 colonies (92.3 percent) were verified as FC. On the other hand, only about 30 percent of the atypical tiny colonies were verified as FC, and the verification rate at 4 hours of incubation (13.3 percent) was very low. On the basis of the results shown in figure 1 and table 7, it is concluded that the optimum time for incubation of PRLB-pre-enriched filters at 35 C is 4 hours.

Two-Step MF versus MPN. The procedures thus far described show that FC detection in chlorinated effluents can be enhanced by the membrane filter type and pre-enrichment with PRLB at an incubation temperature of 35±0.5 C for 4 hours followed by culturing on M-FC agar at 44.5±0.2 C for 18±2 hours. It was hoped that this two-step M-FC agar-MF technique would be comparable to the MPN procedure for FC recovery from chlorinated effluents.

The results of parallel tests of the two-step MF and MPN procedures are depicted in figure 2. For the 126 assayed effluent samples from six plants, 47 of the two-step MF results are higher and 68 are lower than concurrent MPN results; 11 observations were found to be identical. Thomas and Woodward[25] state that the MPN tends to overestimate the true density. Incorporating the corrected MPN bias as described by Thomas[26] shows that 71 of the MF points plotted are above, 47 are below, and 8 are on the reference line (figure 2).

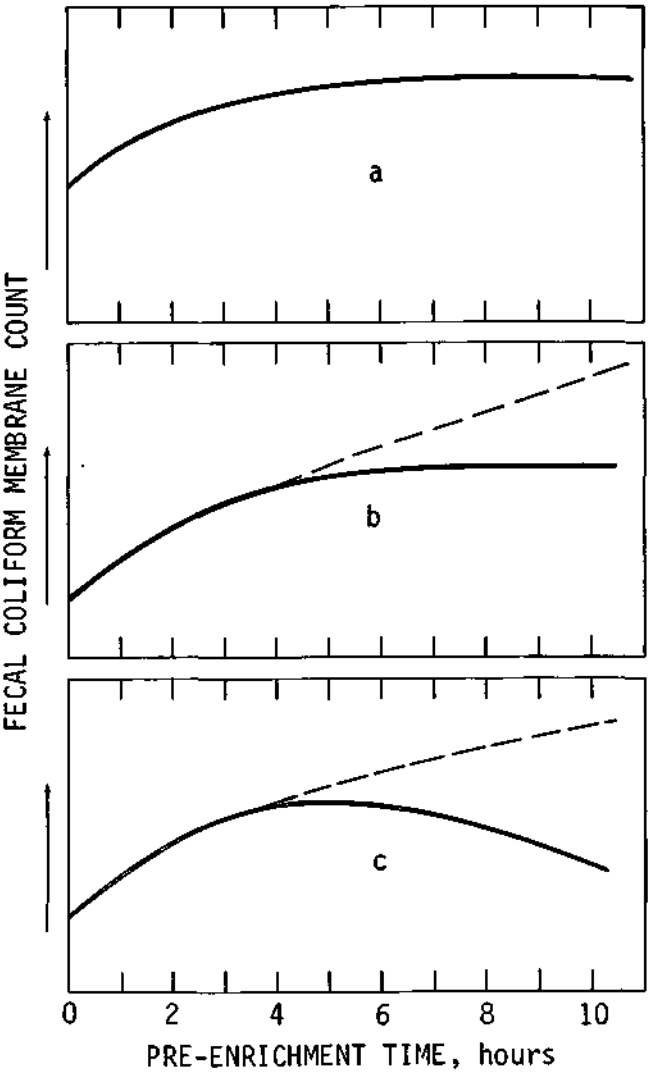

Figure 1. Relationship of fecal coliform counts in chlorinated effluents and pre-enrichment periods

Table 7. Verification of Blue Colonies from PRLB Pre-Enriched M-FC Agar Cultures

Pre-enricbment incubation time (hour)	*Number of colonies tested*	*Colonies verified as fecal coliform*	*Percent verified*
Typical sized colonies			
4	**170**	**157**	**92.3**
5	**45**	**43**	**95.6**
6	**139**	**127**	**91.4**
Overall	**354**	**327**	**92.3**
Atypical (tiny) colonies			
4	**30**	**4**	**13.3**
5	**14**	**4**	**28.5**
6	**136**	**45**	**33.1**
7	**90**	**29**	**32.2**
8	**105**	**32**	**30.5**
Overall	**375**	**111**	**29.6**

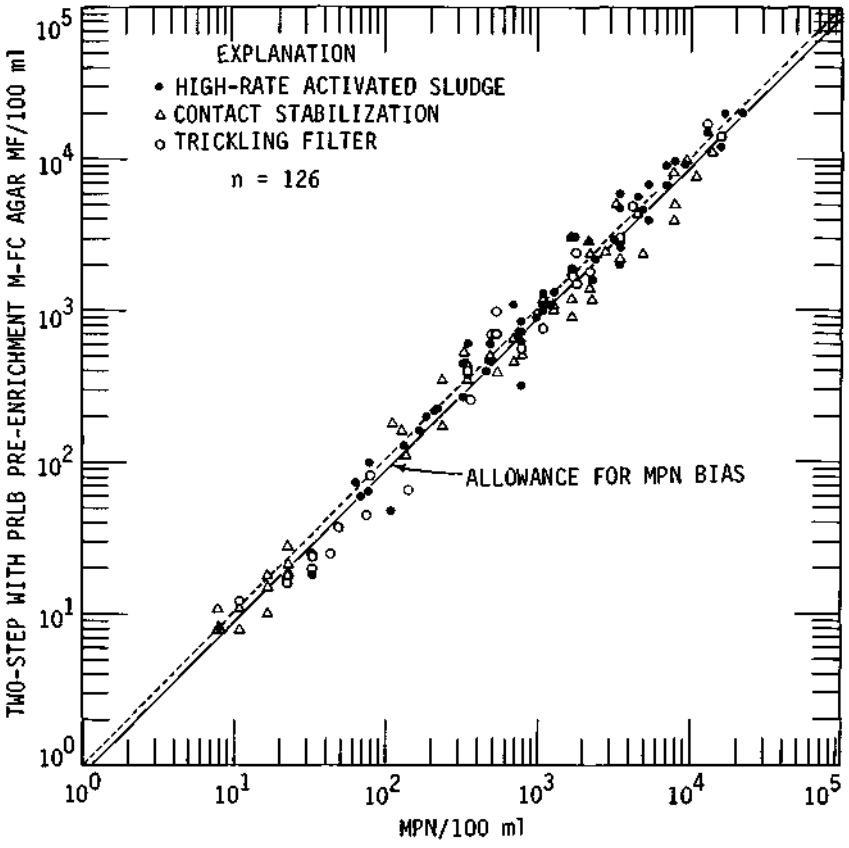

Figure 2. Comparison of fecal coliform densities in chlorinated effluents determined by MPN and two-step M-FC agar MF methods

For the two-step MF technique, including all data for comparative purposes, the geometric mean is 610 FC/100 ml, the geometric standard deviation is 8.30. Similarly, the MPN data reflected a geometric mean of 650 FC/100 ml and a geometric standard deviation of 7.92. All MF values are within the 95 percent confidence limits of the concurrent MPN values. There is not a statistical difference in the mean values of FC densities as determined by the two methods.

The values of the ratio of MF to MPN FC densities ranged from 0.41 to 1.77, with an average of 0.99 and a median of 0.94. On the basis of these comparisons, it is reasonable to conclude that FC recovery with the two-step M-FC agar-MF procedure agrees well with FC recovery obtained by the MPN method.

Comparison of 10 MFs for the Two-Step MF Method

The mean fecal coliform counts on the 10 tested membranes are listed in table 8. Attempting to have membrane filter counts within the desired 20 to 60 FC colony range was difficult. In many cases the counts were either too high or too low. About half of the tests have been discarded. Nevertheless, for comparison purposes, the data in table 8 show the different recoveries of FC from chlorinated effluents among the tested membrane filters.

To determine statistical differences among membranes, mean values shown in table 8 were subjected to statistical analyses by Duncan's multiple range test [27, 28] with four series. The first 32 runs were designated as Series 1 for 7 (membranes A through G) comparisons. The next 10 runs, Series 2, included 9 membrane performances. The rest of the runs, named Series 3, also covered 9 membrane comparisons. Series 4 combined all 61 runs for 6 membrane filters (A through E, and G). There was no parallel test for all 10 membrane types because some types were received late and Nuclepore (F) ran out of supply after run 42. The results of Duncan's multiple range tests for the four series are shown in figures 3 through 6. Average FC counts for each membrane for each series are also listed in the figures.

Figures 3 through 6 include an array of membrane types with FC counts decreasing from left to right. There is no significant difference in FC recovery for any two or more membrane types underscored by the same line (figures 3b through 6b); similarly, significantly different FC recoveries exist for any two or more membrane types not underscored by the same line. Figures 3a through 6a are another way to

Table 8. Mean Fecal Counts* on the 10 Tested Membrane Filters

Run No.	Membrane filter type A	B	C	D	E	F	G	H	I	J
1	100	76	62	100	68	78	110			
2	39	24	17	46	20	24	42			
3	94	50	24	99	51	45	83			
4	80	34	18	91	45	44	97			
5	64	50	11	73	32	43	83			
6	45	23	10	45	17	44	39			
7	42	31	20	45	39	45	57			
8	37	33	14	53	29	16	56			
9	35	21	16	49	32	26	54			
10	100	60	30	128	76	50	130			
11	120	76	46	142	86	68	132			
12	100	62	50	116	94	74	126			
13	43	19	15	46	26	41	40			
14	61	41	38	46	38	48	64			
15	46	39	20	55	34	46	46			
16	52	38	21	50	52	31	58			
17	67	42	44	71	49	52	82			
18	33	28	15	32	31	35	41			
19	62	46	23	60	61	60	58			
20	66	27	24	46	42	49	65			
21	36	23	11	35	28	31	44			
22	71	22	17	65	42	75	87			
23	56	26	21	52	36	49	66			
24	72	41	28	59	42	38	64			
25	60	48	21	48	43	40	60			
26	96	63	36	54	27	59	101			
27	21	15	11	18	14	12	28			
28	69	39	19	56	19	69	49			
29	92	40	22	42	22	60	66			
30	68	26	15	36	18	46	47			
31	96	43	18	41	23	72	63			
32	92	58	16	37	19	56	78			
33	56	26	21	52	36	49	66	29	56	
34	72	41	28	59	42	38	64	31	59	
35	60	48	21	48	43	40	60	42	59	
36	96	63	36	54	27	59	101	60	84	
37	21	15	11	18	14	12	28	20	17	
38	69	39	19	56	19	69	49	35	70	
39	92	40	22	42	22	60	66	55	82	
40	68	26	15	36	18	46	47	26	52	
41	96	43	18	41	23	72	63	69	91	
42	92	58	16	37	19	56	78	62	91	
43	32	12	5	14	11		27	12	19	19
44	112	84	26	96	71		98	76	109	128
45	104	66	43	93	76		156	112	156	89
46	70	28	21	39	41		76	47	69	57
47	72	37	20	42	44		71	59	70	54
48	56	29	12	29	25		42	44	56	34
49	94	83	60	94	86		72	85	94	61
50	84	58	30	74	53		88	61	104	69
51	82	74	45	77	63		84	80	90	82
52	119	57	18	45	49		101	80	70	92
53	109	63	50	71	83		119	113	118	59
54	83	31	19	42	32		52	58	74	33
55	26	19	11	27	12		21	16	29	19
56	75	53	34	50	52		81	56	63	57
57	116	68	52	76	58		110	86	90	70
58	56	44	26	44	41		56	49-	43	40
59	58	41	40	41	49		53	45	48	40
60	54	30	26	46	25		36	27	30	30
61	40	23	23	39	24		32	27	32	23

*Each figure represents average of three replicates.

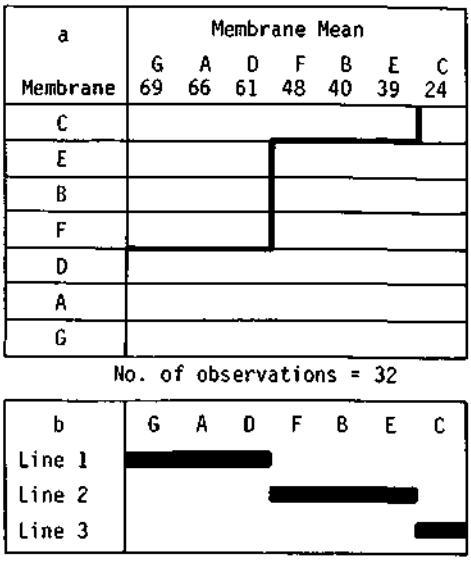

Figure 3. Series 1, results of Duncan's multiple range test

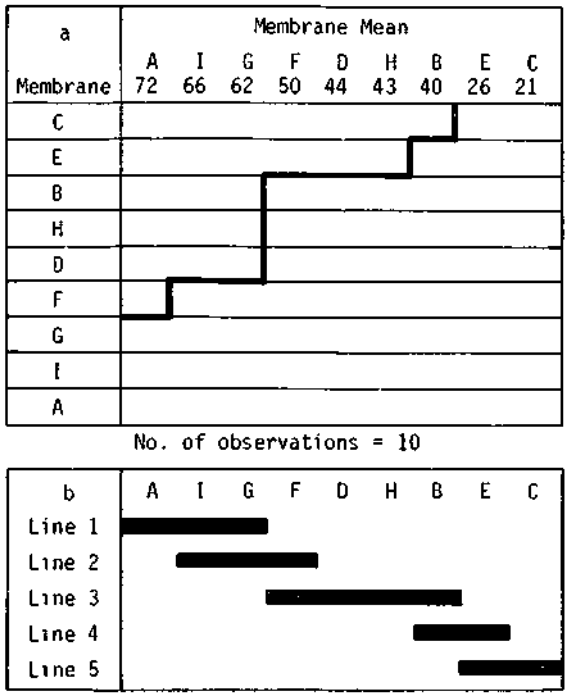

Figure 4. Series 2, results of Duncan's multiple range test

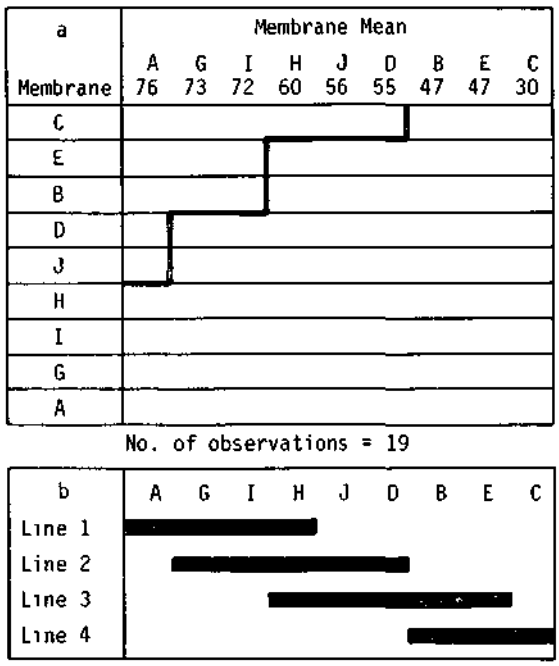

Figure 5. Series 3, results of Duncan's multiple range test

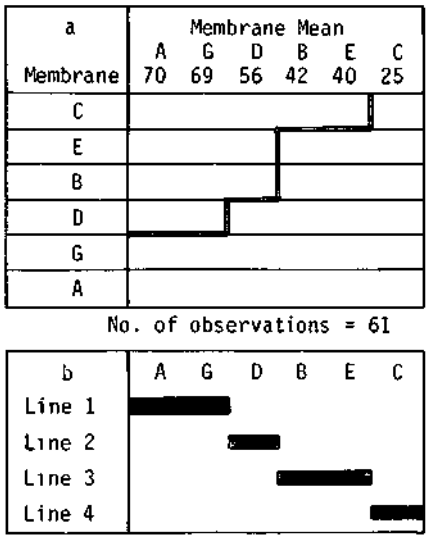

Figure 6. Combined series 1,2, and 3 results of Duncan's multiple range test

demonstrate whether differences exist among membrane performances.

Figure 3 indicates there are not statistical differences in FC recovery from chlorinated effluents by membranes G, A, and D. The same exists for membranes F, B, and E. It is also concluded that membranes G, A, and D are superior to membranes F, B, E, and C. Similarly, membranes F, B, and E are better than membrane C.

From line 1 of figure 4b, it is apparent that there are no significant differences in FC recovery by membrane types A, I, and G. Similar statements can be true for other membranes underscored by other lines. Lines 1 and 2 of figure 4b, or from figure 4a, show that recovery of FC by membrane A is significantly higher than that by membranes F, D, H, B, E, and C. Furthermore, with other lines figure 4 also suggests membranes I and G are superior to membranes D, H, B, E, and C; membranes F, D, and H are superior to membranes E and C; membrane B is superior only to membrane C; and there are equivalent results between membranes E and C for FC recovery.

Figure 5 demonstrates that there are no significant differences for FC recovery by membranes A, G, I, and H and by membranes B, E, and C. Membrane A is superior to membranes J, D, B, E, and C. Similarly, membranes G and I are superior to membranes B, E, and C; and membranes H, J, and D are significantly better than membrane C.

With a large sample size of 61 comparisons for six membranes, figure 6 shows that there are equivalent results by membranes A and G for FC recovery. These two filters give significantly greater FC recoveries than the others. Membrane D is significantly better than membranes B, E, and C. FC recoveries by membranes B and E are significantly higher than that by membrane C also.

In summary, the 10 tested membrane filters can be divided into four groups based on their efficiences of FC recovery in chlorinated effluents. The best ones are membranes A, G, and I. The next best group includes membranes D, F, H, and J. Membrane filters B and E are in another group, while membrane C gives the lowest FC recovery. There are significant differences for FC recovery among the groups but not within the group.

Fecal coliform confirmatory results for each type of membrane filter are presented in table 9. The percentage of confirmation ranged from 89.6 for membrane J to 96.6 for membrane E with an average of 93.2. There are no significant differences among the membrane filters for FC verification.

DISCUSSION

As mentioned earlier, nonlethally injured fecal coliforms are difficult to recover by standard MF techniques with M-FC broth. However, they must be considered in any FC evaluation. To enhance their recovery from chlorinated wastewaters, pre-enrichment, temperature acclimation, and a modification in culturing medium have proved useful. The results reported here suggest that a combination of these methods is comparable to MPN procedures in FC recovery efficiency.

During the course of previous work,[14] lauryl tryptose broth was used as an enrichment medium for FC recovery from chlorinated secondary sewage effluents. The results were not fruitful. Four other media were tried for pre-enrichment purposes in this study. PRLB produced the highest recovery and is therefore the pre-enrichment medium of choice. Greene et al.[16] also found that the M-Endo agar LES two-step MF procedure increased the recovery of FC from both chlorinated and unchlorinated wastewaters. They

Table 9. Confirmation of Fecal Coliform on Membrane Filters Tested

Membrane filter		*Number of colonies tested*	*Confirmation %*
Symbol	*Type*		
A	Millipore HC	190	93.2
B	Johns-Manville, sterilized	100	96.0
C	Johns-Manville, radiation	102	90.2
D	Helena Titan GH	103	92.2
E	Schleicher & Schuell B-9	88	96.6
F	Nuclepore N 040	45	95.5
G	Gelman GN-6	107	92.5
H	Sartorius SM-13756	93	95.7
I	Sartorius SM-13806	97	91.8
J	Schleicher & Schuell BC 07	77	89.6
Overall		1002	93.2

reported that pre-enrichment at an incubation temperature of 25°C for 2 to 6 hours enhanced FC recovery.

Dutka et al.[7] compared Gelman and Millipore autoclaved membrane filters for toxicity to *E. coli* and found that at 35°C both membranes were able to recover 92 percent of the test organisms. At 44.5°C neither filter was able to recover more than 40 percent of the test organisms.

Temperature acclimation is therefore a very important consideration in culturing chlorine-injured cells. The results in table 6 support the observation by Hufham[s] and demonstrate the detrimental effect of a 44.5°C incubation temperature. The adverse effect at 44.5°C probably accounts for the low FC recovery efficiency for stressed organisms with the direct M-FC broth in the MF method compared with MPN procedures.[14] A pre-enrichment incubation temperature of 35 C is considered the temperature of choice for FC recovery using MF techniques.

The pattern of colony count versus pre-enrichment incubation periods (figure 1) is presumably due to residual chlorine concentration and chlorine contact time. The higher residual chlorine and longer contact time produce low bacterial densities, shown in figure la. In contrast, for a high bacterial density note the curve in figure lc. For the sewage effluents examined, a pre-enrichment incubation period of 4 hours is considered optimum.

The MPN of the fermentation tubes is a biased estimator of the true density, and the amount of bias depends on the number of tubes (N) used in each dilution. Consequently, a factor must be applied to correct for this bias.[26] The correcting factor, C, for an estimation of the true density suggested by Thomas[26] is: $C = E^{-0.805/n}$. Data from this study showed that the two-step MF method resulted in higher FC counts than the corresponding MPN bias-corrected values.

One might ask why actual chlorinated effluents were not used in this study. There are four reasons for this. l)Most of the actual chlorinated plant effluents gave no or very low FC counts due to either high chlorine dosages or prolonged contact times. 2) Wide ranges of FC concentrations (1 to 10^s counts/100 ml) in chlorinated effluents are needed for comparison purposes. 3) Four out of five sewage plants tested have no facility for bacterial analysis. Finally, 4) the experimental design of this study, which includes dosing sewage effluents with calcium hypochlorite in the laboratory, is not very different from actual chlorination in sewage works.

The comparison results for membrane filter performances will be different, as expected, if the experimental designs are different. Membrane type, test organisms, and medium used are the major factors influencing the differences. Some works listed in table 1 did not include the type of membrane and mentioned only the manufacturer. Schaeffer et al.[8] stated their method was according to *Standard Methods.*[2] The reader has to guess that it was the one-step method for TC determination. Either M-FC broth or M-FC agar was used for FC detection by most studies listed in table 1. An absorbent pad is needed for the M-FC broth method but not for the one-step M-FC agar procedure. There will be a different capillary action of nutrient transportation.

Levin et al.,[3] Presswood and Brown,[4] Hufham,[s] and Harris[6] reported that Gelman filter was superior to Millipore filter for fecal coliform detection. However, Hufham[s] claimed neither brand gave adequate results. He used Gelman type GA-6 instead of type GN-6. Differences in the type used from the same brand could be significant for those showing inadequate results. Dutka et al.[7] reported conflicting results with autoclaved and ethylene oxide-sterilized Gelman, Millipore, and Sartorius membranes in two studies in March and June with river water and with the same experimental design. The source of test organisms influences the membrane filter performance. This is confirmed by the work of Brodsky and Schiemann.[10] Schaeffer et al.[8] disagreed with the statistical method used by Presswood and Brown[4] and concluded that Gelman was superior to Millipore (HA) for TC detection and both brands were equivalent for FC recovery. Recently Green et al.[9] found that the new Millipore HC is the best filter for FC recovery in unchlorinated waters.

Characteristics of absorbent pads used in this study are apparently different. The Sartorius pad (used with only membrane H) and the S & S pad (used with membranes E and J) need a little more than 2.0 ml of PRLB to wet the whole pad. Pads of the other brands were saturated with approximately 1.8 to 2.0 ml of pre-enrichment broth. The role of amount of impregnating broth has been studied by Sartorius investigators. In comparison with the series with 2 ml plate count broth, they found that the series with 2.2 ml gave an increase in the total count of 15 percent; and the series with 2.4 ml gave an average increase of 21 percent (unpublished data). They recommended that 2 ml of the impregnating broth is the absolute minimum and a certain amount of excess of fluid apparently is necessary to minimize antagonism.

Sartorius SM-13756 (membrane H) filters, in many instances, were found to have hydrophobic areas which reduced the true filtering area. This phenomenon was also observed by Dutka et al.[7] However, the hydrophobic characteristic was not present for the new Sartorius SM-13806 green filter (membrane I). In several instances, the white membrane filters of all brands turned to a beige-yellow background that made counting difficult. This was also reported by Presswood and Brown[4] and Dutka et al.[7]

Three different lots of Helena Titan GH (membrane D) were used for this study. The different appearances of packages, membrane filters, absorbent pads, and printings of the three lots made them very easy to distinguish. This was not the case for Millipore, Schleicher & Schuell, Johns-Manville, and Gelman filters where more than two lots were used (table 2). The Helena Titon GH lot 280 filters were used for the first 28 runs of Series 1 (table 8). After run 29, either lot 296 or 311 was used. Table 8 indicates that

FC counts of membrane D are much lower than that of membranes A and G from run 29 to run 61. Figure 3 shows membranes G, A, and D (mostly lot 280 results) are equivalent for FC recovery. Series 2 and 3, and the overall results (table 3 and figures 4, 5, and 6) indicate membrane D is not comparable to membranes A and G. The quality control of Helena Titan GH was found to be inconsistent.

Many differences also could be observed when membranes were placed on M-FC agar. Nuclepore N 040 is very thin, strong, and soft and contacts well to the agar. In contrast, both types of Johns-Manville filters are thicker and stiff and membrane C is especially difficult to place on M-FC agar. The other brands of filters can be placed equally well.

The sizes of colonies were different among the membranes tested. Generally Nuclepore grew the largest colonies (2 to 3 mm in diameter were common) compared with all other (cellulosic) filters. This might be caused by its unique characteristics which make the nutrients easier to pass through the membrane. The thickness of Nuclepore membrane is about 1/15 that of cellulosic membranes. Pores in Nuclepore membranes are round cylinders normal to the surface with a screen-like pore geometry. Nevertheless, Nuclepore N 040 is found to be inferior to Gelman GN-6 and Millipore HC for FC recovery in chlorinated effluents (figure 3).

Fecal coliform colonies grown on the Gelman filters were generally smaller than those on Millipore filters. This is caused by smaller pore openings, although the two membranes have similar pore morphology.[11] Colony sizes grown on the other brands are mostly comparable to those on Millipore.

As to the reasons for differences between membrane filters in their abilities to recover and grow bacteria, the investigators suspected or proposed that the differences might be caused by inhibiting toxic effect,[3,5,7] pH,[4] sources of test organisms,[5,10] means of sterilization,[7] statistical analysis,[8] and pore morphology.[11,13] A difference in FC recovery in chlorinated effluent was observed between two Johns-Manville filter types, and this was caused by the sterilization method (table 2). Irradiated membranes produced significandy lower FC recovery.

Both Sartorius SM-138 06 green filter (membrane I) and S & S BC 07 are newly introduced by the manufacturers. Schleicher & Schuell claims that the type BC 07 (0.7-µm pore size) should be equivalent to Millipore HC for FC recovery. However, this study found this was not the case for FC recovery in chlorinated effluents. Type BC 07 (membrane J) improved recovery efficiency from conventional S & S type B-9 (or BA 85/21, membrane E, see figure 5).

Sartorius SM-138 06 green filter (membrane I) was developed with a special pore structure on the basis of the theory proposed by Sladek et al.[13] Its pore size is 0.45 µm. The manufacturer claims that the extraordinarily uniform and spongy structure of this membrane filter provides for optimal diffusion and for good possibilities of development of even non-lethally injured microorganisms. That statement is confirmed by this study. No hydrophobic phenomenon was observed for the new green filter, but it was observed for the old SM-137 56 filter during the course of this study. The new green filter (membrane I) is found to be superior to conventional filter (membrane H) and is equivalent to Millipore HC and Gelman GN-6 (figures 4 and 5). Phase 1 of this study found that the two-step M-FC agar MF method using Millipore HC filters was comparable to the MPN procedure of FC enumeration in chlorinated effluents. No attempts were made to compare the two methods with Gelman GN-6 and Sartorius green filters. It is believed that with use of these two filters for the two-step MF and the MPN, results will be in close agreement.

Inspection of the membranes in table 2 shows that Millipore HC, Gelman GN-6, and Sartorius green are cellulosic with different pore size and were sterilized by different procedures. Membranes of 0.45 µm pore size are usually used for bacteria enumeration in the U.S. However, in Europe, 0.2 µm filters are often used for the bacterial analysis of waters. The high FC recovery rates for these three membrane filters are believed to be caused by their similar specific pore structure. It seems there is a need for a detailed study of all other brands of filters in respect to pore morphology. The number of pores in a unit area might be important too.

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