

# Enhanced Accuracy of Coliform Testing in Seawater by a Modification of the Most-Probable-Number Method

BETTY H. OLSON

*Program in Social Ecology, University of California, Irvine, California 92717*

Received for publication 5 July 1978

A 1-year study of marine water sample from six beach locations showed that the most-probable-number method failed to recover significant numbers of coliforms. Modifying this method by transferring, after 48 h, presumptive negatives (growth and no gas production) to confirmed and fecal coliform media significantly improved recovery. Tests which were presumptive negative but confirmed as fecal coliform positive were designated as false negatives. Most-probable-number method false negatives occurred throughout the year, with 143 of 270 samples collected producing false negatives. More than 50% of fecal coliform false-negative isolates were *Escherichia coli*. Inclusion of false-negative tubes into the coliform most-probable-number method data resulted in increased violation of the California ocean water contact sports standard at all sites. More than 20% of the samples collected were in violation of this standard. These data indicate that modification of the most-probable-number method increases detection of coliform numbers in the marine environment.

The use of the most-probable-number (MPN) method for the assessment of indicator organisms in both potable and recreational water has proven to be a useful tool for evaluating water quality. A number of environmental factors (sunlight, bacteriophages, predators, sedimentation, toxic substances, and lack of nutrients) injure or kill coliform bacteria in seawater (4). Furthermore, salinity has been shown to be detrimental to the survival of *Escherichia coli* type I and other coliform bacteria, with aged seawater being more toxic than fresh seawater (6). Savage and Hanes (13) found that increases in biochemical oxygen demand decreased the toxicity of seawater to coliform and fecal coliform bacteria. Carlucci et al. (5) have shown that increasing concentrations of available nutrients (peptone or glucose) increased the survival of *E. coli* in seawater. The foregoing studies focused on the die-off of coliform organisms. However, because the above factors do not always result in death in the marine environment, unique problems exist which hamper the recovery of indicator organisms.

Recent work in freshwater environments has shown that injured coliforms and fecal streptococci are present (2). Recovery of these injured organisms on selective media has been shown to be poor, indicating that growth is inhibited (3). Additionally, lower recovery efficiencies of these organisms may be due to the inhibition of biochemical reactions producing typical positive results in both multiple-tube and membrane filter

assay methods. These findings indicated the need to determine whether injured coliforms are present in the marine environment. The present study examined the ability of the multiple-tube coliform test to enumerate injured coliforms. Traditional testing procedures were compared with a modification involving the transfer of presumptive tubes which showed growth but no gas production.

## MATERIALS AND METHODS

**Sample areas.** Marine water samples were collected weekly from six beaches in Orange County, Calif. from 15 September 1975 through 8 September 1976. The sampling sites are shown in Fig. 1, and descriptions of these locations are given in Table 1.

**Microbial methods.** Surface water samples were collected several feet from the shore at a water depth of 0.5 m. All samples were returned to the laboratory within 2 h of collection and tested immediately.

Volumes of 10, 1, and 0.1 ml of sample were inoculated into the presumptive medium, lauryl tryptose broth (Difco), using the five-tube most-probable-number technique. The tubes were incubated at  $35.5 \pm 0.5^\circ\text{C}$  and read at 24 and 48 h after inoculation. All presumptive tubes which were positive as defined in *Standard Methods for the Examination of Water and Wastewater* (1) were transferred into brilliant green lactose bile broth (Difco), Eijkman lactose broth, and EC broth (Difco) and incubated for 48 h at  $35.5 \pm 0.5$ ,  $45.5 \pm 0.2$ , and  $44.5 \pm 0.2^\circ\text{C}$ , respectively. In addition, a modification of the MPN method was carried out by transferring all presumptive tubes which showed growth but no gas production into brilliant green lactose bile broth and Eijkman lactose

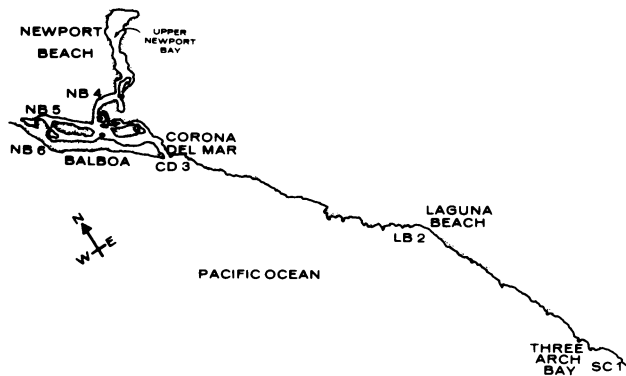


FIG. 1. Sampling locations in Orange County, Calif.

TABLE 1. Description of sampling locations

Site	Characteristics
SC 1	Open-to-ocean beach; heavy wave action; San Juan Capistrano outfall located 5 km south of the site; outfall extends 1,200 m off shore at a depth of 12 m; public beach
LB 2	Open-to-ocean beach; heavy wave action; Laguna City outfall located 750 m north of the site; outfall extends 950 m off shore at a depth of 25 m; public beach
CD 3	Protected beach; little wave action; boats anchor outside bathing area; public beach
NB 4	Protected beach; little wave action; located on main channel of upper Newport Bay; private beach
NB 5	Protected beach; little wave action; located on large channel of lower Newport Bay; boats anchor outside bathing area; private beach
NB 6	Open-to-ocean beach; heavy wave action; Orange County Santa Ana outfall located 5 km north of the site; outfall extends 8 km off shore at a depth of 67 m; public beach

broth or EC broth. The transfers were incubated for 48 h at  $35.5 \pm 0.5$ ,  $45.5 \pm 0.2$ , or  $44.5 \pm 0.2^\circ\text{C}$ , respectively. All confirmed and elevated-temperature tubes were read at 24 and 48 h. The MPN values were recorded for both the standard MPN technique and the modified MPN method. Positive scoring of the modified MPN method required growth but no gas production in the presumptive portion of the test, followed by growth and gas production in both the confirmed and elevated-temperature portions of the MPN method. Presumptive tubes which were negative by the standard MPN method and positive by the modified MPN method were classified as false negatives.

**Isolation and identification of false negatives.** Bacteria from confirmed false negatives were isolated from EC and Eijkman lactose broth (lauryl tryptose

broth negative and brilliant green lactose bile broth and EC or Eijkman lactose broth positive). The bacteria from the positive Eijkman lactose broth tubes were streaked onto eosin-methylene blue agar (Difco) and incubated at  $35.5^\circ\text{C}$  for 24 h. The predominant colony type was then selected, restreaked onto eosin-methylene blue agar, and incubated at  $35.5 \pm 0.5^\circ\text{C}$  for an additional 24 h. This process was repeated until a pure culture was obtained. The isolated bacteria were then streaked onto nutrient agar (Difco) plates and later transferred to nutrient agar slants. The bacterial isolates were stored at  $4^\circ\text{C}$  until identification by screening through the API-20 Enteric System (Analytab Products, Plainview, N.Y.).

## RESULTS

The impact which the modification of the MPN method had on total coliform counts at each sampling time is shown in Fig. 2, 3, and 4. These data indicate that incorporating false negatives into the coliform numbers increased the counts by varying orders of magnitude ( $\leq 1$  to 3 logs). The magnitude of the corrected coliform counts varied with sampling location and month of year. A slight seasonal pattern appeared, with higher corrected counts occurring during the winter months, the period of greatest rainfall. Monthly geometric means of fecal coliform counts for both the standard coliform test and the modified coliform test are shown in Table 2. These results indicate that in all cases the geometric mean is considerably increased by the inclusion of false negatives in the coliform MPN data.

However, when the Spearman rank correlation was used, no relationship was found between the occurrence of false negatives and rainfall ( $r_s = 0.107$ ;  $df = 10$ ). At all sampling locations except CD 3 and NB 6 the number of false negatives was lowest for the months of June through September. The maximum occurrence of false negatives occurred during November and December at site CD 3 and during Decem-

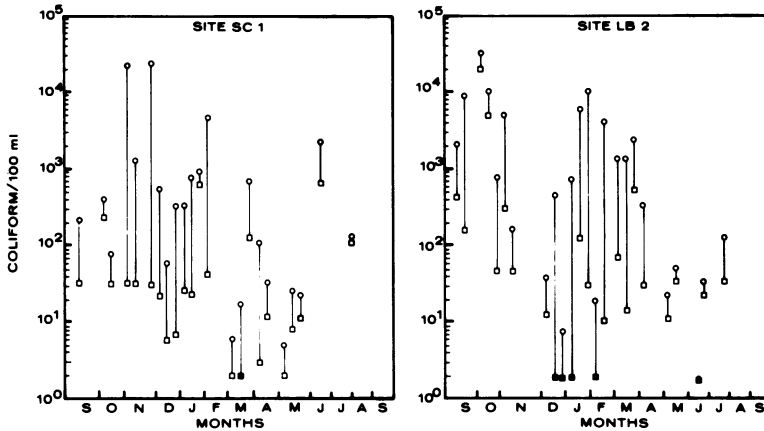


FIG. 2. Increase in MPN coliform count at sites SC 1 and LB 2 produced from the incorporation of false negatives. Symbols: □, standard MPN method; ○, modified MPN method; ■, standard MPN method <2.

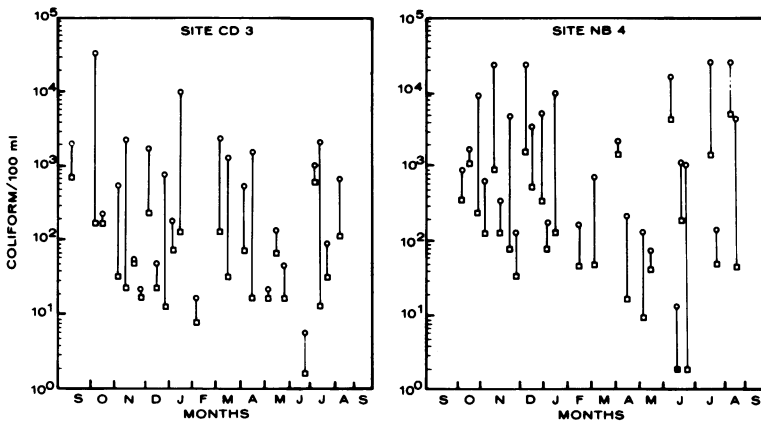


FIG. 3. Increase in MPN coliform count at sites CD 3 and NB 4 produced from the incorporation of false negatives. Symbols: □, standard MPN method; ○, modified MPN method; ■, standard MPN method <2.

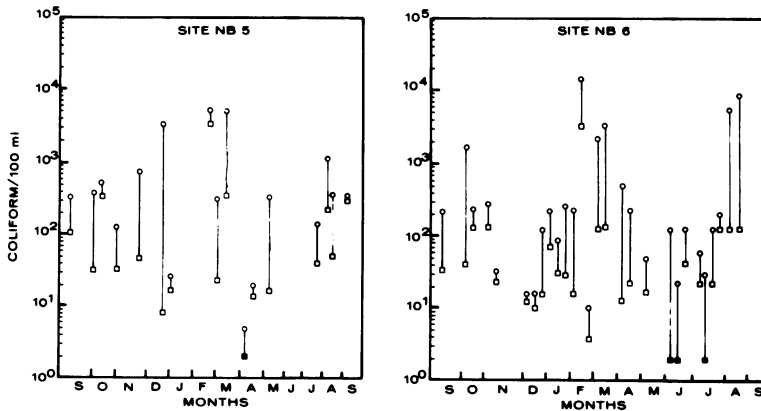


FIG. 4. Increase in MPN coliform count at sites NB 5 and NB 6 produced from the incorporation of false negatives. Symbols: □, standard MPN method; ○, modified MPN method; ■, standard MPN method <2.

TABLE 2. Monthly geometric mean of fecal coliforms from all sampling sites

Month	Method	Geometric mean of fecal coliforms (no./100 ml) at:					
		SC 1	LB 2	CD 3	NB 4	NB 5	NB 6
9/75	MPN	15.2	175.1	136.5	199.5	29.2	20.7
	Modified MPN	25.7	271.2	152.2	— <sup>a</sup>	52.9	53.5
10/75	MPN	29.5	1,258.9	189.5	237.1	42.7	68.8
	Modified MPN	36.7	3,349.7	841.4	827.0	88.9	197.6
11/75	MPN	12.2	35.5	28.2	188.4	20.3	30.9
	Modified MPN	93.6	97.7	348.7	2,238.7	28.5	41.0
12/75	MPN	36.7	3.7	41.7	670.9	184.8	13.6
	Modified MPN	1,813.2	135.9	404.3	7,703.1	501.2	34.1
1/76	MPN	46.8	40.9	21.1	10.6	9.4	25.1
	Modified MPN	96.6	1,995.3	309.0	105.9	45.2	75.0
2/76	MPN	38.9	58.4	190.5	66.8	72.0	158.5
	Modified MPN	113.9	368.7	275.4	89.1	—	562.3
3/76	MPN	8.6	100.0	251.2	266.1	76.3	56.3
	Modified MPN	31.2	758.6	473.2	530.9	473.2	86.7
4/76	MPN	4.3	177.8	19.9	264.5	125.8	10.5
	Modified MPN	20.5	225.2	100.0	1,154.8	167.9	47.7
5/76	MPN	4.3	9.2	19.9	63.1	28.2	13.6
	Modified MPN	15.9	12.6	33.4	141.3	94.4	17.7
6/76	MPN	17.1	107.9	10.6	39.8	45.9	6.2
	Modified MPN	29.4	125.9	17.8	668.3	—	50.0
7/76	MPN	7.4	21.6	37.6	251.2	18.1	19.3
	Modified MPN	7.7	31.1	199.5	707.9	24.8	97.6
8/76	MPN	16.8	6.8	19.9	508.9	71.9	70.8
	Modified MPN	—	—	43.0	541.2	223.9	179.7

<sup>a</sup>—, No false negatives occurred during monthly sample period.

ber at site NB 4 (Fig. 3). There were no seasonal differences in the distribution of false negatives at site NB 5. At NB 6 a rise in false negatives (Fig. 4) occurred from December 1975 through February 1976, and a second increase was found in June and July 1976.

The percentage of samples in which false negatives were detected during the study is shown in Table 3. False negatives were found in more than 50% of the samples taken at sample locations LB 2, CD 3, NB 4, and NB 6. Sampling location NB 5 had the lowest number of false negatives. No difference in occurrence of false negatives was found between open coastal (53.7%) and protected (52.5%) beaches.

The inclusion of false negatives increased the coliform counts by  $\leq 1$  log in 62.3% of the samples tested and by  $\leq 2$  logs in 34.6% of the samples. The data indicated that increases of  $>2$  but  $\leq 3$  logs were relatively infrequent. A mean increase

of 10.4% in the total coliform count occurred at all sites in this range, LB 2 having the maximum increase in this range (21.7%). Sampling locations LB 2 and NB 4 had the smallest increase in the range of  $>1$  but  $\leq 2$  logs but the greatest increase in the range of  $>2$  but  $\leq 3$  logs. The frequency of false negatives (Table 2) at a site had no relationship to the magnitude of increase in the coliform MPN number after the inclusion of the false negatives.

Seasonal effects on the magnitude of increase (Table 4) indicate an even distribution of samples falling into the lower ranges of  $>0$  but  $\leq 1$  and  $>1$  but  $\leq 2$  logs. However, increases of  $>2$  but  $\leq 3$  logs predominantly occurred from October 1975 through January 1976.

Isolations of bacteria from the elevated-temperature test (Table 5) indicated that *E. coli* was the major species present. *Klebsiella pneumoniae* occurred at all sites except SC 1. Other

species identified were *Proteus mirabilis*, *Serratia liquefaciens*, *Salmonella enteritidis*, and *Klebsiella ozaenae*.

Incorporation of false negatives into coliform MPN results increased violations of the ocean water contact sports standard for the state of California (Fig. 5). There was a two- to fivefold increase in violations when the modification procedure was applied. These increases in violations would have caused the closing of the beaches to swimming at LB 2, CD 3, and NB 4.

### DISCUSSION

The adverse effect of the marine environment on coliforms has been well documented and is manifested by decreased survival of coliform and fecal coliform bacteria. Bissonnette et al. (2) referred to three subpopulations of coliform bacteria: those which are able to withstand the stresses of the environment and are detected by traditional testing; those which die off; and those which can be recovered by special testing methods. The results of this study indicate that substantial proportions of the fecal coliform population in seawater fall into this latter category. These data suggest that the enzyme formic hydrogenlyase (which produces hydrogen gas from formic acid) may be impaired or not induced in environmentally stressed cells. An injury of this nature could explain the absence of gas production in the presumptive portion of the MPN test. The enzyme system appears to be repaired or

induced during this phase of the test, with gas production following in the subsequent tests (confirmed and elevated-temperature). Additional explanations for these results may be due to the inhibitory action of sodium lauryl sulfate (8) or of other microorganisms on gas production in coliforms.

The incorporation of false negatives into the coliform MPN method numbers indicated that the magnitude of increase varied among the different sampling sites. The data indicate that, even with a 1-log difference accounted for by the confidence limits about a MPN method value, a significant percentage of samples showed an increase of more than 1 log. Sites CD 3, NB 5, and NB 6 showed a 31.3 to 42.9% increase in fecal coliform counts in the range of >1 but ≤2 logs and a 0 to 4.0% increase in the range of >2 but <3 logs. Environmental factors such as temperature and rainfall appeared to have no significant influence on the occurrence of false negatives. However, variations in the detection of false negatives during the study period indicated

TABLE 3. Percentage of samples which produced false negatives

Site	No. of samples collected	% of samples producing false negatives
SC 1	45	46.6
LB 2	44	52.3
CD 3	45	62.2
NB 4	45	57.7
NB 5	45	37.8
NB 6	45	62.2

TABLE 4. Magnitude of monthly increase due to the incorporation of false-negatives into coliform numbers for all sampling locations

Month	% Increase in coliform no. in the range of:		
	>0 but ≤1 log	>1 but ≤2 logs	>2 but ≤3 logs
9/75	100.0	0.0	0.0
10/75	50.0	28.6	21.4
11/75	53.3	40.0	6.7
12/75	56.3	31.3	12.5
1/76	29.4	41.2	29.4
2/76	66.6	33.3	0.0
3/76	36.4	54.5	9.1
4/76	61.5	38.5	0.0
5/76	84.6	15.4	0.0
6/76	81.8	0.0	18.2
7/76	66.7	33.3	0.0
8/76	57.1	43.9	0.0
9/76	100.0	0.0	0.0

TABLE 5. Distribution of bacterial groups isolated from elevated-temperature tests

Site	No. of isolates	% of isolated strains						
		<i>E. coli</i>	<i>Enterobacter aerogenes</i>	<i>Enterobacter cloacae</i>	<i>Enterobacter agglomerans</i>	<i>K. pneumoniae</i>	<i>Citrobacter freundii</i>	Other
SC 1	25	72.0	— <sup>a</sup>	—	—	—	—	28.0
LB 2	37	70.2	5.4	2.7	2.7	2.7	2.7	13.5
CM 3	41	70.3	—	4.8	2.4	7.3	7.3	7.3
NB 4	28	67.8	3.5	—	—	10.7	—	17.8
NB 5	29	51.7	6.8	3.4	—	12.0	3.4	24.1
NB 6	31	83.9	—	—	—	3.2	3.2	9.6

<sup>a</sup> —, No strains isolated.

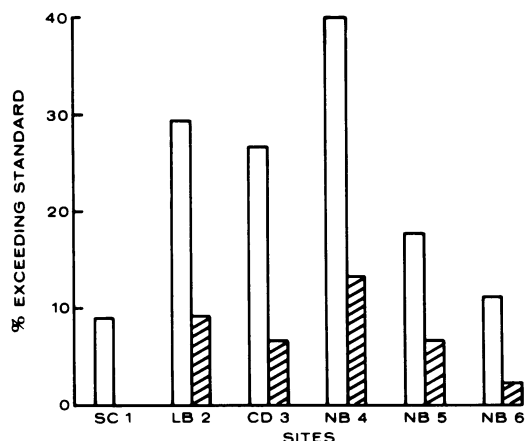


FIG. 5. Percentage of samples which violated the California ocean water contact sports standard. □, Standard MPN method; ▨, modified MPN method.

that environmental factors must affect fecal coliform survival in these areas, but the exact nature of these factors must still be elucidated. Some biological factors which promote injury or death of coliform populations in the marine environment include competition, mutations, sunlight, parasitism, predation, and antimicrobial compounds (7, 9-13). Also, the population density of the marine microflora has been shown to adversely affect coliform survival (11), as do the types of microorganisms present (12, 13). In the marine environment, biotic factors such as colloids sorbed to the surface of coliforms may protect them from predation or parasitism (11). In aquatic environments one or a combination of these factors may be present, and, therefore, variation in the occurrence of false negatives should be anticipated.

Although the true numbers of coliform bacteria present in seawater are never known, the data from this study indicate that substantial increases in coliform counts can be obtained by a modification of the present testing methods. False negatives were not found in all samples, indicating that in certain instances coliform organisms in these water samples either came from recent pollution discharge to the marine receiving waters or had been too damaged by environmental factors to be recovered by the modified method. The damaged recoverable population was present in approximately 50% of the samples at all sites except NB 5. These data indicate that this damaged subpopulation was constant and did not undergo large seasonal fluctuations.

The incorporation of false-negative data into coliform MPN method results produced an increase in the MPN method results of more than

1 log in 44.6% of the samples from all sites, but no increases greater than 3 logs. This latter finding may be due to the three inoculation volumes used in this study.

Several coliform species were predominant in the elevated-temperature isolations. These data reflect frequent isolation of *E. coli*. The damage to these isolated organisms was repaired between the atypical presumptive test result and the typical elevated-temperature and confirmed test results. This study did not determine whether more severely damaged bacteria which showed growth and no gas production in the confirmed and elevated-temperature coliform tests were fecal coliforms. If this had been done, an additional subpopulation which did not recover when standard media were used might have been identified.

These data indicated that inclusion of false-negative fecal coliforms in the MPN data increased violations of the California primary contact standard. Importantly, the increase in coliforms can be easily obtained by transferring all presumptive tubes which showed growth but no gas production to the confirmed media. This procedure will definitely increase the detection of coliform organisms in the environment. The data presented herein indicate that the isolates would be members of the coliform group. The public health significance of these damaged coliforms must still be elucidated by further study.

#### ACKNOWLEDGMENTS

I thank Donald J. Reasoner and Edwin E. Geldreich for reviewing this manuscript.

#### LITERATURE CITED

1. American Public Health Association. 1973. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Inc., New York.
2. Bissonnette, 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.
3. Bissonnette, G. D., 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.
4. Carlucci, A. F., and D. Pramer. 1959. Factors affecting the survival of bacteria in sea water. *Appl. Microbiol.* **7**:388-392.
5. Carlucci, A. F., P. V. Scarpino, and D. Pramer. 1961. Evaluation of factors affecting survival of *Escherichia coli* in sea water. V. studies with heat- and filter-sterilized sea water. *Appl. Microbiol.* **9**:400-404.
6. Gameson, A. L., and J. G. Saxon. 1967. Field studies on the effect of daylight on mortality of coliform bacteria. *Water Res.* **1**:279-295.
7. Hanes, N. B., and R. Fragala. 1967. Effect of sea water concentration on survival of indicator bacteria. *J. Water Pollut. Control Fed.* **39**:97-104.
8. Hill, M. N. 1967. Action of bile salts on bacterial cell walls. *Nature (London)* **214**:1152-1154.

9. **Johannesson, J. K.** 1957. Nature of the bactericidal agent in seawater. *Nature (London)* **180**:285-286.
10. **Mitchell, R.** 1971. Destruction of bacteria and viruses in sea water. *J. Sanit. Eng. Div. Proc. Am. Soc. Civ. Eng. SA4*:425-432.
11. **Roper, M. M., and K. G. Marshall.** 1974. Modification of the interaction between *Escherichia coli* and bacteriophage in saline sediment. *Microb. Ecol.* **1**:1-13.
12. **Roper, M. M., and K. G. Marshall.** 1977. Lysis of *Escherichia coli* by a marine myxobacter. *Microb. Ecol.* **3**:167-171.
13. **Savage, H. P., and N. B. Hanes.** 1971. Toxicity of sea water to coliform bacteria. *J. Water Pollut. Control Fed.* **43**:954-861.