Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach

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Currently used methodologies for estimating fecal coliform densities in marine waters include the American Public Health Association (APHA) most probable number (MPN) procedure,[1,2] the A-1 method,[3] also an MPN technique, and the M-FC method,[2,4] a membrane filtration (MF) procedure. Of these methodologies, only the MPN is used in the 23 coastal states to classify shellfish harvest waters. Because of poor quantification and low original MF procedure selectivity, it has been at least 15 years since any state has used membrane filtration in this way.

Recently, the mTEC membrane filtration technique,[5] which is endorsed by the U. S. Environmental Protection Agency (EPA) for examining environmental waters, including shellfish growing areas,[6] has been proposed for the enumeration of thermotolerant (fecal) coliforms and *Escherichia coli*. The method incorporates a primary, selective, differential medium for enumerating thermotolerant, gram-negative, lactose-fermenting bacteria followed by an *in situ* urease test to distinguish *E. coli* colonies. It provides the best overall performance among several MF techniques, including both the standard and resuscitation modified[7] M-FC methodologies, for the enumeration of fecal coliforms in fresh waters.[8] Studies conducted in marine waters off the coasts of New York and New Jersey demonstrate similar results.[9] Fecal coliform recoveries by the mTEC method were consistently higher than those of both the standard and modified M-FC procedures. It was concluded that the M-FC procedure was inappropriate for marine water use. Moreover, *E. coli* densities cannot be determined by the M-FC procedure unless individual colonies are subcultured and speciated.

The objective of this study was to compare the mTEC and APHA-MPN procedures for fecal coliform and *E. coli* recovery from various marine and estuarine waters. The advantages of using an MF approach rather than the APHA-MPN technique are: results are obtained much more rapidly; less time is required to conduct an assay; the method is more precise than the MPN, especially when plates with 20 to 80 target colonies are enumerated; and the cost per assay and the labor required are dramatically decreased.

MATERIALS AND METHODS

Sampling sites and procedures. Marine and estuarine water samples for bacteriological analyses were collected at Burley Lagoon, Wash., in August, 1981, and January, 1982; New Haven Harbor, Conn., in October, 1983; Gray's Harbor, Wash., in December, 1983; Yaquina Bay, Oreg., in May, 1984; Parrots Creek, Va., in November, 1984; Buttermilk Bay, Mass., in July, 1985; Morro Bay, Calif., in October, 1985; and Narragansett Bay, R. I., in November, 1985. Samples were also taken monthly at estuarine sites in southeastern Massachusetts (Westport) in February, March, and April, 1984. Waters were collected in sterile 1-L polypropylene containers at about 0.5 m depths and held on ice before analysis. The intervals between sample collection and assay did not exceed 6 hours. All bacteriological analyses were performed using aliquots obtained from the same, well mixed, 1-L container.

Paired t-test and the critical ratio test were performed on 574 observations from fecal coliform analyses.

MPN analyses. Either a 3- or 5-tube, multiple dilution MPN procedure for fecal coliform analysis was conducted through the confirmed or completed test according to APHA recommended procedures.[2] Appropriate volumes and decimal dilutions of water samples were inoculated into lauryl tryptose broth (LST) and incubated up to 48 hours at 35°C. All gas-positive LST tubes were subcultured to tubes of EC medium and incubated at 44.5°C for 24 hours. Gas-positive EC tubes were considered positive for fecal coliforms. Selected EC-positive tubes were streaked for colony isolation on EMB plates and incubated at 35°C for 24 hours. Bacterial colonies of differing morphology were subcultured and further identified by routine IMViC procedures.[1] An *E. coli* MPN was then calculated.

MF analyses. The mTEC procedure[5] was performed on appropriate dilution volumes so that when possible, 20 to 80 fecal coliform colonies resulted. Following filtration, the cellulose membrane filters (Gelman GN-6, Gelman Sciences, Ann Arbor, Mich.) were placed on the primary isolation medium and the plates were incubated at 35°C for 2 hours (a resuscitation step). After 20 to 22 hours additional incubation at 44.5°C, all yellow colonies were counted as fecal coliforms. The filters were then transferred to filter pads saturated with urease reagent[5] and incubated for an additional 10 minutes at room temperature. Colonies that remain yellow are *E. coli;* those that turn pink to purple are urease positive and are not counted as *E. coli.*

Statistical analyses. The data were analyzed using two different statistical procedures. The parametric procedure assumed a normal distribution of the target population. The nonparametric test presupposed that the target bacteria distribution was unknown. Differences in fecal coliform and *E. coli* recoveries

for individual observations were tested with a paired t-statistic. The critical ratio test,[10] based on a normal approximation to the binominal distribution, was used to determine if the proportion of paired observations with values that did not fall into the same 0.5 $\log_{10}$ category was significantly different from the expected value.

RESULTS AND DISCUSSION

A summary of the fecal coliform recoveries by the mTEC and MPN procedures is presented in Table 1. A total of 574 samples, collected at various times of the year from marine and estuarine waters and at stations along the Northeast, Southeast, and Pacific Coasts of the U. S., were examined. Results of a paired t-statistic test showed no significant difference (at the 95% confidence level) in fecal coliform recoveries by the two methods at eight of the nine sites examined. The relative percent recovery (mTEC/MPN) was between 59 and 118% with a mean percent recovery (all samples) of 92%.

The mTEC method specificity has been comprehensively examined in two previous studies.[5,9] Of 1300 presumptive fecal coliform colonies, less than 90% were confirmed as such when isolated from various aquatic environments. Therefore, it was not considered necessary to pick and confirm colonies for this study's purposes. Positive EC tubes (other than those selected for *E. coli* enumeration) were not further tested to confirm fecal coliform presence.

Paired MF-MPN fecal coliform data were also examined by using the critical ratio test (Table 2). Bacterial densities were segregated by 0.5 $\log_{10}$ increments. The number of paired observations not falling in the same density categories were tested to determine if the proportion where one method was greater

Table 1—Comparison of fecal coliform densities determined by the mTEC and MPN procedures.

Site	Number of samples[a]	Mean fecal coliform recovery[b] mTEC	MPN	mTEC/MPN
New Haven, Conn.	94	28.8[c]	24.5	1.18
Westport, Mass.	30	35.5[c]	36.3	0.98
Burley Lagoon, Wash.	50	44.8[c]	53.0	0.85
Gray's Harbor, Wash.	42	26.9[d]	45.7	0.59
Yaquina Bay, Oreg.	36	26.3[c]	30.2	0.87
Parrots Creek, Va.	103	66.1[c]	70.9	0.93
Buttermilk Bay, Mass.	86	10.1[c]	11.9	0.85
Narragansett, Bay, R.I.	24	58.9[c]	58.9	1.00
Morro Bay, Calif.	109	53.9[c]	56.6	0.95
All sites	574	40.2[c]	43.5	0.92

[a] Samples were collected from different stations and days at each site.
[b] Geometric mean density/100 mL.
[c] Not significantly different (paired t statistic) from MPN value (P = 0.05).
[d] Significantly different (paired t statistic) from MPN value (P = 0.05).

Table 2—Critical ratio test for fecal coliform recoveries using paired membrane filtration—MPN data for nine marine and estuarine sites.

Site	Number of paired observations (n)	Proportion[a]	Critical ratio[b]
New Haven, Conn.	94	18/36[c]	0.0
Westport, Mass.	30	8/16[c]	0.0
Burley Lagoon, Wash.	50	14/27[c]	0.19
Gray's Harbor, Wash.	42	12/18[c]	1.41
Yaquina Bay, Oreg.	36	11/18[c]	0.94
Parrots Creek, Va.	103	20/35[c]	0.85
Buttermilk Bay, Mass.	86	28/40[d]	2.53
Narragansett Bay, R.I.	24	5/10[c]	0.0
Morro Bay, Calif.	109	34/57[c]	1.46

[a] Proportion of all paired MPN-MF values not falling in the same 0.5 $\log_{10}$ category where the MPN value was greater than the MF value.

[b] Critical Ratio (Z) = $\dfrac{\mu - \pi o}{\sqrt{\dfrac{\pi o(1-\pi o)}{n}}}$, where μ = proportion[a] and πo = ½.

[c] Proportion not significantly different from expected proportion (½, P = 0.05).
[d] Proportion significantly different from expected proportion (½, P = 0.05).

Table 3—Comparison of paired observations of *E. coli* recoveries using the mTEC and APHA-MPN procedures.

Site	Number of paired observations (n)	mTEC[a]	MPN[a]	mTEC/MPN
Parrots Creek, Va.	55	60.3[b]	64.1	0.96
Buttermilk Bay, Mass.	86	9.2[b]	10.4	0.88
Narragansett Bay, R.I.	24	43.1[b]	44.3	0.97
Morro Bay, Calif.	109	50.6[b]	53.9	0.94
All Sites	274	38.9[b]	41.4	0.94

[a] Geometric mean *E. coli* density/100 mL.
[b] Not significantly different (paired t-statistic) from MPN value (P = 0.05).

Table 4—Critical ratio test for *E. coli* recoveries using paired membrane filtration—MPN data from four marine and estuarine sites.

Site	Number of paired observations (n)	Proportion[a]	Critical ratio[b]
Parrots Creek, Va.	55	9/17[c]	0.24
Buttermilk Bay, Mass.	86	20/32[c]	1.41
Narragansett Bay, R.I.	24	7/14[c]	0.0
Morro Bay, Calif.	109	30/59[c]	0.13

[a] Proportion of all paired MPN-MF values not falling in the same 0.5 $\log_{10}$ category where the MPN value was greater than the MF value.

[b] Critical Ratio (Z) = $\frac{\mu - \pi o}{\sqrt{\frac{\pi o(1-\pi o)}{n}}}$, where μ = proportion and $\pi o = ½$.

[c] Proportion not significantly different from expected proportion (½, P = 0.05).

than the other was significantly different from that expected (0.5) if there was no difference between the bacterial densities measured by each method. No differences were found at eight of the nine sites examined. Interestingly, the Buttermilk Bay, Mass., site at which recoveries were significantly different using the critical ratio test is not the same as that at Gray's Harbor, Wash., where significant differences were found using the paired t-statistic.

E. coli recovery comparisons from the MPN and mTEC procedures were conducted at four of the study sites (Table 3). The results of a paired t-statistic test showed that geometric mean *E. coli* densities recovered were not significantly different. The recovery of *E. coli* on mTEC relative to the MPN exceeded 88% at all individual sites, and the overall relative recovery was 94% for all sites examined.

Lactose-positive, urease-negative colonies (*E. coli*) from the mTEC medium were not confirmed for several reasons. Dufour *et al.*[5] subjected the mTEC method to an extensive evaluation during its development and found that the presence of *E. coli* was confirmed in more than 95% of 370 presumptive colonies isolated from estuarine and marine samples collected from northeastern U. S. waters. Vasconcelos and Anthony[11] reported that more than 92% of 295 presumptive *E. coli* isolates (obtained from the mTEC medium) were verified as such from samples taken from recreational waters in the Pacific Northwest. Because of these findings, it was not considered necessary to pick and confirm individual isolates from the mTEC medium. Moreover, for this study, the MPN values obtained were treated as accurate, unbiased estimates of both the fecal coliform and *E. coli* densities, even though the MPN value for a 5-tube multiple dilution technique reportedly overestimates the true bacterial density from a given test sample by about 23%.[12]

Paired MF-MPN *E. coli* data were examined by the critical ratio test (Table 4). Data were treated in the same manner as that described for the fecal coliform analyses noted above. No differences (P = 0.05) were found at any of the four sites examined; therefore, the methods apparently measured the same population.

The mTEC membrane filtration technique has several distinct advantages. The enumeration of both fecal coliforms and *E. coli* is completed within 24 hours, whereas the APHA-MPN procedure requires 3 days to obtain results for fecal coliforms and up to 10 days for *E. coli*. The precision inherent to the conventional MPN approach is very poor. The 95% confidence limits for a 5-tube, 3 dilution technique are 0.3 to 3 times the MPN obtained. The confidence interval is even larger for a 3-tube multiple dilution procedure. These broad confidence limits are unacceptable for work that requires precise bacterial density determinations in aquatic environments. In contrast, the confidence interval for a membrane filtration approach is a function of the number of colonies of the target organism on the filter and the number of dilution replicates. For routine aquatic environment monitoring, when one filtration per dilution is performed, the 95% confidence interval (assuming 20 to 80 target organisms per filter) is 23 to 40%. This degree of precision is considerably greater than that for the MPN. Membrane method precision can be further increased with additional numbers of dilution replicates. Generally it is necessary to perform filtrations on 0.5 log dilutions of sample (that is 1, 3, 10, 30, 100 mL) when examining waters in which the degree of fecal contamination is unknown.

Another distinct advantage of the mTEC approach is the savings in both cost and manhours for conducting assays. On a per assay basis, supplies for the mTEC procedure cost approximately 50% less than those for the APHA-MPN. Moreover, the time required for preparing materials, processing laboratory samples,

Table 5—Advantages of membrane filtration approach for enumeration of fecal coliforms and *E. coli* in marine and estuarine waters.

Method	Assay interval, hour: Fecal coliform	Assay interval, hour: *E. coli*	Precision, percent	Supply cost/assay	Assay time[a] minute
mTEC	24	24	±35[b]	1.50	15
APHA-MPN	72	240[c]	±300[d]	2.80	45

[a] Includes time for media preparation, sample processing and cleanup. Assumes supplies are disposable.

[b] Based on one filtration/dilution.

[c] Through IMViC tests.

[d] Five-tube, multiple dilution procedure.

reading and recording results, and the clean-up following this process is about 30% that of the APHA-MPN procedure. These labor savings must also be factored into the costs for performing either assay. The advantages of the mTEC technique are summarized in Table 5.

The data generated from this study strongly suggest that the mTEC method is a viable alternative to the APHA-MPN procedure for the enumeration of both fecal coliforms and *E. coli* in marine and estuarine waters. The differences between these techniques for enumerating these organisms are statistically indistinguishable. This fact, coupled with the advantages of the mTEC procedure previously noted, provides a strong argument for further investigation of procedure usage because it is a more rapid, facile, precise, and economical alternative to the APHA-MPN technique.

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