APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2894–2896
0099-2240/87/122894-03$02.00/0

Vol. 53, No. 12

Recovery of a Marker Strain of *Escherichia coli* from Ozonated Water by Membrane Filtration

GORDON R. FINCH,[1] MICHAEL E. STILES,[2] AND DANIEL W. SMITH[1]*

Departments of Civil Engineering[1] and Food Science,[2] University of Alberta, Edmonton, Alberta, Canada T6G 2G7

Received 20 April 1987/Accepted 8 August 1987

Selective and nonselective growth media were evaluated at two incubation temperatures, 35 and 44.5°C, for the recovery of a nalidixic acid-resistant marker strain of *Escherichia coli* ATCC 11775 by membrane filtration from ozonated 0.05 M phosphate buffer (pH 6.9). There were significantly fewer bacteria recovered with the standard m-FC agar when compared with the same growth medium prepared without bile salts and rosolic acid. This effect was particularly noticeable at the elevated incubation temperature of 44.5°C. These findings are contrary to previous work which concluded that the standard American Public Health Association membrane filtration procedure is suitable for recovery of fecal coliform indicator bacteria from ozonated wastewater.

Recovery of stressed coliform bacteria from disinfected waters and wastewaters has been of interest because of evidence that stressed organisms do not grow well on the selective media normally used in sanitary microbiology, thereby underestimating the density of surviving organisms (2, 3, 5, 8, 9, 13). Several modifications have been proposed for improving the recovery of stressed fecal coliforms by membrane filtration (MF) including elimination of rosolic acid from m-FC agar (11), the use of a two-temperature incubation procedure (6), and the use of various resuscitation media to allow growth of injured cells (8, 12, 13).

The recovery of chlorine-stressed coliforms has been well documented (5, 6, 11, 13). One study which investigated the density estimates of ozonated coliforms by the most-probable-number and MF procedures concluded that there was no statistically significant difference between these methods (10).

The objective of this study was to determine whether the standard American Public Health Association (APHA) MF procedure (1) underestimates the postozonation density of *Escherichia coli* when compared with growth on a nonselective growth medium incubated at a noninhibitory temperature.

MATERIALS AND METHODS

Cultures. *E. coli* ATCC 11775 from the American Type Culture Collection (ATCC) was used to obtain a nalidixic acid-resistant (Nal^r) mutant strain suitable for seeding and subsequent recovery from disinfected natural waters containing other strains of *E. coli*. An overnight culture of the nalidixic acid-sensitive (Nal^s) parent strain was grown in 5 ml of tryptone soya broth (TSB; Oxoid Canada Inc., Nepean, Ontario, Canada) at 35°C and inoculated, undiluted, onto nutrient agar (NA; Oxoid) containing 50 μg of nalidixic acid per ml (NA_{50}) and incubated overnight at 35°C. Colonies were selected, subcultured in TSB, and inoculated onto NA containing 100 μg of nalidixic acid per ml (NA_{100}) to obtain a Nal^r *E. coli* strain resistant to 100 μg of nalidixic acid per ml. The parent and Nal^r strains of *E. coli* ATCC 11775 were inoculated onto API 20E strips (Analytab Products, Plainview, N.Y.) for biochemical identification. Both strains were stored on NA slants at 4°C.

* Corresponding author.

Stock ozone solution. Oxygen carrier gas containing 4.8% (wt/wt) ozone at standard temperature and pressure (273 K, 101.33 kPa) was bubbled for a minimum of 0.7 h through 400 ml of 20°C Milli-Q water (Millipore Corp., Bedford, Mass.) in a 500-ml gas absorption flask. Ozone concentration in the Milli-Q water was determined in duplicate by the iodometric method for ozone residual (1). The ozone concentration in the stock solution ranged from 20 to 23 mg/liter.

Experimental design and analysis. The nonozonated control experiment for Nal^s *E. coli* consisted of a full, two-level factorial design investigating two factors. The variables were growth medium and incubation temperature. The growth media were the standard m-FC agar (1) and a nonselective agar (TLPY) prepared with the same components as m-FC agar, but excluding bile salts no. 3 and rosolic acid. All ingredients used in the media were from Difco Laboratories (Detroit, Mich.) except sodium chloride and aniline blue indicator, which were from BDH Chemicals Canada Ltd. The two incubation temperatures were 35 and 44.5°C. The treatment combinations for the Nal^s *E. coli* control experiment are indicated in Table 1.

The control experiment for the Nal^r strain was a full, two-level factorial design examining three factors: growth medium, incubation temperature, and presence or absence of 100 μg of nalidixic acid per ml of growth medium. Medium preparation was identical to that for the Nal^s control experiment except that half of each medium contained nalidixic acid. Incubation temperatures were the same as for the Nal^s strain. The details of the treatment combinations are shown in Table 2. Postozonation recovery of the Nal^r *E. coli* strain was investigated by using the same factorial design as in the nonozonated Nal^s control experiment shown in Table 1. The growth media did not contain nalidixic acid.

The differences between treatments were analyzed by one-way analysis of variance (ANOVA) (4), using the $\log_{10}$ transformation of the observed *E. coli* densities. Residuals from the ANOVA model were examined to verify the underlying assumptions of ANOVA (4).

Procedure. The Nal^r and Nal^s strains of *E. coli* on NA slants were subcultured twice in TSB and grown for 18 h at 35°C. A 2.0-ml sample of an 18-h-old culture was centrifuged for 10 min in a benchtop centrifuge (model SPX; Ivan

TABLE 1. Treatments for recovery of the nonozonated Nal^s *E. coli* strain and for recovery of the ozonated Nal^r *E. coli* strain

Treatment	Growth medium	Incubation temp (°C)	Density of nonozonated Nal^s strain (log CFU/100 ml)
1	TLPY	35	7.19
2	m-FC	35	7.14
3	TLPY	44.5	7.14
4[a]	m-FC	44.5	7.14

[a] APHA fecal coliform method (1).

Sorvall, Inc., Norwalk, Conn.), and the resulting pellet was washed once and suspended in 2 ml of sterile ozone demand-free 0.05 M phosphate buffer. This was diluted 1:100 in sterile ozone demand-free phosphate buffer, and 10 ml of this suspension was added to 1,000 ml of ozone demand-free sterile 0.05 M phosphate buffer solution (pH 6.9) in a 2,000-ml ozone demand-free Erlenmeyer flask. The suspension of bacteria was constantly stirred with a magnetic stirrer, taking care not to create a turbulent vortex in the flask. This procedure produced a mean *E. coli* density of 1.29×10^7 CFU/100 ml.

The nonozonated control sample was taken before adding 27 ml of stock ozone solution. The mean ozone dose was 0.19 mg/liter. The solution was stirred for 60 s, at which time the remaining ozone was neutralized with an excess of sterile 1% sodium thiosulfate solution. The test solution was sampled randomly and in duplicate for each treatment in Tables 1 and 2. The samples were filtered by a standard MF procedure (1) with HAWG47 membrane filters (Millipore). The filters were placed on the appropriate growth medium, incubated in accordance with the conditions specified in Tables 1 and 2, and counted after 24 ± 2 h of incubation.

RESULTS AND DISCUSSION

Nonozonated control samples. The biochemical profiles of the Nal^s and Nal^r strains of *E. coli* determined with the API 20E strips were identical. The results for the nonozonated control experiments for the Nal^s and Nal^r strains are tabulated in Tables 1 and 2, respectively. From the data in Table 1, it can be seen that the Nal^s strain is recovered equally well with all treatment combinations. Similarly, from the data in Table 2, it is apparent that the Nal^r strain is recovered equally well with the same treatment combinations as the Nal^s strain (treatments 1 to 4). However, it was found that treatment 8 recovered significantly fewer *E. coli* than the other seven treatments ($P \leq 0.01$). It was postulated that this was the result of an antagonistic interaction between nalidixic acid and rosolic acid at the elevated temperature. This hypothesis was confirmed in a paired trial with nalidixic acid-containing m-FC agar with and without rosolic acid. It was found that the m-FC agar with rosolic acid supported the growth of significantly fewer colonies ($P \leq 0.05$). Consequently, nalidixic acid was not used in growth media during the ozonation trials.

TABLE 2. Treatments for recovery of the nonozonated Nal^r *E. coli* strain from phosphate buffer

Treatment	Growth medium	Incubation temp (°C)	Nalidixic acid in medium	Density of Nal^r *E. coli* (log CFU/100 ml)	
				Run 1	Run 2
1	TLPY	35	No	7.11	7.15
2	m-FC	35	No	7.08	7.04
3	TLPY	44.5	No	7.15	7.15
4[a]	m-FC	44.5	No	7.08	7.04
5	TLPY	35	Yes	7.11	7.11
6	m-FC	35	Yes	7.11	7.28
7	TLPY	44.5	Yes	7.11	7.08
8	m-FC	44.5	Yes	6.45	6.67

[a] APHA fecal coliform method (1).

TABLE 3. Postozonation Nal^r *E. coli* densities determined by the four treatments

Run	*E. coli* density (log CFU/100 ml)			
	Treatment 1 (TLPY, 35°C)	Treatment 2 (m-FC, 35°C)	Treatment 3 (TLPY, 44.5°C)	Treatment 4 (m-FC, 44.5°C)[a]
1	1.99	1.86	1.94	1.54
2	1.94	1.79	1.48	1.48
3		1.00	1.53	0.95
4	1.93	1.65	1.83	1.42
5	2.18	2.11	2.26	2.08
6	1.82	1.45	1.68	1.20
7	2.18	2.08	2.30	2.04
$\bar{x}$	2.01	1.71	1.86	1.53
SEM	0.0586	0.147	0.124	0.156

[a] APHA fecal coliform method (1).

Post-ozonation recovery. Seven ozonation trials were performed, and Nal^r *E. coli* was recovered by the four treatments in Table 1. The observed densities for each treatment are tabulated in Table 3. ANOVA of the results (Table 4) indicated that there was a difference between treatments ($P \geq 0.90$). Examination of residuals from the ANOVA confirmed that the assumptions underlying the procedure were valid. Detailed investigation of the differences between individual treatments was done by a graphical technique described by Box et al. (4). It was found that there was a highly significant ($P \leq 0.01$) difference between the treatment pairs 1 and 4, 1 and 2, and 3 and 4. A less significant ($P \leq 0.05$) difference was observed between treatment pair 2 and 4. No significant difference was observed between treatment pairs 1 and 3 and 2 and 3.

Treatment 4 is the standard APHA method for enumerating fecal coliforms. The method uses an agar incorporating bile salts and rosolic acid and an elevated incubation temperature to inhibit nonfecal organisms. As shown elsewhere (9), bile salts inhibit the recovery of stressed coliform bacteria. The difference between treatments 3 and 4 is probably caused by the bile salts. Also, a marked temperature inhibition is apparent when comparing treatments 2 and 4, in which incubation temperature is the only difference between treatments. The m-FC agar incubated at the elevated temperature supported the growth of fewer colonies. In contrast, treatments 1 and 3 used the nonselective medium and had no detectable difference in recovery of bacteria between the reduced and elevated temperature

TABLE 4. ANOVA of the observed postozonation densities of Nal^r *E. coli*

Source	df	Sum of squares	Mean square	F-statistic
Between treatments	3	0.819	0.273	2.35[a]
Within treatments	23	2.67	0.116	
Total	26	3.49		

[a] $P \leq 0.10$.

treatments. Both treatments recovered significantly more bacteria than the standard APHA method.

Examination of the individual treatment means and their standard errors (Table 3) suggests that the most precise estimate of the mean *E. coli* density was obtained with the nonselective medium at the noninhibitory temperature (treatment 1) followed by the same medium at the elevated temperature (treatment 3). The least precise estimate of the mean density was achieved by the standard APHA method (treatment 4). This observation was consistent with the findings of others who postulated that the recovery of stressed coliforms by standard methods may contribute to a reduction in the precision of the MF method (7).

The results reported here indicate that the standard MF method for enumerating fecal coliform bacteria in ozone-disinfected water may underestimate the bacterial density. Fecal coliform bacteria are the most commonly used indicator organisms for measuring the performance of wastewater disinfection processes, for both research and regulatory purposes. Therefore, it is necessary that the significance of underestimating fecal coliform indicator bacteria in disinfected waters be understood before using the standard MF procedure.

ACKNOWLEDGMENTS

We are indebted to Beverly Uibel for her technical assistance.

This work was supported by the Alberta Environmental Research Trust (grant T0937) and the Natural Sciences and Engineering Research Council of Canada (grant A1010).

LITERATURE CITED

1. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stress on the enumeration of indicator bacteria in natural waters. Appl. Microbiol. **29**:186–194.
3. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1977. Evaluation of recovery methods to detect coliforms in water. Appl. Environ. Microbiol. **33**:590–595.
4. **Box, G. E. P., W. G. Hunter, and J. S. Hunter.** 1978. Statistics for experimenters. John Wiley & Sons, Inc., New York.
5. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. **37**:633–641.
6. **Green, B. L., E. M. Clausen, and W. Litsky.** 1977. Two-temperature membrane filter method for enumerating fecal coliform bacteria from chlorinated effluents. Appl. Environ. Microbiol. **33**:1259–1264.
7. **Haas, C. N., and B. Heller.** 1986. Statistics of enumerating total coliforms in water samples by membrane filter procedures. Water Res. **20**:525–530.
8. **Lin, S. D.** 1976. Membrane filter method for recovery of fecal coliforms in chlorinated sewage effluents. Appl. Environ. Microbiol. **32**:547–552.
9. **McFeters, G. A., S. C. Cameron, and M. W. LeChevallier.** 1982. Influence of diluents, media, and membrane filters on detection of injured waterborne coliform bacteria. Appl. Environ. Microbiol. **43**:97–103.
10. **Meckes, M. C., and A. D. Venosa.** 1978. Comparison of MPN and MF techniques of enumerating coliform bacteria in ozonated wastewater effluent, p. 136–143. *In* A. D. Venosa (ed.), Progress in wastewater disinfection technology. EPA-600/9-79-018. Environmental Protection Agency, Cincinnati.
11. **Presswood, W. G., and D. K. Strong.** 1978. Modification of M-FC medium by eliminating rosolic acid. Appl. Environ. Microbiol. **36**:90–94.
12. **Rose, R. E., E. E. Geldreich, and W. Litsky.** 1975. Improved membrane filtration method for fecal coliform analysis. Appl. Microbiol. **29**:532–536.
13. **Stuart, D. G., G. A. McFeters, and J. E. Schillinger.** 1977. Membrane filter technique for the quantification of stressed fecal coliforms in the aquatic environment. Appl. Environ. Microbiol. **34**:42–46.