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Comparison of APHA-MPN and mTEC Methods for Detecting Indicator Bacteria through a Sanitary Survey of Greenwich Bay, Rhode Island, U. S. A.

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The APHA-MPN procedure is the only officially accepted method for classifying shellfish growing areas in U. S. A. The method estimates the levels of fecal coliforms and *E. coli*, indicators of the sanitary quality of environmental waters. However, the MPN has several disadvantages requiring far more time, labor and expense for assay, as well as providing relatively poor precision. Several membrane filtration procedures have been developed to enumerate these indicators in waters. Of these, the mTEC technique has been shown to provide recoveries of fecal coliforms and *E. coli* comparable to those of the MPN method. In an abbreviated sanitary survey for Greenwich Bay in Rhode Island, U. S. A., classified as an approved shellfish growing area, the mTEC and conventional MPN methods were again compared for their recoveries of the indicator bacteria. It was found that the recoveries of fecal coliforms and *E. coli* provided by the mTEC technique are 1.08 and 1.27 times higher than those produced by MPN for water monitoring, respectively, and that the membrane filtration method appears to be a possible alternative to APHA-MPN.

Introduction

Shellfish is traditionally being consumed as preference food in the United States of America with proper sanitary control since 1924 (FDA, 1992) at which there occurred a widespread food poisoning, typhoid fever, in a large scale by contamination of bacteria. However, shellfish that continue to be subjected to negative reaction in the U. S. are a big issue with the safety guarantee (Committee on Evaluation of the Safety of Fishery Products, 1991).

In the U. S., coastal areas producing shellfish are thoroughly evaluated by sanitary survey for their water quality, and then are classified as approved, conditionally approved, restricted, conditionally restricted, or prohibited, as provided by compliance criteria of the National Shellfish Sanitation Program (NSSP) (FDA, 1992). In contrast, the classification of growing areas in Korea has been very limited, with only four such areas located near Chungmu and Yeosu city being so designated. These classifications in Korea were under taken, so that the shellfish (oyster, blue mussel, and ark clam) produced from these designated areas could be exported to the U. S. under provisions of the 'Shellfish Sanitation Treaty' established in 1972 between Korea and the U. S. (Central Fishery Products Inspection Station, 1973) and renewed to MOU (Memorandum of Understanding) between Korea NFA (National Fisheries Administration) and U. S. FDA (Food and Drug Administration) (1987).

Estimation of indicator bacteria to determine water quality for the purpose of classifying growing areas is achieved using the American Public Health

Association (APHA) most probable number (MPN) procedure (APHA, 1970, 1981), the only method officially approved by U. S. FDA and the Interstate Shellfish Sanitation Conference (ISSC). Howerver, the MPN method has significant disadvantages, in that it requires greater labor, and expense, provides extreamly poor precision, with 95% confidence limits ranging from approximately 30 to 300% of MPNs determined. In spite of these disadvantages, the MPN method has been exclusively used for estimating indicator bacteria in marine waters because there has been no accepted alternative method to the MPN.

Dufour et al. (1981) developed the mTEC (membrane thermotolerant *E. coli*) membrane filtration technique in 1981. This method enumerates thermotolerant fecal coliforms and *Escherichia coli*, has been endorsed by the U. S. Environmental Protection Agency (EPA) (1985) for examining recreational enviromental waters in which many locations include shellfish growing area. The method is considered to be the best among several membrane filtration (MF) techniques (Green et al., 1977; Pagel et al., 1982; Santiago-Mercado and Hazen, 1987) available for enumerating fecal coliforms. It incorporates a primary, selective, differential medium for enumerating thermotolerant, gram-negative, lactose-fermenting bacteria, followed by an in situ urease test to detect *E. coli* colonies. Studies conducted in marine waters of some coastal areas in U. S. have demonstrated comparable results between this mTEC and the standard MPN method (Rippey et al., 1987a). The mTEC procedure has several important advantages, in that it is rapid, inexpensive, requires less analyst time, and is far more precise than the MPN method.

Greenwich Bay, classified as an approved area, lies between Warwick and East Greenwich as a part of Narragansset Bay in Rhode Island, and is one of the country's major producers of quahogs (hard-shell clam), *Mercenaria mercenaria*. However, Greenwich Bay contains four coves classified as prohibited areas, and one of them, Greenwich Cove, receives the effluent from the East Greenwich Sewage Treatment Plant (EGSTP). Because there are actual and potential sources of contamination in this Bay, it is necessary to revaluate the classification of the area periodically, according to NSSP criteria for conducting sanitary surveys. Therefore, an abbreviated sanitary survey for Greenwich Bay was performed under the above background, as well as the recoveries of fecal coliforms and *E. coli* afforded by mTEC and MPN methods through the survey were compared so that whether or not mTEC is possible alternative to MPN.

Materials and Methods

Sampling sites and procedures. To estimate the general condition of Greenwich Bay ten stations (No. 1-10) were established for sampling, including one in Greenwich Cove, a closed area receiving the effluent from EGSTP (Fig. 1). On the basis of the results from the first survey work, sample stations were readjusted to other ten sites (No. 4-16). Sampling was performed on four separate dates and times under varying conditions of tide (flood and ebb) and weather (clear and rain) during the period from August 11th to 18th, 1992. Surface waters were collected in sterile 500*ml* polypropylene containers at about 0.3*m* depth, and held on ice for up to 8 hours until analyses. All bacteriological analyses were performed using aliquots obtained from sample containers after they had been well mixed by shaking.

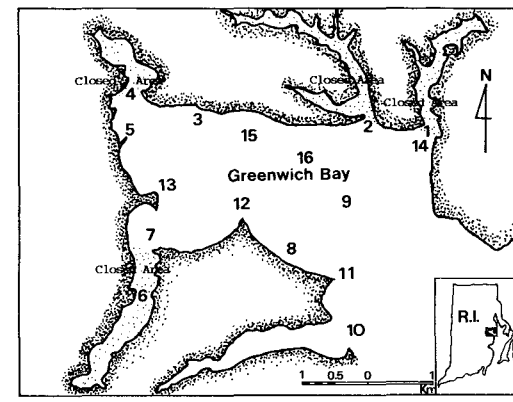

Fig. 1. Reference map of Greenwich Bay, Rhode Island, U. S. A.

MPN method. A five-tube, multiple dilution MPN procedure for fecal coliform analysis was performed through the confirmed test according to APHA recommended procedures (APHA, 1981). Appropriate volumes and decimal dilutions of water samples were inoculated into lauryl tryptose broth (LST) and incubated up to 48 hours at 35℃. All gas-positive LST tubes were subcultured to tubes of the confirmed medium-4-methylumbelliferyl-β-D-glucuronide (EC-MUG) medium (Rippey et al., 1987b) and incubated for 24 hours at 44.5℃ to distinguish fecal coliforms and *E. coli* simultaneously. Gas-positive EC tubes were considered positive for fecal coliforms and were examined under long wave (366nm) ultraviolet light source (Black Ray, UVP, Inc., San Gabriel, CA) and scored for fluorescence to confirm *E. coli*. The most probable number of fecal coliforms and *E. coli* per 100*ml* were then calculated.

mTEC method. The mTEC procedure (Dufour et al., 1981; EPA, 1985) was performed with using cellulose membrane filters (Millipore HC, Millipore Corp., Bedford, MA) and filtration of appropriate volumes so that less than 100 colonies resulted. The cellulose membrane filters were placed plates of mTEC medium (Difco) and incubated in ethafoam blocks so that temperatures rose gradually to about 35℃ in 2 hours (a resuscitation step) and to 44.5℃ in about 3 hours, at which they remained for 18 to 22 hours. After incubation, yellow colonies were counted as fecal coliforms, and densities were determined. Filters then were transferred to filter pads saturated with urease reagent and incubated for an additional 10~20 minutes at room temperature. Colonies remaining yellow are urease negative and counted as *E. coli*. Those that turn pink to purple in color are urease positive and not counted as E. coli.

mCP method. Clostridium perfringens, an anaerobic, spore-forming bacterial species considered to be a conservative indicator of fecal contamination (Akama an Otani, 1970; Haenal, 1970; Bisson and Cabelli, 1980), was enumerated using the mCP procedure (Bisson and Cabelli, 1979), a membrane filtration method. Appropriate volumes of samples were filtered so that, when possible, less than 100 colonies were obtained on membranes. The filters were placed on mCP medium and then incubated anaerobically (nitrogen, hydrogen, and carbon dioxide gas mixture) 45 ± 0.2℃ for 18~22 hours. All yellow or pale yellow colonies were counted as presumptive as *C. perfringens* and confirmed as such using an in situ alkaline phosphatase test; colonies, that turn pink to magenta in color are alkalase positive, and confirmed as *C. perfringens*.

Data analysis. For comparison of the recoveries obtained by both methods were compared using the binomial distribution linear regression analysis technique (Snedecor and Cochran, 1980).

Results and Discussion

Environmental conditions during the survey period

Table 1 shows the temperature and salinity data obtained during sampling, and denotes the tide and environmental conditions encountered. Significant rainfall occurred prior to and during both sampling trials on 18th August, 1992.

Revaluation of the growing area with MPN data

The results obtained by APHA-MPN method for fecal coliform and *E. coli* densities clearly show that during adverse (rainfall) conditions, all stations except 11 exhibit water quality unacceptable for approved status (Table 1), exceeding the NSSP criteria (FDA, 1992) of fecal coliform median or geometric mean MPNs of 14 per 100 *ml*, and more than 10 percent of the samples exceed MPNs of 43 per 100 *ml* for a 5-tube decimal dilution test (Table 2). It seems almost certain that Greenwich Bay should be reclassified in a near future. However, these conclusions are derived from only four sampling surveys. Proper reevaluation of this Bay requires that a full-scale sanitary survey be performed.

C. perfringens densities (Table 3) show a tendency to occur proportional to those of fecal coliforms, and it suggests that this species may provide

Table 1. Daily temperature, salinity, and fecal coliforms and *E. coli* determined by MPN for sea water collected at Greenwich Bay during August 11 to 18, 1992

Date	Time	Station	Temp.(℃)	Sal.(‰)	Fecal coliforms	*E. coli*	Remarks
92. 8.11	10 : 25	1	–	–	17	17	Clear,
	10 : 34	2	–	–	14	9	low
	10 : 42	3	–	–	17	17	ebb
	10 : 46	4	–	–	330	170	tide
	10 : 53	5	–	–	23	13	
	11 : 07	6	–	–	49	23	
	11 : 12	7	–	–	130	130	
	11 : 18	8	–	–	17	8	
	11 : 24	9	–	–	79	33	
	11 : 32	10	–	–	330	330	
92. 8.12	12 : 00	4	24.5	24.5	79	79	Clear,
	11 : 52	7	23.5	25.0	14	11	low
	11 : 38	9	23.5	25.0	<2	<2	ebb
	11 : 26	10	23.0	28.0	2	2	tide
	11 : 33	11	24.0	27.0	7	<2	
	11 : 47	12	23.5	25.0	<2	<2	
	11 : 56	13	23.5	26.0	5	5	
	12 : 10	14	24.5	27.5	8	8	
	12 : 04	15	23.5	27.0	5	2	
	11 : 42	16	23.5	26.0	2	2	
92. 8.18	07 : 55	4	–	22.0	3,500	3,500	Rain,
	07 : 51	7	–	26.0	330	330	high
	07 : 45	9	–	27.0	240	130	tide
	07 : 40	10	–	28.0	79	79	flood
	07 : 43	11	–	28.0	6	6	
	07 : 48	12	–	28.0	8	8	
	07 : 52	13	–	28.0	1,300	790	
	08 : 04	14	–	29.0	130	130	
	08 : 00	15	–	29.0	240	240	
	07 : 47	16	–	28.0	170	110	
92. 8.18	16 : 45	4	–	15.0	1,100	700	Rain,
	16 : 36	7	–	22.0	700	700	high
	16 : 28	9	–	26.0	11	11	tide
	16 : 20	10	–	16.0	1,300	1,300	flood
	16 : 25	11	–	26.0	5	5	
	16 : 34	12	–	26.0	110	79	
	16 : 42	13	–	24.0	2,400	2,400	
	16 : 58	14	–	27.0	490	330	
	16 : 51	15	–	27.0	79	49	
	16 : 31	16	–	26.0	33	33	
Tatal		40					

Table 2. Overall for fecal coliforms determined by MPN for sea water collected at Greenwich Bay

Station	No. of samples	Fecal coliforms/100 *ml*		% of >43/100 *ml*	Maximum value
		Median	G. mean[1]		
1	1	17	17	0	17
2	1	14	14	0	14
3	1	17	17	0	17
4	4	720	560	100	3,500
5	1	23	23	0	23
6	1	49	49	100	49
7	4	230	140	75	700
8	1	17	17	0	17
9	4	45	25	50	240
10	4	210	90	75	1,300
11	3	6	5	0	7
12	3	8	11	33	110
13	3	1,300	250	67	2,400
14	3	130	79	67	490
15	3	79	45	67	240
16	3	33	22	33	170
Total	40				
NSSP criteria		≤ 14	≤ 14	≤ 10	

[1]Geometric mean

a good indication of contamination emanating from human fecal wastes (Akama and Otani, 1970; Haenal, 1970; Bisson and Cabellip, 1980).

Comparison of MPN and mTEC

Fecal coliform recoveries by the mTEC and MPN methods were summarized in Table 3. The results from a total 40 samples are variable depending on the weather, tide, and location. For this reason, the relative percent recovery of mTEC to MPN was individually indicated (ratio) for each sample. Overall recovery results were between 40 and 445%, with a mean percent recovery of 108%. Examined by regression analysis, fecal coliform densities obtained by the MPN and mTEC methods are shown in Fig. 2. The results of both methods are a highly comparable, having a Pearson correlation coefficient of 0.9570.

Similar results were obtained for *E. coli* recoveries by EC-MUG and mTEC, and these are shown in Table 3. Individual relative percent recovery of mTEC to MPN ranged from 42 to 445%, with a mean recovery percent of 127%. When the results of *E. coli* from both methods are plotted on a regression curve (Fig. 3), they also show high comparability, having a Pearson correlation coefficient of

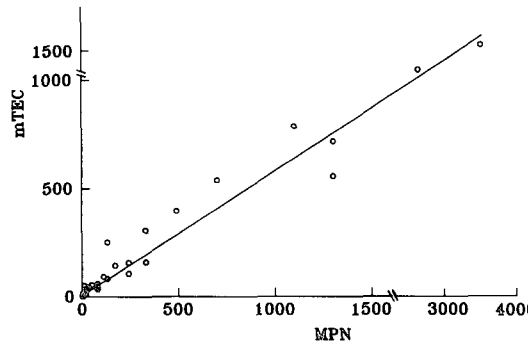

Fig. 2. Correlation of fecal coliform densities obtained by the MPN and mTEC methods.

Table 3. Results obained by APHA-MPN, mTEC, and mCP methods for sea waters collected at Greenwich Bay during August 11 to 18, 1992

Sampling date	Station	Fecal coliforms		Ratio	*E. coli*		Ratio	*C. per*[1]
		MPN	mTEC	(mTEC/MPN)	MPN	mTEC	(mTEC/MPN)	mCP
92. 8.11	1	17	10	0.59	17	9	0.53	10
	2	14	21	1.50	9	21	2.33	9
	3	17	33	1.94	17	33	1.94	17
	4	330	159	0.48	170	152	0.89	20
	5	23	33	1.43	13	29	2.23	6
	6	49	53	1.08	23	47	2.04	<1
	7	130	83	0.64	130	75	0.58	11
	8	17	27	1.59	8	27	3.38	14
	9	79	46	0.58	33	45	1.36	12
	10	330	158	0.48	330	155	0.47	19
92. 8.12	4	79	33	0.42	79	33	0.42	3
	7	14	18	1.29	11	18	1.64	5
	9	<2	1	0.56	<2	1	0.56	5
	10	2	2	1.00	2	2	1.00	3
	11	7	4	0.56	<2	1	0.56	5
	12	<2	2	1.11	<2	2	1.11	4
	13	5	15	3.00	5	14	2.80	3
	14	8	5	0.63	8	5	0.63	3
	15	5	2	0.40	2	2	1.00	2
	16	2	3	1.50	2	3	1.50	6
92. 8.18	4	3,500	1,556	0.44	3,500	1,552	0.44	125
	7	330	307	0.93	330	281	0.85	21
	9	240	156	0.65	130	150	1.15	5
	10	79	37	0.47	79	34	0.43	4
	11	6	8	1.33	6	6	1.00	3
	12	8	16	2.00	8	15	1.88	4
	13	1,300	557	0.43	790	541	0.68	42
	14	130	251	1.93	130	239	1.84	12
	15	240	107	0.45	240	106	0.44	4
	16	170	143	0.84	110	140	1.27	9
92. 8.18	4	1,100	786	0.71	700	686	0.98	37
	7	700	539	0.77	700	470	0.67	74
	9	11	49	4.45	11	49	4.45	13
	10	1,300	717	0.55	1,300	675	0.52	52
	11	5	11	2.20	5	11	2.20	3
	12	110	93	0.85	79	81	1.03	23
	13	2,400	1,146	0.48	2,400	1,086	0.45	75
	14	490	398	0.81	330	365	1.11	64
	15	79	59	0.75	49	56	1.14	15
	16	33	42	1.27	33	39	1.18	15
Total	40			1.08			1.27	

[1]*Clostridium perfringens*

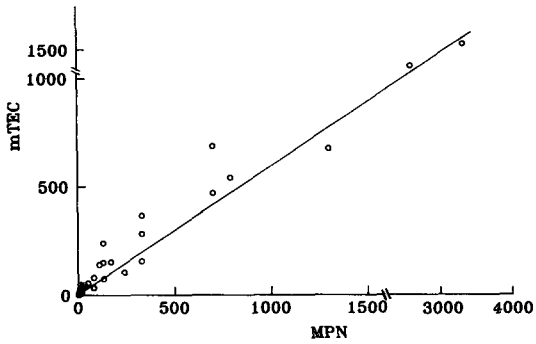

Fig. 3. Correlation of *E. coli* densities obtained by the EC-MUG and mTEC methods.

0.9420.

These results indicate that the mTEC procedure is a distinctly possible alternative to the MPN for estimating indicator bacteria in routine environmental monitoring and classification studies of shellfish growing area. Rippey et al. (1987a) have described several distinct advantages of the mTEC membrane filtration procedure. They cite that the enumeration of fecal coliforms and *E. coli* by mTEC are completed within 24 hours, whereas the APHA-MPN procedure requires 3 days to obtain equivalent results, even EC-MUG (Rippey et al., 1987b) is used, and up to 10 days for *E. coli* when conventional confirmation methodology is used. The precision inherent to the MPN is very poor (0.3 to 3 times values) at the 95% confidence limits for a 5-tube technique, contrasted to a 95% confidence interval of 23 to 40% for mTEC when only one filter per dilution is used. This degree of precision is typical of direct enumeration procedures, and is remarkably greater than that of the MPN for specially less than 100 colonies ranging of NSSP criteria (Table 3). In addition, the lower cost per assay and savings in analyst are significant advantages afforded by the mTEC method over the MPN technique.

The results obtained from this sanitary survey for Greenwich Bay support the suggestion recommended by Rippey et al. (1987a) that the mTEC method is a viable alternative to the APHA-MPN procedure for the enumeration of indicator bacteria, fecal coliforms and *E. coli*, in aquatic environmental monitoring. If the mTEC is adopted as an official procedure, this method would be very helpful and advantageous not only for the management of designated areas, but for classification of shellfish growing areas in Korea.

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위생지표세균 검출을 위한 APHA-MPN과 mTEC법의 비교－미국 Rhode Island주 Greenwich Bay의 위생조사를 통하여

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APHA-MPN(American Public Health Association-Most Probable Numbers)은 환경수중에 존재하는 위생지표세균 추정에 사용되는 미국 FDA의 유일한 공인방법이기는 하나 결과를 얻기까지 많은 시간, 인력 및 경비가 소요될 뿐 아니라 일반적으로 정밀도도 낮다는 결점이 있다. 이러한 문제를 극복하기 위하여 개발된 막여과 방법중 mTEC(membrane thermotolerant *E. coli*)은 분변계대장균 및 *E. coli*의 회수율이 좋을 뿐 아니라 경비도 절약할 수 있는 방법으로서 알려져 있어, 미국 Rhode Island주의 Greenwich Bay의 위생조사를 통하여 MPN과 비교하여 보았다. 그 결과 mTEC의 분변계 대장균 및 *E. coli* 회수율은 MPN보다 평균치로써 각각 1.08 및 1.27배 높게 나타나, MPN의 대체방법으로서의 가능성이 확인되었으며, 따라서 이 방법이 앞으로 위생지표세균의 검출방법으로서 공인된다면 우리나라 수출용 패류생산 지정해역의 관리는 물론 패류양식장의 위생학적인 분류도 효율적으로 할 수 있으리라 기대된다.