Estimation of Coliform Density by the Membrane Filter and the Fermentation Tube Methods

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That procedures in public health practice do not remain static or routine is suggested by this extensive research in the application of two different methods for measuring water contamination.

Statistical analyses of three extensive investigations comparing the membrane filter and fermentation tube technics for the quantitative estimation of coliform density [1-3] are summarized. The results indicate that for a wide variety of natural waters the estimates of coliform density obtained by using the MF technics employed in these investigations are, for practical purposes, in agreement with those obtained by the MPN method. The precision of the new method is two to five times greater than that of the standard procedure. It is undoubtedly true that the MF method may be improved through further developmental effort and that, at present, the two test procedures do not measure precisely the same groups of bacteria. However, the statistical analyses reported here point out certain deficiencies of the MPN method and present evidence that different standard confirmatory procedures have different coliform recovery efficiences and do not measure precisely the same groups of bacteria. In view of inherent limitations of the interpretative value of the coliform group as a whole, it is doubtful whether minor differences in the results obtained by two technics should weigh more heavily in the selection of standard methods than other relevant aspects of the tests, such as reproducibility, speed, convenience, and cost.

Method of Analysis

The laboratory examinations upon which these analyses are based were conducted with meticulous attention to detail by skilled bacteriologists. The precision of these density measurements is probably better than that commonly attained in routine water quality control and stream surveys. In addition to the published results [1-3] the authors have had access to original data from the Robert A. Taft Sanitary Engineering Center and the Millipore Filter Corporation.

The most extensive body of data analyzed pertains to a group of sampling stations near Boston (summarized in Table 1), for which the technics have been described by Yee, et al.[2] Re-

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This paper was prepared for and approved by the National Research Council, Division of Medical Sciences, Committee on Sanitary Engineering and Environment, Abel Wolman, Dr.Eng., chairman, and by the Subcommittees on Waste Disposal and on Water Supply, June, 1955.

Table 1—Coliform Densities in Streams and Ponds of Eastern Massachusetts

Station	Membrane Filter Tests							Fermentation Tube Tests							
	No. Replicate Filter	Vol. Sample per Filter ml	Mean Count per 100 ml	Mean Count per Filter	Std. dev. per Filter	Coef. of Variation	Lexis Ratio	No. Replicate Tests	Mean MPN/ 100 ml	Std. dev. MPN/ 100 ml	Coef. of Variation	$^{s}mpn/^{s}t$	Relative Coliform Recovery MF/MPN	Relative Precision $^{s}mf/^{s}mpn$	Total Bacteria Mean Count/ 100 ml
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Concord R. (1)	25	10	44.4	4.4	2.42	0.54	1.15	10	89.6	71.1	0.79	1.29	0.50	0.34	6,870
Lincoln Pond	25	50	13.7	6.8	2.96	0.43	1.13	10	12.0	14.5	1.20	1.95	1.13	0.41	84
Wilmington Brk	25	25	29 3	7.3	2.95	0.40	1.09	10	64.2	34.5	0.54	0.88	0.46	0.34	712
Concord R. (2)	21	10	57.2	5.7	2.79	0.49	1.17	10	16.6	10.4	0.63	1.02	3.45	2.69	15,500
Charles R. (1)	100	1	1,139	11.4	3.45	0.30	1.02	5	2,320	1,050	0.45	0.73	0.49	0.33	..
Great Pond	25	100	11.6	11.6	6.07	0.52	1.78	10	5.6	4.4	0.78	1.27	2.06	1.38	552
Stony Brk (1)	25	100	13.0	13.0	4.68	0.36	1.30	10	14.0	6.8	0.48	0.78	0.93	0.69	5,470
Muddy R.	25	1	1,372	13.7	10.4	0.76	2.81	10	3,150	3,952	1.25	2.04	0.44	0.26	18,000
Assabet R.	23	1	1,456	14.6	9.04	0.62	2.37	10	617	525	0.85	1.38	2.36	1.72	22,700
Neponset R.	25	1	1,668	16.7	5.80	0.35	1.42	10	887	732	0.82	1.33	1.88	0.79	7,400
Fresh Pond	20	25	78.8	19.7	4.83	0.24	1.09	9	99	59.0	0.60	0.97	0.80	0.33	9,730
Sudbury R.	25	10	666	66.6	15.0	0.22	1.84	10	826	590	0.71	1.16	0.81	0.25	11,000
Stony Brk (2)	25	100	71.6	71.6	13.0	0.18	1.54	10	66.2	77.3	1.17	1.90	1.08	0.17	2,400
Merrimac R.	25	1	8,388	83.9	19.0	0.23	2.07	10	5,153	5,450	1.06	1.72	1.54	0.35	81,300
Charles R. (2)	25	1	9,808	98.1	13.8	0.14	1.40	10	9,890	7,420	0.75	1.22	0.99	0.19	112,300
Dracut Well	23	5	2,295	115	30.9	0.27	2.88	10	2,912	4,885	1.68	2.72	0.79	0.13	15,320
Charles R. (3)	40	1	14,160	142	26.1	0.18	2.19	5	15,580	10,130	0.65	1.06	0.91	0.26	..
Mystic R.	25	1	14,290	143	41.5	0.29	3.47	10	5,221	11,370	2.17	3.52	2.74	0.36	329,000
Median						0.32	1.48				0.78	1.27	0.96	0.34	

Col. 7 = col. 6 ÷ col. 5.

Col. 8 = col. 6 ÷ square root of col. 5. The Lexis ratio is the quotient: Sample standard deviation/square root of mean count per filter. It is a measure of "overdispersion" relative to a Poisson model.

Col. 12 = col. 11 ÷ col. 10.

Col. 13 = col. 12 ÷ 0.616. The sample standard deviation/theoretical standard deviation.

When 5 tubes in each of 3 or more decimal dilutions are used the theoretical coefficient of variation (reference 4):

$$\sqrt{\exp.\left(\frac{1.27}{5^{1/2}}\right)^{2} - 1} = 0.616.$$

The ratios of col. 13 reflect overdispersion relative to the theoretical distribution.
Col. 14 = col. 4 ÷ col. 10.
Col. 15 = [col. 6 × 100] ÷ [col. 11 × col. 3].

sults are given for 18 samples collected from 14 streams and ponds during the period January, 1953, to February, 1955. For each sample 20 or more replicate filters were prepared using modified Endo medium with oxine, 10 replicate fermentation tube tests yielding 10 MPN's were made on each sample. Each MPN was based upon five tubes in each of three or more decimal dilutions with confirmation on EMB agar. Fresh sterile pipettes were used for each dilution in each bank of five tubes to insure that the resulting precision would accord with that routinely attained with individual MPN's. In a few instances indeterminate MPN values occurred and the means and standard deviations were calculated by analysis with log-probability paper.[5] In all other samples the usual statistical computations were employed.

From Table 1 (columns 4, 10, and 16) it is evident that a broad spectrum of bacterial densities was measured. The relative coliform recovery of the two technics for the various samples, as expressed by the ratio of the mean MF to the mean MPN density (column 14), ranged from 0.50 to 3.45 with an over-all median of 0.96. In 10 of 18 samples the standard procedure gave higher results.

The total bacterial densities in Table 1 (column 16) were calculated from counts on membrane filters using a non-selective medium. With each sample a sufficient number of colonies was counted so that the reported mean density has a standard counting error of no more than 5 per cent.

Table 2 presents a summary of the principal results of the analyses of all three investigations. Essentially the same statistical methods were employed throughout and Table 2 is arranged to parallel Table 1. The Cincinnati study was designed and carried forward in a manner similar to that in Boston. However, the confirmatory medium used at the Sanitary Engineering Center[6] differed somewhat from that with which the data of Table 1 were obtained. Improvements in medium and technics used in the MF test were made subsequent to the survey summarized in Table 2; these have been described by Kabler.[7] In the interpretation of the relative MPN dispersions in the columns of Table 2 it is pertinent to note that the 10–10–10-tube test used in the Cincinnati investigation inherently

Table 2—Coliform Data—MPN vs. MF: Summary of Results of Three Comparative Investigations

Location	Description: Reference	Description: No. of Sampling Stations	Description: No. of Samples	Results: Median for All Samples: MF Coefficient of Variation (7)	MF Lexis Ratio (8)	MPN Coefficient of Variation (12)	MPN $^{a}mpn/^{a}t$ (13)	Relative Coliform Recovery MF/MPN (14)	Relative Precision $^{a}mf/^{a}mpn$ (15)
Boston and vicinity	(2) and Table 1	14	18	0.32	1.48	0.78	1.27	0.96	0.34
Cincinnati and vicinity	(1)	12	18[a]	0.26	1.63	0.38[b]	0.90	0.71	0.54
Woods Hole, Massachusetts and vicinity	(3)	3	(i) 46	0.32	..	0.74[b]	1.20	0.77[c]	0.33
			(ii) 45	0.25	..	0.64[b]	1.04	0.52[c]	0.20
			(iii) 48	0.24	..	0.69[b]	1.12	1.00[c]	0.35

a. On most samples more than one quantity of water was filtered.

b. Computed in accordance with formula in footnote (col. 13) of Table 1. Ten tubes in each of 3 or more dilutions were used in the investigation at Cincinnati; five tube tests were used at Woods Hole and Boston.

c. Ninety-five per cent confidence limits for the population medians of relative recovery are: (i) 0.56 and 1.0; (ii) 0.42 and 0.64; and (iii) 0.60 and 1.10.

yields more reproducible MPN's than the 5–5–5-tube test used in the other studies.

The experimental design of the Woods Hole investigation[3] differed from that of the others. An extended time series of density measurements were made at three ocean water sampling stations. Fewer replicate filters were prepared with each sample than in the other studies and consequently the sampling errors of replicate measurements (Table 2, columns 7, 12, 13, and 15) cannot be regarded as being as firmly established as those of the other studies. However, these data have especial merit in that it is possible with many measurements at regular intervals at a given station to evaluate rather precisely the relative sensitivity of the MPN and MF technics (Table 2, column 14 and footnote c). The 95 per cent confidence limits for the population median recovery factor were computed by a nonparametric statistical method[8] and therefore are not predicated on arbitrary assumptions as to the precise mathematical frequency distribution function of the median densities. While the confidence zones are broad, they would appear to leave little doubt but that the densities obtained by the MPN method are generally somewhat larger than those obtained by the membrane filter technic. Woodward's analysis[1] corroborates this conclusion. While such differences may be statistically significant, it does not follow logically that the differences are of much practical import. In the following section are set forth various considerations relating to a practical evaluation of the observed coliform recoveries by the two methods.

Coliform Recovery

From Table 2 the over-all relative recovery factor, mean MF/ mean MPN, ranged from 0.52 to 1.00. At nearly all of the sampling stations the mean recovery factors fell in the range 0.3–3.00. This latter range corresponds approximately to the theoretical range into which 95 per cent of replicate 5–5–5-tube MPN's may be expected to fall. That is, if many replicate samples are tested from a water having a true mean density of 100 coliforms per 100 ml, 95 per cent of the resulting MPN's may be expected, according to the theoretical considerations of Halvorsen and Ziegler,[4] to fall in the range 30–300. The theoretical analysis, however, is predicated on an ideally random (Poisson) distribution of bacteria and does not take into account the effect of various experimental errors, such as imprecise pipetting and the action of predatory protozoa on coliform growth. There is some indication in the Boston and Woods Hole MPN data (Tables 1 and 2, column 13) that a small amount of "overdispersion" occurred * and that the theoretical 95 per cent confidence limits in practice might properly be regarded as really being 85 or 90 per cent confidence limits.

A large part of the differences in the MPN sensitivity and reproducibility between the Boston and Cincinnati data may undoubtedly be attributed to inherent differences in the standard confirmation technics employed—eosin-methylene blue plates (Boston); brilliant green bile tubes (Cincinnati). Examination of the MPN data in the Boston study indicated the occurrence of a disportionately high number of

* Similar overdispersion was noted in all investigations for the MF counts (Tables 1 and 2, column 8). The Lexis ratio (observed standard deviations divided by the Poissonian standard deviation) indicated that the dispersion of replicate counts actually attained was about 50 per cent larger than that expected from water having ideally distributed microorganisms and with hypothetical technics free from experimental sources of variance. It is noteworthy that the overdispersion observed was about the same as that reported by many workers for bacteria plate counts (Lexis ratios of 1.5–2.0).

low dilution (high concentration) tubes that failed to confirm and an excessive number of "skips" corresponding to improbable MPN's resulted. In flagrant instances of this occurrence the low dilution results were ignored in the computation of the MPN's so that the disparity between the EMB and BGB technics reported is not as great as otherwise would have been the case. With some of the Boston samples both EMB and BGB confirmation were carried out in parallel. The latter were found rather consistently to yield larger MPN values. From a statistical viewpoint it appears that the two standard confirmatory procedures do not measure precisely the same groups of bacteria.

A further point in this connection is relevant. While the median ratios of densities by the two methods indicate that MF counts on the average are undoubtedly smaller than corresponding MPN's, a sizable portion of the difference may be attributed simply to the fact that the most probable number, mathematically considered, is a biased estimator of the true density. The arithmetic means of replicate MPN's tend to be too high by a factor of 23 per cent in a 5–5–5–tube test and a factor of 43 per cent in a 3–3–3–tube test. If a large number of replicate 5–5–5–tube MPN tests are made from water containing 100 coliform per 100 ml the arithmetic mean MPN will be 123 instead of 100. If the method suggested [6] for correcting for the bias of the MPN test had been used in the statistical analysis of Tables 1 and 2, the reported recovery factors (column 14) would have been more closely centered around unity. In this respect, therefore, the disparity in coliform recovery between the two technics is more apparent than real.

In Figure 1 is shown a scatter-diagram of the Woods Hole density measurements by the two methods. In this plot the MF values are plotted against the MPN values with correction for bias. The correction factor (0.81 = 1/1.23) pertains to the 5–5–5–tube test used in the investigation. With this adjustment for bias the plotted points exhibit an average recovery factor of approximately 100 per cent.

As a matter of interest confidence zones of 50 per cent, 75 per cent, 95 per cent based on the Halvorsen-Ziegler theory for the dispersion of replicate MPN's have been constructed on Figure 1. These zones are applicable (neglecting overdispersion) to points obtained from pairs of replicate 5–5–5–tube MPN's. Thus on a scatter diagram of duplicate MPN values, 95 per cent of

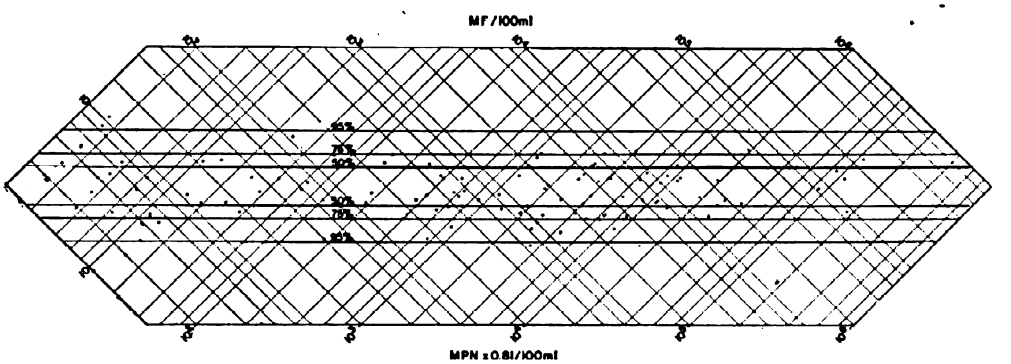

Figure 1—Log-Log Plot of Coliform Densities by the MF and MPN Methods, Woods Hole Series (Presnell, Arcisz, and Kelly [3])

the points may be expected to fall between the parallel lines delimiting the 95 per cent confidence zone. In Figure 1 in which MF is plotted against MPN, 132 of the 139 points (94.97 per cent) fall within the 95 per cent confidence zone. The implication is that for the Woods Hole samples the correlation between MPN and MF is about the same as that which would be obtained between duplicate MPN values.

The foregoing discussion may be summarized in the following statement: The disparity in the coliform sensitivities of the MF and MPN in a wide variety of natural waters does not have much practical significance in view of the inherently low degree of reproducibility of the standard dilution test and the bias of MPN estimates and in view of comparable differences in sensitivities existing between permissible alternate procedures of the latter.

Comparison of Precision

In assessing the precision of the two methods the following points are relevant: (1) The precision of a MPN value obtained from a multidilution fermentation tube test depends chiefly upon the number of tubes observed in each dilution and not upon the bacterial density.[4] The standard error of sampling may be made very small if a large number of replicate tubes are tested. (2) The precision of a MF value from a single filter depends primarily upon the number of sheen colonies counted. Hence, the reproducibility of a MF value depends on the product of the bacterial density and the volume of sample filtered. (3) The standard error of a mean density obtained from replicate tests depends upon the foregoing factors and in addition varies inversely with the square root of the number of replicates. The standard deviations tested in Table 1 pertain to individual measurements and depend only upon factors (1) and (2).

From Table 1, column 15, it may be seen that the precision of the MF test was superior in all but three of the samples tested. From Table 2, column 15, the median ratio of the standard deviation of the individual MF test to that of the MPN for 5–5–5–tube test is seen to be about one-third, and for the 10–10–10–tube test about one-half.

In assessing the practical aspects of the relative reproducibility, the results (Table 3) of a comparative computation may be helpful. Formulating the frequency dispersion of replicate of 5–5–5–tube MPN's by the log normal distribution [4, 5] and that of replicate MF's by the negative binomial distribution [9] with a constant Lexis ratio of 1.65, estimates have been made of the

Table 3—Sizes of MPN and MF Tests Having Equal Precision

	MF Test One Filter		MPN *
Number of Colonies Counted on Individual Membrane Filter	No. of ml of 1 Coliform/100 ml Water Filtered	No. of ml of 5,000 Coliform/100 ml Water Filtered	No. of Tubes Required in Each of 3 Decimal Dilutions
1	100	0.02	2
10	1,000	0.2	9
100	10,000	2.0	69

* One multidilution tube test yielding a single determinate MPN. In the case of potable waters this requires the use of 100 ml fermentation tubes.

number of tubes needed in multidilution MPN tests to provide a precision equal to MF tests involving various numbers of counted sheen colonies.

From Table 3 it is evident that with waters of high coliform density the advantage of the MF technic is especially marked. With densities in the potable water range the advantage is not as great unless larger volumes of water are filtered. However, such waters usually will fiter rapidly and precise counts may readily be obtained. With the assumptions underlying the calculations summarized in Table 3 it may be shown that 42 ml of water just complying with the drinking water standard of 1 coliform per 100 milliliter must be filtered so that the resulting MF count will yield a precision equal to that obtained with the usual single-dilution test of five 10 ml tubes.

Conclusions

Results of statistical analyses of three extensive investigations on a wide variety of natural waters comparing the MPN and MF technics indicate that, on the average, the former gave higher indications of density by a factor 1.0–1.9 with an average of 1.3 for the specific technics used in these investigations. However, the difference is not regarded as important from a practical viewpoint because of the inherent lack of precision of the individual MPN value. Moreover, the disparity between MF and MPN values for most of the water samples tested was not significantly larger than discrepancies between results obtained with permissible variations of the standard dilution method. A considerable part of the disparity between the MF and MPN values may be attributed to the fact that mathematically considered, the MPN tends to overestimate the true density; on the average MPN values are greater than the true density.

With nearly all of the samples listed the precision attained with a single filter was found to be two to five times greater than that of a 5–5–5–tube MPN. The reproducibility of the MF test as measured by the coefficient of variation of replicate tests was found to vary inversely with the square root of the number of colonies counted.

ACKNOWLEDGMENT—The authors acknowledge with appreciation the collaboration of Dr. Paul W. Kabler of the Robert A. Taft Sanitary Engineering Center whose advice was most helpful in planning and interpreting the statistical treatment of the data.

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