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Robert H. Ruskin, Jr.
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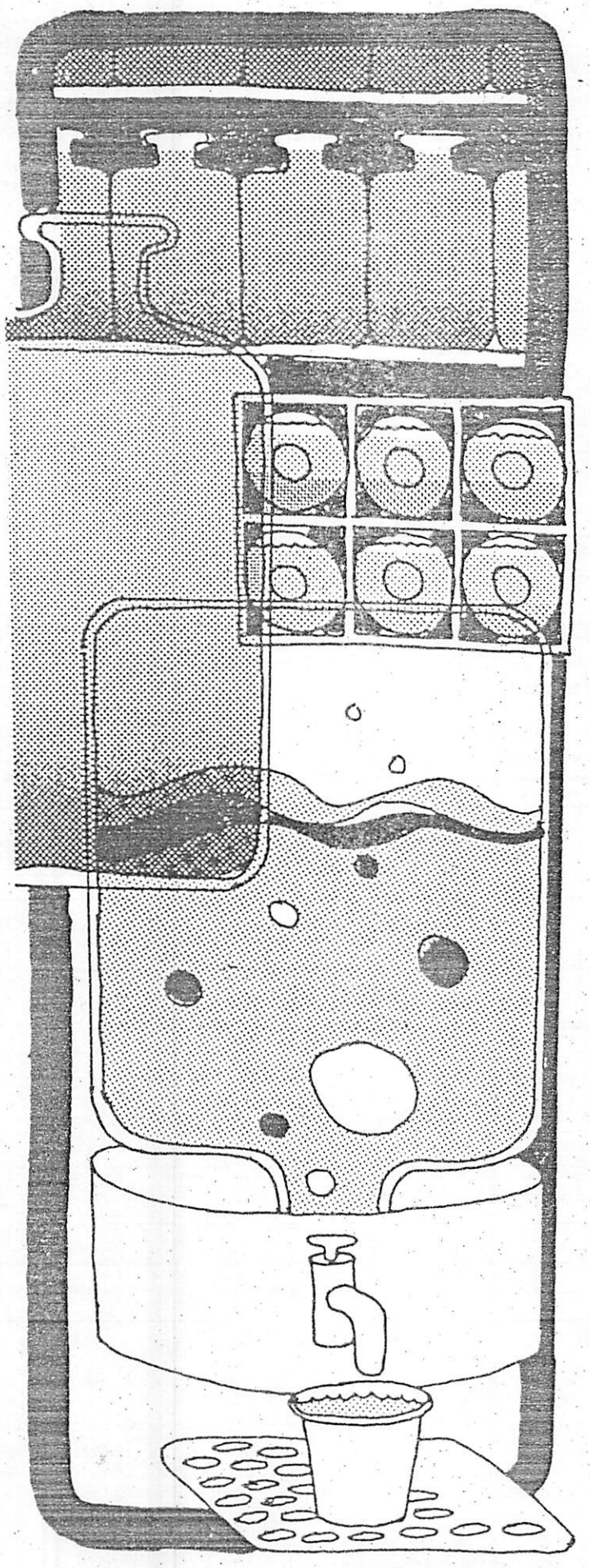

The Necessity of Controlling Bacterial Populations in Potable Waters—Bottled Water and Emergency Water Supplies

Edwin E. Geldreich, Harry D. Nash, Donald J. Reasoner, and Raymond H. Taylor

An article based on a paper presented at the annual meeting of the Amer. Soc. for Microbiology, May 12-17, 1974, Chicago, Ill., and contributed to the JOURNAL on Dec. 18, 1974, by Edwin E. Geldreich (Active Member, AWWA), consult. bact.; Harry D. Nash, res. bact.; Donald J. Reasoner (Active Member, AWWA), res. bact.; and Raymond H. Taylor, res. bact. (Active Member, AWWA); all of the Wtr. Sply. Res. Lab., NERC, ORD, USEPA, Cincinnati, Ohio.

Part of the Safe Drinking Water Act stipulates that communities provide for emergency supplies of water that are safely free of bacteria. This article, in addition to documenting findings about bacterial counts for bottled water, provides data and recommendations concerning emergency water supplies.

The growing public concern over water pollution, the fact that numerous municipal water supplies use polluted streams for source water, opposition to the addition of fluoride to some municipal water supplies, and the taste and odor problems in some community water supplies have stimulated growth of the bottled-water industry in the US.

In 1972 there were an estimated 700 water-bottling plants in the US, which accounted for at least $107 million in total sales. In southern California, where the sales of bottled water are the largest in the nation, more than 100 mil gal of bottled water are sold yearly to an estimated 930 000 customers.[1]

Some of the large bottled-water companies now reach large regional markets throughout the US. With the increased geographical distribution and growth in bottled-water sales, an increasing proportion of the population is exposed to a potable-water source of uncertain bacteriological quality.

The need for systematic bottled-water-quality surveillance is a growing concern. Some states require that bottled-water-quality standards comply with the USPHS;[2] however, most states either have no regulations, or the existing regulations are ineffective or not enforced. The state regulations range from programs with a poorly defined sampling frequency to others that specify a minimum of five samples per month if the population served is fewer than 6 000 and require an increase in sampling frequency proportional to the increase in the population served.[3] The number of samples required is based on the sampling frequency curve from the USPHS Drinking Water Standards, as shown in Fig. 1. Unfortunately, few states specify a definite sampling frequency for bacteriological examination or repeat testing when the initial sample results are unsatisfactory.

The assumption that bottled water is of unquestionably good quality simply because the source water is a spring or artesian well is untenable. The labeling of some bottled waters may even imply that the bottled waters are derived from pristine sources that do not require treatment and consequently are superior to municipal water supplies that utilize polluted raw water sources. The implication is that bottled-water source waters are initially of better quality and that the finished products will retain this high quality during shelf life before purchase or when used in water cooling dispensers.

The purpose of this study, conducted over a 1 1/2-year period, was four fold:

1. Investigate the bacteriological quality of a variety of brands of bottled water purchased from retail outlets
2. Investigate the variability in bacteriological quality of freshly bottled water
3. Characterize changes in the bacterial density of bottled water during storage
4. Examine the bacteriological quality of stored emergency water (civil defense).

Materials and Methods

Sample collection. Half- or full-gallon (1.9–3.8-l) containers of available brands of bottled water were purchased from local retail outlets for bacteriological examination. After initial examination, some bottled water samples were examined repeatedly over a period of 30 days or longer to evaluate changes in bacterial density during storage.

In addition, samples of freshly bottled water, obtained directly from the bottler, were examined for bacteriological quality. A portion of these samples was collected in conjunction with a nationwide bottled-water study conducted by the Water Supply Div. USEPA,[4] in which 25 water bottlers were surveyed. Five water bottlers in the Cincinnati, Ohio, area were included in that survey.

Source-water samples collected in sterile, 1-l polypropylene bottles also were obtained from the water bottlers. All bottled water and source-water samples were transported (unrefrigerated) to the laboratory within 6 hr for analysis. Some samples of freshly bottled water were held in the laboratory at 23 ± 2C for repeat sampling to determine how the bacterial populations changed during storage. After the initial examination, repeat examinations were conducted over a period of 63 days.

To obtain samples of civil defense emergency drinking

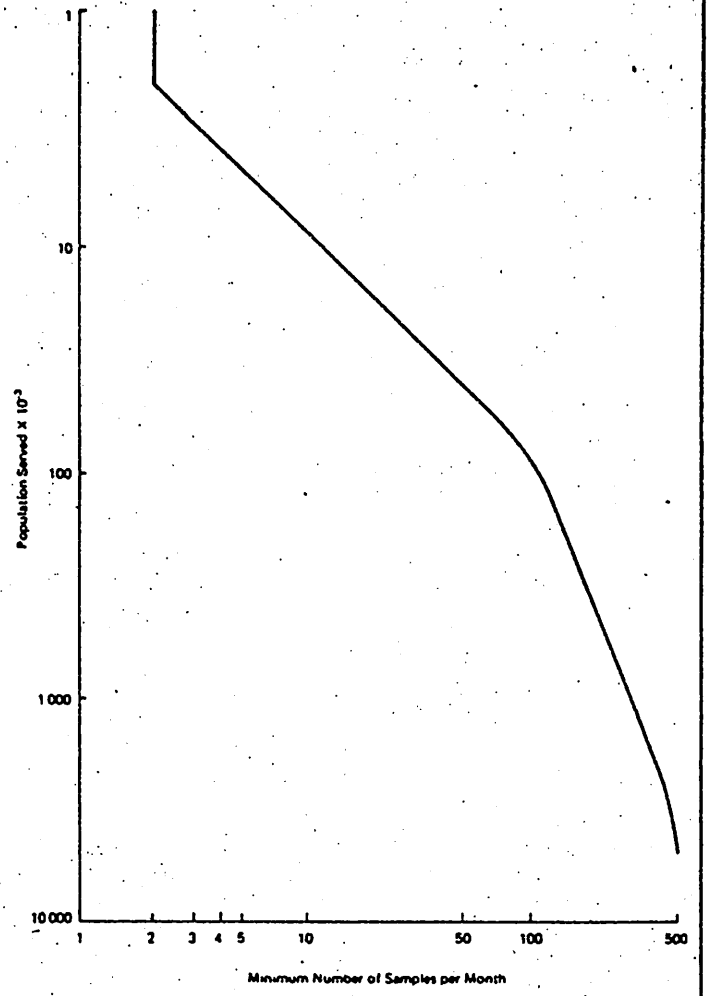

Fig. 1. Recommended Minimum Monthly Samples per Population Served by Water Supply

TABLE 1
Effect of Incubation Time on Bottled Water Plate Counts at 35C

Count SPC/ml	No. of Analyses	Ratio 24:72 Hr	No. of Analyses	Ratio 48:72 Hr	No. of Analyses	Ratio 120:72 Hr
30–100	21	0.25	37	0.53	17	1.32
101–500	14	0.25	28	0.68	14	1.23
501–1 000	10	0.22	13	0.68	3	1.09
1 001–10 000	33	0.20	44	0.60	12	1.28
10 000–50 000	28	0.23	37	0.80	7	1.11
50 000–100 000	11	0.31	17	0.81	6	0.99
>100 000	12	0.06	19	0.62	8	1.03
Total	129		180		51	
Average ratio		0.22		0.67		1.15

TABLE 2
*Ranges of Standard Bacterial Plate Counts in Freshly Bottled Water Samples**

Count SPC/ml	No. of Samples	Samples per cent	Cumulative per cent
<1	23	17.8	17.8
1–10	56	43.4	61.2
10–100	28	21.7	82.9
100–500	8	6.2	89.1
500–1 000	5	3.9	93.0
1 000–10 000	7	5.4	98.4
<10 000	2	1.6	100.0

*All samples were plated within 48 hr of bottling.

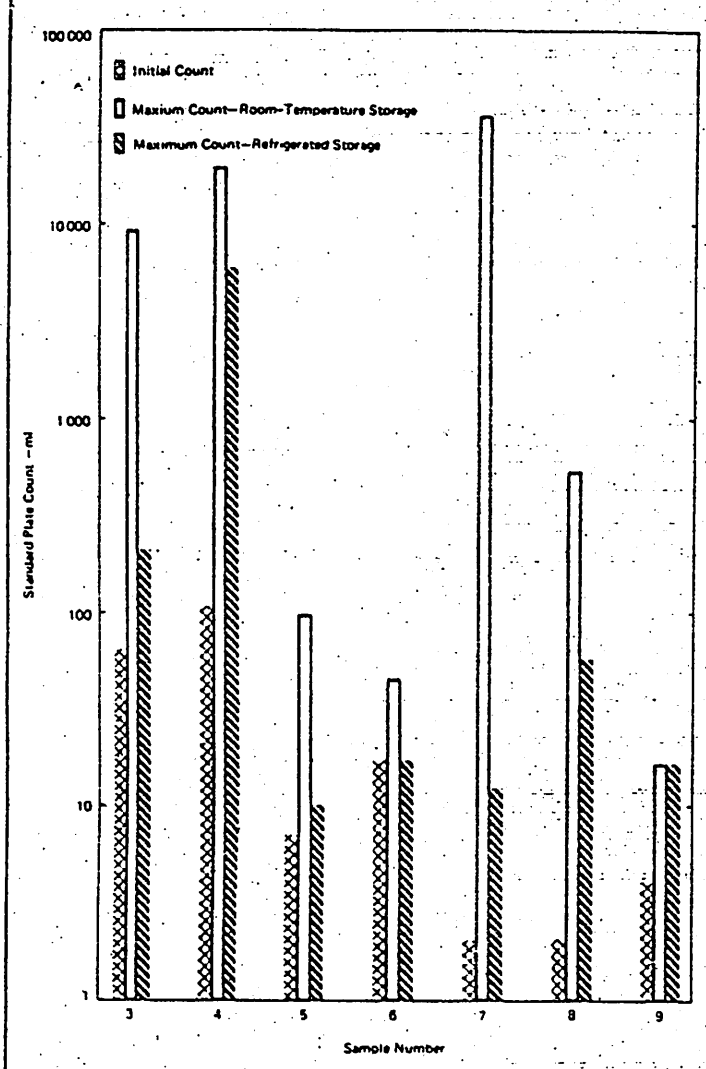

Fig. 2. The Effect of Refrigerated and Room-Temperature Storage on the Standard Plate Count of Bottled Water

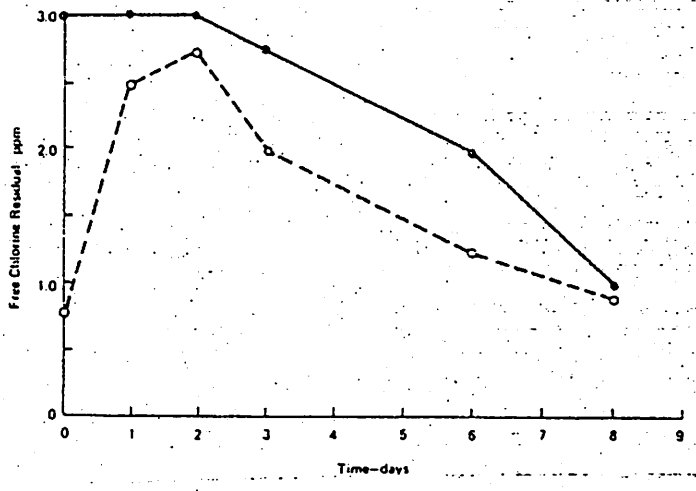

Fig. 3. Free-Chlorine Residual From Chlorine Tablets and Liquid Bleach in Civil Defense Shelter Water-Storage Drums

Liquid bleach: ——; tablet: ----

water, the drum lid was removed and the top of the inner polyethylene storage bag was swabbed with ethanol; a 2.5-cm cut was made in the polyethylene bag with a sterile knife blade; approximately 400 ml of water was pipetted (using a sterile 100-ml volumetric pipet) from the storage drum into a sterile 500-ml flask; the opening in the polyethylene liner was resealed with tape; and the drum lid was replaced. Sample analyses were initiated within 1 hr after collection.

Bacteriological tests. 1. *Standard plate count (SPC).* The SPC of bottled (freshly bottled and retail-purchased)-and-stored emergency drinking-water samples was determined using the pour plate procedure described in *Standard Methods*.[5]

Sample aliquots of 1.0, 0.1, and 0.01 ml were planted, five replicates for each dilution, in disposable 15- × 100-mm plastic petri dishes. A 15-ml volume of sterile plate-count agar (used for all SPC determinations), tempered to 44-46C, was poured into each plate and thoroughly mixed with the sample aliquot. The agar was allowed to solidify and the plates inverted and incubated at 35 ±0.5C. The plates were examined and colonies counted after 24, 48, 72, and 120 hr incubation. Results were expressed as SPC/ml.

2. Total and fecal coliform. Total- and fecal-coliform-count determinations were conducted according to *Standard Methods* membrane filtration procedures.[5] Separate 250-ml volumes of samples were examined for fecal and total coliform determinations for bottled water. For each fecal- and total-coliform determination of source-water samples, a 100-ml volume was examined. Results were calculated and expressed as total coliforms/100 ml and fecal coliforms/100 ml.

3. Pseudomonas aeruginosa *counts.* The membrane-filter method of Levin and Cabelli,[6] utilizing M-PA agar, was used to examine bottled, source, and stored emergency-drinking-water samples for the presence of *Pseudomonas aeruginosa.* Sample volumes of 250 ml of bottled water and 100 ml of source water and emergency drinking water were examined for *Pseudomonas aeruginosa.*

Results

Incubation time. At the start of this study, considerable difficulty was encountered in determining the SPC because of the slow development of the bacteria on plate-count agar after an incubation period of only 24 hr at 35C. Consequently, the incubation period at 35C was extended to as long as 120 hr (five days). Colony counts were made after incubation periods of 24, 48, 72, and 120 hr to determine the optimum incubation period needed to obtain valid counts.

The data summarized from SPC analyses of 180 bottled-water samples are shown in Table 1. Data were derived from initial examinations of bottled waters and subsequent repeat examinations after various storage intervals at 23 ± 2C. For bacterial count used was an average of five replicate plates, using only plates that had counts of 30–300 colonies after a 72-hr incubation at 35C were included.

The data in Table 1 show that the bacterial counts after a 24- and 48-hr incubation averaged only 22 and 67 per cent, respectively, of the 72-hr count. Increasing bacterial densities generally had little effect on these ratios. The notable exception was the 24:72-hr count ratio for densities >100 000 bacteria/ml. This indicates that when the bottled-water bacterial density is very large, the 24-hr count at 35C approximated fewer than 10 per cent of the 72-hr count.

The incubation period for the bacterial plate count was extended to 120 hr (five days) to determine if a longer incubation period would result in further increases in the plate counts of

TABLE 3

*Standard Bacterial Plate Counts in Bottled Water Obtained From Retail Outlets**

Count SPC/ml	No. of Samples	Samples per cent	Cumulative per cent
<1	11	10.9	10.9
1–10	20	19.8	30.7
10–100	19	18.8	49.5
100–500	9	8.9	58.4
500–1 000	6	5.9	64.3
1 000–10 000	16	15.9	80.2
> 10 000	20	19.8	100.0

*Samples purchased after undetermined periods in stock

bottled-water samples. Data from 51 samples (Table 1) indicate that increasing the incubation period from 72 to 120 hr produced only a 15 per cent increase in count. The sample-to-sample variation in the percentage increase in bacterial count was found to be greater between the 72- and 120-hr incubation than between the 48- and 72-hr incubation time. Because of the relatively small increase in bacterial count, extending the incubation period from 72 hr to 120 hr had little advantage for most samples.

Freshly bottled water. A total of 129 freshly bottled water samples was collected directly from 25 different bottlers. Of them, only 14 (10 per cent) had an initial SPC greater than 500 bacteria/ml (Table 2). Total coliforms were detected in six samples; only two of them exceeded the USPHS Drinking Water Standards coliform limit (for an individual sample) of 4 coliforms/100 ml. Also, one of the two coliform-positive samples contained fecal coliform bacteria, and the other coliform-positive sample contained 42 *Pseudomonas aeruginosa*/100 ml.

Bottled-water samples of unknown age. The bacterial densities of bottled water purchased from retail outlets were highly variable. The data in Table 3 show that 42 of 101 samples (41.6 per cent) contained more than 500 bacteria/ml, and about 11 per cent contained fewer than 1 bacterium/ml. Three samples had total coliform counts that exceeded the USPHS Drinking Water Standards. Fecal coliform bacteria were not detected, but one sample contained *Pseudomonas aeruginosa* at a concentration of 13/100 ml.

Table 4 shows the variability in bacterial density and the frequency of coliform-positive samples among the 19 brands of bottled water analyzed during this study. Examination of the density ranges of bacteria reveals the variability of the SPC from brand to brand as well as the variability within the same brand where multiple samples were analyzed.

Comparison of the data in Tables 2–4 shows that the bacteriological quality of bottled water examined within 48 hr of bottling was not representative of the quality of bottled water obtained from the retail shelf after an undertermined period in stock.

Forty-four bottled-water samples purchased from retail stores were stored at room temperature for 30 days and sampled weekly. The maximum SPC/ml recorded for stored bottled-water samples are grouped by range in Table 5. The SPC in 33 of the 44 stored samples (75 per cent) increased to more than 500 bacteria/ml during the storage period; this indicates that sufficient nutrients were present in the bottled waters to support significant increases in bacterial densities.

In addition, changes in bacterial populations of six freshly bottled water samples stored at 23 ± 2C were observed for a period of 63 days. These samples were collected and examined in conjunction with a nationwide pilot survey of water bottlers and bottled water.[4] The data in Table 6 show the bacterial den-

TABLE 4

Comparison of Standard Plate-Count Variability Among Brands of Bottled Water

Brand	Sample Type*	No. of Samples	Count Range† SPC/ml	Coliform Positive Samples‡
A	fresh	91	<10–25 000	3/91
	retail	16	<10–28 000	0/16
B	retail	6	<10–260 000	0/4
C	fresh	1	<10	0/1
	retail	11	1 200–160 000	0/11
D	retail	5	31–650	0/5
E	fresh	1	<10	0/1
	retail	2	100 000–390 000	2/2
F	retail	2	<10–12 000	0/2
G	fresh	2	<10	0/2
	retail	1	<10	0/1
H	retail	9	<10–390	1/9
I	retail	2	<10	0/2
J	retail	2	<10–2 000	1/2
K	retail	2	<10–12	1/2
L	retail	4	13–4 300	0/4
M	retail	1	<10	0/1
N	retail	1	<10	0/1
O	retail	1	1×10^6	1/1
P	retail	1	8 600	0/1
Q	retail	1	<10	0/1
R§	retail	1	<10	0/1
S§	retail	1	12	0/1

*Fresh = samples direct from bottler, examined within 24 hr;
retail = samples of unknown age purchased from retail outlets.
†Standard plate count (SPC) range values represent average count of bacteria/ml, calculated from five replicate plates incubated for 72 hr at 35C using plate count agar.
‡One or more coliforms/100 ml.
§Imported bottled water, carbonated.

TABLE 5

Maximum Standard Plate Counts Attained in Retail Purchased Bottled Waters During Room Temperature Storage for 30 Days

Maximum Count† SPC/ml	No. of Samples	Samples per cent	Cumulative per cent
<1	2	4.5	4.5
1–10	4	9.1	13.6
10–100	4	9.1	22.7
100–500	1	2.3	25.0
500–1 000	1	2.3	27.3
1 000–10 000	13	29.5	56.8
> 10 000	13	43.2	100.0

TABLE 6

*Variation of SPC in Fresh Bottled Water Samples From The Cincinnati, Ohio, Area During Storage at 23C ± 2C**

Storage Period, days	Sample Number					
	1	2	3	4	5	6
0	4†	2	3	1	1	3
3	20 000	350	18 000	5	3	4
5	52 000	250	21 000	31	39	4
7	43 000	1 300	11 000	36	25	69
10	76 000	670	70 000	130	7	2 700
14	47 000	710	18 000	1 100	39	29 000
21	40 000	1 700	13 000	2 200	2 200	100 000
28	35 000	2 100	2 200	1 200	1 300	53 000
35	33 000	1 400	1 400	690	880	56 000
42	44 000	1 900	1 400	650	1 100	53 000
49	48 000	4 900	660	700	1 100	46 000
56	46 000	1 000	490	310	460	50 000
63	61 000	1 200	490	240	360	44 000

*Storage study period for all samples began within 24 hr of bottling.
†Average counts/ml, calculated from five replicate plates incubated for 72 hr at 35C with the use of plate count agar.

TABLE 7

Standard Plate Counts in Stored Civil Defense Emergency Water

Count SPC/ml*	72-Hr Incubation†		Five-day Incubation‡		Avg. Count Ratio 72:120 Hr
	No. Samples	per cent	No. Samples	per cent	
0–10	4	13.3	2	9.5	1.00
10–100	6	20.0	2	9.5	0.78
100–500	11	36.7	3	14.3	0.50
500–1 000	2	6.7	6	28.6	0.38
1 000–10 000	1	3.3	2	9.5	0.10
> 10 000	6	20.0	6	28.6	0.44

*Each value used was an average of five replicate plates, incubated for either 72 hr or 120 hr (five days) at 35C.
†Includes all samples for which 72-hr SPC was determined.
‡Includes only samples for which both the 72-hr and five-day SPC were determined.

TABLE 8

Changes in Bacterial Quality Following Initial Disinfection of Civil Defence Emergency Stored Water

Count SPC/ml	Occurrence—per cent								
	Months						Years		
	1	3	4	6	8	12	5	6	9
<1	45.1	39.2	29.2	29.6	36.4	29.4	71.8	73.6	10.0
1–50	17.2	22.8	9.7	16.2	12.7	19.1	4.9	none	10.0
51–100	3.1	1.3	8.3	8.1	3.2	2.9	1.0	5.3	15.0
101–500	9.4	10.1	13.9	2.7	14.3	13.2	5.8	none	30.0
501–1 000	3.1	6.3	4.2	2.7	3.2	4.4	1.0	none	5.0
1 001–5 000	6.3	8.9	13.9	20.3	12.7	17.7	8.7	5.3	none
5 001–10 000	1.7	6.3	8.3	6.8	3.2	1.5	4.9	none	10.0
10 001–50 000	9.4	1.3	11.1	6.8	7.9	7.4	1.0	10.5	15.0
> 50 000	4.7	3.8	1.4	6.8	6.4	4.4	none	5.3	5.0
Total samples	64	79	72	74	63	68	103	19	20

TABLE 9

Emergency Disinfection Treatment for Stored Supplies of Drinking Water

Commercial Product	Approximate of Available Disinfectant per cent	Treatment		Source Availability
		Disinfectant Quantity*	Volume Treated—gal	
High-test hypochloride tablets	70	1 tablet	80	janitorial, hotel, restaurant, dairy, and swimming pool supplies
Iodine or chlorine tablets	—	2 tablets	1/4	drug store and sporting goods store
Iodophore†	1.0–1.6	1 tbs	4–6.5	drug store, dairy and chemical supplies
Liquid chlorine laundry bleach	5.3	1 tbs	20	grocery store
Tincture of iodine	2	1 tbs	8	drug store
Chlorine based antiseptic powder‡	1.0	1 tbs	4	drug store

*Ten-mg/l dose, 30-min contact time
†Isodine, Blair Co; Wescodyne, West Chemical
‡Zonite, Norcliss Co.

sity changes that occurred in each of the six bottled water samples starting within 24 hr of bottling. Each standard plate count in the table represents the average of five replicate plates incubated for 72 hr at 35C.

These results indicate that bacterial densities in bottled water increase appreciably during storage and raise questions concerning (1) possible interference with coliform detection, and (2) the possibility of increased risk of human exposure to bacteria known to be secondary pathogens.

Storage effects. To determine if the increase in bacterial density in bottled water could be minimized during storage, seven pairs of bottled-water samples were purchased. One bottle from each pair was stored at refrigerator temperature (4 ± 2C) and the other stored at room temperature (23 ± 2C) for the duration of the storage experiment. Samples from each bottle were examined weekly for eight weeks to observe changes in bacterial density.

Results from six of the paired samples showed that the maximum bacterial density attained in the stored refrigerated sample was significantly lower than that attained in the stored unrefrigerated sample (Fig. 2). In the remaining paired sample, maximum counts of less than 20 bacteria per ml were observed in both the room temperature and refrigerated samples. The peak bacterial density in the refrigerated samples occurred after an average storage of 26 days, as opposed to only eleven days storage for the room-temperature samples.

Emergency water. Water samples were collected from 17.5-gal (66.2) drums of civil defense emergency water that had been stored for ten years. The ranges and the distribution of SPCs for 30 emergency water samples collected during the period Jul. 1972 to Apr. 1973 are listed in Table 7. SPCs after incubation at 35C for 72 hr and 120 hr (five days) and the average 72:120-hr count ratios for the plate count ranges are also shown. The data show that 21 of 30 samples (70 per cent) had a SPC of fewer than 500 bacteria/ml after 72-hr incubation at 35C. Of the samples for which SPCs were determined after both 72-hr and five-day incubation, however, only 7 of 21 (33.3 per cent) contained fewer than 500 bacteria/ml. These data indicate that the five-day-incubation SPC at 35C provides a better estimate of the bacterial density of stored emergency water than does the 72-hr incubation. Total coliform, fecal coliform, and *Pseudomonas aeruginosa* were not detected in any of the 30 emergency water samples analyzed.

Discussion

Bottled water, fresh and stored. Concern over the bacteriological quality of bottled water dates back at least to 1916.[7] However, there is little documentation of the variability of bottled-water quality.

These results indicate (Table 1) that bottled water samples should be incubated at 35C for 72 hr to obtain reliable SPC data. Many of the bacteria detected in bottled water grow slowly when inoculated into the nutrient-rich medium used for the SPC analysis. Therefore, SPC analyses of bottled water samples using 35C for 24 hr or 20C for a 48-hr incubation generally will not provide an accurate estimate of the bacterial densities in the samples. The same conclusion applies to the examination of stored, emergency water samples (Table 7), except that incubation for 120 hr (five days) at 35C is needed to obtain reliable counts. Therefore, minimum incubation periods of 72 hr for SPC analyses of bottled-water samples and 120 hr for stored emergency water samples at 35C are recommended. The plating medium should be plate-count agar or tryptone glucose extract agar.[5]

Although a specific statement may not be present on the brand label of a bottled-water product, the label may imply that the bottled-water source is of better quality than the municipal supply source and that the finished product will retain the initial high quality throughout its shelf-life. The results obtained do not support this implication: the bacteriological quality of the freshly bottled product varied greatly from brand to brand and from sample to sample within the same brand. Although the data are not presented here, source-water samples from brands A, C, E, and G exhibited wide variability in bacterial content. The method of treatment before bottling appeared to be more important than source-water quality in determining the bacterial quality of the finished product.

The type of treatment used for the production of bottled water was variable. The data collected during the bottled-water survey[4] show that the use of a single means of treatment was infrequent—usually a series of treatment steps was used. The bottled-water treatment process ranged from no treatment to a combination of treatment steps that might include softening or ion exchange, filtration, and disinfection.

Ozonation or ultraviolet irradiation was most frequently used as the final disinfection step before bottling. The bacterial quality of bottled waters disinfected by either of these methods was highly variable among bottlers and for a given bottler at different times.

Of the various bottled waters examined in the Cincinnati area, one brand was pasteurized and its usually good quality was maintained during storage. In general, bottled waters treated by an ion-exchange column (or bed) or by filtration alone were poorest in quality because of the bacterial growth on the ion exchanger or filter medium as a result of infrequent or inadequate maintenance of the equipment.

Wallis and associates[8] have shown that charcoal filters used to remove objectionable tastes and odors from drinking water can support large bacterial populations. The charcoal filters concentrate both bacteria and organic nutrients and then provide a place for the bacteria to multiply. Bacterial concentrations as high as 7.0×10^6 cells/100 ml were detected in the effluent from a charcoal filter only six days after installation. Beds or columns of ion-exchange resins or activated carbon may support similar growths of bacteria unless properly maintained and serviced. Good quality bottled water was generally produced by a combination of filtration or distillation and ozonation or ultraviolet irradiation.

The use of protective sealed caps on bottles and covered storage during transit to the consumer have reduced the risk of bottled-water contamination. Unprotected storage in business establishments and homes, poor handling techniques, and water dispensers of questionable cleanliness may, however, result in rapid deterioration of bottled-water quality.

Few coliform bacteria were recovered from bottled water during this study. During a study conducted in 1936,[9] coliform bacteria were detected in 32 per cent of 212 bottled-water cooling dispensers, and 8.5 per cent of the samples contained approximately 1 000 coliforms/100 ml. A more recent study reported coliform bacteria in only 6.4 per cent of 1 040 bottled-water cooling dispensers, and counts ranged from 2.2 to 240 coliforms/100 ml.[10] Apparently, better cap protection, improved dispenser designs, nonreturnable plastic containers, and improved handling and sanitation of water-cooler dispensers have reduced the coliform occurrence.

In some cases, however, new nonsterile plastic bottles can be a source of contamination. The bottles are shipped by the manufacturer in unsealed cardboard boxes containing six uncapped bottles; the caps, packaged in plastic bags, are shipped separately in bulk quantities.

Eight unused bottles were examined by rinsing each bottle with a volume of sterile buffered water followed by (1) plating 1-ml aliquots in plate-count agar and incubating at 35C for 72 hr or (2) filtration of 50-ml aliquots through sterile 0.45-μm membrane filters that were then placed on a modified plate-count medium and incubated at 35C for 72 hr. These new plastic bottles contained a low density of bacteria (counts ranged from <1 to 5 bacteria/ml) that contributed to the contamination of the finished product and resulted in decreased product quality.

Persistence of initial bacterial contamination, whether coliform or noncoliform, will be determined by the availability of bacterial nutrients, water temperature, pH, and antagonism or competition of other bacteria present in the water. In one study of bottled spring water, coliform bacteria in the contaminated bottled-water samples persisted for periods of fewer than two weeks.[10] A die-away interval of nine to fourteen days was noted for fecal coliforms in spring water from another geographical area,[11] but the total coliform population persisted for four to six weeks, undergoing a declining cyclic survival pattern. Numbers of parallel studies in different types of water have shown that fecal coliform and *Salmonella* survival rates are essentially identical;[12] therefore the occurrence of fecal coliforms in spring water could indicate the possible occurrence of pathogens.

In addition to indicator bacteria, a large, general, bacterial population may occur in bottled water; this population depends on the kinds of bacteria present and the availability of nutrients in the water. Pigment-producing bacteria are frequently found in bottled, distilled, and stored civil-defense water.[13–16]

When bottled-water samples examined during this study contained pigmented bacteria, the proportion of the standard plate count represented by the pigmented forms ranged from only a few per cent to nearly 100 per cent. In some bottled-water samples, the bacterial population included yellow, orange, or pink pigmented forms. Yellow forms were most often encountered, but in two instances a pink pigmented bacterium accounted for nearly 100 per cent of the bacterial population. The pigmented forms were predominantly gram-negative rods that varied widely in size.

Bacteriological results (Table 7) obtained during this study from stored emergency water-supply samples were in good agreement with results obtained for a previous study.

In this previous study the quality of emergency water supplies located in twenty cities was examined over a twelve-month period to determine the effects of long-term storage.[17] Periodic coliform and SPC analyses were performed on samples from twenty 17.5-gal (68-l) water-storage drums during the first year of storage. The water, which was obtained from the local community water supply, was stored in a polyethylene bag surrounded by an outer polyethylene liner. At the time of filling, 7 ml (1 teaspoon) of 5.25 per cent chlorine bleach was added to each drum of water to protect against accidental contamination during filling and handling operations.

The long-term protection afforded by chlorine disinfection is, however, limited by the chlorine demand of (1) organic and inorganic substances in the water, (2) the polyethylene bag, and (3) dust contamination accidentally introduced during long-term storage. Studies indicate that effective free chlorine residuals resulting from liquid laundry bleach or chlorine tablets placed into civil defense water-storage drums[17] probably persists for several weeks (Fig. 3). These data also indicate that the liquid chlorine solution was instantly soluble and gave a slightly

higher concentration of available chlorine than did the chlorine tablets.

In samples from a total of 87 storage drums in the twenty cities, unsatisfactory coliform results were recorded for only six samples. One supply had coliform densities of 15/100 ml in the first month, 38/100 ml in the fourth and 9/100 ml in the twelfth month. Coliforms were not detected in the initial sample or in the three-, five-, or eight-month samples, which suggests that contamination of that particular water drum may have recurred at times during the twelve-month storage period.

The general bacterial quality of stored emergency water supplies was variable and related directly to the quality of the source waters used. Source-water quality was examined in 62 instances during initial filling of civil defense water drums in 1962. The community water supply in only one city had an excessive bacterial count, which ranged from 2 100 to 10 000/ml. Thirty source waters (48.4 per cent) contained 1 organism/ml, and 24 samples (38.7 per cent) had bacterial counts that ranged from 1 to 16 organisms/ml. A significant decline in the overall quality of water in these stored supplies occurred during the first year of storage (Table 8). Apparently, the bacterial flora initially present adjusted to this particular water environment and, with changes in dominance of microbial species, reached a stabilized density.

Stored-water samples were obtained after five and six years' storage from two different metropolitan areas not included in the earlier investigation. The bacteriological data obtained from these samples generally agreed with the data from the previous study. The results indicate that long-term storage produced a substantial increase in the number of stored water supplies that had less than 1 bacterium/ml. However, occasional samples from five- and six-year-old stored-water supplies had appreciable bacterial densities.

Examination of the bacterial populations that persisted in stored water after approximately five years revealed that 22.8 per cent of 167 samples contained species of *Flavobacterium* and 16.2 per cent contained species of *Pseudomonas*. Flavobacteria were frequently dominant, and counts ranged from 10 to 26 000/ml.

Recommendations

Bottled water. The results from the bottled-water survey and the storage studies support two recommendations for the bottled-water industry and retailers. First, refrigerated storage will minimize bacterial multiplication in bottled water from the time of bottling to sale. The consumer should also be advised to keep bottled water refrigerated. Second, each container should be marked with the bottling date or lot number or both to assist the retailer and consumer in determining freshness. The lot number is desirable as an aid to the recall of a given lot of bottled water from retail shelves if the bacteriological quality is found unacceptable. These measures which are designed to discourage retail overstocking and shorten storage time would minimize increases in bacterial populations in bottled-water products.

Bottled drinking water should be analyzed for bacteriological quality at the same frequency per month as is required by the USPHS Drinking Water Standards (Fig. 1) and include a repeat sampling program and a follow-up sanitary survey when the results are unsatisfactory.[2]

At the time of bottling, the water should contain less than one coliform per 100 ml and have an SPC of fewer than 500 bacteria/ml. Samples taken from supermarket, drugstore, or restaurant supplies should have fewer than 1 coliform/100 ml and an SPC of fewer than 1 000 organisms/1 ml. Establishment of an SPC limit is suggested for control of bacterial quality deterioration during storage. The storage limit of 1 000 bacteria/ml for bottled water is higher than the 500-bacteria/ml limit suggested for treated distribution water[18] because bottled waters may normally be stored for a longer period than the residence time for community finished waters in distribution lines.

In addition to this, bottled water usually does not contain residual chlorine to control bacteria and is not subject to other problems associated with distribution lines such as sediment buildup, line breaks, back siphonage due to low pressure, or other related problems.

Concern over high bacterial counts in bottled-water relates to (1) the possible loss of coliform test sensitivity in waters with excessively high bacterial populations and (2) the increased risk of human exposure to organisms that are considered secondary pathogenic invaders.[16] The bottled-water standards adopted by the USFDA, which became effective on May 22, 1974,[19] do not include an SPC limit for bacteria, although the coliform limit is similar to that of the present USPHS Drinking Water Standards. This is unfortunate because in many cases bottled water is recommended for use in preparing baby formulas, prescriptions, and fruit juices, coffee, tea, and other beverages. Thus, some infants and many older people who use bottled water are in a position of increased health risk because of possible exposure to bacteria that may be secondary pathogens. Both young and elderly individuals are relatively more susceptible to secondary pathogens than the rest of the population.

Stored emergency water. The requirements for stored emergency water supplies set aside for use at time of natural disasters or during war-time devastation are unusual. These stored supplies may be used for drinking water or for medical treatment of injured personnel. Water used to treat the injured must be of good overall bacteriological quality, and quality surveillance requires an examination of the water for more than fecal contamination. Radiation exposure may reduce the patient's white-cell count, impair body resistance to bacterial invasion, and thus enable apparently harmless bacteria to become serious pathogenic invaders.[20-23]

Ensuring the continual availability of a high-quality emergency water supply over an extended storage period is difficult. Initially a high quality source water must be used and care must be exercised to prevent contamination when filling and sealing storage containers.

Nutrient concentrations in stored water supplies, although low, are sufficient to permit slow growth of some bacteria initially present in the source water or introduced by contamination during storage. For comparison, the nutrients available in distilled water may be present in trace amounts, yet examination of these waters shows that sizable bacterial populations can develop.[13-15, 19] Because the organisms in stored water supplies have adjusted to slow growth rates, the need to incubate pour plates for 120 hr (five days) to obtain a reliable bacterial count is re-emphasized.

The data available on stored, emergency drinking-water supplies indicate that water-quality deterioration occurred in a significant number of the filled water drums. Contamination during storage may occur, but it is probable that most bacterial occurrences relate directly to the initial source-water quality. In either case, disinfection is recommended before emergency use, particularly if the water must be used for first-aid treatment, for drinking, and for bathing purposes by disaster casualties. In emergencies, disinfection is best accomplished by

boiling the water for at least 1 min or by using commonly available disinfectants[24] such as household laundry bleach, swimming-pool disinfectants, tincture of iodine or various trade-name iodine or chlorine tablets. The data in Table 9 indicate the quantity of some of the products required to release 10 mg of Cl_2 or I_2 per litre of water. Recommended contact time should be 30 min to ensure maximum kill rates for various organisms that might be present.

Finally, emergency water stockpiles must receive periodic inspections for evidence of water leakage and evaporation. Occasional inspection of some supplies revealed empty or partially filled water drums. One recommendation proposes a continual monitoring program for these supplies, including semiannual bacteriological examination[17] of samples taken at random from each supply: two, three, or four drums per shelter would be examined when the stockpile contains 1–100, 100–1 000, or 1 000–5 000 drums, respectively.

A critical need exists to reduce the bacterial population to the lowest level possible in all emergency supplies. During an annual or semiannual inspection a systematic effort should be made to reestablish a free chlorine residual, preferably 10 mg/l, in each water drum. Also water drums depleted because of water loss or fouled in dirty or flooded storage areas should be routinely refilled. Such inspections would involve carefully opening and reclosing the plastic water bags to avoid accidental contamination of the water.

Summary

Although coliforms were infrequently detected in bottled water, the general bacterial population often exceeded 1 000 organisms/ml. The initial quality of any bottled water is related, in part, to the quality of source water, but the type and effectiveness of disinfection are much more important. Contamination during bottling or storage may result from poor plant sanitation, reuse of unclean glass bottles, or improper maintenance of disinfection or other treatment equipment.

In spite of the possibilities for contamination, low bacterial densities found in many freshly bottled water samples indicate that good quality bottled water containing fewer than 500 bacteria/ml can be produced. There will generally be some deterioration in bacterial quality during storage before sale, but by use of good plant sanitation and refrigerated storage, the bacterial densities in bottled water can be held to fewer than 1 000/ml. Thus, the authors recommend a working limit of 500 bacteria/ml for freshly bottled water and a permissible increase during storage to a maximum of 1 000 bacteria/ml.

In general, the pasteurized bottled water tested was of excellent quality and did not deteriorate during storage. Based on data from a pilot survey of 25 bottlers,[6] ultraviolet irradiation or ozonation or both, however, most frequently was used to disinfect source water, and the bacterial quality of bottled water treated by either method was highly variable. Bottled waters prepared using ion-exchange columns (or beds) were poorest in quality because of bacterial growth in the ion-exchange media and infrequent or inadequate maintenance of the equipment. This problem is easily remedied but often overlooked or ignored until a regulatory agency, such as a state health department, enforces proper replacement or maintenance of deionizing media and equipment.

The lack of systematic surveillance of bottled waters is a matter of growing concern. These sources of drinking water should be analyzed at the same monthly sampling frequency as that required by the USPHS Drinking Water Standards for community water supplies, including repeat sampling and a follow-up sanitary survey when results are unsatisfactory.

The bacteriological quality of emergency water supplies may deteriorate during storage and may present a health hazard if such supplies are ever needed. Because the quality of stored water may be difficult to protect and maintain over indefinite storage periods, all emergency stored water supplies should be given supplemental disinfection during annual inspections and before use in an emergency.

References

1. HETHERINGTON, B. O. Bottled Water in California. *Jour. Envir. Health*, 29:256 (Nov.–Dec. 1966).
2. USPHS Drinking Water Standards, Pubn. No. 956. Dept. of HEW, Govt. Prntg. Ofce. Washington, D. C. (Rev. 1962).
3. BUMSTEAD, J. C.; SYROTYNSKI, S.; & MCKEEFE, F. Owners and Operators of Bottled and Bulk Water Facilities. Public Wtr. Sply. Guide, New York State Dept. of Health (Jan. 1971).
4. A Pilot Survey of Water Bottlers and Bottled Water. Bottled Wtr. Rprt. in Bottled Water Study. Wtr. Sply. Div., USEPA (Sep. 1972).
5. *Standard Methods for the Examination of Water and Wastewater*, APHA, AWWA, WPCF, Washington, D.C. (13th ed., 1971).
6. LEVIN, M. A. & CABELLI, V. J. Membrane Filter Technique for Enumeration of *Pseudomonas aeruginosa*. *Appl. Microbiol.*, 24:6:861 (Dec. 1972).
7. OBST, M. M. Bacteria in Commerical Bottled Waters. *Jour. Bact.*, 1:103 (Jan. 1916).
8. WALLIS, C.; STAGG, C. H.; & MELNICK, J. L. The Hazards of Incorporating Charcoal Filters into Domestic Water Systems. *Wtr. Res.*, 8:111 (Feb. 1974).
9. ZOBELL, C. E. & FORD, G. C. A Sanitary Survey of 212 Bottled Drinking Water Dispensers. California State Dept. Public Health Weekly Bull., 15:141 (Oct. 3, 1936).
10. CONSOLE, O. M. A Study of Sanitary Conditions in Bottled Water Cooling Dispensers. *Jour. Envir. Health*, 26:326 (Mar.–Apr. 1963).
11. GELDREICH, E. E. & CLARKE N. A. Bacterial Pollution Indicators in the Intestinal Tract of Freshwater Fish. *Appl. Microbiol.*, 14:429 (May 1966).
12. GELDREICH, E. E. ET AL. The Bacteriological Aspects of Stormwater Pollution. *Jour. WPCF*, 40:1861 (Nov. 1968).
13. LEIFSON, E. The Bacterial Flora of Distilled and Stored Water. Part 1: General Observations, Techniques and Ecology. Intl. Bull. Bacterial Nomenclature and Taxonomy, 12:133 (1962).
14. LEIFSON, E. The Bacterial Flora of Distilled and Stored Water. Part 2: *Caulobacter vibrioides* (Henrici and Johnson 1935) in Distilled Water. Intl. Bull. Bacterial Nomenclature and Taxonomy, 12:155 (1962).
15. LEIFSON, E. The Bacterial Flora of Distilled and Stored Water. Part 3: New Species of the Genera *Corynebacterium*, *Flavobacterium*, *Spirillum*, and *Pseudomonas*. Intl. Bull. Bacterial Nomenclature and Taxonomy, 12:161 (1962).
16. GELDREICH, E. E. & CLARK, H. F. Distilled Water Suitability for Microbiological Applications. *Jour. Milk and Food Technol.*, 28:351 (Nov. 1965).
17. TAYLOR, F. B. Shelter I. USPHS–Office of Civil Defense Study of Storage of Drinking Water in Fallout Shelters. Dept. of HEW, Govt Prntg. Ofce., 1–220. Washington, D. C. (Nov. 1963).
18. GELDREICH, E. E. ET AL. The Necessity of Controlling Bacterial Populations in Potable Waters: Community Water Supply. *Jour. AWWA*, 64:596 (Sep. 1972).
19. Bottled Water Quality Standards: Addition of Fluoride, and Current Good Manufacturing Practice Regulations. Dept. of HEW, *Fed. Rgtr.* Pt. III, 38:226:32558 (Nov. 26, 1973).
20. SMITH, W. W. Survival after Radiation Exposure—Influence of a Disturbed Environment. *Nucleonics*, 10:80 (Nov. 1952).
21. GONSHERY, L.; MARSTON, R. Q.; & SMITH, W. W. Naturally Occurring Infections in Untreated and Streptomycin-Treated X-Irradiated Mice. *Amer. Jour. Physiol.*, 172:359 (Feb. 1953).
22. WENSINCK, F.; VAN BEKKUM, D. W.; & RENAUD, H. The Prevention of *Pseudomonas aeruginosa* Infections in Irradiated Mice and Rats. *Radn. Res.*, 7:491 (Nov. 1957).
23. KOVAL, B. Physicians (and Engineers) Save Radiation Victims. *Chem. Engrg.*, 64:245 (Apr. 22, 1968).
24. Safe Drinking Water in Emergencies. Dept. of HEW, USPHS Pubn. No. 387, Govt. Prntg. Ofce. Washington, D. C. (Rev. 1964).

Committee Report: Microbiological Considerations for Drinking Water Regulation Revisions

AWWA Organisms in Water Committee

Because of the importance of the microbiological quality of water, the AWWA Organisms in Water Committee elected to review the various options and approaches that had been proposed in connection with revised drinking water regulations. This report addresses these proposals, as well as current practice, and summarizes the committee's concerns and recommendations.

As required by the Safe Drinking Water Act, the US Environmental Protection Agency (USEPA) is involved in a comprehensive reassessment of federal drinking water regulations.[1] A significant part of this reassessment deals with microbial degradation of source water quality, waterborne disease outbreaks (*Giardia, Yersinia, Campylobacter, Legionella*, and viral agents), adequacy of the coliform indicator, the impact of alternative treatment processes, measurement of microbial quality, monitoring of public water supplies, and the approach to public notification. Many of the proposed options and new approaches relating to these issues were addressed in the NATO/CCMS Drinking Water Pilot Project,[2] the USEPA Microbiology Workshop,[3] and the AWWA Research Foundation–USEPA Workshops on the Revised Primary Drinking Water Regulations.[4] Because of the importance of these issues, the AWWA Organisms in Water Committee elected to review these proposals and existing practices and to present a synthesis of concerns and recommendations.

Treating and protecting water supplies

Increasing urban populations often place a burden on water suppliers, both in terms of the quantity demanded and in terms of contamination potential through the use of less desirable raw waters derived from polluted rivers, lakes, and groundwater. Wastewater treatment plants, urban and rural runoff, feedlots, and a host of other activities often discharge to a watercourse that may be the source for a public water supply. Even the most pristine watershed is potentially contaminated with organisms pathogenic to humans.

Surface waters. Surface water is at risk from microbiological contamination. *Giardia lamblia*, for example, has been recovered from isolated lakes and streams where there is little human activity. This pathogenic protozoan is less susceptible to conventional disinfection practices than are coliforms and other bacteria. Further, particulate matter can physically protect even susceptible pathogenic bacteria and viruses from the disinfecting agent. The combined effect of these two factors has resulted in outbreaks of waterborne disease involving surface supplies, even when disinfection produces water meeting the coliform standard.

The AWWA committee agreed that a dual barrier is desirable to provide a consistently high quality water supply. Minimal treatment of surface waters from restricted watersheds should consist of coagulation and rapid sand filtration to entrap cysts and turbidity, followed by disinfection to inactivate any remaining cysts, coliforms, pathogenic bacteria, and viruses. Slow sand or diatomaceous earth filtration, in conjunction with disinfection, is a viable treatment alternative, which would in addition eliminate the need for chemical pretreatment. Exceptions to these treatment requirements would be based on a past record of no giardiasis caused by the public water supply, periodic monitoring of treated water for *Giardia*, a watershed management program to minimize the potential for contamination by enteric viruses and *Giardia* cysts in the source water, demonstration that the water characteristics will not interfere with effective disinfection, and alternatives to filtration that will meet the requirement of a thousandfold reduction in microbial contamination.

Wastes must be treated properly to minimize pathogen releases. Steady progress is being made to bring on-line more waste treatment facilities nationwide. Most important, treated wastewaters should be disinfected, unless it can be demonstrated on a case-by-case basis that there will be no adverse environmental effects. Variance from this requirement should depend on considerations of time and distance, water temperature, the microbiological nature of the discharge (e.g., pathogenic bacteria, viruses, or parasitic protozoans), and the efficiency of the available water treatment barriers.

Several factors justify the disinfection of wastewater effluents. First is the philosophy of maximizing public health protection through a multiple barrier concept. Disinfection of treated wastewater provides the initial barrier to the transmission of waterborne disease. Removal of this barrier simply transfers an additional burden to the potable water purveyor. The second concern is that wastewater treatment is often not precise and reliable, with some municipal plants being unable to meet permit limitations, thus making disinfection less effective. Efficient disinfection of the treatment plant effluent ensures controlled minimal protection for downstream water users and reduces microbiological contamination during periods when treatment processes are not operating properly. Where feasible, chlorinated sewage effluent should be retained on the premises until the chlorine residual has dissipated or, upon release of the effluent, is not detectable in the discharge for more than 330 ft (100 m) downstream. Trihalomethanes formed during chlorination of wastewater may be significantly dissipated in the receiving stream provided there is adequate dilution or volatilization.

When there is concern over the effect of chlorine-based disinfectants on aquatic life, alternative processes or disinfectants, e.g., dechlorination, ozone, or ultraviolet light, can protect both the native aquatic biota and the downstream water supplies. The AWWA committee believes that the overriding concern is public health protection and that the water purveyor should not be forced to demonstrate a public health threat to make upstream disinfection of wastewater mandatory.

Surface waters receiving discharges from municipal wastewater treatment plants and industrial and agricultural activities require more complete treatment, including coagulation, flocculation, sedimentation, filtration, and disinfection to minimize organics and fluctuating densities of microbial contaminants. Such serial treatment provides the multiple barriers needed to

ensure the microbiological quality of the water. In the case of source waters that need special treatment to reduce suspected carcinogens, the use of aeration, ozonation, activated carbon adsorption, or another effective process before disinfection may be desirable. However, microbial activity does occur in granular activated carbon (GAC) processes, possibly resulting in regrowth related to biodegradable organics, especially in warm-water periods. In addition, carbon fines may transport bacteria in GAC product water during stabilization of a new bed of virgin GAC or replacement with reactivated GAC. Neither problem is beyond control if reasonable treatment precautions are taken.

Groundwaters. Groundwaters derived from deep aquifers are generally of good bacteriological quality because percolation of water through soil results in the removal of much microbial pollution. As

Given the widespread potential for groundwater contamination, it is prudent to assume that, unless otherwise demonstrated, groundwater supplies also contain microbiological contaminants and need to be treated. Specifically, all treatment plants for public groundwater supplies should incorporate disinfection or at least have disinfection capability. An allowable variance to this requirement would be based on increased bacteriological monitoring, no record of an outbreak with the current operational configuration, and annual sanitary surveys to demonstrate continued integrity of the system.

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Finished waters. For either surface water or groundwater, the ideal disinfectant should be capable of maintaining a residual throughout the distribution system to control microbiological regrowth and protect against other contamination events such as cross-connection or low line pressure. Disinfection has been one of the most effective public health practices of modern times; this fact must not be lost in the current controversy surrounding chlorination by-products and carcinogenesis.

Whatever raw water quality problems are corrected by adequate treatment, all is for naught unless proper distribution system water protection practices are followed. Maintaining a disinfectant residual in the distribution system is critical, but it is just one step in proper system management. To ensure delivery of a high quality water to each consumer, managers of public water supply systems must be vigilant for any contamination in the distribution network and for evidence of water quality degradation. This is made difficult by the complexity of a distribution system, which is a network of mains, fire hydrants, valves, auxiliary pumping or chlorination substations, storage reservoirs, standpipes, and service lines.

Properly constructed storage reservoirs are needed to ensure that inadvertent, or even intentional, contamination does not occur. The well-known problems associated with animals and children around finished water reservoirs have been increased by acts of vandalism. Today, more than ever before, all finished water storage reservoirs should be covered, with all access ports locked and all vents screened.

In addition, there should be an adequate program for flushing the distribution system, reservoir cleaning, and disinfecting all new and repaired water mains, and a viable cross-connection control program. This preventive maintenance program is critical to the continued protection of water during distribution to the consumer. Because cross-connections may be widespread, it is not only desirable to identify them at the water treatment plant (short-circuiting of process waters) and throughout the distribution system but also to eliminate them through repairs and backflow preventer devices. High risk locations, e.g., hospitals, mortuaries, car washes, and wastewater treatment plants, should be priorities.

It would, of course, be prudent to select a nonpolluted raw water source. Such sources are rare, if they exist at all, but the best available source should be used. A safe water supply, therefore, requires proper treatment, effective operation, and safe passage through the distribution system to the consumer's faucet.

Types of pathogens

Special attention is being focused on viral agents, *Giardia lamblia, Legionella,* and opportunistic pathogens, which are among the newly identified waterborne pathogens. Consideration has been given to including these organisms in the Primary Drinking Water Regulations, but no recommendation to do so is now being made except for recommendations pertaining to additional treatment processes to control pathogen occurrences.

Viruses. Viral agents may be important because of the growing reuse of water, particularly when reuse is associated with decreased wastewater disinfection. Numerous public surface water supplies are neither filtered nor disinfected. Some water utilities (small groundwater supplies in Illinois, for example) are even reducing chlorination of potable water. Still other communities are shifting to combined chlorine disinfection to achieve trihalomethane reduction. Changed disinfection practices and other treatment modifications should be evaluated as microbial barriers wherever the risk of human viral contamination in source waters is real. It would also be desirable for the state authority to conduct prospective epidemiological surveys of the incidence of viral disease in such communities to identify special risks. Additional research is needed on virus methodology. The usefulness of a surrogate such as the coliphage test is unproven at this time.

Giardia. Detection of *Giardia* in source water, treatment processes, and finished drinking water is labor intensive, is variable in recovery efficiency, and requires a special professional skill that may not yet be available to many utilities. No appropriate surrogate indicator has been found. Because of this problem of analytical methodology, it is more appropriate to use the best available treatment technology to control infection and to make periodic prospective epidemiological surveys. Although properly operated treatment schemes, including chemical additional, filtration, and chlorination with a free residual, are capable of significant *Giardia* reductions, water suppliers are also advised to use turbidity monitoring for filtration performance evaluation. If continuous turbidity monitoring is used, the turbidity value at 4-h intervals (or a shorter regular time interval) is critical for determining compliance with performance criteria. For systems using slow sand or diatomaceous earth filtration, the sampling frequency for turbidity could be limited to one sample per day. It must be remembered, however, that breakthrough in filter beds is greater in small plants and that *Giardia* outbreaks have occurred in small systems using improper filtration. Furthermore, monitoring of filtered water for *Giardia* cysts (where washwater is recovered) could lead to a decision not to recover backwash water in order to prevent cyst contamination of the processed water.

***Legionella* and other opportunistic pathogens.** Anxiety about *Legionella* may represent only the tip of the iceberg as far as unknown pathogens are concerned. Classic water treatment practice has focused on reducing enteric pathogens. The coliform group has been used as an indicator of either source water acceptability or disinfection efficiency, because if coliforms are present, enteric pathogens may also be present and may have survived treatment. Such is not the case

with all pathogens. *Legionella* and *Legionella*-like organisms are pathogens that are transmitted through aerosolization and inhalation rather than by ingestion. Other pathogens with potential for transmission through inhalation of drinking water aerosols are *Mycobacterium, Pseudomonas,* and *Klebsiella.* Microbial allergens (a cause of allergic-type reactions from industrial air filters used in humidification devices) may also represent a problem. The committee feels that if the water treatment plant operator has responsibility for minimizing microbial dissemination via the aerosol route, then acceptable indicator systems and required levels of treatment to be applied at sites of high risk need to be defined. Because few data are available to define the infectious dose of such respiratory pathogens, a rational water quality criterion needs to be developed.

When they come into body contact, opportunistic pathogens also are a concern, especially for hospital patients. Waterborne organisms that have caused secondary infections in hospitals include *Pseudomonas putida, P. multophila, P. aeruginosa, Acinetobacter calcoaceticus, Alcaligenes faecalis,* and *Flavobacterium* species. The problem may be complicated further if opportunistic pathogens become antibiotic resistant through acquisition of plasmids from resistant bacteria, thereby making patient treatment more difficult.

Drinking water treatment is intended to produce water that is free from pathogenic microorganisms but not sterile. Bacterial regrowth in the distribution system may not be of significance for public health but may be of concern in hospitals or kidney dialysis clinics. Thus, it may be necessary for hospitals to consider installation of booster chlorinators to restore lost disinfectant residuals for improved reduction of all heterotrophic organisms, including opportunistic bacteria, and to maintain an in-house flushing program to prevent colonization. Particular attention should be given to routine monitoring for disinfectant residuals in the building plumbing network and to periodic flushing of all reservoirs and hot water tanks to remove sediment accumulations that protect bacterial survivors. Hospital personnel need to establish effective monitoring programs for water supplies and to plan corrective action. Although water quality deterioration beyond the service lines is not a utility responsibility, better communication is desirable between water purveyors and special user groups, e.g., hospitals and food processing and pharmaceutical plants. The AWWA committee suggested that periodic meetings address the utility's water quality objectives, review facility plumbing flushing and corrosion control programs, and identify specialized treatment approaches to be implemented by user groups to minimize amplification of organisms (*Legionella*, opportunistic bacteria, and fungi) associated with water inhalation and body contact infections.

Conventional criteria of treatment effectiveness

Total coliforms. The total coliform group of bacteria remains the best available indicator of treatment effectiveness. Although there is substantial evidence that coliforms are not an adequate indicator of *Giardia* occurrences and there are documented instances in which disease outbreaks occurred in the absence of any indication of a problem by coliform results, it is likely that similar situations would occur regardless of the indicator used. No indicator is absolute in its ability to predict the presence or absence of pathogens or to predict the occurrence of a waterborne outbreak. The most effective preventative measure would be to expand treatment barriers. Testing larger sample volumes of plant effluents and reemphasizing use of the sanitary engineering survey would be valuable adjuncts.

Heterotrophic bacteria (standard plate count). The heterotrophic plate count (HPC) is a good operational tool for measuring microbial breakthrough, evaluating process modifications, and detecting loss of water main integrity. This general measure of bacterial water quality can also be an important early sign of excessive microbial growth on distribution system pipe walls and in sediments, and it can indicate the presence of opportunistic pathogens. It is recommended that baseline data be gathered on the density of heterotrophic bacteria in the distribution system and that the information be utilized to measure treatment train effectiveness and bacterial quality changes. The committee recommends that a minimum of two samples or 10 percent of the coliform samples, whichever is greater, be examined quarterly, including one sample from the plant effluent and one from storage facilities in the distribution system.

The density of heterotrophic bacteria in the plant effluent can easily be maintained at very low levels (<10 organisms/mL) with adequate disinfection. When normal background heterotrophic plate counts in the distribution system become greater than 10^3 organisms/mL (a hundredfold increase) and this is confirmed by a second sample, action should be initiated immediately to resolve the microbial regrowth problem. One committee member disagreed with the recommendation to establish a limit for the heterotrophic bacterial population, claiming that colonization exists in the distribution system at all times and cannot be prevented.

Turbidity. Particles in water protect bacteria and promote their growth in the distribution system. For this reason, turbidity in finished water should be minimized. Well operated treatment plants consistently achieve a finished water turbidity <1 ntu. Where *Giardia* cysts or asbestos fibers may be present in the raw water, a 0.2-ntu standard may be necessary (depending on the treatment applied) to ensure minimal risk. Good quality groundwater supplies can be expected to have a turbidity of <0.5 ntu when they enter the distribution network. Exceptions for cases of turbidity >1 ntu may be made as long as the turbidity does not interfere with coliform detection or contribute to disinfectant demand in the finished water. Turbidity monitoring in the distribution system, although not required by regulation, is a good quality control practice; the goal should be 2 ntu. Values ≥5 ntu would signal the need to flush the distribution system and to search for areas of pipe corrosion that must be brought under control.

Disinfectant residuals. Measuring residual disinfectant is undoubtedly the most important test used in water supply monitoring. Emphasis should be on observing and interpreting sudden changes in the disinfectant residual of finished water and water in the distribution system. Disinfectant measurements should be used as a routine diagnostic tool. The practice of adding ammonia to convert free chlorine to combined chlorine to minimize trihalomethane formation will increase $C \cdot T$ disinfectant values. Several very large water systems, however, have used chloramines for years with no evidence of quality deterioration or public health problems. Recent case histories documenting coliform colonies in distribution networks have renewed interest among water suppliers in maintaining disinfectant residuals to the ends of the system, including dead-end sections, in the attempt to reduce microbial growth in such places.

Measuring microbial quality

The bateriological quality of water was initially measured by the multiple tube or most probable number (MPN) method. Regulations defined an unsatisfactory sample as one producing gas positive results in more than three tubes of a five-tube test. A water supply was out of compliance when >10 percent of the tubes in all MPN tests performed on distribution system samples over a 30-day period were positive. With the introduction of the membrane filter (MF) procedure, quantitative density limits were recognized: An unsatisfactory sample was defined as one containing more than 4 coliforms/100 mL, and a water supply was out of compliance

when the monthly average of all MF tests exceeded 1 coliform/100 mL.

Since the mid-1960s, many laboratories have replaced the MPN procedure with the less labor-intensive MF technique for total coliforms. With more than 50 percent of all laboratories now using the MF procedure, there is greater emphasis on the quantitative aspects of coliform occurrences. To equalize the sample volumes for testing by either the MF or MPN procedure, the sixteenth edition of *Standard Methods* has introduced a 10-tube multiple tube test and includes a table so that expanded MPN values can be calculated. This expanded multiple tube test extends quantitative results from a maximum value of 16/100 mL to 23/100 mL and improves the 95 percent confidence range. The committee suggests using the expanded multiple tube test as a quantitative measurement that is more comparable to the MF technique in precision and sample volume (100 mL) requirement.

Observation of coliform bacteria has received recent attention, particularly in regard to resolving problems of infrequent and minimal sampling of small water supplies (serving 25–3300 people). Regulating the percentage occurrences for any density of coliforms found by either testing procedure over 12 months, rather than limiting coliform density to a 30-day period, needs to be fully evaluated in different geographical areas of the United States. Frequency of occurrence is measured by a presence–absence test, the result of which is only qualitative. A new presence–absence test medium may improve recovery of stressed coliform bacteria and could be designed for use in a delayed incubation procedure after samples have been transmitted via mail to a central laboratory. Statistical interpretation of the results would be based on a 12-month moving average; the water supply would be in compliance if <5 percent of all presence–absence tests were positive. The committee believes that before the frequency of occurrence concept is adopted, more water supply data from different geographical areas and different water treatment processes should be analyzed to determine the compliance equivalency of frequency of occurrence with present regulation requirements. This could be done by simply recalculating MPN or MF "presence" results for any coliform occurrences and "absences" from all negative tests.

Monitoring public water supplies

Monitoring the quality of public water supplies is essential. Water plant operators and microbiologists agree that there continues to be a need for operational monitoring methods that will rapidly characterize product water. Unfortunately, rapid methods are unavailable, or they fail to detect coliforms at the concentration of 1 organism/100 mL.

Finished waters. Although large water systems monitor the plant effluent for coliforms, turbidity, and chlorine residual several times a day, smaller systems may make such analyses once every 24 h or only several times a week. The required frequency of coliform analysis for water plant effluents (population served, 10 000 or more people) should be at least one sample per day, collected at different times within the operational period. A more desirable approach might be composite sampling of the plant effluent over a 24-h period, with analysis of 0.25–1.25-gal (1–5-L) portions by the MF procedure. The purpose of this would be the identification of low density coliform breakthroughs. The frequency of monitoring plant effluents from small water systems would be determined by the state authority and would be intensified if the system had a water quality problem.

The turbidity of the finished water produced from surface water sources should be measured continuously or once per operating shift. The turbidity value reported for maximum contaminant levels would be the average value per day. For small systems serving <1000 persons, a grab sample, preferably taken toward the end of the filter run or once per shift, would be adequate. Aside from the mandatory requirement to measure finished water turbidity, it is desirable to measure turbidity at all stages of the treatment train to determine process effectiveness.

Distribution water. Water quality monitoring of the distribution system should continue to be based on population served, but consideration should also be given to the length of the distribution system and to any other mitigating circumstances that might dictate additional sampling. Stringent quality control and sample collection guidelines need to be developed and imposed. For small water systems, minimum sampling should be increased to five samples per month, with state discretion permitted to reduce the number on the basis of knowledge and experience. The current requirement of only one sample per month (or only one sample per quarter) does not provide adequate information if there is a water quality problem. The highest priority, however, should be placed on providing good water quality, not just on increased monitoring.

Samples must be taken at representative sites in the system. A portion of these sites should be fixed locations that are selected with reference to pressure zones, potential sources of contamination, high risk areas (hospitals, clinics), and the past history of coliform occurrences. Other sampling locations should be varied so that all parts of the distribution system are sampled. Routine monitoring should include measuring coliforms, turbidity, and, with lesser frequency, heterotrophic bacteria.

Turbidity sampling of the distribution system should be done at 10 percent of the sites used in the coliform monitoring program, the turbidity value being indexed to corrosion, chlorine demand, bacteriological regrowth, and the need to flush the distribution lines. With respect to monitoring the heterotrophic bacterial population, a data base must be established for all seasons and for all sites where samples are collected for coliform analyses; then testing can be reduced to 10 percent of the sampling frequency recommended for total coliform determinations. During periods of observed microbial regrowth, sampling for coliform and heterotrophic bacteria should be intensified until appropriate measures are found to be effective in eliminating the colonization.

Public notification

The National Primary Drinking Water Regulations require public notification, through announcements included with water bills or through other appropriate means, whenever a violation occurs. This requirement is applied whether the violation is minor or major, isolated or persistent. Maximum contaminant level (MCL) violations in addition require publication of the notice in newspapers and provision of the notice to the electronic media.

Public notification relates to the bacteriological MCL in two ways: (1) public notice is required of systems that fail to monitor as prescribed, and (2) public notice is required of systems whose samples fail to meet the bacteriological MCL. The net effect of this requirement from the consumer's point of view is that all violations are given equal weight, when, in fact, only MCL violations imply any potential health risk. Thus, the current requirement appears to be counterproductive. Whatever benefits are gained by public awareness of a water system's lack of sampling are more than offset by either public perception that such failure constitutes a serious health hazard or, worse, public belief that notices by water purveyors are routine and should not be considered significant. In either case, such notice does not achieve the principal desired effect, namely, community awareness of a potential health hazard. Public notice should be used with discretion to ensure that legitimate threats to public health receive the required visibility and attention.

There are two levels of concern and each requires a different level of action: (1) utility accountability to the public

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served, and (2) the prompt notification of a potential health problem. At the first level, any adverse trend in water quality should be reviewed by utility, state, and federal water supply representatives and state and local health officials. A joint action plan would then be formulated that should result in the intensification of data gathering (including monitoring for any fecal coliform occurrences), a sanitary (engineering) survey of the system, and daily review of the results with appropriate corrective measures. The second level of action would be immediate public notification if an outbreak occurs, if fecal contamination is detected in the water, or if the utility fails to follow corrective strategy defined by joint agreement of designated authorities.

In short, the AWWA committee concluded that public notices should be applied only when some risk to community health is definable. They should not be used as a regulatory punishment for minor or non-health-related violations. The key question that needs to be addressed to protect public health through safe water supplies is not whether the regulations have been violated, but rather the cause and significance of these violations in terms of public health.

References

1. US Environmental Protection Agency. National Interim Primary Drinking Water Regulations. EPA-570/9-76/003. Office of Water Supply, Washington, D.C. (1976).
2. Committee on the Challenges of Modern Society. Drinking Water Pilot Study Summary. NATO/CCMS Drinking Water Pilot Project Series CCMS 130. EPA 570/9-82-007. Office of Drinking Water, USEPA, Washington, D.C. (1983).
3. Assessment of Microbiology and Turbidity Standards for Drinking Water (P.S. Berger and Y. Argaman, editors). Proc. of a Workshop, December 2-4, 1981. EPA-570/9-83-001. Office of Drinking Water, USEPA, Washington, D.C. (1983).
4. AWWA Research Foundation-USEPA Workshops on Revising the Regulations. Philadelphia, Pa., Sept. 21-23, 1983; St. Louis, Mo., Oct. 4-6, 1983; Reno, Nev., Oct. 31-Nov. 4, 1983; Winter Park, Fla., Nov. 27-30, 1983.

About the authors: *This report was prepared by the AWWA Organisms in Water Committee. Committee members were E.E. Geldreich (chairman), A.E. Greenberg, C.N. Haas, J.C. Hoff, R.J. Karlin, J. Martin, E. Means, R.H. Moser, P. Regunathan, K. Reich, and H. Victoreen.*

Monitoring for Indicator Bacteria in Small Water Systems*

Edwin E. Geldreich, Eugene W. Rice and Eleanor J. Read**

The problem of monitoring for water quality in small or rural water supplies is a great concern because these are the public water supplies that experience most of the waterborne disease outbreaks. The reasons for the unsatisfactory state of public health may be found either in the use of poor quality raw source waters that are not adequately treated or in the ineffectual operation of existing plant processes.

Major waterborne agents contaminating water supplies include pathogenic bacteria, virus, protozoa and blood flukes, any of which cause a variety of gastroentestinal illnesses. These organisms originate in the feces discharged from infected humans, animal pets, farm animals and wild life and are transported by sewage and stormwater to the receiving waters that may be used as a raw source water in water supply. Engineered process barriers are designed to intervene in the passage of waterborne risks through public water supply. How effective these barriers are in prevention of pathogen passage in drinking water is the key purpose of the monitoring program.

While monitoring for pathogens in water is desirable, it is not practical for many reasons. First, the number of waterborne pathogens identified in water is estimated to be several hundred or more when consideration is given to the numerous species of Salmonella and types of viral agents. This list of pathogens has expanded in recent years with the discovery of new bacterial and viral agents that include Yersinia, Campylobacter, rotavirus, reovirus, parvovirus and the protozoans, Giardia and Cryptosporidium. Other pathogens will be discovered in the future as new breakthroughs in methodology unravel the mysteries of unidentified agents associated with waterborne outbreaks. In the United States alone (Table 1), unidentified etiologic agents accounted for 46.5% of all outbreaks during 1961-1983 and caused 86,740 individual illness cases (Craun, 1985; Lippy and Waltrip, 1984).

The problem of pathogen monitoring is further complicated by the lack of a single test that will detect all bacterial pathogens, viral

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**EPA Senior Microbiologist; Research Microbiologist; Water Engineering Research Laboratory and Senior Statistician, Computer Sciences Corporation, Cincinnati, Ohio, respectively.

agents or pathogenic protozoans and the knowledge that a negative pathogen test is inconclusive, using current state-of-the-art techniques. Monitoring sewage for pathogens currently prevalent in the community is not completely adequate because there will be no input from pathogen shedders in the infected farm animal and wildlife population. While some methods are available for specific pathogens (Salmonella, Yersinia, Campylobacter and enterovirus) these tests are best done in the specilaized laboratory, not by the average technician using the limited resources available in the small laboratory. Cost becomes the other limitation on direct monitoring for pathogens. Estimates for bacterial pathogen examination in water samples can range from $20 to $50 per sample while a virus or Giardia examination can cost $100 or more. Obviously, these costs can not be reconciled to small water system operating budgets. Therefore, the only viable alternative is to select a surrogate organism or bacterial indicator of fecal contamination that can be detected in a simple, inexpensive laboratory test.

Table 1. Waterborne Outbreaks in the United States During 1961-1983*

Etiologic Agents	Outbreaks	Cases	Deaths
Bacterial			
Shigella	52	7,462	6
Salmonella	37	19,286	3
Campylobacter	5	4,773	0
Toxigenic E. coli	5	1,188	4
Vibrio	1	17	0
Yersinia	1	16	0
Viral			
Hepatitis A	51	1,626	1
Norwalk	16	3,973	0
Rotavirus	1	1,761	0
Protozoan			
Giardia	84	22,897	0
Entamoeba	3	39	2
Chemical			
Inorganic (metals, nitrate)	29	891	0
Organic (pesticides, herbicides)	21	2,725	7
Unidentified agents	266	86,740	0
TOTAL	572	153,394	23

*Data adapted from Craun (1985); Lippy and Waltrip (1984).

The perfect surrogate would be one that completely mimics the occurrence and survival pattern of all fecal pathogens that might be waterborne and traceable through inadequate treatment barriers. Unfortunately, such an organism or indicator system does not exist. What is available are several indicator systems (total coliform, fecal coliform and fecal streptococcus) that are found in all warm-blooded animal feces with densities ranging from 10^6 to 10^8 organisms per gram (Kowal, 1982; Geldreich, 1978). Historical studies of these candidates has placed general emphasis on the wide spectrum of intestinal organisms that comprise the total coliform population. Their detection in water supply would suggest fecal contamination has occurred and this contamination very likely includes some intestinal pathogen risk to water consumers. Furthermore, adequate treatment will remove all coliforms detectable in 100 mL, including those that are of fecal origin as well as the more ubiquitous environmental strains. While this assumption has generally been accurate, the concept is occasionally flawed by virus or protozoan occurrences not associated with detection of coliforms in 100 mL samples. However, an overwhelming data base from systems world-wide has supported the general assumption that coliform absence in public water supply indicates there is reasonable assurance that the water is safe and carries minimal public health hazard from pathogens (World Health Organization, 1984).

Basic Principals for Coliform Detection

Total coliform detection in water supply is not complicated nor does it require a large amount of specialized equipment or a professional microbiologist to perform the test. There are two approaches: measurement of coliform density in a 50 or 100 mL test portion or the simple determination of the presence or absence of coliforms in 100 mL sample volume (Figure 1). For quantitative measurements, many laboratories use a membrane filter (MF) technique which involves the cultivation of differentiated coliform colonies on the filter surface using a lactose type medium and incubation at 35°C for 24 hours before colony counts are established (American Public Health Association, 1985). The multiple tube fermentation test (FT) has been in use by some laboratories for many years as the alternative method for detecting coliforms (American Public Health Association, 1985). This procedure is based on detecting gas produced by coliforms growing in a medium containing lactose. Five tubes of the sterile medium are inoculated with 10 mL sample portions and then the set of tubes is incubated for 24-48 hrs at 35°C to permit any coliforms present to ferment the lactose with release of gas into the medium and entrappment in a fermentation vial. If growth and gas are produced, those individual positive cultures are verified by growth transfers into a second, more restrictive lactose medium for further evidence of gas production. This confirmation provides assurance the sample contained coliform bacteria, not some other bacteria causing a false positive reaction. The density of coliform bacteria per 100 mL is then estimated from the number of tubes positive by using a most probable number statistical table. The FT procedure requires at least 48 to 96 hours to determine a test result and is more labor intensive than the MF procedure.

A more simplified test (Figure 1) and one that can be a very attractive operational test for small water plant operators is the presence-absence (P-A) test (American Public Health Association, 1985). While this procedure appears to be somewhat similar to a multiple tube fermentation test, the involvement in laboratory work is much less complicated. Basically, the procedure consists of inoculating 100 mL of sample into a bottle containing the appropriate concentration of a lactose type medium and a fermentation tube for gas entrappment. The bottle, with 100 mL sample, is incubated for 24 to 48 hours at 35°C and inspected for growth and gas production. If gas is noted in the fermentation tube or a color change (acid reaction) is observed, a small inoculumn of the culture is transferred to a tube of brilliant green lactose broth for verification that gas production again occurs and was related to coliform occurrence. Results of this test are completed within 96 hrs and reported as coliform present or absent. The only equipment needed is a 35°C incubator, sterile sample containers, bottles of P-A medium and a supply of sterile brilliant green lactose broth in culture tubes containing fermentation vials for gas entrappment. Media could be prepared and sterilized in a small autoclave at the water plant or obtained prepared for use from a central laboratory. If the P-A culture bottles are carefully premarked at 150 mL capacity (100 mL for sample plus 50 mL for medium with adjustment made for fermentation tube displacement in liquid) the samples could be added directly into the P-A bottle at the site of sample collection. With this accomplished, sterile sample containers would not be a necessary item in the test. Any chlorine residual in the water would be immediately neutralized by the medium constituents.

P-A Concept vs P-A Test

It is important to separate two different aspects associated with presence-absence information. The P-A concept is concerned with the frequency of coliform occurrence in a water supply over a specified time span. Such data can be obtained from conventional bacteriological tests using either the membrane filter or multiple tube procedure, simply by translating any coliform count or positive tube results into a coliform occurrence. This concept places equal emphasis on all positive samples, regardless of density, with a limit defined by a specific percentage of positive coliform occurrences permitted. For example, the presence-absence record of compliance could be based on degree of treatment: 5% positive occurrences permitted if the system uses complete treatment, 3% if treatment consists only of filtration and disinfection of surface waters and 1% for water supplies using disinfection as the only treatment control barriers. For the public health authority, information on how often there are coliform occurrences, over the long term, is an important indication of treatment effectiveness and operator skill in providing a continuous supply of safe drinking water.

From the operators viewpoint, it is desirable to have the capability for a simple bacteriological test which provides frequent checks on water quality produced. Before acceptance of the P-A test, two questions need to be addressed: (1), what are the technical considerations in performing the test and (2), how do the results of the P-A test compare with the more exacting laboratory procedures using either the

membrane filter or multiple tube test? In a review of technical considerations, the P-A test ranks high for ease of examination. The sample can be added directly to the culture bottle at the field site, thus dispensing with the sterile sample bottle and dechlorinating agent. Preparation of medium in sterile culture bottles could be delegated to a cooperating public health laboratory and sufficient supplies stored in a dark, cool storage area for no more than four weeks. As another option, at least one commercial venture is exploring the potential for manufacturing prepared medium in a disposable culture bottle so that no medium preparation need be done in the water plant. The answers to the second question can be found in a statisitcal review of several evaluation studies now completed (Jacobs, et al., 1986; Pipes, et al., 1986; Caldwell and Seidler, 1987).

How Valid is the P-A Test

Operational tests used by the small water plant operator should not only be inexpensive and easy to perform but also acknowledged to produce data that is equivalent or better in precision to that of the standard laboratory procedures. It therefore becomes important to understand the validity of P-A test results obtained from a variety of water supplies in different geographical areas. This consideration is critical to both the operator proposing to use the test and the public health authority who needs to evaluate the water supply quality. Test sensitivity to coliform detection and parallel examinations by the P-A test and either the MF or FT procedure (or both) were done in three widely divergent geographical areas. A data base of 1,483 samples in Vermont (Jacobs, et al., 1986), 1,560 samples in Oregon (Caldwell and Seidler, 1987) and 2,601 samples in eastern Pennsylvania (Pipes, et al., 1986) obtained from small water systems, were analyzed statistically to determine if significant differences could be established between any of the three testing procedures. Comparative data in the Pennsylvania study did not include parallel examination by the FT test. The McNemars statistical test (Fleiss, 1981) was selected as the most appropriate way to compare the different coliform detection methods, since each water sample was examined in parallel by more than one method.

As noted in Figures 2 and 3, the P-A test significantly outperformed both the multiple tube and membrane filter tests for coliform detection in water samples from supplies in Vermont and Oregon. Details of the statistical analyses are given in Table 2. Further analysis of these data also revealed that the multiple tube fermentation test detected more samples containing coliforms than the conventional membrane filter procedure using M-Endo medium. The statistical analysis of the eastern Pennsylvania data (Figure 4) indicated that there was no significant difference in coliform detection by the P-A test and the MF method. The Chi-square value was only 1.27, signifying that comparative results between the P-A and MF test results were essentially equivalent. Why there was no clear cut superiority to the P-A test as the best method for coliform detection in all instances may be related to the state of vigor for coliforms in the water supply. For the eastern Pennsylvania study, many of the positive results were obtained from samples of a small water system that did not chlorinate or other-

wise treat the groundwater supply. Regardless of this fact, these field studies on test performance do demonstrate that the P-A method equals or exceeds the results obtained by the recognized conventional procedures. Therefore, there should be no reason not to use the P-A test as either an operational tool in the small water plant or as part of the official monitoring data.

Table 2. McNemar's Test Results

Method Comparison	Study	McNemar's x^2	p-value
P-A vs FT	Vermont	5.3	0.02
	Oregon	12.7	<0.01
P-A vs MF	Vermont	52.0	<0.01
	Oregon	39.9	<0.01
	Pennsylvania	1.3	0.26
FT vs MF	Vermont	31.4	<0.01
	Oregon	8.3	<0.01

Operator Tests for Water Quality

Lacking information on plant effluent quality seriously restricts the rural water plant operator's ability to promptly respond to unsatisfactory water quality conditions through treatment adjustments. Perhaps this position leads to blind faith that water treatment can run automatically and produce a satisfactory water supply of uniform quality. This may be true for protected groundwater supplies but in many small water systems, surface and ground raw water quality does fluctuate because of poorly designed wells that do not protect water quality from contamination by improperly operated wastewater treatment systems, stormwater runoff and animal activity in the area adjacent to water supply intakes.

Monitoring finished water quality for several key characterisitcs is not beyond the realm of possibility in small water systems. There are available several basic techniques that water plant operators can learn to use with minimal training that will give them a measure of water quality being released as public water supply. Much critical information of immediate value can be obtained by frequent measurements for free chlorine residual, turbidity and coliform bacteria in the plant effluent. These three measurements will provide information respectively on the continued maintenance of a treatment control process, interference and protective shielding of microorganims and verification that the treatment process is effectively removing coliform bacteria. Chlorine and turbidity measurements can be made at frequent intervals in the day and provide immediate information using basic test kits that are readily available and easy to use. While

coliform testing requires a minimum of 24 hours of processing time it is an important record of treatment effectiveness for controlling the microbial quality of water produced and should be done at least once per week.

Bacteriological testing of public water supplies in rural or remote areas is not beyond the reach of operator capability. Both the MF and FT procedures have been packaged into commercially available field kits that may be used. For sheer simplicity, however, the presence-absence (P-A) coliform test may be the method of choice. Adapting the P-A test into a simplified operational test would be ideal for on-site monitoring although it does not quantify the extent of contamination events that might suggest treatment breakdown, loss of distribution integrity or biofilm development. The procedure tests a single 100 mL sample, not 50 mL which must be divided into five replicate portions of 10 mL required in the multiple tube test. Materials required for the P-A test (media and associated glassware items) are more readily available and cost less in many third world countries than membrane filters and associated filtration equipment. While the P-A concept was originally developed with a modified lactose broth (P-A broth), there is no reason why the test could not be applied to other coliform broth formulations (lactose broth, lauryl tryptose broth or MacConkey broth) to which bromcresol purple is added to indicate acid production. To further simplify the P-A test application to small water systems use, it should be permissable to utilize positive test information obtained from culture bottles after 24 to 48 hours incubation at 35°C without any further positive result confirmation. While these positive results might occasionally contain a false positive reaction (gas produced by a non-coliform organism) the error would be on the conservative side for safety considerations. These options would provide the opportunity to use the P-A test in remote locations in the world, where regional suppliers of laboratory materials carry limited choices of bacteriological media. The only critical consideration would be in the preparation of triple strength medium to be dispensed to the culture bottles in 50 mL volume. Medium dilution by the addition of 100 mL sample would create normal strength medium.

P-A Test For Remote Monitoring

Regional or national public health authorities need to monitor the water quality of small systems in all areas of the District or Nation. This essential program should define a minimal number of water samples to be collected (on a monthly basis) from all public water supplies, regardless of their remote locations and submitted to the central laboratory for examination. Unfortunately, some samples may be in transit for several days before they reach the laboratory. During this time, the microbial flora in the water changes, often leading to adverse, uncharacteristic test results that suggest the water sample meets national drinking water standards when indeed it does not (McDaniels and Bordner, 1983).

Since preservation of bacteriological samples is difficult to achieve without loss of low density coliform occurrences through nutrient depletion, extended contact time with chlorine, toxicity of

heavy metal impurities or microbial flora antagonism, the only other approach is to permit controlled growth during transit.

The P-A test could be a solution to this problem. The sample is added directly to the medium, then the culture bottles transported (maximum of 5 days) back to a central laboratory. The procedure is uncomplicated and requires very minimal effort by the water plant operator to perform (Pipes, et al., 1986). During transit, ambient air temperatures will influence the magnitude of sample culture growth, being slow during winter temperatures below 10°C and accelerated at summer or tropical temperatures that may reach normal (35°C) incubation temperature. Upon arrival in the laboratory, observation for gas production is made to determine what processing will be required. If turbidity and gas production is evident, the culture is confirmed in brilliant green lactose broth for evidence of coliform occurrence. If no gas production is noted, the culture bottle is incubated for 24 to 48 hrs at 35°C; then confirmed, if positive. Those culture bottles with no acid production or no visible turbidity but gas in the inner tube indicate air was shaken into tube during shipment, so these must be inverted to release the entrapped air and incubated 24 to 48 hrs as for the other negative cultures received for processing. The procedure is not labor intensive and would provide information on any coliform occurrences that were related to the original water sample.

Summary

Development of a practical microbiological monitoring program for small water systems is urgently needed at the local level for prompt detection of contamination followed by appropriate remedial actions. Three operational measurements are within the capability of the water plant operator: chlorine residual, turbidity and a basic test for coliform occurences using a presence or absence (P-A) concept. Evaluation of the P-A test in three different geographical areas demonstrated the procedure to outperform both the membrane filter and fermentation tube (MPN) tests for coliform detection. The use of a P-A test approach is recommended for small water system personnel because of its simplicity and adaptability to minimal resources available to the public system sector in rural and remote regions world-wide. Further adaptation of the test can be made to initiate field inoculation of the sample to be sent to a central laboratory for final processing and data gathering on water quality nationwide.

References

American Public Health Association (1985) Standard Methods for the Examination of Water and Wastewater. 16th ed., 1268 pp., Washington, D.C.

Caldwell, B. A. and Seidler, R. J. (1987) Comparison of Bacteriological Assays and Sampling Regimes for Increased Coliform Detection in Small Public Water Supplies. Appl. Environ. Microbiol. (In Press).

Craun, G. (1985) An Overview of Statistics on Acute and Chronic Water Contamination Problems, p 5-15. In Fourth Domestic Water Quality

Symposium: Point-of-Use Treatment and Its Implications. Water Quality Assoc., Lisle, Ill.

Fleiss, J. L. (1981) Statistical Methods for Rates and Proportions. 2nd ed. John Wiley and Sons, New York.

Geldreich, E. E. (1978). Bacterial Populations and Indicator Concepts in Feces, Sewage, Stormwater and Solid Wastes. In Indicators of Viruses in Water and Food, G. Berg ed., 422 pp., Ann Arbor Science Publishers Inc., Ann Arbor, MI.

Jacobs, N. J., Zeigler, W. L., Reed, F. C. Stukel, T. A. and Rice, E. W. (1986) Comparison of Membrane Filter, Multiple-Fermentation-Tube, and Presence-Absence Techniques for Detecting Total Coliforms in Small Community Water Systems. Appl. Environ. Microbiol., 51:1007-1012.

Kowal, N. E. (1982) Health Effects of Land Treatment: Microbiological. U.S. Environmental Protection Agency, EPA-600/1-82-007, Health Effects Research Laboratory, Cincinnati, Ohio.

Lippy, E. and Waltrip, S. (1984) Waterborne Disease Outbreaks, 1946 - 1980: A Thirty-Five-Year Perspective. Jour. Amer. Water Works Assoc., 76:60-67.

McDaniels, A. E. and Bordner, R. H. (1983) Effects of Holding Time and Temperature on Coliform Numbers in Drinking Water. Jour. Amer. Water Works Assoc., 75:458-463.

Pipes, W. O., Minnigh, H. A., Moyer, B. and Trog, M. A. (1986) Comparison of Clark's Presence-Absence Test and the Membrane Filter Method for Coliform Detection in Potable Water Samples. Appl. Environ. Microbiol. 52:439-443.

Pipes, W. O., Minnigh, H. A. and Troy, M. (In press). Field Incubation Of Clark's P-A Test for Coliform Detection. Proc. Water Quality Technology Confr., Portland, OR.

World Health Organization (1984) Guidelines for Drinking Water Quality, Vols. 1-3. Geneva, Switzerland.

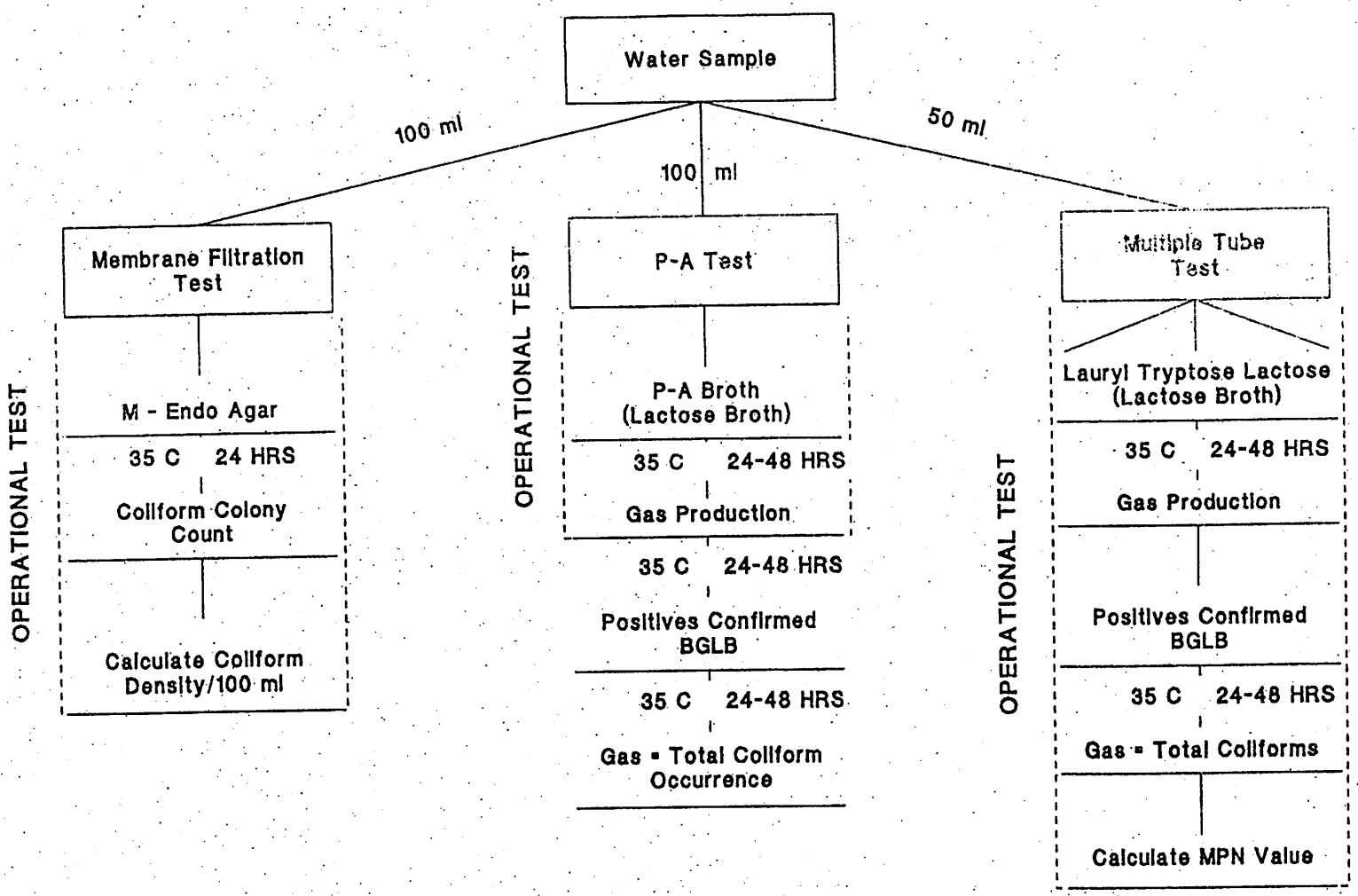

FIGURE 1. PATHWAYS FOR TOTAL COLIFORM DETECTION IN WATER SUPPLY

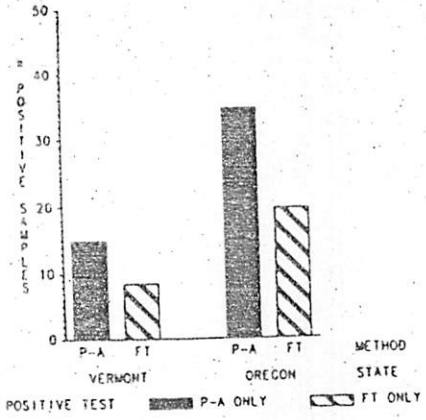

FIGURE 2. P-A VS FT METHOD COMPARISONS

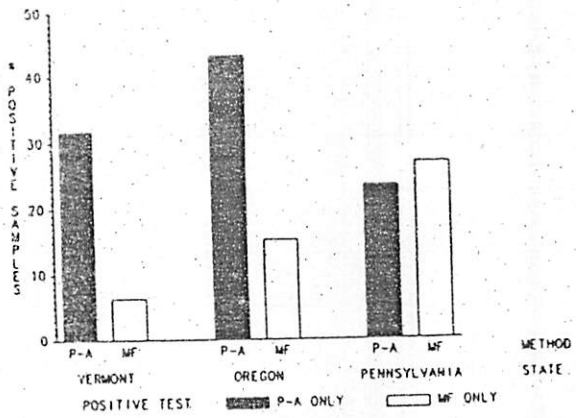

FIGURE 3. P-A VS MF METHOD COMPARISONS

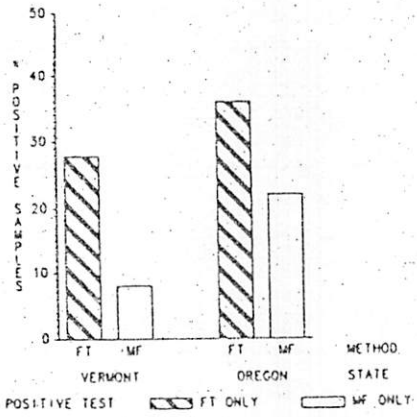

FIGURE 4. FT VS MF METHOD COMPARISONS

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Comparison of Membrane Filter, Multiple-Fermentation-Tube, and Presence-Absence Techniques for Detecting Total Coliforms in Small Community Water Systems

NICHOLAS J. JACOBS,[1]* WANDA L. ZEIGLER,[1] FRANK C. REED,[2] THERESE A. STUKEL,[3] AND EUGENE W. RICE[4]

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03756[1]; Connecticut River Watershed Council, Easthampton, Massachusetts 01027[2]; Department of Community and Family Medicine, Dartmouth Medical School, Hanover, New Hampshire 03756[3]; and Drinking Water Research Division, Water Engineering Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268[4]

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Methods for detecting total coliform bacteria in drinking water were compared using 1,483 different drinking water samples from 15 small community water systems in Vermont and New Hampshire. The methods included the membrane filter (MF) technique, a 10-tube fermentation tube (FT) technique, and the presence-absence (P-A) test. Each technique was evaluated using a 100-ml drinking water sample. Of the 1,483 samples tested, 336 (23%) contained coliforms as indicated by either one, two, or all three techniques. The FT detected 82%, the P-A detected 88%, and the MF detected 64% of these positives. All techniques simultaneously detected 55% of the positives. Evaluation of the confirmation efficiency of the P-A technique showed 94% of the presumptive positives confirming as coliforms. Thirteen different species of coliforms were identified from the 37 tests in which the P-A was positive but the MF and FT were negative. The P-A test was simple to inoculate and interpret and was considerably more sensitive than the MF and slightly more sensitive than the FT in detecting coliforms in this type of drinking water supply.

Currently, the membrane filter (MF) technique and the multiple-fermentation-tube (FT) technique, as described in *Standard Methods for the Examination of Water and Wastewater* (1), are the only procedures approved for monitoring drinking water systems for total coliforms under the Safe Drinking Water Act (14). Both techniques have been evaluated and compared in several studies (4, 13, 19, 20, 22, 24). The conclusions from these studies have suggested modifications of the media and procedures of both methods for more accurate results (11, 13, 17, 18, 22). Other investigators have proposed a presence-absence (P-A) technique as an alternative (7, 8, 23, 25).

The P-A technique, a basic simplification of the FT procedure, was developed by J. A. Clark as a qualitative means of monitoring drinking water systems. Although it was tested in parallel with the MF on different drinking water systems and found to be as sensitive (7), it has not been compared with both the FT and MF together.

In this study, the sensitivity of each of the methods (MF, FT, and P-A) was compared using drinking water samples from a variety of small community water systems. The methods used were the conventional MF technique, a 10-tube FT technique, and the most recently proposed P-A technique (1). Small water systems located in rural areas are quite variable and are often less well protected than municipal supplies. In addition, appropriate laboratory facilities for monitoring are not readily available. The results presented in this study should assist in developing appropriate methods for improving this type of water supply.

MATERIALS AND METHODS

Samples. Over a 1-year period, a total of 15 small community water systems in Vermont and New Hampshire were sampled. These water systems (Table 1) each serve less than 1,000 persons and have more than 10 service connections. Sources of water for these systems include shallow wells, deep wells, and springs. Most systems did not chlorinate or filter their water. Samples were collected from one location in each system on a weekly basis. Once a month, five samples were collected from five sites in the distribution network of each system, including the weekly site.

Samples were collected according to the guidelines in *Handbook for Evaluating Water Bacteriological Laboratories* (16). Sterile 500-ml plastic bottles (polymethylpentene) containing sodium thiosulfate (16) were used as sample containers. Samples were kept in an ice chest, transported to the laboratory within 2 to 3 h, and analyzed within 5 to 6 h after collection.

Microbiological procedures. Each sample was analyzed by each of the techniques, using 100-ml water portions for each test. The MF procedure was performed by methods detailed in references 1 and 5. HA membrane filters (Millipore Corp., Bedford, Mass.) were placed on sterile pads (Millipore Corp.) saturated with M-endo broth (Millipore Corp.; Difco Laboratories, Detroit, Mich.) and incubated for 24 h at 35°C. A minimum of five of the typical green-metallic sheen colonies from each positive sample were transferred to lauryl tryptose broth (LTB; Difco) and brilliant green bile lactose broth (BGLB; Difco) for verification as coliforms (5). Production of gas in LTB and BGLB within 48 h was considered a positive test (5). At least one colony per positive sample was examined for lactose fermentation after growth on Levine eosin methylene blue (EMB) agar (Difco).

The FT technique involved 10 tubes, each containing 10 ml of double-strength LTB and a fermentation tube. The addition of a 10-ml sample of water to each tube allowed a total of 100 ml of water to be examined. For each positive sample, all presumptive positive tubes up to a maximum of

* Corresponding author.

TABLE 1. Description of the 15 drinking water systems

System no.	Year established	No. of service connections	Approx. no. of users	Water source[a]	Chlorination[b]	Filtration[c]
011	1928	11	30	1	−	−
021	1930	70	240	1	−	−
031	1960	20	55	1/5	+	−
041	1946	238	910	1	+	−
051	1965	38	90	2/5	+	−
061	1960	17	70	3	−	−
071	1965	18	55	1/5	−	−
081	1900	250	650	1	+	−
091	1949	330	930	1/5	−	−
1011	1977	40	75	1	+	+
1021	1968	30	65	1/5	−	−
1031	1952	13	30	1/5	−	−
1041	1935	70	325	2	+	−
1051	1970	98	350	2	+	+
1061	1948	135	420	1/5	−	+

[a] Water source: 1, deep well; 2, shallow well; 3, springs; 4, wells to reservoirs; 5, wells to covered reservoirs.
[b] Chlorination: −, none; +, chlorination with hypochlorite.
[c] Filtration: −, none; +, yes.

five were confirmed for gas production in BGLB. As indicated in Results, these water samples demonstrated a high confirmation efficiency, and therefore the confirmation of five tubes was sufficient to judge a given sample as contaminated with confirmed coliforms. At least one BGLB tube was transferred to Levine EMB agar. The number of coliforms per 100 ml was estimated from a 10-tube most-probable-number (MPN) table (1). The procedure was the same as previously described (5), with the omission of the final step of the completed test, in which typical colonies on EMB agar are transferred to LTB. To assess the validity of this modified procedure, a resampling scheme was conducted in which 49 positive samples (14% of the total number of positive samples of this study) were carried through to the final step of the completed test (see results below).

The P-A technique used was a recent modification of the original test proposed by Clark (7). The test consisted of a single culture bottle (250-ml milk dilution bottle), containing a 50-ml portion of triple-strength medium plus a fermentation tube (12 by 75 mm). The formulation of the medium at single strength was: 13.0 g of lactose broth (Difco), 17.5 g of LTB, and 0.0085 g of bromocresol purple (Difco) (dissolved in 10 ml of 0.1 N NaOH before addition to broth) in 1 liter of distilled water. The prepared bottle was autoclaved for 12 min at 121°C and was stored until used. Before samples were added, the bottle was inverted to empty the medium out of the fermentation tube. After the bottle had been inoculated with a 100-ml sample portion, it was inverted to fill the fermentation tube. The bottle was incubated at 35 ± 0.5°C and inspected after 24 and 48 h for production of acid or acid plus gas. Bottles showing any degree of color change from purple to yellow or brownish yellow were subcultured for confirmation. Inoculum from a presumptive positive test was transferred to BGLB for confirmation and then to Levine EMB agar for detection of lactose-fermenting colonies.

In cases in which the P-A test was positive and the MPN (FT) and MF methods were negative, organisms were isolated in pure culture from Levine EMB agar and identified using API 20E identification test strips (Analytab Products, Inc., Plainview, N.Y.). The species names given are those obtained from the API identification scheme.

A quality assurance program was performed as outlined in reference 5.

Statistical methods. McNemar's test (15) was used to compare the overall proportion of positive samples detected by different methods. This test does not take into account the disagreement on individual samples. To study patterns of agreement between methods, kappa measures of inter-rater agreement (2, 15) were used. Kappa is an index taking values between −1 and 1, indicating the relative agreement between two techniques beyond chance agreement. $\kappa = 1$ indicates maximum possible agreement; values greater than 0.75 represent excellent agreement, and values below 0.4 represent poor agreement beyond chance. $\kappa = 0$ indicates chance agreement only.

A further analysis was performed to study where disagreements of the P-A and the FT with MF occurred. This analysis was based on the obviously unproven assumption that MF gives the correct conclusions. The MF technique was chosen as the standard for comparison because of its wide usage among water laboratories. Conditional kappa measures of agreement (2) with FT were calculated given MF counts of 0, 1 to 4 (inclusive), and ≥5 per 100 ml. By collapsing the 1 to 4 (inclusive) and ≥5 categories into a ≥1 category, an additional measure of agreement was computed conditional on a positive MF count. Due to the dichotomous nature of the P-A test, conditional kappa measures could only be computed given zero and positive MF counts.

RESULTS

Comparison of the MF, FT, and P-A methods. Of the 1,483 samples analyzed in this study, 336 (23%) confirmed positive samples were detected by either one, two, or all three of the techniques. The total coliform counts of these samples ranged from 1 to over 300 organisms per 100 ml. A comparison of the three methods in detecting the presence of coliforms is shown in Fig. 1. The FT technique detected 275 positive samples (19% of the total samples, 82% of all positives), whereas the MF technique detected 216 positive samples (15% of the total samples, 64% of all positives) and the P-A technique detected 296 positive samples (20% of the total samples, 88% of all positives). All three techniques simultaneously detected coliforms in 185 samples. There was a statistically significant difference, when analyzed by McNemar's test (15), in the detection rate of positive samples between each pair of methods. The MF detected fewer

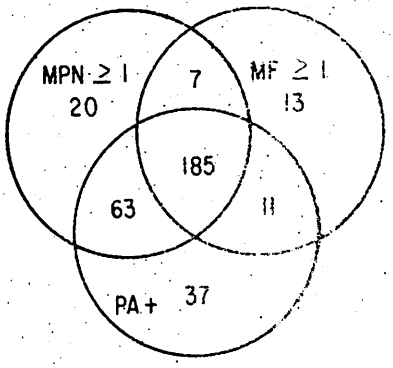

FIG. 1. Comparison of three methods for detection of water samples positive for coliforms. The number of positive samples detected by each technique is given in the text. Positive samples are defined as at least 1 coliform per 100 ml of the MPN (FT) and MF tests, or a positive reaction in the P-A test.

positive samples than either the MPN (FT) ($p < 0.001$) or the P-A ($p < 0.001$); the P-A detected slightly more positives than the FT ($p = 0.02$).

To obtain a comparison of samples with both low and high coliform densities, the coliform counts for the FT and the MF test were divided into three categories: negative, ≥5, and 1 to 4 (inclusive) per 100 ml. The latter represents an arbitrary figure for identifying systems with low coliform densities. Figure 2 indicates the cross-classified coliform counts for each pair of techniques. These data were analyzed statistically (see below), but one conclusion is obvious: the P-A test was always positive when the MF count was ≥5

A

PA	MPN VALUES 0	1-4	≥5	
−	1160	21	6	1187
+	48	76	172	296
	1208	97	178	1483

B

PA	MF COUNTS 0	1-4	≥5	
−	1167	20	0	1187
+	100	114	82	296
	1267	134	82	1483

C

MPN VALUES	MF COUNTS 0	1-4	≥5	
0	1184	24	0	1208
1-4	53	42	2	97
≥5	30	68	80	178
	1267	134	82	1483

FIG. 2. Cross-classified coliform counts for each pair of microbiological techniques.

TABLE 2. Kappa measures of agreement and their standard errors for pairwise agreement between the three techniques

Comparison	Observed κ (SE)
PA vs MPN (FT) (0 vs ≥1)[a]	0.83 (0.02)
PA vs MF (0 vs ≥1)	0.72 (0.03)
MF vs MPN (FT) (0 vs ≥1)	0.74 (0.02)
MF vs MPN (FT) (overall)[b]	0.59 (0.02)

[a] (0 vs ≥1) indicates negative versus positive.
[b] Classification of samples into negative, 1 to 4 (inclusive), and ≥5 categories.

(Fig. 2B). Furthermore, there were 30 samples where the MPN (FT) value was ≥5, but the MF was negative (Fig. 2C).

The statistical analysis for the data in Fig. 2 is presented as kappa values (see Methods) in Table 2. The agreement between P-A and FT for a simple negative-positive classification (0 versus ≥1) was excellent. Agreement between MF and the other two techniques for the same binary classification was not as strong but still good. Moderate agreement only was found between FT and MF for overall classification of samples into negative, low-coliform (1 to 4), and high-coliform (≥5) categories.

In Fig. 2C the counts below the diagonal are much higher than those above the diagonal: of the 177 total disagreements, 151 (85%) occurred below the diagonal. This indicates that when there was disagreement on any particular sample, coliform counts for FT were usually higher than those for MF. The same pattern of disagreement occurred between the P-A and MF (Fig. 2B): there were 120 disagreements, 100 (83%) of which showed MF negative and P-A positive. It should be noted, however, that agreement between FT and P-A was very strong (Fig. 2A and Table 2). There were fewer (75) overall disagreements; 48 of these (64%) showed P-A positive and FT negative.

The lower sensitivity of the MF test is also apparent from the data in Fig. 1, which compares all three techniques as either positive or negative. There were 63 samples (19% of the positives) for which both the FT and P-A tests together were positive while the MF test was negative. In addition, for 120 samples (36% of the positives) the MF was negative and either the MPN (FT) or the P-A gave positive results. Furthermore, there were only 13 samples for which the MF was positive alone, 7 samples for which the MF and FT alone were positive, and 11 samples where the MF and P-A tests alone were positive.

In summary, both the MPN (FT) and the P-A methods appear to be more sensitive than the MF test. That the FT and P-A strongly agree gives some evidence that the MF could be less sensitive. In addition, the P-A appears to be a more sensitive test than the FT.

A formal analysis of the disagreements between the MF and the P-A and FT techniques is shown in Table 3, using conditional kappa values (see Materials and Methods). When MF ≥ 5, agreement with the FT was excellent, i.e., MPN value of ≥5. When MF ≥ 1, agreement was very good, i.e., P-A positive; MPN value of ≥1. However, when MF = 0, agreement (i.e., P-A negative; MPN = 0) was only moderate. In summary, when the MF technique indicated a positive sample, the other two techniques showed strong agreement; when the MF technique indicated a negative sample, agreement was not as strong. Furthermore, the higher the coliform count for the sample as determined by MF, the stronger the agreement with the FT technique. Very poor agreement was found between FT and MF when MF density was between 1 and 4 (inclusive) (see Fig. 2C and

TABLE 3. Conditional kappa measures of agreement with MF as the standard[a]

MF count	Observed κ (SE)	
	MPN (FT) agreement	PA agreement
0	0.65 (0.03)	0.61 (0.02)
1–4	0.26 (0.02)	N.A.[b]
>4	0.86 (0.03)	0.88 (0.03)
>8	0.92 (0.04)	N.A.

[a] See Materials and Methods for explanation of conditional kappa measures and for the use of MF as the standard for comparison.
[b] N.A., Not applicable.

Table 3), although 110 of 134 (82%) of these samples tested positive by FT, 68 of the 134 (51%) samples had >8 coliforms by the FT test. Again, it was seen that the FT consistently read more coliforms for a sample than the MF.

Confirmation efficiency and importance of gas production. A useful screening test should exhibit a high ratio of presumptive positives to confirmed positives. We calculated the percentage of presumptive positive tests that were subsequently confirmed (confirmation efficiency) for each technique (Table 4). The confirmation efficiency of the MF technique was 93%, with 654 of 702 suspected colonies picked from the membrane filters confirmed as coliforms. The confirmation efficiency of the FT technique was 93%, with 1,043 of 1,125 presumptive positive tubes confirmed as coliforms.

The efficiency of the P-A test compared favorably. Of the 316 presumptive positive P-A tests that showed acid (either strong or weak) and gas, 94% were confirmed as coliforms. Two categories of positive P-A tests were noted. The vast majority showed both a strong acid (yellow color) and gas reaction. Of 277 of these tests, only 8 were not confirmed, giving a 97% confirmation efficiency for this category. However, there were 39 borderline tests which had relatively slight color changes (a brownish yellow) with a small amount of gas (4- to 6-mm gas bubble in fermentation tube). Of these 39 cases, 27 were confirmed, giving a 69% confirmation efficiency. In contrast, there were 24 tests in which only acid reactions and no gas production was observed. None of these was confirmed, indicating the critical importance of examining the P-A test for gas production.

A resampling scheme was conducted to assess the validity of our modification of the FT technique. As indicated in Materials and Methods, we omitted the final step of the completed test, in which typical isolated colonies on EMB agar are transferred to LTB. Each of the 15 water systems was resampled an equal number of times. A total of 49 positive samples were obtained for which at least one tube carried through to the entire completed step (5). In this resampling scheme, we subcultured all BGLB tubes positive at the confirmed stage. These samples showed a total of 147 tubes that carried through to the first step of the completed test, where typical colonies were observed on EMB agar. All of these typical colonies were transferred to LTB, the final step of the completed test, and produced gas within 48 h. These results indicate that the observation of typical colonies on EMB agar was a true reflection of the presence of coliforms. Therefore, for the water samples we investigated, our modification of the completed step of the FT procedure was valid.

Organisms isolated from the P-A test. Organisms were isolated and identified from the 37 P-A tests that were positive when the MF and FT tests were negative. The API system for identification and nomenclature was used. Table 5 lists the organisms along with the number of times they were isolated. *Citrobacter freundii* was the most frequently isolated organism, followed by *Enterobacter agglomerans*, and *Serratia plymuthica*. Most (81%) of these 37 presumptive positive P-A tests were noticed on day 2 of incubation, rather than at 24 h. The color of these positives ranged from brown to yellow, and gas was detected in each case. Each of the pure isolates which was designated as a species of the genus *Serratia* was reinoculated into lactose broth to confirm its ability to produce gas from lactose. All these organisms produced gas upon retesting. The identities of the *S. plymuthica* species were confirmed by the Analytab Aerobe Laboratory (Analytab Products, Plainview, N.Y.).

Comparison of the 10-tube FT with the 5-tube FT tests. The FT technique tested in this study used 10 10-ml tubes, as opposed to the FT technique which utilizes only 5 10-ml tubes (1). As a means of comparing the 10-tube with the 5-tube FT, we separately tabulated the number of water samples which would have tested positive if only 5 tubes had been analyzed instead of 10. This comparison was done by separating the 10-tube method into two sets of 5 tubes, consisting of the even- and odd-numbered tubes. Of the 275 water samples positive by FT, both sets of five tubes showed

TABLE 4. Confirmation efficiencies of the P-A, MPN (FT), and MF techniques

Test	Description of test result	No. subjected to confirmation	No. confirmed	% Confirmed
P-A	Strong acid, gas	277	269	97
	Slight acid,[a] gas	39	27	69
	Strong or slight acid, gas[b]	316[b]	296[b]	94
	Strong or slight acid, no gas	24	0	0
MPN (FT)	Tubes with gas	1,125	1,043	93
MF	Metallic-sheened colonies	702	654	93

[a] Slight acid was indicated by a brownish-yellow color.
[b] Sum of the two lines above.

TABLE 5. Organisms isolated from positive P-A tests when the MF and MPN (FT) tests were negative

Isolate[a]	No. of times isolated
Enterobacter agglomerans	6
E. cloacae	3
E. aerogenes	2
Citrobacter freundii	8
Escherichia coli	1
Klebsiella pneumoniae	5
K. ozaenae	1
K. oxytoca	2
Serratia plymuthica	6
S. fonticola	1
S. rubideae	1
S. oderifera	1
Hafnia alvei	1

[a] Identified according to the profile numbers determined by API.

TABLE 6. Positive samples detected by MF, FT, and P-A techniques in each of the 15 water systems

System	No. of positive samples by:		
	MF	FT	P-A
011	18	33	38
021	17	21	22
031	55	57	62
041	0	1	0
051	1	2	2
061	82	100	96
071	6	15	16
081	6	2	5
091	9	12	12
1011	2	4	8
1021	15	24	25
1031	4	2	3
1041	0	4	4
1051	0	0	0
1061	1	1	3

at least one positive tube in 207 (76%) of the samples. On the other hand, there were 68 (24%) samples for which only one set of five tubes showed one or more positive tubes. Thus, assuming an equal distribution of coliform in the tubes, on average 88% of the positives from the 10-tube method would have been detected using only 5 tubes (88% = 76% + 1/2 24%).

Comparison of the three methods in individual water systems. Approximately 100 samples were collected from each system. The results for each individual system are compared in Table 6. Systems 061 and 031 were the most frequently contaminated, and the MF technique compared favorably with the P-A technique in detecting positives in these two systems (Table 6). In contrast, the P-A technique detected many more positives than the MF technique in some of the less frequently contaminated systems. For instance, system 011 was positive 38 times by the P-A but only 18 times by the MF procedure (Table 6).

DISCUSSION

The results indicate that, under the circumstances of our tests, the P-A is much more sensitive than the MF and slightly more sensitive than the FT. There are several factors that may account for this observation.

The MF technique gave negative readings in many samples for which the MPN (FT) and P-A methods were positive. Previous studies have also reported the lower sensitivity of the MF technique in recovering coliforms. Reasons suggested for this failure have included the survival of coliforms on a membrane filter surface compared to survival when in broth (23), the failure to revive injured coliforms or weakened cells (19, 24), or the possibility that the M-endo broth used in the MF test is a selective medium which may be inhibitory to stressed coliforms (3, 4, 24). Inoculation of a sample into an enriched broth-based medium with a prolonged incubation period may enhance the recovery of indicator organisms (3, 24). These organisms may have been injured coliforms that needed more time to grow.

The 10-tube FT technique appears as an optional procedure in the 16th (1985) edition of *Standard Methods for the Examination of Water and Wastewater* (1). This 10-tube technique provides more precise MPN values than the 5-tube FT. With the particular set of drinking water samples that we studied, 12% of the positive samples on average would have been read as coliform negative if the 5-tube FT had been utilized instead of the 10-tube method.

Since this 10-tube FT procedure uses a 100-ml sample of water, it is comparable to the MF and P-A methods, which also use 100-ml samples. The FT was considerably more sensitive in terms of recovery than the MF procedure. It was surprising that the FT was slightly less sensitive than the P-A test, since they are both broth-based methods relying on gas production and are both incubated for up to 48 h. A possible explanation is the slight difference in medium composition between the P-A and FT tests. There is a slightly higher lactose concentration in the P-A (0.75%) than in the FT (0.5%). It has previously been found that isolates anaerogenic in the FT medium (LTB) were aerogenic in a broth containing a slightly higher lactose concentration (12). Members of the genera *Citrobacter*, *Enterobacter*, and *Klebsiella* were most frequently isolated (12). These organisms were also isolated in the present study from the positive P-A tests when the MF and FT were negative. Other possible explanations for the failure of the FT technique to detect coliforms are given in other studies (6, 11, 13, 17, 21).

The P-A test showed a high confirmation efficiency, which is an important characteristic for a screening technique. This high confirmation efficiency was found only in tests that showed both acid and gas production. P-A tests that showed acid but no gas were not confirmed and probably contained lactose-fermenting species other than coliforms (8–10).

Another important characteristic for a useful screening technique is the ease of detection of a positive test. With the P-A test, we found that a large percentage of all positive samples (91%) showed a distinctive yellow color and gas. Clear negatives (77% of all samples) were readily noted by no change in the purple color and no gas. However, there were a small percentage (3% of all samples) which showed only a borderline color change (yellow-brown or brown), but did show some gas production. These were usually true positives, with a 69% confirmation efficiency. However, when no gas was observed, these borderline color changes did not confirm. These findings have important practical implications for the reading of borderline P-A tests in the field. Borderline color changes which showed a small amount of gas represented approximately 10% of our positive P-A tests. Only 69% of these borderlines were confirmed as coliforms. Therefore, it is particularly important to carry all P-A tests with borderline color changes through to the confirmation step before making any decision about the presence of coliforms.

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LITERATURE CITED

1. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Inc., Washington, D.C.
2. **Bishop, Y. M., W. E. Fienberg, and P. W. Holland.** 1975. Discrete multivariate analysis: theory and practice. MIT Press, Cambridge, Mass.
3. **Bissonette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stress on enumeration

of indicator bacteria from natural waters. Appl. Microbiol. 29:186–194.
4. **Bissonette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1977. Evaluation of recovery methods to detect coliforms in water. Appl. Microbiol. 33:590–595.
5. **Bordner, R., and J. Winter (ed.).** 1978. Microbiological methods for monitoring the environment—water and wastes. U.S. Environmental Protection Agency, Cincinnati.
6. **Chambers, C. W.** 1950. Relationship of coliform bacteria to gas production in media containing lactose. Public Health Rep. 65:619–627.
7. **Clark, J. A.** 1968. A presence-absence (P-A) test providing sensitive and inexpensive detection of coliforms, fecal coliforms and fecal streptococci in municipal drinking water supplies. Can. J. Microbiol. 14:13–18.
8. **Clark, J. A.** 1969. The detection of various bacteria indicative of water pollution by a presence-absence (P-A) procedure. Can. J. Microbiol. 15:771–780.
9. **Clark, J. A.** 1980. The influence of increasing numbers of non-indicator organisms upon the detection of indicator organisms by the membrane filter and presence-absence tests. Can. J. Microbiol. 26:827–832.
10. **Clark, J. A., and L. T. Vlassoff.** 1973. Relationships among pollution indicator bacteria isolated from raw water and distribution systems by the presence-absence (P-A) test. Health Lab. Sci. 10:163–172.
11. **Evans, T. M., M. W. LeChevallier, C. E. Waarvick, and R. J. Seidler.** 1981. Coliform species recovered from untreated surface water and drinking water by the membrane filter, standard, and modified most-probable-number techniques. Appl. Environ. Microbiol. 41:657–663.
12. **Evans, T. M., R. J. Seidler, and M. W. LeChevallier.** 1981. Impact of verification media and resuscitation on accuracy of the membrane filter total coliform enumeration technique. Appl. Environ. Microbiol. 41:1144–1151.
13. **Evans, T. M., C. E. Waarvick, R. J. Seidler, and M. W. LeChevallier.** 1981. Failure of the most-probable-number technique to detect coliforms in drinking water and raw water supplies. Appl. Environ. Microbiol. 41:130–138.
14. **Federal Register.** 1975. National interim primary water regulations. Fed. Reg. 40:59566–59588.
15. **Fleiss, J. L.** 1981. Statistical methods for rates and proportions, 2nd ed. John Wiley and Sons, New York.
16. **Geldreich, E. E.** 1975. Handbook for evaluating water bacteriological laboratories, 2nd ed. U.S. Environmental Protection Agency, Cincinnati.
17. **Geldreich, E. E., H. D. Nash, D. J. Reasoner, and R. H. Taylor.** 1972. The necessity of controlling bacterial populations in potable waters: community water supply. J. Am. Water Works Assoc. 64:596–602.
18. **LeChevallier, M. W., S. C. Cameron, and G. A. McFeters.** 1983. New medium for improved recovery of coliform bacteria from drinking water. Appl. Environ. Microbiol. 45:484–492.
19. **McFeters, G. A., S. C. Cameron, and M. W. LeChevallier.** 1982. Influence of diluents, media, and membrane filters on the detection of injured waterborne coliform bacteria. Appl. Environ. Microbiol. 43:97–103.
20. **McFeters, G. A., and D. G. Stuart.** 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane filter chambers. Appl. Microbiol. 24:805–811.
21. **Meadows, P. S., J. G. Anderson, K. Patel, and B. W. Mullins.** 1980. Variability in gas production by *Escherichia coli* in enrichment media and its relationship to pH. Appl. Environ. Microbiol. 40:309–312.
22. **Morgan, G. B., P. Gubbins, and V. Morgan.** 1965. A critical appraisal of the membrane filter technic. Health Lab. Sci. 2:227–237.
23. **Pipes, W. O., and R. D. Christian.** 1984. Estimating mean coliform densities of water distribution systems. J. Am. Water Works Assoc. 76:60–64.
24. **Shipe, E. L., and G. M. Cameron.** 1954. A comparison of the membrane filter with the most probable number method for coliform determinations from several waters. Appl. Microbiol. 2:85–88.
25. **Weiss, J. E., and C. A. Hunter.** 1939. Simplified bacteriological examination of water. J. Am. Water Works Assoc. 31:707–713.

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SWIMMING-ASSOCIATED GASTROENTERITIS AND WATER QUALITY[1]

V. J. CABELLI,[2] A. P. DUFOUR,[3] L. J. McCABE,[3] AND M. A. LEVIN[4]

Cabelli, V. J. (Dept. of Microbiology, U. of Rhode Island, Kingston, RI 02881), A. P. Dufour, L. J. McCabe and M. A. Levin. Swimming-associated gastroenteritis and water quality. *Am J Epidemiol* 1982;115:606-16.

A direct, linear relationship between swimming-associated gastrointestinal illness and the quality of the bathing water was obtained from a multi-year, multiple-location prospective epidemiologic-microbiologic research program conducted in New York City, 1973–1975, Lake Pontchartrain, Louisiana, 1977–1978, and Boston, Massachusetts, 1978. Several microbial indicators were used in attempting to define the quality of the water; and, of those examined, enterococci showed the best correlation to total and "highly credible" gastrointestinal symptoms. The frequency of gastrointestinal symptoms also had a high degree of association with distance from known sources of municipal wastewater. A striking feature of the relationship was the very low enterococcus and *Escherichia coli* densities in the water (10/100 ml) associated with appreciable attack rates (about 10/1000 persons) for "highly credible" gastrointestinal symptoms. Moreover, the ratio of the swimmer to nonswimmer symptom rates indicated that swimming in even marginally polluted marine bathing water is a significant route of transmission for the observed gastroenteritis.

gastroenteritis; swimming; water microbiology

In an earlier report (1), the authors presented evidence from a prospective epidemiologic-microbiologic study that there are measurable health effects associated with swimming in sewage-polluted waters. In some cases, these effects were observed even in waters that were in compliance with existing recreational water quality guidelines and standards (2). The swimming-associated illness observed was an acute, relatively benign gastroenteritis which had a short incubation period and duration. The accompanying symptoms, as pointed out in another report (3), suggested that the etiologic agent might be the human rotaviruses or Norwalk-like viruses. The water-related nature of one of these agents, the Norwalk-like virus, recently has been confirmed in a shellfish-associated outbreak of gastroenteritis in Australia of some 2000 cases (4) and in several outbreaks associated with drinking water (5).

The objective of the overall research program was to determine if there are illnesses associated with swimming in sewage-polluted water and, if so, whether their rates can be quantitatively related to some measure of the quality of the bathing water. This question has been the subject of controversy since the 1950s

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[1] From the US Environmental Protection Agency, Health Effects Research Laboratory, Marine Field Station, West Kingston, RI.

[2] Present address, Department of Microbiology, University of Rhode Island, Kingston, RI 02881 (Reprint requests to Dr. Cabelli.)

[3] Present address, Health Effects Research Laboratory, US Environmental Protection Agency, Cincinnati, OH.

[4] Present address, Office of Research and Development, US Environmental Protection Agency, Washington, DC.

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when Stevenson (6) and Moore (7) obtained seemingly contradictory results. Therefore, studies similar to those reported for New York City beaches (8, 9) were conducted at two other locations in the United States: Lake Pontchartrain, Louisiana and Boston, Massachusetts. The results obtained at these two sites were essentially the same as those found in the New York City study.

This report describes the quantitative relationship of the swimming-associated gastroenteritis to the mean enterococcus density of the water as obtained from all the epidemiologic-microbiologic studies conducted in the United States.

Materials and methods

Study sites. Studies were conducted at three general locations: New York City (beaches on Coney Island and the Rockaways) in 1973–1975; Lake Pontchartrain, Louisiana (Levee Beach and Fontainbleau Beach) in 1977 and 1978; and Boston, Massachusetts (Revere Beach and Nahant Beach) in 1978. The beaches were chosen because they were near large metropolitan areas and, hence, used by large numbers of individuals who swam on weekends but not during midweek days. This was an essential requirement of the experimental design for reasons to be given.

The sources of pollution reaching the beaches in the New York City study were reasonably well defined as those emerging from the mouth of the Hudson River. They were less defined in the Boston study, and least defined in the Lake Pontchartrain study. Moreover, in order to determine which, if any, symptoms or groups of symptons were both swimming-associated and pollution-related (a major objective of the first two years of the New York City study), a relatively unpolluted beach at the Rockaways was paired with a barely acceptable beach on Coney Island. The latter was adjacent to a beach area classified by local authorities as unsafe for swimming. The paired beaches were also chosen so that they would have demographically similar populations. The results obtained with reference to this objective of the New York City study have been published (1).

Study design. A prospective cohort design was used in all the studies. The essential features of the design, which have been described previously (1, 9, 10), are as follows:

1. Discrete trials were conducted only on Saturdays and Sundays. Potential participants were recruited at the beach, preferably as family groups. Trials were limited to weekend days to maximize the size of the beachgoing population, especially the portion that comes to the beach only on weekends. By excluding from the study those individuals who swam in the five midweek days before and after the weekend in question or at other locations on either weekend day, exposure to bathing water was limited to that at the specific beach during a single day, or two days at the most. This decreased the confounding effect of beach-to-beach and day-to-day variability in pollution levels on the illness-pollution relationships sought. In addition, it allowed the analyses of the data by trials (study days) or by groups of trials when the pollution levels as indicated by the mean indicator densities in the water were similar.

2. Demographic information was obtained at the initial beach interview and during the subsequent telephone follow-up survey. The information included age, sex, ethnicity and socioeconomic status, as determined from a persons-to-rooms ratio.

3. Information on bathing activity was obtained at the initial beach interview. Swimming was stringently defined as complete exposure of the head to the water. This characteristic was determined by direct inquiry and by observation, i.e., whether or not the hair of the subject was wet. Individuals who did not

tinal symptoms and gastroenteritis will be used synonomously.

Water quality monitoring. Water samples were collected periodically on trial days during the time of maximum swimming activity at the beaches. This was generally between the hours of 11 a.m. and 5 p.m. Usually, three to four samples were collected at two or three sites from each beach at chest depth approximately four inches below the surface of the water. Upon collection, the samples were iced and delivered to the laboratory, where they were assayed within eight hours of collection. Potential water quality indicators that were examined are shown in table 2. Total coliform and fecal coliform densities were obtained using the most probable number or membrane filter methods, as described (11). The densities of total coliforms and the component genera of that group (*Escherichia*, *Klebsiella*, *Citrobacter-Enterobacter*) were also measured using the membrane filter procedure for coliforms (mC) of Dufour and Cabelli (12). After 1974, *Escherichia coli* densities were determined by the membrane filter method for thermotolerant *E. coli* (mTEC) (13). Enterococci (14), *Pseudomonas aeruginosa* (15), *Aeromonas hydrophila* (16), *Clostridium perfringens* (17) and *Vibrio parahemolyticus* (18) were assayed using membrane filter methods.

TABLE 2

Potential water quality indicators used at the New York City, Lake Pontchartrain, Louisiana and Boston, Massachusetts beaches, 1973–1975

Indicators	New York City	Lake Pontchartrain	Boston
Enterococci	+*	+	+
Escherichia coli	+	+	+
Klebsiella sp.	+	+	
Enterobacter sp.	+		
Citrobacter sp.	+		
Total coliforms	+		
Clostridium perfringens	+		
Pseudomonas aeruginosa	+	+	
Fecal coliforms	+	+	
Aeromonas hydrophila	+		
Vibrio parahemolyticus	+		

* + shows that measurements were made for this indicator at the specified location.

Analysis. The relationship of swimming-associated (swimmer minus nonswimmer) gastrointestinal symptom rates to the mean indicator densities in the water was examined by regression analysis. Because the participants were recruited at the beach on weekends and individuals who were swimming in the midweeks before and after the one in question were eliminated from the study, the symptom rates for a given weekend day (trial) and the associated mean indicator density could have been analyzed as a point on the regression line. In fact, this was not possible with most of the trials because the number of nonswimming participants was too small. This problem was circumvented by grouping the trials. Single-day trials were arrayed according to increasing indicator densities. Groups were selected by utilizing "natural breaks" in the array; in this way, those trial days with similar indicator densities formed a group of data from which a geometric mean density and the associated rates for gastrointestinal and highly credible gastrointestinal symptoms could be calculated. This arbitrary grouping of trials was done for each of the indicator organisms.

The attack rates for gastrointestinal and highly credible gastrointestinal symptoms were regressed against the mean indicator density. The log-linear regression equation

$$Y = a + b \log X \qquad (1)$$

was used in which X was the mean indicator density and Y the gastrointestinal symptom rate.

RESULTS

Studies were conducted over several years at three locations in the United States. The locations, beaches, study years, follow-up percentages and number of usable responses are shown in table 3.

TABLE 3

Location of beaches and the number of usable responses by beach and study year, 1973–1978

Location	Beaches	% Follow-up during study year			No. of usable responses during study year		
		1	2	3	1	2	3
New York City*	Coney Island	82.3	78.3	78.3	641	3146	6491
	Rockaways	86.6	82.9		681	4923	
Lake Pontchartrain, LA†	Levee	77.2	77.9		3432	2768	
	Fontainbleau		—§			551	
Boston, MA‡	Revere	81.2			1824		
	Nahant	81.2			2229		

* Coney Island, 1973–1975; Rockaways, 1973–1974

† Levee, 1977–1978; Fontainbleau, 1978.

‡ Revere, 1978; Nahant, 1978.

§ Included with Levee Beach.

The degree of association of the mean indicator densities to swimming-associated gastrointestinal symptoms in the three years of the New York City study was used to reduce the number of indicators examined in subsequent studies. The correlation coefficients for the various indicators obtained from these regression analyses are shown in table 4. It can be seen that enterococci was the best indicator of those examined. Equally important, fecal coliform densities, the basis for most federal and state guidelines and standards (2), correlated very poorly with swimming-associated gastrointestinal symptoms.

The rates for total and highly credible gastrointestinal symptoms among swimmers and nonswimmers and the residuals (swimmer minus nonswimmer rates) for the grouped trials are given in table 5. Also included are the corresponding means and ranges of the enterococcus densities and the number of trials (days) in each group. Similar data for *E. coli* are given in table 6 by way of contrast. In a number of instances, the swimmer and nonswimmer rates were significantly different from each other. This was more frequent for residual rates associated with high enterococcus densities and with total gastrointestinal symptoms.

The regression lines obtained from the data given in tables 5 and 6 are shown in figure 1 along with their correlation coefficients (r). In addition to having higher r values, the enterococcus regression lines differ from those for *E. coli* in two other ways. The *E. coli* lines have shallower slopes and intercept the X axis at much lower densities. However, in the regression lines for both indicators, rather low densities are associated with appreciable attack rates. Attack rates for highly credible gastrointestinal symptoms of about

TABLE 4

Correlation coefficients for total gastrointestinal (GI) symptoms and the "highly credible" gastrointestinal (HCGI) portion against the mean indicator densities for studies at New York City beaches, 1973–1975

Indicator	Correlation coefficients (r)		No. of points
	HCGI	GI	
Enterococci	0.96	0.81	9
Escherichia coli	0.58	0.51	9
Klebsiella	0.61	0.47	11
Enterobacter-Citrobacter	0.64	0.54	13
Total coliforms	0.65	0.46	11
*Clostridium perfringens**	0.01	−0.36	8
Pseudomonas aeruginosa	0.59	0.35	11
Fecal coliforms	0.51	0.36	12
Aeromonas hydrophila	0.60	0.27	11
*Vibrio parahemolyticus**	0.42	0.05	7

* No data for 1973.

TABLE 5

Summary of the mean enterococcus density - gastrointestinal (GI) symptom rate relationships among swimmers, nonswimmers and residuals obtained from clustered trials in studies on swimming-associated gastrointestinal illness, 1973 - 1978

Study	Beach	Year	Enterococcus density/100 ml		Trials (days) clustered	No. of swimmers	No. of non-swimmers	Symptom rates in cases/1000					
			Mean	Range				Total GI			Highly credible GI		
								Swimmers	Non-swimmers	Residuals	Swimmers	Non-swimmers	Residuals
New York City	Rockaways	1973†	21.8	1-2-59	8	484	197	81	46	35	30.4	15.2	15.2
	Coney Island		91.2	6-186	8	474	167	72	24	48*	46.4	18.0	28.4
		1974	3.6	2-5	3	1391	711	27	23	4	7.6	4.2	3.4
			7.0	7	3	951	1009	38	34	4	10.5	6.9	3.6
			13.5	10-17	2	625	419	42	17	25*	16.0	2.4	13.6
			31.5	30-33	2	831	440	43	23	20	18.1		18.1*
		1975	5.7	2-11	14	2232	936	63	55	8	18.8	19.3	-0.5
			20.4	14-38	10	1896	678	59	37	22*	14.8	7.4	7.4
			151	86-298	4	579	191	60	31	29	34.6		34.6*
Lake Pontchartrain, LA	Levee	1977	14	9-7-88	8	874	451	86	51	35*	32.0	11.1	20.9*
			224	190-249	4	720	456	108	50	58**	31.9	8.8	23.1*
			495	344-711	2	895	464	108	54	54**	35.8	8.6	27.2**
	Levee	1978	11.1	3-30	8	1230	415	75	34	41**	36.6	14.5	22.1*
	Fontainbleau		14.1	3-34	5	248	303	81	63	18	44.3	23.1	21.2
	Levee		112	67-303	4	801	322	112	50	62**	42.4	15.6	26.9*
Boston, MA	Revere	1978	4.4	2-6	3	697	529	83	66	17	23.0	11.0	12.0
	Nahant		7.1	6-9	2	1130	1099	71	67	4	33.0	28.0	5.0
	Revere		12.0	12	1	222	376	108	74	34*	41.0	13.0	28.0*

* $p < 0.05$; ** $p < 0.01$

† Study population too small to cluster trials by similar indicator densities.

TABLE 6

Summary of the mean E. coli density – gastrointestinal (GI) symptom rate relationships among swimmers, nonswimmers and residuals obtained from clustered trials in studies on swimming-associated gastrointestinal illness, 1973–1978

Study	Beach	Year	*E. coli* density/100 ml			Trials (days) clustered	No. of swimmers	No. of non-swimmers	Symptom rates in cases/1000					
									Total GI			Highly credible GI		
			Mean	Range					Swimmers	Non-swimmers	Residuals	Swimmers	Non-swimmers	Residuals
New York City	Rockaways	1973	24.8	4	34	8	484	197	81	46	35	30.4	15.2	15.2
	Coney Island		174	50	708	8	474	167	72	24	48*	46.4	18.0	28.4
		1974	2.2	1	4	6	2514	1641	25	34	-9	8.0	3.7	4.3
			13.3	9	19	4	1304	1045	38	29	9	14.1	5.7	8.4*
			30.5	26	35	2	600	425	65	33	32*	23.3	2.4	20.9
		1975	46.5	22	89	10	1945	1099	55	51	4	13.4	17.8	-4.4
			142	115	169	6	775	194	76	41	35	24.5	10.3	14.2
			278	208	356	8	1049	330	55	24	31*	21.0	3.0	18.0*
			514	441	659	4	937	271	68	55	13	24.5	7.4	17.1
Lake Pontchartrain, LA	Levee	1977	44	33	54	2	372	222	132	45	87**	32.3	9.0	23.3
			161	112	221	3	910	306	120	65	55**	52.7	22.8	29.9*
			497	445	556	4	574	307	85	45	40*	32.8	13.0	19.8
			3091	1030	4270	5	419	204	88	83	5	31.0	4.9	26.1
	Fontainbleau	1978	9.0	1	23	8	248	303	81	63	18	44.3	23.1	21.2
	Levee		32.6	17	87	5	1123	382	78	44	34*	38.3	20.9	17.4
			93.7	53	177	4	918	355	103	36	67**	39.2	8.5	30.7
Boston, MA		1978	5.5	4	7	2	541	874	72	63	9	39.0	29.0	10.0
			7.0	5	9	2	477	410	86	68	18	23.0	10.0	13.0
			17.5	13	22	2	589	225	70	67	3	27.0	27.0	0.0
			29.5	28	31	2	442	495	93	71	22	32.0	14.0	18.0

* $p < 0.05$; ** $p < 0.01$

† Study population too small to cluster trials by similar indicator densities.

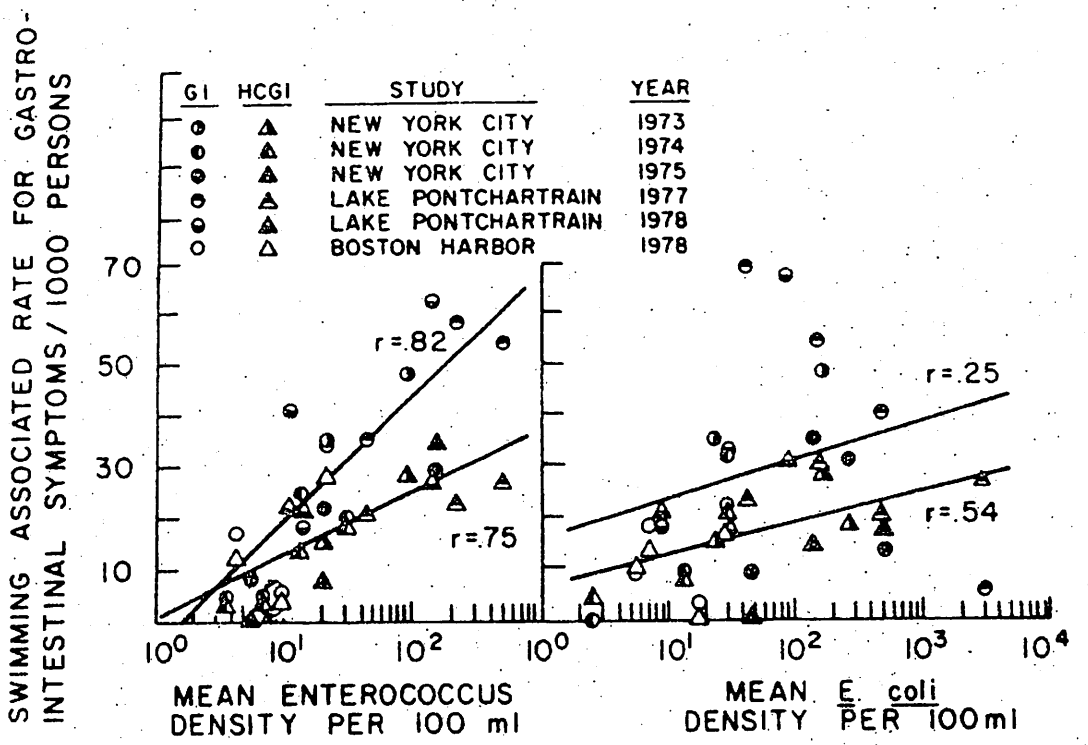

FIGURE 1. Regression of swimming-associated (swimmer minus nonswimmer) rates for gastrointestinal (GI) symptoms on the mean enterococcus and *E. coli* densities in the water. Coordinate points are from tables 5 and 6. Correlation coefficients (*r*) are as given. HCGI, highly credible gastrointestinal.

10/1000 (1 per cent) are associated with enterococcus densities of about 10/100 ml.

The enterococcus regression lines, their formulae, the *r* and *p* values and 95 per cent confidence limits for the lines are shown in figure 2. These relationships predict the illness rates from the mean enterococcus densities.

The relative importance of swimming in sewage-polluted water as a route of transmission for enteric illness was determined by examining the ratio of swimmer to nonswimmer gastroenteritis rates against the mean enterococcus density. It was assumed that all the cases acquired by all the routes other than swimming in sewage-polluted waters were included in the nonswimmer rates. The regression lines obtained for the trials clustered by indicator densities are shown in figure 3. It can be seen that the rates for both total and highly credible gastrointestinal symptoms were equal at a mean enterococcus density of about 1/100 ml. At a level of 10/100 ml, the rates for total and highly credible gastrointestinal symptoms were 1.5 times for swimmers and twice those for nonswimmers, respectively. The higher ratios for highly credible than for total gastrointestinal symptoms are of interest because of their implications concerning the reliability of the respondents' information to the illness queries.

DISCUSSION

The results clearly show that the risk of gastroenteritis associated with swimming in marine waters impacted with municipal wastewaters is related to the quality of the water as indexed by the mean enterococcus density in the water. More-

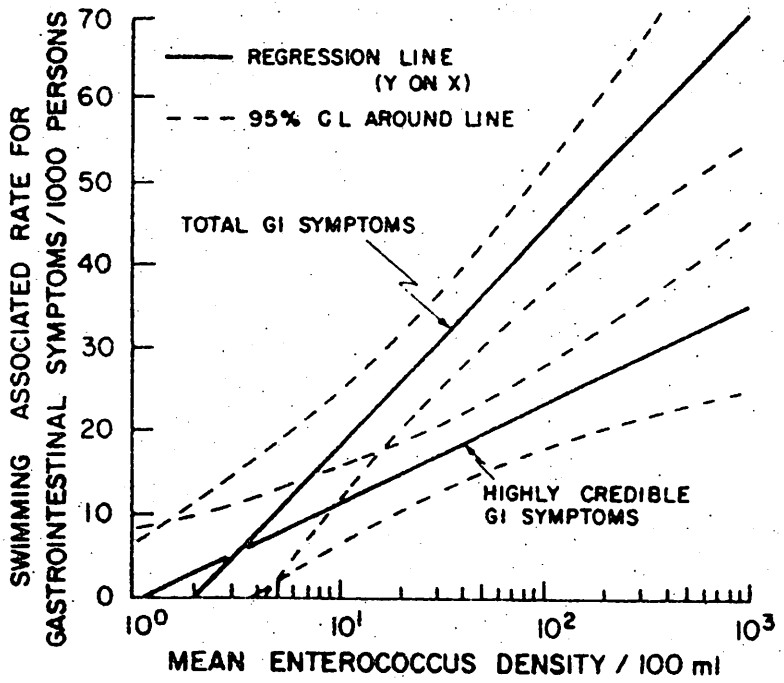

FIGURE 2. Regression of swimming-associated gastrointestinal (GI) symptom rates on the mean enterococcus densities in the water. Data are from all US studies. The 95 per cent confidence limits (CL) for the lines are as shown. The slopes, intercepts, r and p values for GI symptoms are, respectively, 24.2, −5.1, 0.82 and <0.0001. For highly credible GI symptoms, they are, respectively, 12.2, 0.2, 0.75 and <0.001.

over, the risk is detectable at extremely low levels of pollution. According to the criteria suggested by Hill (19), there is a strong suggestion of causality. First, the association is a good one; in some trials, the swimming-associated gastroenteritis rate was three to four times greater than the nonswimming rate. Second, there was a consistency in the association in that it was observed at multiple locations over multiple years. Third, the association between enteric disease and fecal contamination is a reasonable one by its very nature. Fourth, the association is a coherent one since there is a precedent for such a relationship by other waterborne routes of transmission, i.e., in shellfish (20) and potable water (21).

It was also understandable that, of the indicators examined, enterococcus densities in the water correlated best with the rates for the swimming-associated gastroenteritis. The two salient indicator characteristics required for the specific association obtained are a consistent fecal source and "good" survival during sewage treatment and transport in the aquatic environment. Of the indicators examined, enterococci and *E. coli* best satisfy the first requirement (22, 23); and, of the two, enterococci have the best survival characteristics (24), although their densities in raw or treated sewage are 1–2 orders of magnitude less than those of *E. coli* (25). These two differences are consistent with those observed in the slopes and X axis intercepts of the regression lines for the two indicators. That is, the slopes of the regression lines should become shallower and the lines should cross the X axis at lower indicator densities as the survival

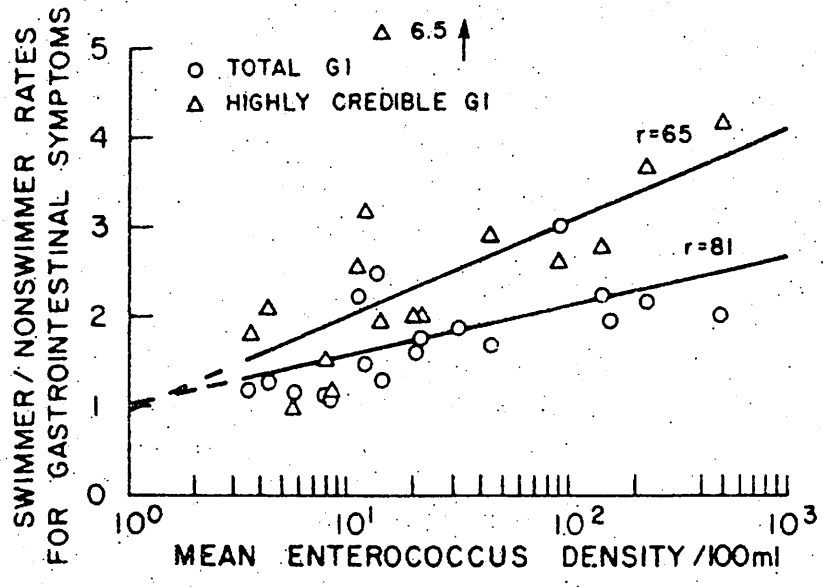

FIGURE 3. The relative risk of swimming in sewage-polluted waters as shown by the regression of the ratio of swimmer to nonswimmer (background) rates for gastrointestinal (GI) symptoms on the mean enterococcus density in the water. Data are from table 5.

characteristics of the indicator become poorer relative to those of the etiologic agents.

There are two implications from the finding of rather high gastroenteritis rates (1 per cent) associated with the ingestion of one to five enterococci (the accidental ingestion of 10–15 ml of water (26) whose enterococcus density was about 10/100 ml). The first is that even enterococci may not survive as well as the etiologic agent for the gastroenteritis. The second is that the agent must be present in the bathing waters and, hence, municipal wastewaters in very large numbers, be highly infectious, survive very well in the marine environment or, most probably, a combination of all three.

The analysis of the ratios of the swimmer to nonswimmer gastroenteritis rates would suggest that, for individuals of "swimming age," swimming in sewage-polluted waters is not an insignificant route of transmission for the disease. Moreover, the risk of gastroenteritis is present even at relatively low pollution levels, as seen from the indicator densities. The higher ratio of the swimmer to nonswimmer rates observed with highly credible as opposed to total gastrointestinal symptoms suggests that nausea, stomachache and even diarrhea are disproportionately reported by nonswimmers. This, in turn, suggests that the swimming-associated rates for total gastrointestinal symptoms are underestimated.

Finally, the finding of swimming-related rates of gastroenteritis associated with very low indicator densities, i.e., the ingestion of one to five enterococci, has some interesting implications with regard to the existence of sporadic cases of this illness by the other potential water-associated routes of transmission, e.g., shellfish, drinking water and even aerosols generated from municipal sewage and its receiving waters. These possibilities should be pursued by prospective epidemiologic investigations.

References

1. Cabelli VJ, Dufour AP, Levin MA, et al. Relationship of microbial indicators to health effects at marine bathing beaches. Am J Public Health 1979;69:690-6
2. US Environmental Protection Agency. Quality criteria for water. Washington, DC: USEPA 1976:42-50.
3. Cabelli VJ. Evaluation of recreational water quality. In: James S, Evison L, eds. Biological indicators of water quality. London: Wiley, 1979:14-1 to 14-22.
4. Murphy AM, Grohmann GS, Christopher PJ, et al. An Australia-wide outbreak of gastroenteritis from oysters caused by Norwalk virus. Med J Aust 1979;2:329-33.
5. Haley CE, Gunn RA, Hughes JM, et al. Outbreaks of waterborne disease in the United States, 1978. J Infect Dis 1980;141:794-7.
6. Stevenson AH. Studies of bathing water quality and health. J Am Public Health Assoc 1953;43:529-38.
7. Moore B. Sewage contamination of coastal bathing waters in England and Wales: a bacteriological and epidemiological study. J Hyg 1959;57:435-72.
8. Anderson AC, Ktsanes VK, Diem JE, et al. Bacterial indicators and health effects among swimmers. Abstracts of the Annual Meeting of the American Society for Microbiology, 1979:222
9. Cabelli VJ. Health effects criteria for marine recreational waters. Cincinnati, OH: HERL, United States Environmental Protection Agency, 1980. EPA-600/1-80-031).
10. Cabelli VJ, Levin MA, Dufour AP, et al. The development of criteria for recreation waters. In: Gameson H, ed. International symposium on discharge of sewage from sea outfalls. London: Pergamon, 1975:63-73
11. American Public Health Association. Standard methods for the examination of water and wastewater, 14th ed. Washington, DC: American Public Health Association, 1976.
12. Dufour AP, Cabelli VJ. Membrane filter procedure for enumerating the component genera of the coliform group in seawater. Appl Microbiol 1975;29:526-33.
13. Dufour AP, Strickland ER, Cabelli VJ. Membrane filter method for enumerating *Escherichia coli*. Appl Environ Microbiol 1981;41:1152-8.
14. Levin MA, Fischer JR, Cabelli VJ. Membrane filter technique for enumeration of enterococci in marine waters. Appl Microbiol 1975;30:66-71.
15. Levin MA, Cabelli VJ. Membrane filter technique for enumeration of *Pseudomonas aeruginosa*. Appl Microbiol 1972;24:864-70.
16. Rippey, SR, Cabelli VJ. Membrane filter procedure for enumeration of *Aeromonas hydrophila*. Appl Environ Microbiol 1979;38:108-13.
17. Bisson JW, Cabelli VJ. Membrane filter enumeration method for *Clostridium perfringens*. Appl Environ Microbiol 1979;37:55-66
18. Watkins WD, Thomas CD, Cabelli VJ. Membrane filter procedure for enumeration of *Vibrio parahaemolyticus*. Appl Environ Microbiol 1976;32:679-84.
19. Hill AB. The environment and disease: association or causation? Proc R Soc Med 1965;58:295-300.
20. Verber JL. Shellfish borne disease outbreaks. Internal report. Davisville, RI: Northeast Technical Service Unit, USFDA, 1972.
21. McCabe LJ. Epidemiological consideration in the application of indicator bacteria in North America. In: Hoadley AW, Dutka BJ, eds. Bacterial indicators/health hazards associated with water. Philadelphia: American Society for Testing & Materials, 1976:15-22.
22. Dufour AP. *E. coli*: the fecal coliform. In: Hoadley AW, Dutka BJ, eds. Bacterial indicators/health hazards associated with water. Philadelphia: American Society for Testing & Materials, 1976:48-58.
23. Cabelli VJ. Indicators of recreational water quality. In: Hoadley AW, Dutka BJ, eds. Bacterial indicators/health hazards associated with water. Philadelphia: American Society for Testing & Materials, 1976:222-38
24. Hanes NB, Fragala R. Effect of seawater concentration on survival of indicator bacteria. J Water Pollut Control Fed 1967;39:97-104.
25. Mueller JJ, Cabelli VJ. Enterococci and other microbial indicators in municipal sewage effluents. J Water Pollut Control Fed 1982 in press.
26. Streeter HA. Bacterial quality objectives for the Ohio River. A guide for the evaluation of sanitary condition of waters used for potable supplies and recreational uses. Cincinnati, Ohio, Ohio River Valley Sanitation Commission, 1951.

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Membrane Filter Technique for Enumeration of Enterococci in Marine Waters

M. A. LEVIN,* J. R. FISCHER, AND V. J. CABELLI

National Marine Water Quality Laboratory, West Kingston, Rhode Island 02892

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A membrane filter procedure is described for the enumeration of enterococci in marine waters. The procedure utilizes a highly selective and somewhat differential primary isolation medium followed by an in situ substrate test for identifying colonies of those organisms capable of hydrolyzing esculin. The procedure (mE) was evaluated with known streptococci strains and field samples with regard to its accuracy, sensitivity, selectivity, specificity, precision, and comparability to existing methods. Essentially quantitative recovery was obtained with seawater-stressed cells of *Streptococcus faecalis* and *S. faecium*. Neither *S. bovis*, *S. equinus*, *S. mitis*, nor *S. salivarius* grew on the medium. The selectivity of the medium was such that a 10,000-fold reduction in background organisms was obtained relative to a medium which contained no inhibitors and was incubated at 35 C. About 90% of those typical colonies designated as enterococci confirmed as such and about 12% of the colonies not so designated were, in fact, identified as enterococci. Plate to plate variability across samples approximated that expected by chance alone. Verified recoveries of enterococci from natural samples by the mE procedure, on the average, exceeded those by the KF method by one order of magnitude.

Shortly after J. P. Laws and F. W. Andrewes first reported streptococci from the gastrointestinal tract, Houston noted that these organisms appeared to be characteristic of sewage and animal fecal wastes. He suggested that they were indicative of dangerous pollution because they are readily demonstrable in recently polluted waters and seemingly absent from waters above suspicion of contamination (18). Since that time, a volume of data has been accumulated categorically demonstrating that fecal streptococci are associated with the fecal wastes of man and lower animals and that they can be isolated from polluted water containing such wastes (14, 20). Nevertheless, this group of organisms has not been generally accepted as an indicator of fecal contamination for at least two reasons. First, coliforms and coliform biotypes have been a more attractive means of identifying fecal contamination because early workers found them easier to quantify and they are present in larger numbers in feces, sewage, and polluted waters. Second, there has been a good deal of confusion concerning the identity of the fecal streptococci, particularly as it relates to their ecological distribution. This is reflected in the fact that those streptococci which can be found in the feces of man and other warm-blooded animals have been referred to variously as enterococci, fecal streptococci and, more recently, group D streptococci. The composition of these three groups is shown in Fig. 1. It can be seen that two organisms not associated with humans, *Streptococcus bovis* and *S. equinus*, and two organisms associated with humans, but not exclusively with fecal wastes, *S. mitis* and *S. salivarius*, are included in one or more of the groups. Ideally, the indicator should be limited to the fewest number of species or biotypes which are most closely or exclusively associated with the fecal wastes of man, i.e., *S. faecalis* and *S. faecium*. Facile methods to do this have not been available. Hence, broader groups of streptococci have been used at times, because these were the biotypes recovered by the procedures available.

The taxonomy and distribution of this group of organisms has been reviewed by Hartman et al. (8), Kjellander (11), and revised recently in *Bergey's Manual for Determinative Bacteriology*, 8th ed. (2).

Early attempts to quantify fecal streptococci relied on enrichment tube procedures associated with the use of the most probable number method. In 1940 Mallmann (15) suggested the use of azide lactose broth. This was later

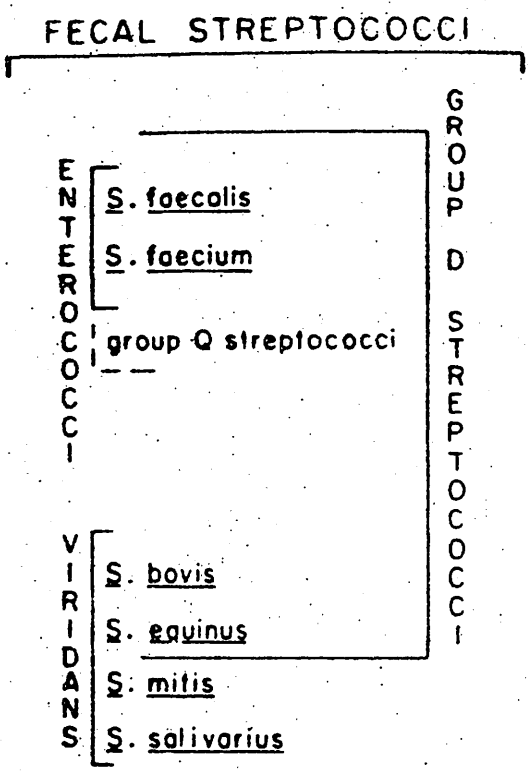

FIG. 1. *Distribution of streptococci species among three groups. This study was completed prior to the release of the 8th edition of Bergey's Manual for Determinative Bacteriology (2), which has classified S. zymogenes as S. faecalis, subspecies zymogenes and S. durans as equivalent to S. faecium.*

modified by Mallmann and Seligmann (16) using Roth's basic azide dextrose medium, followed by a confirmation in ethyl violet azide broth as described by Litsky et al. (13). This procedure has been evaluated in marine waters (3). Hajna and Perry (7) developed the SF medium; and Winter and Sandholzer (23) described presumptive and confirmatory media which use sodium azide and/or high concentrations of NaCl as inhibitors.

The quantification of enterococci by a membrane filter procedure was first reported by Slanetz and Bartley in 1957 (21); and, in 1961, Kenner et al. (10) described the KF method for the enumeration of fecal streptococci. In 1966 Isenberg et al. (9) reported a plating procedure (PSE) for the quantification of group D streptococci. This medium offered increased recoveries and easier differentiation based on the use of the esculin reaction. The recoveries by these and other procedures have been compared by a number of workers (19, 22).

Impetus for the present investigation developed from a comparison of the KF methods and PSE medium (used in conjunction with membrane filtration) for the isolation of fecal streptococci from marine waters at beaches in the vicinity of New York City. In this preliminary investigation, it was observed that the confirmed recoveries of fecal streptococci by the modified PSE procedure exceeded those by the KF method by about one order of magnitude. However, the modified PSE procedure as used was deficient in that overgrowth by background microorganisms was a serious problem. The present report describes the evaluation of a procedure for the enumeration of enterococci designed to obviate the problems noted above. In addition, an attempt was made to make the method more specific, i.e., to eliminate the recovery of the viridans group (*S. mitis*, *S. salivarius*) and the two organisms exclusively associated with animal feces (*S. equinus* and *S. bovis*). The method (mE) was evaluated against the following criteria: (i) accuracy, recovery of at least 75% of the viable *S. faecalis* and *S. faecium* cells following a stress imposed by exposure to sea water for 48 h at 4 C; (ii) selectivity, the reduction of background organisms in naturally polluted waters by at least three orders of magnitude (1,000-fold); (iii) specificity, colonies designated as positive should verify as such at least 75% of the time, and no more than 10% of those designated as negative should verify as enterococci; (iv) precision, with field samples, the D^2 (4) value distribution approximates that estimated by chance; and (v) comparability, the accuracy and sensitivity of the method be equal to or greater than existing membrane filter methods.

MATERIALS AND METHODS

Cultures and field samples. The recovery and accuracy studies were performed with cultures of *S. faecalis*, *S. faecium*, *S. bovis*, *S. equinus*, *S. mitis*, and *S. salivarius* provided by R. Facklam (Center for Disease Control, Atlanta, Ga.), and with a strain of *S. faecalis* isolated from New York Habor. Suspensions of these organisms were prepared from brain-heart infusion broth (BHI, Difco) cultures incubated at 37 C for 20 h. After incubation, the organisms were washed three times in sterile phosphate-buffered saline (NaH_2PO_4, 0.58 g; Na_2HPO_4, 0.25 g; NaCl, 0.95 g; distilled water, 100 ml). Aliquots of the resultant suspensions were delivered into flasks of sea water passed through a 0.2-nm membrane filter. These were held at 4 C and sampled periodically by the mE method and a control procedure (spread plates on BHI agar) to determine the number of recoverable organisms.

Field samples were collected from marine and

estuarine waters in sterile containers held at 4 to 6 C and assayed within 12 h of collection.

Membrane filtration. Appropriate volumes of the test suspensions or water samples used in evaluating the experimental medium were passed through membrane filters (47-mm diameter, 0.45-nm pore size). When the portion of the water sample to be filtered was less than 20 ml, it was brought to at least that volume with phosphate-buffered saline to wash residual organisms onto the membrane. The glass filter holders were sterilized for 2 min in an ultraviolet sterilizing apparatus (Millipore Filter Corp.). The membrane filters were obtained presterilized from the manufacturer.

Recovery media: mE. The formulae and methods of preparation of mE medium and the esculin-iron agar substrate used in the in situ esculin test are given in Table 1. After a basal medium containing peptone, sodium chloride, yeast extract, esculin, and ferric ammonium citrate was shown to quantitatively recover the organisms, a search for appropriate inhibitors was initiated. A number of candidates were screened for this purpose initially by using a modification of the gradient plate method of Szybalski as described by Levin and Cabelli (12). Nalidixic acid and sodium azide are used to inhibit gram-negative organisms and actidione to inhibit fungi. Triphenyl tetrazolium chloride in the concentration used colors the colonies, differentiates enterococci from other streptococci based upon its reduction, and has a slight inhibitory effect on some background microorganisms. Esculin is included to induce the enzyme catalyzing its hydrolysis. The mE plates are incubated for 48 h at 41 C; the elevated incubation temperature also inhibits some of the indigenous microbial flora.

In situ esculin substrate. The hydrolysis of esculin is used in the characterization of enterococci. Initially, the esculin indicator system was included in the primary medium. This resulted in dark red colonies approximately 2 mm in diameter with black halos in the medium resulting from the reaction of cumarin with the ferric chloride. When more than 80 colonies were present, however, the zones coalesced making it impossible to determine which of the colonies was positive. This problem was overcome by the use of an in situ test in which, after incubation, the membrane is transferred to an esculin-iron agar plate (Table 1). After 20 to 30 min at 41 C, small black spots appear under the positive colonies, permitting enumeration of at least 80 enterococcus colonies per plate without problems of coalescence.

Control media. KF (Difco) and PSE (Pfizer) media were prepared and used following instructions from the manufacturers and *Standard Methods for the Examination of Water and Wastewater* (1). As noted earlier, the PSE medium was used in a membrane filter procedure.

Verification of colonies. Verification of colonies as enterococci was accomplished by using the bile-esculin medium of Schwan in combination with a modification of the procedure of Facklam and Moody (5), i.e., (i) growth at 45 C in BHI broth; (ii) a negative catalase test; (iii) esculin hydrolysis; (iv) growth on 40% bile-blood agar; (v) an acid reaction in litmus milk; and (vi) a positive Gram stain.

TABLE 1. *Preparation of mSD medium and EIA substrate*

mSD		EIA	
Ingredients*	g/liter	Ingredients*	g/liter
Agar	15.0	Agar	15
Peptone	10.0	Esculin	1.0
NaCl	15.0	Ferric citrate	0.5
Esculin	1.0	Distilled water	1,000 ml
Yeast extract	30.0		
Actidione	0.050		
Sodium azide	0.150		
Distilled water	1,000 ml		

* Autoclave at 121 C for 15 min. After autoclaving, add nalidixic acid, 0.24 g, and triphenyl tetrazolium chloride, 0.15 g. Adjust pH to 7.1 ± 0.1 and pour in 3.5-ml amounts to 50-mm membrane filter plates.

* Adjust pH to 7.1 ± 0.1 before autoclaving at 121 C for 15 min. Pour in 3.5-ml amounts to 50-mm membrane filter plates.

RESULTS

The accuracy of the mE method was determined by comparing the recoveries obtained by this procedure to those observed when eight species of streptococci were spread plated on BHI agar. The suspensions, whose initial cell densities varied between 10^3 to 10^6 per ml, were prepared in filtered estuarine water and held at 5 C for periods up to 9 days. The average relative recovery of *S. faecalis* and *S. faecium* over the 9-day period was 102%; *S. bovis*, *S. equinus*, *S. mitis*, and *S. salivarius* recoveries were 0.0001% or less (Table 2).

The selectivity of the mE method was such that a 10,000-fold reduction in background organisms was obtained relative to that observed when the inhibitors were omitted from mE medium and the plates were incubated at 35 C. This 99.99% reduction was obtained with samples whose initial background densities were 10^4 to 10^6 cells per 100 ml.

The specificity of the mE procedure was examined by determining (i) the percentage of typical colonies which did not verify as members of the enterococci group (false positives) and (ii) the percentage of other colonies, those which did not possess the typical colonial characteristics which, in fact, were enterococci (false negatives). The 2,231 colonies examined were isolates from polluted marine and estuarine water samples collected at six locations along the east coat of the United States. Most of the

TABLE 2. *The recovery of stressed[a] fecal streptococci on mSD medium*

Organism	% Recovery[b] after exposure (days)				
	0	1	3	6	9
S. faecalis	107	ND[d]	94	123	87
S. faecium	102	ND	126	73	109
S. faecium[c]	114	ND	108	46	100
S. faecalis subsp. *zymogenes*	117	ND	118	ND	113
S. bovis	0.001	<0.0001	ND	ND	ND
S. equinus	<0.0001	ND	ND	ND	ND
S. salivarius	<0.0001	ND	ND	ND	ND
S. mitis	<0.0001	ND	ND	ND	ND

[a] Suspensions held in seawater at 5 C for number of days as indicated.
[b] Relative to recovery on BHI spread plates.
[c] Obtained in 1973 from R. Facklam as *S. durans*.
[d] No data.

isolates came from samples collected at beaches in the vicinity of New York City. In general, all the colonies on a given plate were examined. Ninety percent of the typical colonies and 11.7% of the other colonies were enterococci (Table 3). Neither *S. bovis*, *S. equinus*, *S. mitis*, nor *S. salivarius* were encountered.

The precision of the mE method was determined from D^2 values for assay variability calculated from the following equation as given by Eisenhart and Wilson (4): $D^2 = N\Sigma X1^2 - (\Sigma Xi)^2/\Sigma Xi$, where ΣXi is the summation of the plate counts $X_1, X_2 \cdots X_n$, and N (the number of replicate plates per sample) was 5. The D^2 values calculated from the examination of 15 polluted, marine, and estuarine water samples are displayed in Fig. 2 along with the expected D^2 control limits for $P = 0.005$, 0.025 and 0.5. In the event of excessive variability among the five replicate determinations (plates), hence, poor reliability of a single determination, the observed D^2 values should have exceeded the control limits more frequently than expected by chance alone. By chance alone, one D^2 value in 40 would have been expected to exceed the $P = 0.005$ limit. However, it can be seen that none of the values exceed the limits, and that they are evenly distributed around the $P = 0.5$ limit (Fig. 2).

Sixteen water samples collected from a variety of sources were assayed in parallel by the KF, PSE, and mE methods. Typical colonies as described for the various procedures were verified as stated in Materials and Methods. Thus, the data presented are derived from verified recoveries. As can be seen from Table 4, neither the PSE nor the KF recoveries approached those obtained with the mE method, although those by the PSE and KF methods did approximate each other. The average number of colonies on the filters in these 13 trials was 70, ranging from 9 to 110. In a second, more extensive set of trials, fecal streptococcus densities obtained by the KF method were compared

TABLE 3. *Specificity of the mSD procedure for the enumeration of group D streptococci*

General sample location	No. of colonies and % verification			
	Typical colonies		Other colonies	
	No. examined	% False positive	No. examined	% False negative
Coney Island N.Y.	1,225	11	107	18
Riis Park N.Y.	577	10	104	11
Miami, Fla.	16	0[a]	16	0[a]
Boston Harb., Mass.	59	3.0	ND[b]	ND
Connecticut	67	4.0	20	0[a]
Rhode Island	30	0[a]	10	0[a]
Overall	1,974	10.0	257	11.7

[a] No false-negative or positive colonies.
[b] No data.

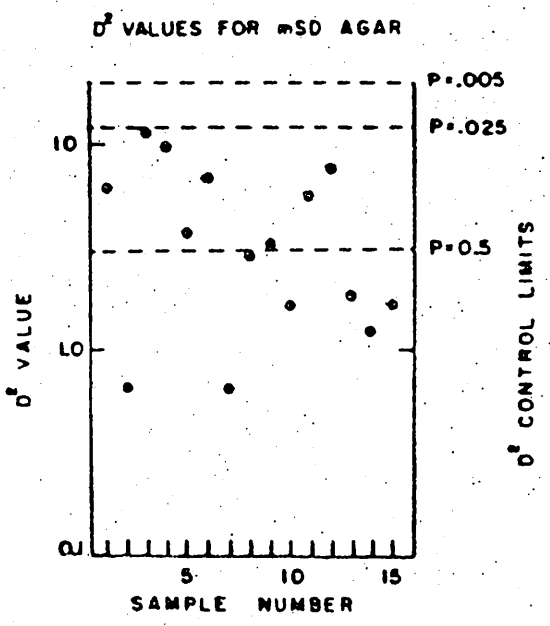

FIG. 2. *Precision of the msD procedure for enterococci as extended from dispersion of D^2 values. Symbols: (●) D^2 value calculated from five replicate plates at each point; ----: control limits when probability is as stated.*

TABLE 4. *Comparison of verified recoveries of group D streptococci by the mSD, PSE and KF methods*

Location	Trial	mE recovery (colonies/plate)	% mSD recoveries* by	
			PSE	KF
Providence River	1	45	82	62
	2	23	70	74
	3	90	68	58
	4	55	78	94
	5	70	49	73
Coney Island	1	110	59	61
	2	82	60	77
	3	21	48	67
Boston Harbor	1	89	86	75
	2	97	59	70
	3	73	47	73
Miami	1	9	ND†	66
	2	107	ND†	33
Stonington Harbor	1	9	39	75
New London Harbor	1	61	25	38
Mystic Harbor	1	85	68	9
Avg		70	60	63

* Values obtained from the average of triplicate plate counts.

† No data because the PSE plates were overgrown with background organisms.

directly to those by the mE procedure (Table 5). In only three instances did the KF recoveries exceed the mE recoveries; on the average, the mE recoveries were about 10 times greater than those by the KF procedure. The samples reported in Table 4 were obtained during the winter months and those reported in Table 5 were obtained in July and August.

DISCUSSION

The mE procedure has satisfied most of the predetermined criteria for a primary, selective-differential method for the enumeration of enterococci in marine waters. The only exception was the rate of false-negative colonies.

Essentially quantitative recovery of *S. faecalis* and *S. faecium* was obtained with unstressed and stressed cells. Although this approach is basically artificial in that the work was done with pure cultures rather than natural samples, it is not logistically feasible to perform such experiments with natural samples. *S. bovis* and *S. equinus* were not recovered in significant numbers on mE medium, nor were they isolated from natural samples. Therefore, it may be assumed that these two species, whose origin is animal feces, are not included in densities of enterococci as obtained by the mE procedure. Relative to other methods, this increases the specificity of the mE procedure for enterococci of human origin. However, *S. faecalis* and *S. faecium* do occur in animal feces (2). In addition, biotypes of *S. faecalis* have been reported (4, 15) as being associated with vegetation unpolluted with human fecal wastes and with insects. The former are differentiated by their ability to hydrolyze starch, a characteristic which could be performed as a subsequent in situ test.

The selectivity criterion for the evaluation of mE methods assumes that the marine recreational waters to be examined for enterococci generally would contain less than 10^3 background organisms per ml (those bacteria which grow in 48 h on the mE medium when the inhibitors are omitted and the plates are incubated at 35 C). The required 1,000-fold (99.9%) reduction in the level of these organisms, to be achieved by the combination of inhibitors and

TABLE 5. *Comparison of verified recoveries of fecal streptococci and other organisms by the mSD and KF methods*

Location	Trial	Recovery per 100 ml by group and method			
		Fecal streptococci		Other colonies	
		mSD	KF	mSD	KF
Coney Island, N.Y.	1	38	<1	54	13
	2	38	ND*	35	TN†
	3	270	6	190	<1
	4	660	ND	TN	TN
	5	670	ND	240	TN
	6	310	20	430	<10
	7	70	4	123	<1
	8	7	1	4	<1
	9	70	4	70	<1
	10	1,510	6	310	<1
Rockaways, N.Y.	1	170	12	940	<1
	2	230	17	1,090	<1
	3	440	8	TN	<1
	4	51	5	450	<1
	5	25	61	56	<10
	6	49	62	24	<10
	7	31	2	<1	<1
	8	16	8	127	<1
	9	32	5	100	<1
	10	47	2	53	<1
	11	36	8	2	<1
	12	12	57	89	<1

* No data, confluent growth of background organisms.

† Too numerous to count.

the elevated incubation temperature, would result in 50 colonies per filter when a 50-ml water sample is assayed. The differential characteristics of the medium then should permit the detection of a single group enterococcus colony on such a filter. The actual reduction (99.99%) exceeded this requirement, thereby permitting the detection of group D streptococci in the presence of 500,000 background organisms deposited on the filter.

Although the rate of false-negative colonies (11.7%) slightly exceeded the specificity criterion (10%), the false-positive rate (10%) was markedly less than the specified limit (25%). Therefore, it may be assumed that verification of a number of typical and other colonies is necessary only when an operator is being trained. In the absence of verification, estimates of enterococci densities should be designated as presumptive. After verification, the estimates would be considered as confirmed.

The results demonstrate (Fig. 2) the precision (reproducibility) of the mE procedure. The plate to plate variability over the samples examined was that expected by chance alone; that is, the D^2 estimates distributed equally on both sides of the $P = 0.5$ limit of 3.2 and none exceeded the $P = 0.025$ limit.

The recovery efficiency of the mE method is comparable to, or better than, that by the KF method for the examination of enterococci in estuarine waters; in addition, it provides higher confirmed recoveries. The difference between recovery ratios (mE/KF) observed at Coney Island in the summmer months (1.5; Table 4) and winter months (54; Table 5) may be a function of a seasonal variation (water temperature, rainfall). As noted previously, the method measures a more specific portion of the fecal streptococcus population and one that appears to be a close association with the fecal wastes of humans.

LITERATURE CITED

1. American Public Health Association. 1971. Standard methods for the examination of water and wastewater. 13th ed. p. 719. American Public Health Association, New York.
2. Buchanan, R. E., and N. E. Gibbons. 1974. Bergey's manual of determinative bacteriology. 8th ed. p. 490-509. Williams and Wilkins Co.
3. Buck, J. D. 1972. Selective detection of enterococci in marine waters. Am. J. Public Health 62:419-421.
4. Eisenhart, C., and P. W. Wilson. 1943. Statistical methods and control in bacteriology. Bacteriol. Rev. 7:57-137.
5. Facklam, R. R., and M. D. Moody. 1970. Presumptive identification of group D streptococci: the bile-esculin test. Appl. Microbiol. 20:245-250.
6. Geldreich, E., and B. A. Kenner. 1969. Concepts of fecal streptococci in stream pollution. J. Water Pollut. Control Fed. Res. 41(Suppl.):R336-362.
7. Hajna, A. A., and C. A. Perry. 1943. Comparative study of presumptive and confirmatory media for bacteria of the coliform group and for fecal streptococci. Am. J. Public Health 33(5):550-556.
8. Hartman, P. A., G. W. Reinbold, and D. S. Saraswat. 1966. Indicator organisms—a review. I. Taxonomy of the fecal streptococci. Int. J. Syst. Bacteriol. 16:197-221.
9. Isenberg, H. D., D. Goldberg, and J. Sampson. 1970. Laboratory studies with a selective enterococcus medium. Appl. Microbiol. 20:433-436.
10. Kenner, R. A., H. F. Clark, and P. W. Kabler. 1960. Fecal streptococci I. Cultivation and enumeration of streptococci in surface waters. Appl. Microbiol. 9:15-20.
11. Kjellander, J. 1960. Enteric streptococci as indicators of fecal contamination of water. Acta Pathol. Microbiol. Scand. 48(Suppl.):136. 1-124.
12. Levin, M. A., and V. J. Cabelli. 1972. Membrane filter technique for enumeration of *Pseudomonas aeruginosa*. Appl. Microbiol. 24:864-870.
13. Litsky, W., W. L. Mallmann, and C. W. Fifield. 1953. A new medium for the detection of enterococci in water. Am. J. Public Health 43:873-879.
14. Litsky, W., W. L. Mallmann, and C. W. Fifield. 1955. Comparison of the most probable numbers of *Escherichia coli* and enterococci in river waters. Am. J. Public Health 45:1049-1053.
15. Mallmann, W. L. 1940. A new yardstick for measuring sewage pollution. Sewage Works J. 12:875-878.
16. Mallmann, W. L., and E. B. Seligmann, Jr. 1950. A comparative study of media for the detection of streptococci in water and sewage. Am. J. Public Health 40:286-289.
17. Papavassilous, J. 1962. Species differentiation of group D streptococci. Appl. Microbiol. 10:65-69.
18. Prescott, S. C., A. C. Winslow, and M. H. Macrady. 1946. Water bacteriology, p. 207-212. John Wiley and Sons, New York.
19. Sabbaj, J., V. L. Sutter, and S. M. Finegold. 1971. Comparison of selective media for isolation of presumptive group D streptococci from human feces. Appl. Microbiol. 22:1008-1011.
20. Schwan, A. 1954. The use of a bile-aesculin medium and of Maxted's technique of Lancefield grouping in the identification of enterococci (Group D streptococci). J. Clin. Pathol. 7:160-163.
21. Slanetz, L. W., and C. H. Bartley. 1957. Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. J. Bacteriol. 74:591-595.
22. Switzer, R. E., and J. B. Evans. 1974. Evaluation of selective media for enumeration of group D streptococci in bovine feces. Appl. Microbiol. 28:1086-1087.
23. Winter, C. E., and L. A. Sandholzer. 1946. Recommended procedure for detecting the presence of enterococci. TL2, U.S. Dept. of Interior, Fish, and Wildlife Service, Washington, D. C., November.

Q 66 Microbial Degradation of High Molecular Weight Polycaprolactones. C.V. Benedict* and J.A. Cameron, University of Connecticut, Storrs, CT

Two polycaprolactones, PCL-700 (MW 40,000) and PCL-300 (MW 10,000) were the sole carbon source in individual enrichment cultures of lake and river sediments. Degradation has been quantitatively monitored using high pressure liquid chromatography. Both mixed enrichment systems and individual isolates obtained from them have been assayed for degradative capacity under a variety of conditions. In the absence of alternate carbon sources, the higher molecular weight polymer appears more readily degraded; 100% degradation has been seen in mixed culture systems after one months' incubation. A yeast, identified as Cryptococcus laurentii, has been shown to extensively degrade both polymers. Preliminary evidence indicates that the degree of crystallinity is an important characteristic affecting polymer degradation. With current polymer preparation techniques, PCL-300 is more crystalline than PCL-700. Most single isolates do not have the degradative capacity of the mixed cultures. Of nineteen organisms, eighteen are coryneforms and one is a yeast, Trichosporon pullulans. Among these, a single coryneform was able to degrade PCL-300 to a greater extent than the mixed culture from which it was obtained.

Q 67 The Effect of Nutrient Conditions upon Biodegradation of a Synthetic Polymer. D.A. Marchuk* and J.A. Cameron, University of Connecticut, Storrs, CT

The effect of nutrient conditions upon the ability of microorganisms to degrade synthetic polymers has been studied. Twelve bacterial and yeast isolates from enrichment cultures were tested for polycaprolactone degradation by zone clearing on an agar containing polymer. Yeast isolates demonstrated growth and degradation of polycaprolactone in a minimal medium, whereas the bacteria did not. Bacterial degradation could be promoted by the addition of mono, di and tri-carboxylic acids and amino acids to the medium. Mono and disaccharides did not promote bacterial degradation of the polymer and inhibited yeast degradation, however inhibition could be overcome by a low concentration of succinate in the medium. Inhibition of degradation also occurred upon the addition of caproate to the medium, suggesting a feedback inhibition mechanism.

Q 68 Scanning Electron-Microscopic Visualization of Biodegradation of Synthetic Polymers by Fungi. W.J. Cook* and P. Jarrett, University of Connecticut, Storrs, CT

The polyesters polycaprolactone 700 (40,000 daltons) and polycaprolactone 300 (10,000 daltons) were used as polymer models to demonstrate the biodegradation process by fungi. Several fungal species, isolated from polycaprolactone 700 enrichment cultures of sewage or pond water showed growth and signs of degradation on agar plates coated with a polycaprolactone film. Specimens for microscopy were prepared by coating basal minimal salts - noble agar plates or acid washed glass slides with a polymer film, inoculating with a fungal isolate, and incubating in a moist chamber for a month. After incubation, samples were removed from the agar and slides, air-dried, sputtered with gold and viewed with a scanning electron microscope (SEM). The molecules of a polymer cast film organize into the typical supramolecular structures called spherulites demonstrating crystalline (ordered) and non-crystalline (unordered) regions. Biodegradation was seen on the PCL700 and PCL300. It appears that deterioration of the amorphous areas of the spherulites preceded the biodegradation of the crystalline areas.

Q 69 A 24-hour Membrane Filter Procedure for Enumerating Enterococci. A.P. DUFOUR, Marine Field Station, HERL-Cin., West Kingston, RI 02892.

A membrane filter procedure (mE) for enumerating enterococci was modified and simplified by eliminating the in situ esculin test and decreasing the incubation period from 48 hours to 24 hours. Indoxyl-β-D-glucoside, a chromogenic cellobiose analogue, was substituted for esculin in the primary medium.

The mE procedure and the modified method were compared for the recovery of enterococci from marine, fresh and estuarine water samples. Slightly increased recoveries were observed with the modified procedure. Colonies isolated by both methods were submitted to biochemical testing to confirm their identity. Ninety-four percent of the isolates examined from each method were confirmed as enterococci. The false negative rate was less than 4% with the modified procedure.

The modified mE procedure is a specific, facile method that can be utilized for enumerating enterococci in marine, estuarine or freshwater environments.

Q 70 The Enumeration and Identification of Streptococci from Landfill Leachates. J. A. DONNELLY,* P. V. SCARPINO, and D. BRUNNER. Univ. of Cincinnati and USEPA, Cincinnati, Ohio.

Increasing use of landfills to deposit sewage sludge, municipal and hospital solid wastes, and others containing pathogenic microbes enhances the possibility that these pathogens will leave the landfill in the leachate and contaminate ground and surface water supplies. Fecal streptococci were found to survive longer in landfills than most Gram-negative rods, including coliforms, and their presence in leachate implied the fecal origin of the waste material in the landfill. Identification of streptococcal species further indicated the vegetable, animal or human origin of the leachate. Several quantitative streptococcal procedures were compared with the Standard Methods (1976) Most Probable Number (MPN) test. Leachates were obtained from laboratory and large scale field lysimeters, and a commercial landfill. As expected violet azide broth from the MPN test was not always specific when used with leachate, it was replaced by streptococcal identifying media as bile esculin azide, KF streptococcal and m-enterococcus agars. With high streptococcal count leachate, all plate count media gave two-fold to ten-fold higher counts than the Standard Methods MPN. Older lysimeters showed a higher percentage of Group D streptococci, while the commercial landfill contained a greater variety of streptococci, including Group T, and viridans.

Q 71 Membrane Filter Technique for the Isolation of Yersinia enterocolitica. T. J. BARTLEY*, J. J. QUAN, M. T. COLLINS, AND S. M. MORRISON. Colorado State University and Center for Disease Control, Fort Collins.

A membrane filter procedure was developed for the isolation of Yersinia enterocolitica from aquatic environments. Primary differentiation was based on the fermentation of sorbitol, the absence of lysine decarboxylase and arginine dihydrolase activities, and the production of urease. Sodium desoxycholate was incorporated as an inhibitor of background organisms. The presumptive identification of Y. enterocolitica was accomplished in 50 hours, at 25°C, and the rate of confirmation of identity of typical positive colonies was 90%. The mean recovery rate of 15 strains of the organism from phosphate buffer suspensions was 91%. The technique used to isolate 33 cultures of Y. enterocolitica from 15 of 27 river water samples and from prechlorinated sewage effluent. Two isolates were identified as serotype 0:4 (or 0:4,32), 2 were 0:17, and the fifth was 0:40.

as of 1 Jan 1981

Media Preparation: mE Medium-Modified

Bacto-Agar	15g
Bacto-Peptone	10g
Yeast Extract	30g
NaCl	15g
Sodium Azide	0.15g
Actidione(cycloheximide)	0.05g
Deionized Water	1000.0ml

Autoclave for 15min, cool to 50 C. Aseptically add the following:

1. Nalidixic Acid 0.24g, weigh into a sterile test tube, add 3ml sterile water. Add 0.2ml 10N NaOH, vortex to dissolve. Add to cooled medium and rinse tube with medium.

2. Triphenyl Tetrazolium Chloride 0.02g, weigh into a sterile test tube. Add to cooled medium and rinse tube with medium.

3. Indoxyl-B-D-Glucoside 0.75g, weigh into a sterile test tube, add 5ml of 95% EtOH. Dissolve with mixing and add 5ml sterile water, vortex. Add to medium and rinse tube with the medium.

Swirl to mix or use stir plate, aseptically adjust pH to 7.1±0.1, dispense 4.5mls into 50x9mm plates using a sterile Cornwall syringe. Store inverted in a foil covered basket at 4 C, will keep about 1 month.

Incubation- place inverted plates in stacks of 2 in 41 C incubator for 48hrs.

Counting- count all typical colonies: pink center, gray fringe, blue halo or those that are dark with blue halos. No minimum size.

References:

1. Levin, M.A., et al, Applied Microbiology vol 30:1 pg. 66-71, 1975.

2. Dufour, A.P., Abstracts Annual Meeting American Society for Microbiology, 1980, abstract number Q69.

TEST METHOD

ENTEROCOCCI IN WATER BY THE MEMBRANE FILTER PROCEDURE

METHOD 1106.1
1985

1. Citation

2. Scope and Application

2.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. The enterococci are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens.

2.2 The enterococci test measures the bacteriological quality of recreational waters. Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on the established relationship between health effects and water quality. The signficance of finding enterococci in recreational water samples is the direct relationship between the density of enterococci in the water and swimming-associated gastroenteritis studies of marine and fresh water bathing beaches (1).

2.3 The test for enterococci can be applied to potable, fresh, estuarine, marine, and shellfish growing waters.

2.4 Since a wide range of sample volumes or dilutions can be analyzed by the MF technique, a wide range of enterococci levels in water can be detected and enumerated.

3. Summary

3.1 The MF method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (2). A water sample is filtered through the membrane which retains the bacteria. Following filtration, the membrane containing the bacterial cells is placed on a selective medium, mE agar, and incubated for 48 h at 41°C. After incubation, the filter is transferred to EIA agar and held at 41°C for 20 min. Pink to red enterococci colonies will develop a black or reddish-brown precipitate on the underside of the filter. These colonies are counted with a fluorescent lamp and a magnifying lens.

4. Definition

4.1 In this method, enterococci are those bacteria which produce pink to red colonies with black or reddish-brown precipitate after incubation

on mE agar and subsequent transfer to EIA medium. Enterococci include Streptococcus faecalis, Streptococcus faecium, Streptococcus avium, and their variants.

5. Interferences

5.1 Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.

6. Safety Precautions

6.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

6.2 Mouth-pipetting is prohibited.

7. Apparatus and Equipment

7.1 Glass lens with magnification of 2-5X or stereoscopic microscope.

7.2 Lamp, with a cool, white fluorescent tube.

7.3 Hand tally or electronic counting device.

7.4 Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets.

7.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 Graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper and sterile.

7.7 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterile.

7.8 Ultraviolet unit for sterilizing the filter funnel between filtrations (optional).

7.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used.

7.10 Flask, filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

7.11 Flask for safety trap placed between the filter flask and the vacuum source.

7.12 Forceps, straight or curved, with smooth tips to handle filters without damage.

7.13 Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.

7.14 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles.

7.15 Thermometer, checked against a National Bureau of Standards (NBS) certified thermometer, or one traceable to an NBS thermometer.

7.16 Petri dishes, sterile, plastic, 50 x 12 mm, with tight-fitting lids.

7.17 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1-100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1-10 dilutions.

7.18 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume.

7.19 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 $\pm$ 0.02 µm pore size.

7.20 Inoculation loops, at least 3-mm diameter, and needles, nichrome or platinum wire, 26 B & S gauge, in suitable holders.

7.21 Incubator maintained at 41 $\pm$ 0.5°C.

7.22 Waterbath maintained at 44-46°C for tempering agar.

7.23 Test tubes, 150 x 20 mm, borosilicate glass or plastic.

7.24 Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes.

7.25 Test tubes, screw-cap, borosilicate glass, 125 x 16 mm or other appropriate size.

8. Reagents and Materials

8.1 Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (3). The agar used in preparation of culture media must be of microbiological grade.

8.2 Whenever possible, use commercial culture media as a means of quality control.

8.3 Purity of Water: Reagent water conforming to Specification D1193, Type II, Annual Book of ASTM Standards (4).

8.4 Buffered Dilution Water

8.4.1 Composition:

Sodium Dihydrogen Phosphate	0.58 g
Sodium Monohydrogen Phosphate	2.50 g
Sodium Chloride	8.50 g

8.4.2 Preparation: Dissolve the ingredients in 1 L of reagent water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 lb pressure) for 15 min. The final pH should be 7.4 ± 0.2.

8.5 mE Agar (Difco 0333-15-1)

8.5.1 Composition of Basal Medium:

Peptone	10.0 g
Sodium Chloride	15.0 g
Yeast Extract	30.0 g
Esculin	1.0 g
Actidione	0.05 g
Sodium Azide	0.15 g
Agar	15.0 g

8.5.2 Preparation of basal medium: Add 71.2 g of dehydrated mE basal medium to 1 L of reagent grade water in a flask and heat to boiling until ingredients dissolve. Autoclave at 121°C and 15 lb pressure for 15 min and cool in a 44-46°C water bath.

8.5.3 Reagents added after sterilization: Mix 0.25 g nalidixic acid in 5 mL reagent grade water, add 0.2 mL of 10 N NaOH to dissolve, and add to the basal medium. Add 0.15 g triphenyl tetrazolium chloride separately to the basal medium and mix.

8.5.4 Preparation of mE Agar: Pour the mE agar into 50 mm petri dishes to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. The final pH of medium should be 7.1 ± 0.2. Store in a refrigerator.

8.6 EIA Substrate Agar (Difco 0488-15-4)

8.6.1 Composition:

Esculin	1.0 g
Ferric Citrate	0.5 g
Agar	15.0 g

Preparation: Add 16.5 g of dehydrated EIA medium to 1 L of reagent grade water in a flask and heat to boiling until ingredients are dissolved. Autoclave the EIA medium at 121°C and 15 lb pressure for 15 min and cool in a 44-46°C water bath. After cooling, pour the medium into 50-mm petri dishes to a depth of 4-5 mm (approximately 4-6 mL) and allow to solidify. The final pH should be 7.1 ± 0.2 before autoclaving. Store in a refrigerator.

8.7 Brain Heart Infusion (BHI) (Difco 0037-02, BBL 11058)

8.7.1 Composition:

Calf Brain Infusion	200.0 g
Beef Heart Infusion	250.0 g
Peptone	10.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	2.5 g
Dextrose	2.0 g

8.7.2 Preparation: Dissolve 37 g of dehydrated brain heart infusion in 1 L of reagent grade water. Dispense in 8-10 mL volumes in screw-cap tubes and autoclave at 121°C (15 lb pressure) for 15 min. If the medium is not used the same day as prepared and sterilized, heat in boiling water bath for several min to remove absorbed oxygen, and cool quickly without agitation, just prior to inoculation. The final pH should be 7.4 ± 0.2.

8.8 Brain Heart Infusion (BHI) Broth with 6.5% NaCl

8.8.1 Composition: Brain heart infusion broth with 6.5% NaCl is the same as BHI broth in 8.7 with additional NaCl.

8.8.2 Preparation: Add 60.0 g NaCl per liter of medium. Since most commercially available dehydrated media contain sodium chloride, this amount is taken into consideration in determining the final NaCl percentage above.

8.9 Brain Heart Infusion Agar (Difco 0418-02, BBL 11064)

8.9.1 Composition: Brain heart infusion agar contains the same components as BHI (see 8.7) with the addition of 15.0 g of agar per L of BHI broth.

8.9.2 Preparation: Heat to boiling until ingredients are dissolved. Dispense 10-12 mL of medium in screw-cap test tubes and sterilize for 15 min at 121°C (15 lb pressure). Slant after sterilization. The final pH should be 7.4 ± 0.2.

8.10 Bile Esculin Agar (BEA) (Difco 0879)

8.10.1 Composition:

Bacto Beef Extract	3.0 g
Bacto Peptone	5.0 g
Bacto Oxgall	40.0 g
Bacto Esculin	1.0 g
Ferric Citrate	0.5 g
Bacto Agar	15.0 g

8.10.2 Preparation: Add 64.5 g of dehydrated BEA to 1 L reagent water and heat to boiling to dissolve completely. Dispense in 8-10 mL volumes in tubes for slants or into flasks for subsequent plating. Autoclave at 121°C at 15 lb pressure for 15 min. Overheating may cause darkening of the medium. Cool to 44-46°C and dispense into sterile petri dishes. The final pH should be 6.6 ± 0.2. Store in a refrigerator.

9. Sample Collection, Preservation and Holding Times

9.1 Sampling procedures are described in detail in the USEPA microbiology methods manual, Section II, A (5). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples should not be analyzed if these conditions are not met.

9.1.1 Storage Temperature and Handling Conditions

Ice or refrigerate bacteriological samples at a temperature of 1-4°C during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

9.1.2 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 6 h between collection and initiation of analyses.

10. Calibration and Standardization

10.1 Check temperatures in incubators daily to insure operation within stated limits.

10.2 Check thermometers at least annually against an NBS certified thermometer or one traceable to NBS. Check mercury columns for breaks.

11. Quality Control

11.1 See recommendations on quality control for microbiological analyses in the USEPA microbiology methods manual, Part IV, C (5).

12. Procedure

12.1 Prepare the mE agar as directed in 8.5.

12.2 Mark the petri dishes and report forms with sample identification and sample volumes.

12.3 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base; the membrane filter is now held between the funnel and the base.

12.4 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.

12.5 For ambient surface waters and wastewaters, select sample volumes based on previous knowledge of pollution level, to produce 20-60 enterococci colonies on membranes. Sample volumes of 1-100 mL are normally tested at half log intervals, for example 100, 30, 10, 3mL, etc.

12.6 Smaller sample size or sample dilution can be used to minimize the interference of turbidity or high bacterial densities. Multiple volumes of the same sample or dilution of sample may be filtered and the results combined.

12.7 Filter the sample and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum and remove the funnel from the filter base.

12.8 Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the ME agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Close the dish, invert, and incubate at 41 $\pm$ 0.5°C for 48 h.

12.9 After incubation, transfer the membranes to EIA agar plates which have been at room temperature for 20-30 min, and incubate at 41°C for 20 min.

12.10 After incubation, count and record colonies on those membrane filters containing, if practical, 20-60 pink-to-red colonies with

black or reddish-brown precipitate on the underside of the membrane. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

13. Calculation of Results

Use the following general rules to calculate the enterococci count per 100 mL of sample:

13.1 Select and count membranes with ideally 20-60 pink to red colonies with black or reddish-brown precipitate on the underside. Calculate the final value using the formula:

$$\text{Enterococci/100 mL} = \frac{\text{No. of enterococci colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

13.2 See the USEPA microbiology manual, Part II, Section C, 3.5, for general counting rules.[5]

14. Reporting Results

14.1 Report the results as enterococci per 100 mL of sample.

15. Verification Procedure

15.1 Pink to red colonies with black or reddish-brown precipitate after incubation on EIA agar can be verified as enterococci. Verification of colonies may be required in evidence gathering, and is also recommended as a QC procedure upon initial use of the test and with changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows:

15.2 Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies into a brain heart infusion broth (BHI) tube and onto a BHI slant. Incubate broth tubes for 24 h and slants for 48 h at 35 ± 0.5°C.

15.3 After 24 h incubation, transfer a loopful of material from each BHI broth tube to:

a. Bile Esculin Agar (BEA) and incubate at 35 ± 0.5°C for 48 h.

b. BHI Broth and incubate at 45 ± 0.5°C for 48 h.

c. BHI Broth with 6.5% NaCl and incubate at 35 ± 0.5°C for 48 h.

15.4 Observe for growth.

15.5 After 48 h incubation, apply a gram stain to growth from each BHI agar slant.

15.6 Gram positive cocci which grow in BEA, BHI Broth at 45°C, and BHI Broth + 6.5% NaCl, and hydrolyze esculin, are verified as enterococci.

16. Precision and Bias

16.1 Performance Characteristics

16.1.1 Precision - The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95% confidence limits of the mean computed from the results of a series of controlled determinations. Precision of the mE method was established by Levin et al. (2) who indicated that the method did not exceed the expected limits for counts having the Poisson distribution.

16.1.2 Bias - The persistent positive or negative deviation of the results from the assumed or accepted true value. The bias of the enterococci MF method with mE Agar has been reported to be +2% of the true value (2).

16.1.3 Specificity - The ability of a method to select and/or distinguish the target bacteria from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false positive and false negative results. The specificity for this medium as reported for various environmental water samples was 10% false positive and 11.7% false negative (2).

16.2 Collaborative Study Data

16.2.1 A collaborative study was conducted among eleven volunteer laboratories, each with two analysts who independently tested local fresh and marine recreational waters and sewage treatment plant effluent samples, in duplicate. The data were reported to the Environmental Monitoring and Support Laboratory - Cincinnati, U.S. Environmental Protection Agency, for statistical analyses.

16.2.2 The results of the study are shown in Figure 1 where S_O equals the pooled standard deviation among replicate counts from a single analyst for three groupings (counts less than 30, counts from 30 to 50, and counts greater than 50) and S_B equals the pooled standard deviation between means of duplicates from analysts in the same laboratory for the same groupings. The precision estimates from this study did not differ with the water types tested.

16.2.3 By linear regression, the precision of the method can be generalized as:

$$S_O = 0.103 \text{ count/100 mL} + 2.42 \text{ (dilution factor) and}$$

$$S_B = 0.152 \text{ count/100 mL} + 5.16 \text{ (dilution factor)}$$

where dilution factor = $\frac{100}{\text{volume of original sample filtered}}$

16.2.4 Because of the instability of microbial populations in water samples, each laboratory analyzed its own sample series and no full measure of recovery or bias was possible. However, all laboratories analyzed a single surrogate sample prepared from a freeze-dried culture of Streptococcus faecalis. The mean count ($\bar{x}$) and the standard deviation of the counts (S_T) (including the variability among laboratories for this standardized enterococci sample) were 32.5 colonies/ membrane and 9.42 colonies/membrane, respectively.

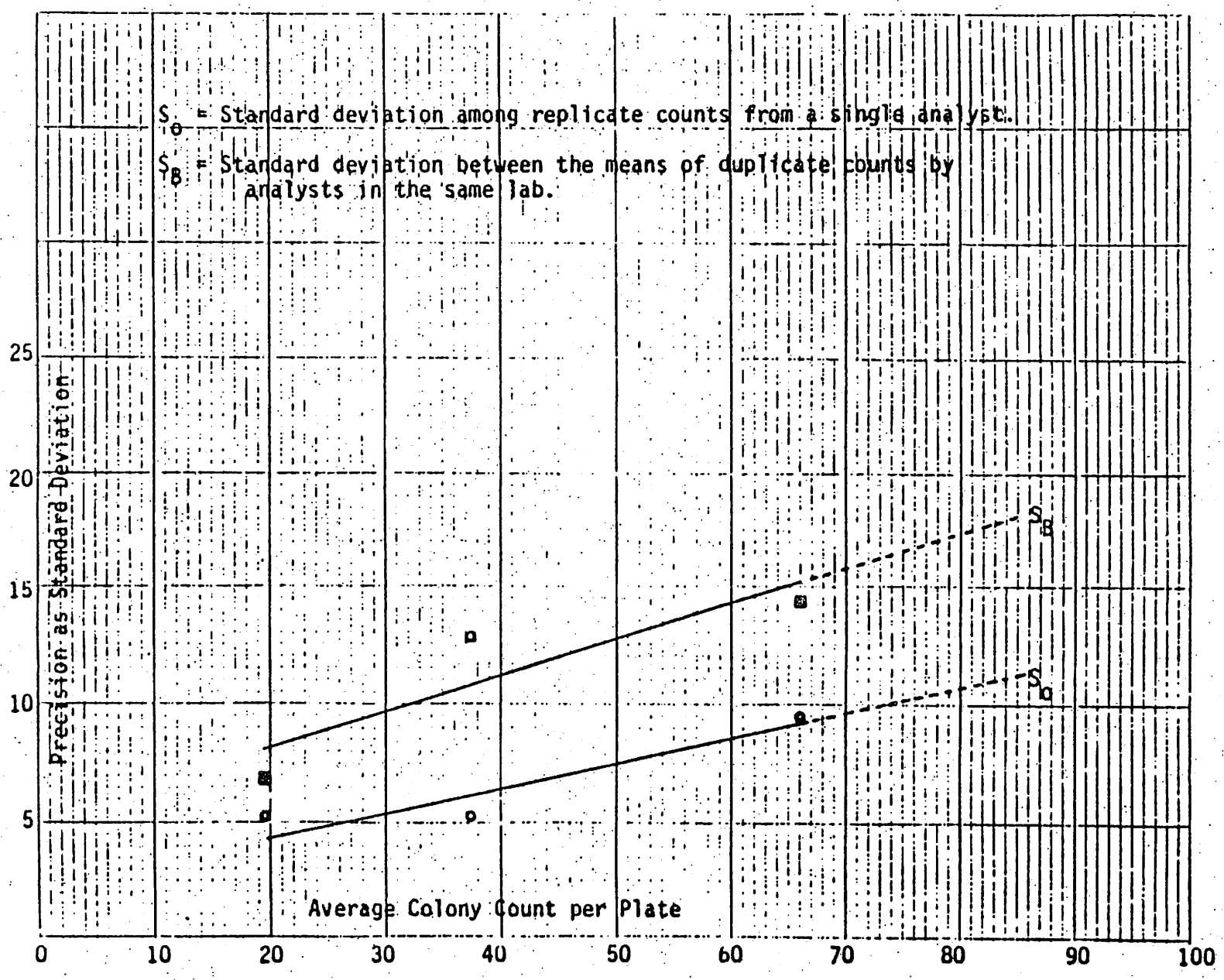

FIGURE 1. Precision Estimates for Enterococci in Water by the Membrane Filter/mE Procedure

References

1. Cabelli, V. J., A. P. Dufour, M. A. Levin, L. J. McCabe, and P. W. Haberman, "Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches," Amer. Jour. Public Health, 69, 690-696, 1979.

2. Levin, M. A., J. R. Fischer and V. J. Cabelli, "Membrane Filter Technique for Enumeration of Enterococci in Marine Waters," Appl. Microbiol. 30, 66-71, 1975.

3. "Reagent Chemicals," American Chemical Society Specifications 6th Edition, Am. Chem. Soc., Washington, D.C., 1981. For suggestions of the testing of reagents not listed by the American Chemical Society, see Reagent Chemicals and Standards, Joseph Rosin, D. Van Nostrand, Co., Inc., Princeton, N.J., 1967, and the United States Pharmacopeia, Nineteenth Edition, United States Pharmacopeial Convention, Inc., Rockville, MD, 1974.

4. Annual Book of ASTM Standards, Vol. 11.01, Water, American Society for Testing and Materials, Philadelphia, PA., 1985.

5. Bordner, R., J. A. Winter and P. V. Scarpino (eds.), "Microbiological Methods for Monitoring the Environment, Water and Wastes," EPA-600/8-78-017, U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory - Cincinnati, Cincinnati, Ohio, 1978.

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Comparative Study of Selective Media for Enumeration of *Pseudomonas aeruginosa* from Water by Membrane Filtration

ANTONIO DE VICENTE,* JUAN J. BORREGO, FRANCISCO ARRABAL, AND PEDRO ROMERO

Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain

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In the present study, mPA-D and mPA-E agar, modifications of mPA-C agar that reduce background fecal streptococci that interfere with the differentiation and enumeration of the *Pseudomonas aeruginosa* colonies grown in other mPA media, are proposed for use in analyzing natural water samples. In addition, the efficiencies of several culture media for the recovery of *P. aeruginosa* in water after membrane filtration and multiple-tube techniques are compared. The degree of selectivity, precision, efficiency, and sensitivity achieved with the proposed media exceeded that achieved by current methods. Furthermore, they yielded equal rates of accuracy and specificity. Incubation at 36°C resulted in an improved recovery of stressed *P. aeruginosa*. In conclusion, we propose the use of mPA-D and mPA-E agar, both incubated at 36°C for 24 to 48 h, for analyzing river water and seawater, respectively.

Pseudomonas aeruginosa is considered to be a ubiquitous and easily detectable microorganism in waters and soils, although it is not autochthonous to these environments (16). Among its most important habitats are human and animal fecal wastes, which are the main source of pollution in natural surface waters (3, 10, 16).

The importance of the study of *P. aeruginosa* in natural surface waters used for swimming is based on its potentiality in originating different kinds of infections, especially otitis (5, 20, 25). It has also been considered to be a water quality indicator microorganism (3, 13, 21).

Several different methods, including the multiple-tube technique (1, 9) and membrane filtration (4, 11, 23, 24), have been developed for the enumeration of *P. aeruginosa* in water (10, 17). However, most of them show some disadvantage, and none of them are completely accepted (10). Generally, the counting techniques based on most probable number (MPN) are considered to be less precise and effective than those based on membrane filtration (6, 14).

In 1972, Levin and Cabelli (23) developed the mPA medium for the isolation and enumeration of *P. aeruginosa* from natural waters that was later modified by Dutka and Kwan (11) and by Brodsky and Ciebin (4). Even so, these media do not provide suitable results, because of the growth of background fermentative microorganisms (A. de Vicente, J. J. Borrego, and P. Romero, Abstr. 9th Congr. Nac. Microbiol. 1983, 436, p. 929–930). To eliminate this problem, mPA-D agar, in which sugars present in mPA-C agar (4) are suppressed, and mPA-E agar, with xylose as the unique sugar, are proposed for analyzing river water and seawater samples, respectively.

TABLE 1. Average percent recovery of *P. aeruginosa* strains from different stressed suspensions[a]

Medium	Incubation (°C/h)	% Recovery of strain:					Avg % recovery
		ATCC10145	ATCC14216	J75	T26	N61	
mPA	41.5/48	5.14	21.41	18.41	5.50	4.90	11.07
mPA-B	41.5/72[b]	96.42	84.44	89.55	87.58	90.10	89.62
mPA-C	41.5/24	94.45	74.55	82.06	85.13	98.49	86.94
mPA-D	36/24	87.55	87.10	74.34	80.80	67.47	79.45
	36/48	94.98	88.51	75.01	82.08	72.26	82.57
	41.5/24	85.42	80.92	76.08	81.22	96.86	84.10
	41.5/48	89.87	83.20	77.05	81.38	99.35	86.17
mPA-E	36/24	87.52	83.77	87.28	91.60	72.34	84.75
	36/48	90.61	85.21	88.26	93.09	76.64	86.97
	41.5/24	92.54	76.44	80.21	90.68	92.53	87.08
	41.5/48	94.32	83.33	82.90	95.27	93.47	89.45

[a] Calculated with equation 1. Ten samples of each strain were used to calculate the average percent recovery.
[b] Similar values were obtained at 96 h.

MATERIALS AND METHODS

Field samples. The number and kinds of water samples provided were as follows: 10 samples of polluted river water, 6 samples of seawater, and 9 samples of sewage water, all collected during an 8-week period. The samples were refrigerated at 4°C and processed within 6 h of collection. All samples were collected from superficial layers (up to 30 cm below the water surface).

Microorganisms. The following five strains of *P. aeruginosa* were used for the evaluation of the accuracy of the

* Corresponding author.

TABLE 2. Effect of exposure to different stressed suspensions on the recovery of *P. aeruginosa* strains[a]

Medium	Incubation (°C/h)	Control[b]	% Recovery of strains from stressed suspensions of: Seawater stressed for (h): 0	6	24	Freshwater stressed for (h): 0	6	24	Distilled water stressed for (h): 0	6	24	Avg % recovery
mPA	41.5/48	0.00	34.29	19.60	34.33	7.57	9.85	0.00	2.25	0.70	2.09	11.07
mPA-B	41.5/72[c]	86.85	92.46	105.73	100.42	82.34	85.98	94.88	59.43	93.60	94.45	89.62
mPA-C	41.5/24	74.10	103.80	88.41	99.12	81.86	94.86	73.51	74.91	99.80	79.00	86.94
mPA-D	36/24	84.86	78.38	80.32	85.68	66.06	74.30	77.32	90.05	93.12	64.43	79.45
	36/48	94.69	80.25	81.43	87.29	68.19	78.91	81.51	93.71	94.14	65.55	82.57
	41.5/24	82.87	87.73	94.35	87.36	74.68	90.99	59.84	87.51	97.75	77.92	84.10
	41.5/48	85.89	90.39	95.46	87.96	75.45	91.12	71.07	88.42	97.85	78.07	86.17
mPA-E	36/24	87.53	87.94	89.41	98.80	74.05	71.94	95.57	76.80	98.34	69.95	84.75
	36/48	90.14	90.48	92.08	99.82	76.36	74.06	96.65	80.12	100.14	72.42	86.97
	41.5/24	93.15	93.24	94.44	91.32	74.59	87.66	68.34	88.61	98.92	80.52	87.08
	41.5/48	96.25	95.96	94.85	91.41	75.91	88.32	80.69	90.57	100.04	80.55	89.45

[a] Calculated with equation 1. Five samples of each strain were used to calculate the average percent recovery.
[b] Unstressed cells in BHI.
[c] Similar values were obtained at 96 h.

different methods: ATCC 10145, ATCC 14216, and J75 from seawater; T26 from river water; and N61 from untreated sewage. Twenty-one *Streptococcus* strains, including 4 *S. faecalis*, 7 *S. faecium*, and 10 *S. avium* strains, isolated from samples of natural waters, and *S. faecium* ATCC 10541 were used for the growth assay.

Recovery media. Drake 10 medium (9) and Favero asparagine broth (1) were used as recovery media in the MPN technique and incubated for 48 h at 36°C. All the tubes that showed growth with greenish-blue pigment, fluorescence, or both under UV light were subcultivated in acetamide agar and milk agar (1) for confirmation as *P. aeruginosa*.

The following recovery media, with membrane filtration, were used: nalidixic acid-cetrimide agar (24), mPA agar (23), mPA-B agar (11), mPA-C agar (4), and mPA-D and mPA-E agar. mPA-D and mPA-E agar are modifications of the mPA-C agar developed by us which suppress all of the sugar compounds (mPA-D) or only lactose and sucrose (mPA-E). These two media were prepared by the procedure of Brodsky and Ciebin (4), with suppression antibiotics sulfapyridine and actidione. Both of these media were incubated at 36 and 41.5°C and examined at 24 and 48 h.

Methods. The MPN assays were carried out as described in *Standard Methods* (1). The filtrations were fivefold for each one of the assayed methods with 0.45-μm membrane filters (HAWG 047; Millipore Corp., Bedford, Mass.). Phosphate-buffered saline (23) was used as diluent solution. The membrane filtration technique was used following the specifications described previously (1).

Control media and suspensions. *P. aeruginosa* cultures were prepared by inoculation of strains into brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) and incubated for 18 to 20 h at 36°C. Suspensions and culture dilutions were prepared in phosphate-buffered saline blank tubes. The concentrations of suspended microorganisms were determined by the spread plate technique with 0.1-ml portions of tryptic soy agar (Difco), BHI agar (BHIA; Difco), and King's A agar (22). The recounts were determined after 48 h at 36°C with three replicate plates. BHIA, plate count agar (Difco), and mPA-B agar without antibiotics (11) were used to quantify the total number of organisms in natural water samples by membrane filtration.

Fermentative colony verification. The fermentative colonies grown on mPA, mPA-B, and mPA-C agars were isolated on nutritive agar containing 0.1% glucose. The colonies were identified by the following tests: morphology and Gram stain; motility; oxidation-fermentation test; catalase and cytochrome oxidase tests; xylose, lactose, and sucrose fermentation; growth in BHI at 10°C, at 45°C, at pH 9.6, and after 30 min at 60°C; growth in milk–0.1% methylene blue, in broth with 6.5% NaCl, and in 0.04% potassium tellurite; and resistance to 0.01% sodium azide (7, 15). Confirmation was accomplished with the API 20 Strep system (Analytab Products, Plainview, N.Y.).

***Streptococcus* qualitative growth test.** *Streptococcus* cultures obtained from BHI containing 0.1% glucose were streaked on the different assayed mPA and *m* Enterococcus (Difco) media. The growth and fermentation results were recorded at 24, 48, and 72 h.

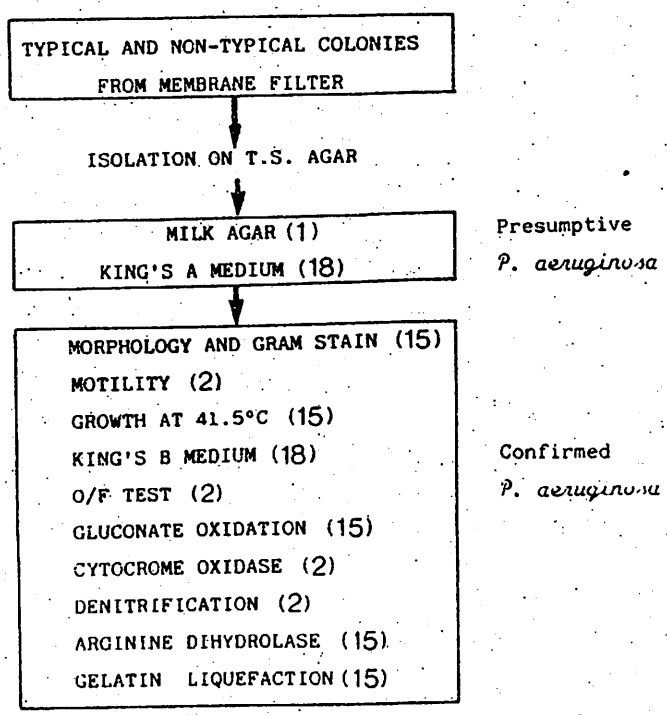

FIG. 1. *P. aeruginosa* identification scheme (numbers in parentheses indicate references in Literature Cited).

TABLE 3. Verification as *P. aeruginosa* of colonies isolated by different methods

Medium	Incubation (°C/h)	No. of colonies examined	Typical colonies		Atypical colonies	
			No. examined	% Verified[a]	No. examined	% Verified[a]
mPA	41.5/48	71	19	84.21	52	1.92
mPA-B	41.5/72	377	162	83.95	215	8.37
mPA-C	41.5/24	263	126	90.48	137	8.76
mPA-D	36/48	443	299	95.65	144	5.55
	41.5/48	101	53	98.11	48	4.16
mPA-E	36/48	287	204	98.04	83	7.23
	41.5/48	114	50	90.00	64	9.37
Nalidixic acid-cetrimide	36/24	283	58	87.93	225	6.67

[a] Verified as *P. aeruginosa*.

RESULTS

Accuracy was determined by comparison of the *P. aeruginosa* recoveries obtained with the mPA media with those obtained with the reference media (BHIA, tryptic soy agar, and King's A agar) by the following equation:

$$\Sigma \frac{\text{number of CFU for each method}}{(\text{number of CFU in BHIA} + \text{tryptic soy agar} + \text{King's A agar})/3} \times 100/\text{number of samples} \quad (1)$$

Cultures of the five *P. aeruginosa* strains, with cell densities of 10^9 to 10^{10} CFU per ml in BHI, were suspended in seawater, distilled water, and river water; all water samples had been sterilized by filtration. Appropriate volumes of *P. aeruginosa* cultures were used as inocula to obtain a concentration of ca. 10^7 to 10^8 cells per ml and were stored at 4°C. Accuracy tests of strain cultures and suspensions were realized at 0, 6, and 24 h by the spread plate method. The counts were done in triplicate, and the arithmetical mean was calculated. The results, with an interval average recovery of 79.41 (mPA-D agar, 36°C for 24 h) to 89.45% (mPA-E agar, 41.5°C for 48 h) for the proposed media, are given in Tables 1 and 2. The accuracy of the methods always exceeded 75% recovery, except in mPA.

Specificity of the methods (culture medium and procedure used) was determined by testing seawater, river water, and sewage water samples from different locations. The typical *P. aeruginosa* colony was flat and dry, and its color varied between greenish grey with a dark center or black with or without a greenish rim and dark brown without a rim and more round (0.8- to 2-mm diameter), with irregular edges. Both typical and atypical colonies obtained from the different enumeration media were biochemically identified (Fig. 1). The confirmation percentages of typical colonies such as *P. aeruginosa* in mPA-D agar were 96.88% and of atypical colonies were 4.85%. In mPA-E agar, the confirmation percents were 90 to 98% for typical colonies, depending on incubation conditions, and 8.3% for atypical colonies. It can also be seen that all of the assayed media exceeded 83% confirmed typical colonies and that they never exceeded 10% false-negative results (Table 3).

mPA, mPA-B, mPA-C, mPA-D, mPA-E, and nalidixic acid-cetrimide agar selectivities for quantitative *P. aeruginosa* recovery from natural waters are shown in Table 4. Such selectivities were obtained by observing the degree of reduction of background microbial flora in the assayed methods. Reduction factors were obtained by calculating the rate between the average number of colonies per 100 ml detected in control media (BHIA, plate count agar, and mPA base agar) and the total number of colonies per 100 ml detected in the different selective *P. aeruginosa* media. These factors are highly variable, depending mainly on the media, the incubation conditions, and particularly the nature of the studied sample. In the two proposed media, these factors varied between 801 for mPA-E agar (36°C for 48 h) and 23,500 for mPA-D agar (41.5°C for 24 h) in freshwater samples. The degree of reduction ranged from 1,820 for mPA-D agar (36°C for 48 h) to 14,500 for mPA-E agar (41.5°C for 24 h) in seawater samples. In sewage samples, minimal and maximum reduction factors were obtained on mPA-E agar, and the values were 1.81×10^7 (36°C for 48 h) and 3.87×10^7 (41.5°C for 24 h), respectively. Generally, the average reduction values of the different water samples, obtained with mPA-D and mPA-E agar, were equal or higher than those with other media.

The precision of the different methods was graphically determined from dispersion of Fisher index D^2 values of the assay variability for mPA-B, mPA-C, mPA-D, and mPA-E agar as calculated from the following equation (12, 23):

$$D^2 = | N\Sigma X_i^2 - (\Sigma X_i)^2 | / \Sigma X_i \quad (2)$$

where X_i is the bacterial number obtained from each plate of

TABLE 4. Selectivity of methods for enumeration of *P. aeruginosa* from natural water samples

Medium	Incubation (°C/h)	Mean background reduction factor[a]		
		Freshwater ($\times 10^3$)	Seawater ($\times 10^3$)	Sewage ($\times 10^3$)
mPA	41.5/48	30.70	16.90	27.20
mPA-B	41.5/72[b]	8.36	1.22	5.81
mPA-C	41.5/24	44.40	4.75	21.50
mPA-D	36/24	1.85	2.11	1.95
	36/48	1.03	1.82	2.03
	41.5/24	23.50	14.20	3.41
	41.5/48	10.60	5.40	2.62
mPA-E	36/24	1.69	3.13	1.83
	36/48	0.80	1.92	1.81
	41.5/24	17.10	14.50	3.87
	41.5/48	7.65	5.81	2.37
Nalidixic acid-cetrimide	36/24	0.40	1.16	0.89

[a] Ratio between total colonies recount on control media (plate count agar, BHIA, and mPA-B base agar) and total colonies recount by each method assayed. Values shown are the arithmetical mean of five samples.

[b] Similar values were obtained at 96 h.

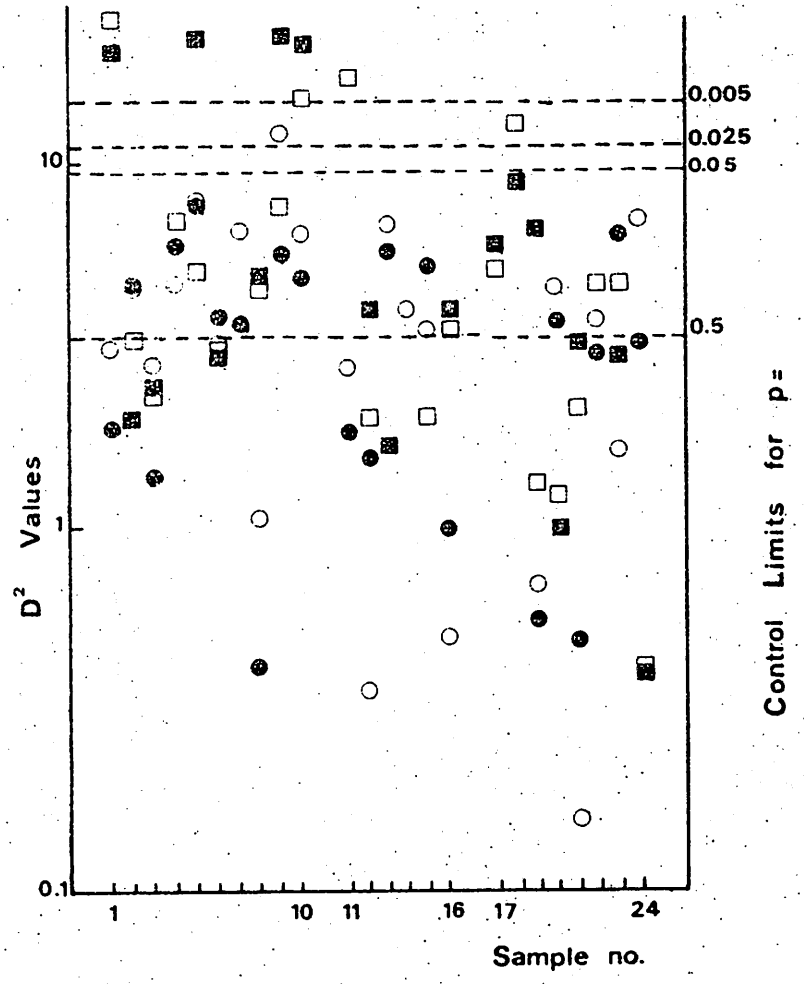

FIG. 2. Precision of mPA-D agar procedures for *P. aeruginosa* as estimated from dispersion of D^2 values (equation 2). Symbols: ●, incubated at 36°C for 24 h; ○, incubated at 36°C for 48 h; ■, incubated at 41.5°C for 24 h; □, incubated at 41.5°C for 48 h; -----, control limits when probability was as stated. Sources of samples: 1 to 10, freshwater; 11 to 16, seawater; 17 to 24, sewage. Data from five replicate plates were used to calculate the D^2 value for each point.

the same portion and sample and N is the number of replicate plates, five for each assayed portion. The D^2 values, determined for the different methods with mPA-D and mPA-E agar and 24 analyzed water samples (river water, numbers 1 to 10; seawater, numbers 11 to 16; sewage water, numbers 17 to 24) and theoretical D^2 values for different probabilities are given in Fig. 2 and 3. D^2 values of mPA-B and mPA-C agar are given in Fig. 4. From mPA-D and mPA-E agar results, it may be deduced that there was a uniform distribution and that, from the recount variability results, there was not a significant effect from plate to plate. On the other hand, mPA-B and mPA-C agar presented an extended dispersion of results because of a more heterogeneous recount of the different replicates.

Twenty-four water samples from different sources were assayed in parallel by the multiple-tube and membrane filtration procedures with the media described previously. The results of each method, expressed as the relative percent recovery with respect to the maximum recount for that sample, given as 100% (equation 3) and as the percentage of samples in which *P. aeruginosa* was detected with respect to the total number of tested samples (equation 4), were compared as follows (12):

$$\Sigma \frac{(P.\ aeruginosa \text{ concentration for assayed method}/P.\ aeruginosa \text{ maximum concentration obtained for that sample})}{\text{number of samples}} \times 100 \quad (3)$$

$$\frac{\text{number of samples in which } P.\ aeruginosa \text{ was detected}}{\text{total number of analyzed samples}} \times 100 \quad (4)$$

Positive *P. aeruginosa* percents recovery in each method (considering that in every sample *P. aeruginosa* was detected by one or more methods) and average *P. aeruginosa* percent levels in each method compared with the maximum level obtained for the same sample are expressed in Table 5. This comparison was also carried out under different culture conditions (incubation temperature and time).

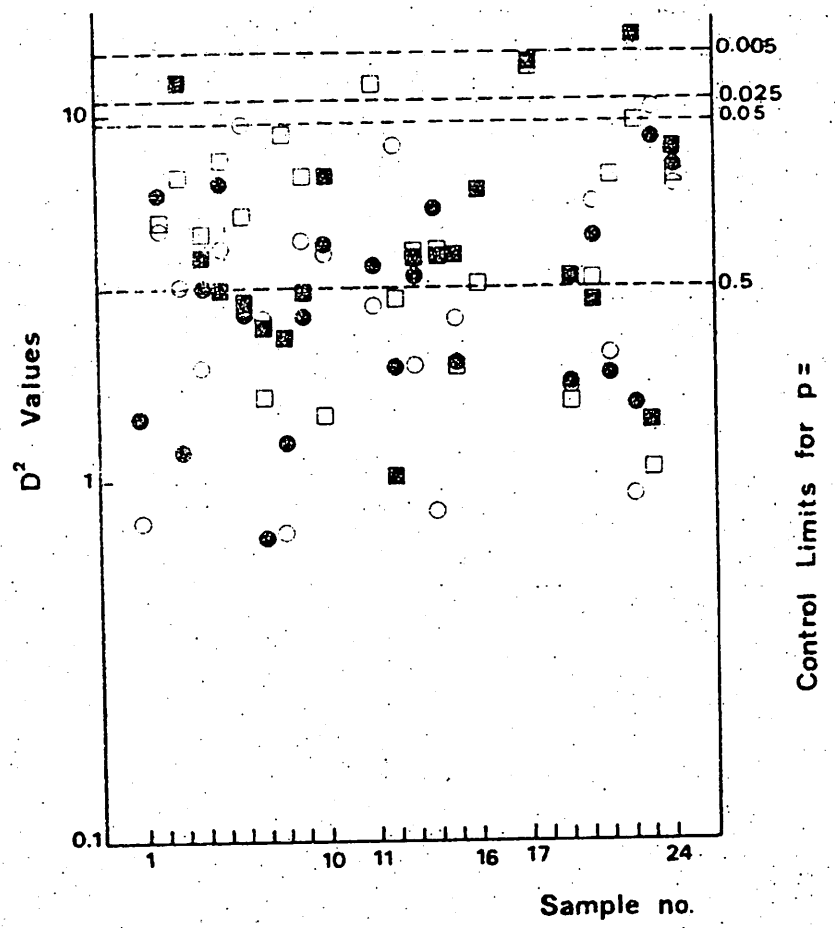

FIG. 3. Precision of mPA-E agar procedures for *P. aeruginosa* as estimated from dispersion of D^2 values (equation 2). Symbols: ●, incubated at 36°C for 24 h; ○, incubated at 36°C for 48 h; ■, incubated at 41.5°C for 24 h; □, incubated at 41.5°C for 48 h; -----, control limits when probability was as stated. Sources of samples: 1 to 10, freshwater; 11 to 16, seawater; 17 to 24, sewage. Data from five replicate plates were used to calculate the D^2 value for each point.

Fermentative colonies that interfered with *P. aeruginosa* recounts appeared after 24 h of incubation on mPA-B and mPA-C agar when different kinds of water were analyzed. When identification tests specified in *Bergey's Manual* (7) were used, these microorganisms appeared to be of the genus *Streptococcus*. The percentages of the isolated microorganisms were as follows: *S. avium*, 35.30%; *S. faecalis*, 23.53%; *S. faecium*, 41.17%. Also, fecal streptococci from the same water samples were investigated with *m* Enterococcus agar; the percentages of fecal streptococci were as follows: *S. avium*, 33.33%; *S. faecalis*, 16.66%; *S. faecium*, 50%. To determine whether these microorganisms were responsible for the interference described above, qualitative growth tests on mPA and *m* Enterococcus agar were carried out. The results show that there was a delay in and inhibition of streptococcal development on the proposed media which improved *P. aeruginosa* colony recount (Table 6).

DISCUSSION

When water samples with high concentrations of bacteria are studied, detection methods for specific organisms must fulfill two basic conditions: high selectivity and optimal recovery efficiency. Several methods have been proposed to detect and quantify *P. aeruginosa* in water samples, but none of them can be considered optimal. The exclusion of sugar by mPA-D agar delayed the growth of *Streptococcus* colonies. Because xylose is metabolized by *P. aeruginosa* but not by *Streptococcus* species, we decided to design mPA-E agar.

mPA-D and mPA-E agar accuracy were evaluated by comparison with media used in other methods (4, 11, 23). Average recoveries with mPA-D agar varied from 79.45 to 86.17% for the different kinds of samples tested, depending on incubation conditions. The range varied from 84.75 to 89.45% for mPA-E agar. These percents are similar to those obtained with mPA-B (89.62%) and mPA-C (86.94%) agar under the same conditions but are much higher than those obtained with mPA agar (11.07%) which also fluctuated, depending on the suspension tested (Table 2). Analyzing the results of the different *P. aeruginosa* strains (Table 1), it can be seen that the recovery interval for mPA-D agar oscillated from 67.47 to 99.35% with the N61 strain. The percents ranged from 72.34% for the N61 strain to 95.27% for the T26 strain when mPA-E agar was used. These values are very similar to those obtained with mPA-B and mPA-C agar but considerably different from those obtained with mPA agar, ranging from 4.90 to 21.14% for N61 and ATCC 14216, respectively, both under culture conditions proposed by

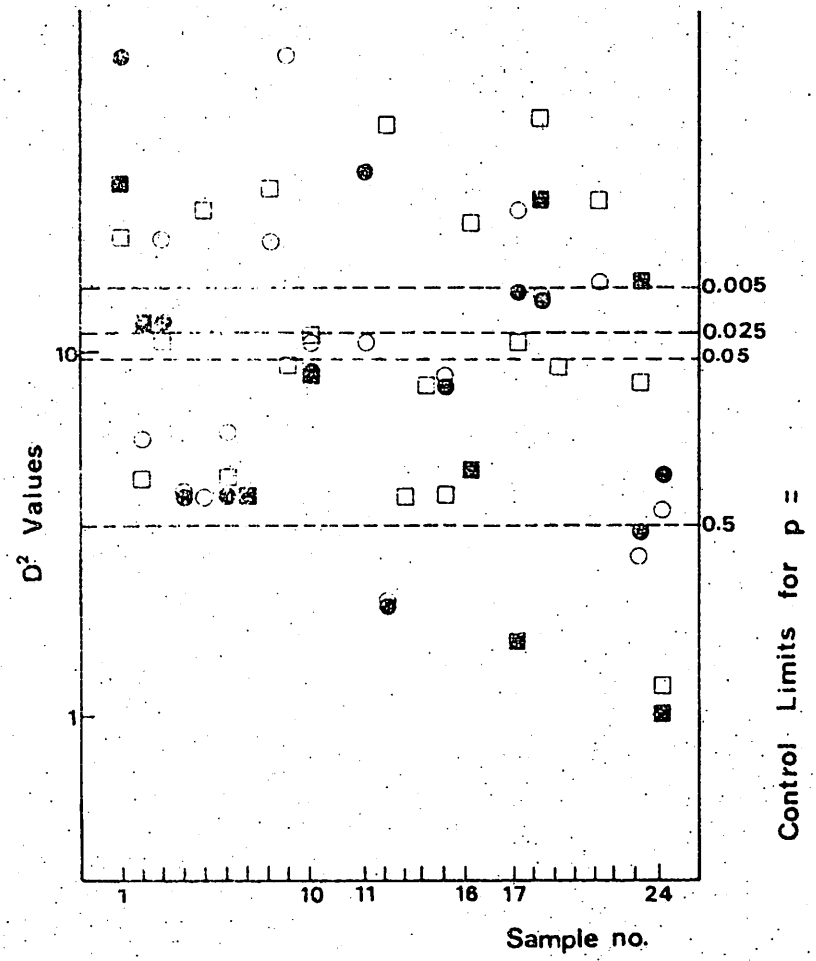

FIG. 4. Precision of mPA-B and mPA-C agar procedures for *P. aeruginosa* as estimated by dispersion of D^2 values (equation 2). Symbols: ●, mPA-B agar incubated at 41.5°C for 48 h; ○, mPA-B agar incubated at 41.5°C for 72 to 96 h; ■, mPA-C agar incubated at 41.5°C for 24 h; □, mPA-C agar incubated at 41.5°C for 48 h; ——, control limits when probability was as stated. Sources of samples: 1 to 10, freshwater; 11 to 16, seawater; 17 to 24, sewage. Data from five replicate plates were used to calculate the D^2 value for each point.

Levin and Cabelli (23). From these results, it can be seen that these average recoveries exceeded the required levels for the correct accuracy of a medium, except in the case of mPA agar. The accuracy results obtained with mPA-B and mPA-C agar agree with the reports of Brodsky and Ciebin (4), but those obtained with mPA agar are in open opposition to those pointed out by Levin and Cabelli (23).

In the most stressful conditions, the *P. aeruginosa* recovery with unselective media was greater than with selective media (Table 2). However, there are instances in which recovery with the selective media assayed increases with time exposed to a stressful environment. These results suggest that injury to the cells occurs during the lag phase and that the percentage of injured cells is lower after a long exposure time to stressful conditions. The decreased percentage of injured cells could be explained by either the repair or the inactivation phenomena subsequent to the cell injury, as previously reported by Hoadley and Cheng and by Hoadley (18, 19). These results occur more frequently when the incubation temperature is not restrictive, as is 36°C.

Specificity of mPA-D and mPA-E agar was high, because the percentage of colonies confirmed as *P. aeruginosa* was above 90% in all culture conditions, and the percentage of false-negative colonies never exceeded 10%, with over 5% of colonies being falsely negative on mPA-D agar and 8% being falsely negative on mPA-E agar. With the remaining methods, the positive verification percentage of typical colonies ranged between 83.95 and 90.48% for mPA-B and mPA-C agar, respectively, and the percentage of atypical colonies identified as *P. aeruginosa* ranged between 1.92 and 8.76% for mPA and mPA-C agar, respectively (Table 3). Even though these results are slightly lower than those obtained by other investigators (4, 11, 23), they have enough reliability to avoid the routine verification of typical colonies. Furthermore, in disagreement with Dutka and Kwan (11), significative changes in colonies cannot be seen when the incubation temperature is modified.

Selectivity criteria of a recount medium state that the background flora of water samples must decrease by at least three orders of magnitude (23). Results of the ratio between the average concentration of microorganisms detected in control media by membrane filtration and the total concentration of microorganisms grown on *P. aeruginosa*-selective media are given in Table 4. These results show that there was a significant decrease of background flora, depending on the level of bacteria in the sample and also on the source of the assayed water. Therefore, recoveries from freshwater and seawater samples were similar and lower than those

TABLE 5. Comparison of the efficiency of different procedures for recovery of *P. aeruginosa* from waters

Medium	Incubation (°C/h)	% Detection or recovery from the following samples:							
		Freshwater (n = 30)		Seawater (n = 5)		Sewage (n = 9)		% Overall (n = 24)	
		Detection[a]	Recovery[b]	Detection[a]	Recovery[b]	Detection[a]	Recovery[b]	Detection[a]	Recovery[b]
mPA	36/48	10.00	5.88	20.00	25.19	55.55	23.98	29.16	21.57
	41.5/48[c]	0.00		0.00		0.00		0.00	
	41.5/72	0.00		0.00		0.00		0.00	
mPA-B	36/48	90.00	46.02	40.00	34.02	100.00	53.39	82.61	47.86
	41.3/48	60.00	10.09	40.00	17.17	55.55	63.72	54.16	31.81
	41.5/72[c]	90.00	12.34	40.00	26.26	66.66	54.45	70.83	28.84
mPA-C	36/24	100.00	74.50	60.00	55.50	87.50	59.02	86.95	66.23
	36/48	100.00	75.46	60.00	65.30	100.00	68.18	91.30	71.24
	41.5/24[c]	50.00	11.66	20.00	4.42	55.55	39.64	45.83	23.72
	41.5/48	90.00	26.68	60.00	6.10	77.77	53.29	79.16	33.13
mPA-D	36/24	100.00	82.16	80.00	50.91	100.00	79.24	95.83	75.58
	36/48	100.00	82.71	100.00	46.81	100.00	82.96	100.00	74.99
	41.5/24	90.00	23.90	60.00	28.85	77.77	40.83	79.16	30.92
	41.5/48	100.00	47.30	60.00	42.46	100.00	57.10	91.66	50.64
mPA-E	36/24	100.00	79.83	100.00	66.36	100.00	81.90	100.00	77.81
	36/48	100.00	77.61	100.00	96.18	100.00	87.85	100.00	85.21
	41.5/24	80.00	51.60	100.00	13.96	88.88	35.76	87.50	36.61
	41.5/48	100.00	65.72	100.00	35.32	100.00	66.32	100.00	59.61
Nalidixic acid-cetrimide	36/24	100.00	43.19	60.00	43.46	88.88	57.29	87.50	48.60
	36/48	90.00	64.43	80.00	43.21	87.50	57.31	86.95	57.70
Favero broth		100.00	32.31	20.00	1.73	100.00	38.22	83.33	33.44
Drake 10 medium		100.00	36.94	0.00		100.00	16.21	79.12	27.12

[a] Percentage of samples in which *P. aeruginosa* was detected (equation 4).
[b] Average relative percent recovery with respect to the maximum recount for each sample, based only on samples positive for *P. aeruginosa* (equation 3).
[c] Originally proposed procedure.

from sewage samples. If selectivities of the different methods are compared, similar behavior is observed, because all of the methods presented high selectivity (decrease above 1,000-fold), except in nalidixic acid-cetrimide and mPA-E agar incubated at 36°C for 48 h, which did not achieve such a decrease in river water samples. In all mPA media incubated at 41.5°C, more than a 1,000-fold reduction of overgrowth was obtained, and in sewage samples this reduction was even higher. But when the incubation temperature was lowered to 36°C, selectivities of mPA-D and mPA-E agar were reduced by one order of magnitude in river water and seawater samples, but in sewage samples this decrease was insignificant. To explain this phenomenon, it can be implied that selectivity is affected by the temperature of the water samples, often over 20°C, and by a high concentration of thermophilic microorganisms (4, 23).

Even though there was not a significant quantitative improvement in selectivity with mPA-D and mPA-E agar, there was a qualitative one, because streptococcal growth that produced fermentative colonies on mPA agar was delayed up to 72 h (Table 6). The *Streptococcus* species isolated from mPA media were the same species that were detected with *m* Enterococcus agar and were obtained from the same samples of polluted natural water. The importance of delaying streptococcal growth on mPA, mPA-B, and mPA-C agar is that these microorganisms present higher concentrations than does *P. aeruginosa* in the same natural polluted water samples (8). Also, by allowing incubation at a temperature of 36°C, detection of stressed *P. aeruginosa* cells, which can be inhibited at 41.5°C, is improved.

Experimental D^2 values in variability assays (Fig. 2 and 3) are approximately equal to the expected estimates, demonstrating the high precision of mPA-D and mPA-E agar. Comparison of D^2 values obtained for mPA-B and mPA-C agar with those obtained for mPA-D and mPA-E agar (Fig. 2 to 4) suggests that mPA-D and mPA-E agar are more precise, especially when incubation is carried out at 36°C. In this study, a higher variability than that obtained by Brodsky and Ciebin (4) can be seen for mPA-B and mPA-C agar. This may be caused by the variation of typical colony morphology (6).

Comparison of mPA-D and mPA-E agar assayed methods with other assayed methods (Table 5) was achieved by means of the efficacy and sensitivity of those methods, designated *P. aeruginosa* recovery efficiency. The techniques based on MPN, with Favero asparagine broth and Drake 10 medium, presented a high detection sensitivity, except with seawater samples, but they showed a poor percent recovery, about 30%. Even so, it must be noted that

TABLE 6. Qualitative growth of *Streptococcus* test strains on mPA media at 36 and 41.5°C[a]

Assayed medium	Time (h)	Growth of test strains[b]			
		S. avium (n = 10)	*S. faecium* (n = 7)	*S. faecalis* (n = 4)	*S. faecium* ATCC 10541
mPA	24	+, F	+, F	+, F	+, F
mPA-B	24	+, F	+, F	+, F	+, F
mPA-C	24	+, F	+, F	+, F	+, F
mPA-D	24	−	−	−	−
	48	−	−	−	−
	72	(+)	(+)	(+)	(+)
mPA-E	24	−	−	−	−
	48	(+)	(+)	(+)	(+)
	72	(+)	(+)	(+)	(+)

[a] Results at 36 and 41.5°C were equal.
[b] +, Growth; F, fermentation; −, no growth; (+), weak growth.

interference which other background flora can produce in the test tubes can produce false results, decreasing the sensitivity of the method. This could also be caused by the individual or global inhibitory effect of processes of amensalism, competition, or toxic substance inhibition of the sample. Another inconvenience of this method is that, because its presumptive test is based on fluorescein production and because for the confirmation test pyocyanine production is required, the existence of apyocyanogenic strains or the nonproduction of fluorescein or both (17) that result in a loss of sensitivity and security of these methods cannot be forgotten.

Efficiency was much higher for membrane filtration methods than for MPN, in agreement with the results reported by other investigators (4, 11, 23). mPA agar, used as described by Levin and Cabelli (23), did not detect *P. aeruginosa* in any of the samples. Incubation at 36°C for 48 h only achieved 29% sensitivity and 21% recovery. Only four assays reached maximum sensitivity detection (100%): mPA-D agar at 36°C for 48 h, mPA-E agar at 36°C for 24 h, mPA-E agar at 36°C for 48 h, and mPA-E agar at 41.5°C for 48 h (Table 5). The highest percent recovery belonged to mPA-E agar at 36°C for 48 h, with an average recovery of 85%; the comparative percent recovery was only above 75% in mPA-D and mPA-E agar incubated at 36°C, so both media in these culture conditions offered higher efficiency in *P. aeruginosa* enumeration from natural water. mPA and mPA-C agar methods presented a low sensitivity and a slight recovery level compared with the methods proposed in this study. Likewise, the mPA-B agar method showed low sensitivity for seawater and a degree of recovery ranging from 12 to 54%, depending on the type of sample. It must be noted that nalidixic acid-cetrimide agar has high sensitivity, about 87%, even though it only gives 50% recovery of *P. aeruginosa*; furthermore, limited definition and concretion of *P. aeruginosa* typical colonies, grown on this medium, must be considered.

Behavior of the media varies with the source of the sample. All methods lose recovery ability or sensitivity or both with seawater samples, except with mPA-E agar incubated at 36°C for 48 h; thus, this medium is recommended for these samples. The method that showed the best results for freshwater samples was mPA-D agar incubated at 36°C for 48 h. Both methods can be used with sewage water.

We must agree with Levin and Cabelli (23) that, with each medium, sensitivity of the method is considerably lower when turbid samples with high contents of particulate matter or with sediments are used. This is because particulate matter produces faulty filtration and disguises the normal morphology and colony color of *P. aeruginosa* in these media.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **American Public Health Association.** 1981. Standard methods for the examination of water and wastewater, 15th ed. American Public Health Association, Washington, D.C.
2. **American Society for Microbiology Committee on Continuing Education.** 1981. Identification of glucose non-fermenting gram-negative rods. American Society for Microbiology, Washington, D.C.
3. **Bonde, G. J.** 1977. Bacterial indication of water pollution, p. 273–364. *In* M. Droop and H. W. Jannasch (ed.), Advances in aquatic microbiology, vol. 1. Academic Press, Inc. (London), Ltd., London.
4. **Brodsky, M. H., and B. W. Ciebin.** 1978. Improved medium for recovery and enumeration of *Pseudomonas aeruginosa* from water using membrane filters. Appl. Environ. Microbiol. 36: 36–42.
5. **Cabelli, V. J., H. Kennedy, and M. A. Levin.** 1976. *Pseudomonas aeruginosa*-fecal coliform relationships in estuarine and fresh recreational waters. J. Water Pollut. Control Fed. 48: 367–376.
6. **Carson, L. A., N. J. Petersen, M. S. Favero, I. L. Doto, D. E. Collins, and M. A. Levin.** 1975. Factors influencing detection and enumeration of *Pseudomonas aeruginosa* by most-probable-number and membrane filtration techniques. Appl. Microbiol. 30:935–942.
7. **Deibel, R. H., and H. W. Seeley, Jr.** 1974. Family II. *Streptococcaceae*, fam. nov., p. 490–517. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **de Vicente, A., J. J. Borrego, F. Arrabal, and P. Romero.** 1983. An initial study of the inactivation of *Pseudomonas aeruginosa* in seawater and its comparison with the usual bacterial indicators of fecal pollution. Rapp. P.-V. Reun. Comm. Int. Explor. Sci. Mer Mediterr. Monaco 28:49–50.
9. **Drake, C. H.** 1966. Evaluation of culture media for the isolation and enumeration of *Pseudomonas aeruginosa*. Health Lab. Sci. 3:10–19.
10. **Dutka, B. J.** 1981. *Pseudomonas aeruginosa*: a controversial indicator pathogen, p. 119–128. *In* B. J. Dutka (ed.), Membrane filtration: applications, techniques and problems. Marcel Dekker, Inc., New York.
11. **Dutka, B. J., and K. K. Kwan.** 1977. Confirmation of the single-step membrane filtration procedure for estimating *Pseudomonas aeruginosa* densities in water. Appl. Environ. Microbiol. 33:240–245.
12. **El-Shaarawi, A. H., and W. O. Pipes.** 1982. Enumeration and statistical interferences, p. 43–66. *In* W. O. Pipes (ed.), Bacterial indicators of pollution. CRC Press, Inc., Boca Raton, Fla.
13. **Foster, D. H., N. B. Hanes, and S. M. Lord, Jr.** 1971. A critical examination of bathing water quality standards. J. Water Pollut. Control Fed. 43:2229–2241.
14. **Geldreich, E. E.** 1975. Handbook for evaluating water bacteriological laboratories, 2nd ed. U.S. Environmental Protection Agency, Washington, D.C.
15. **Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.).** 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
16. **Hoadley, A. W.** 1977. *Pseudomonas aeruginosa* in surface waters, p. 31–57. *In* V. M. Young (ed.), *Pseudomonas aeruginosa*, ecological aspects and patient colonization. Raven Press, New York.
17. **Hoadley, A. W.** 1977. Potential health hazards associated with *Pseudomonas aeruginosa* in water, p. 80–114. *In* A. W. Hoadley and B. J. Dutka (ed.), Bacterial indicators/health hazards associated with water. American Society for Testing and Materials, Philadelphia.
18. **Hoadley, A. W.** 1981. Effect of injury on the recovery of bacteria on membrane filters, p. 413–450. *In* B. J. Dutka (ed.), Membrane filtration: applications, techniques and problems. Marcel Dekker, Inc., New York.
19. **Hoadley, A. W., and C. M. Cheng.** 1974. The recovery of indicator bacteria on selective media. J. Appl. Bacteriol. 37: 46–57.
20. **Hoadley, A. W., and D. E. Knight.** 1975. Outer (external otitis) ear infections among swimmers and nonswimmers. Arch. Environ. Health 30:445.
21. **Kenner, B. A., and H. P. Clark.** 1974. Detection and enumeration of *Salmonella* and *Pseudomonas aeruginosa*. J. Water

Pollut. Control Fed. 46:2163–2171.
22. King, E. O., M. K. Ward, and A. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301–307.
23. Levin, M. A., and V. J. Cabelli. 1972. Membrane filter technique for enumeration of *Pseudomonas aeruginosa*. Appl. Microbiol. 24:864–870.
24. Lilly, H. A., and E. J. L. Lowbury. 1972. Cetrimide-nalidixic acid agar as a selective medium for *Pseudomonas aeruginosa*. J. Med. Microbiol. 5:151–153.
25. Pipes, W. O. (ed.). 1982. Bacterial indicators of pollution, p. 1–19. CRC Press, Inc., Boca Raton, Fla.

mPA - A

l-Lysine Hydrochloride	0.5 g
NaCl	0.5 g
Yeast Extract	0.2 g
Xylose	0.25 g
Sodium Thiosulfate	0.68 g
Sucrose	0.125 g
Lactose	0.125 g
Phenol Red	0.008 g
Ferric Ammonium Citrate	0.08 g
Agar	1.5 g

1. Suspend in 100 mL deionized water
2. Adjust the pH to 7.2 $\pm$ 0.1
3. Autoclave at 15 lbs for 15 minutes
4. Cool to 50 -60 C. and add the following dry antibiotics

0.0176 g	Sulfapyridine
0.00085 g	Kanamycin
0.0037 g	Nalidixic Acid
0.0150 g	Actidione

5. Adjust the final pH if necessary to 7.1 $\pm$ 0.1 and dispense into 50 x 9 mm petri dishes

l-Lysine Hydrochloride	0.5 g
NaCl	0.5 g
Yeast Extract	0.2 g
Xylose	0.125 g
Sucrose	0.125 g
Lactose	0.125 g
Phenol Red	0.008 g
Ferric Ammonium Citrate	0.08 g
Sodium Thiosulfate	0.5 g
Mg SO4 - 7H2O	0.15 g
Agar	1.5 g

1. Suspend in 100 mL deionized water
2. Adjust the pH to 7.1 $\pm$ 0.1
3. Autoclave at 15 lbs for 15 minutes
4. Cool to 50 -60 C. and add the following dry antibiotics

0.0176 g	Sulfapyridine
0.00085 g	Kanamycin
0.0037 g	Nalidixic Acid
0.0150 g	Actidione

5. Adjust the final pH if necessary to 7.1 $\pm$ 0.1 and dispense into 50 x 9 mm petri dishes

mPA - C

l-Lysine Hydrochloride	0.5 g
NaCl	0.5 g
Yeast Extract	0.2 g
Xylose	0.125 g
Sodium Thiosulfate	0.5 g
Sucrose	0.125 g
Lactose	0.125 g
Phenol Red	0.008 g
Mg SO4 - 7H2O	0.15 g
Ferric Ammonium Citrate	0.08 g
Agar	1.5 g

1. Suspend in 100 mL deionized water
2. Adjust the pH to 7.1 $\pm$ 0.1
3. Autoclave at 15 lbs for 15 minutes
4. Cool to 50 -60 C. and add the following dry antibiotics

0.00085 g	Kanamycin
0.0037 g	Nalidixic Acid

5. Adjust the final pH if necessary to 7.1 $\pm$ 0.1 and dispense into 50 x 9 mm petri dishes

mPA - D

l-Lysine Hydrochloride	0.5 g
NaCl	0.5 g
Yeast Extract	0.2 g
Sodium Thiosulfate	0.5 g
Phenol Red	0.008 g
Mg SO4 - 7H2O	0.15 g
Ferric Ammonium Citrate	0.08 g
Agar	1.5 g

1. Suspend in 100 mL deionized water
2. Adjust the pH to 7.1 $\pm$ 0.1
3. Autoclave at 15 lbs for 15 minutes
4. Cool to 50 -60 C. and add the following dry antibiotics

0.00085 g	Kanamycin
0.0037 g	Nalidixic Acid
0.0150 g	Actidione

5. Adjust the final pH if necessary to 7.1 $\pm$ 0.1 and dispense into 50 x 9 mm petri dishes

mPA - E

l-Lysine Hydrochloride	0.5 g
NaCl	0.5 g
Yeast Extract	0.2 g
Xylose	0.125 g
Sodium Thiosulfate	0.5 g
Phenol Red	0.008 g
Mg SO4 - 7H2O	0.15 g
Ferric Ammonium Citrate	0.08 g
Agar	1.5 g

1. Suspend in 100 mL deionized water
2. Adjust the pH to 7.1 $\pm$ 0.1
3. Autoclave at 15 lbs for 15 minutes
4. Cool to 50 -60 C. and add the following dry antibiotics

0.00085 g	Kanamycin
0.0037 g	Nalidixic Acid

5. Adjust the final pH if necessary to 7.1 $\pm$ 0.1 and dispense into 50 x 9 mm petri dishes

m - CX

Bacto Peptone	4.0 g
Potassium Sulfate	2.0 g
Xylose	1.0 g
Mg SO4 - 7H2O	0.28 g
Cetrimide	0.06 g
Bacto Agar	1.36 g

1. Suspend in 100 mL deionized water
2. Adjust the pH to 7.1 $\pm$ 0.1
3. Add 1.5 mL of Glycerol
4. Heat to boiling in a water bath
5. Autoclave at 15 lbs for 15 minutes
6. Cool to 50 - 60 C. and add the following dry antibiotics

0.00085 g	Kanamycin
0.0037 g	Nalidixic Acid

7. Adjust the final pH if necessary to 7.1 $\pm$ 0.1 and dispense into 50 x 9 mm petri dishes