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Comparison of Methods for Enumeration of Selected Coliforms Exposed to Ozone

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mT7 medium performed no better than m-Endo medium in enumerating cells of *Escherichia coli* and *Citrobacter freundii* exposed to ozone. Also, there was no difference in the plate count of heterotrophic bacteria in ozonated raw water determined on modified Henrici agar or R2A agar. Statistically significant differences were seen between bacteria and the type of water in which they were suspended during ozonation.

The use of ozone in water treatment is widespread in Europe and is now receiving considerable attention in the United States (11). It has been known for many years that ozone can be used to kill *Escherichia coli* (7, 8, 9, 17). Furthermore, it has been determined that a threshold concentration of 0.19 mg of ozone per liter is necessary to kill *E. coli* (2). Also, the effect of ozone on *E. coli* at 1°C has been studied (5). It has been shown that a longer contact time is necessary to kill *E. coli* suspended in secondary effluent from a wastewater treatment plant than *E. coli* suspended in phosphate-buffered saline (3). Moreover, it has been determined that growing cells of *E. coli* are more resistant to ozone than are nongrowing cells (18).

There has been concern that standard methods for enumerating coliform bacteria exposed to disinfectants such as chlorine may not recover stressed cells (12). A comparison of most probable number methodology with membrane filter methodology for the enumeration of coliform bacteria in ozonated effluents from a wastewater treatment plant found no statistically significant differences between methodologies, indicating that cells are killed and not stressed by ozone (12). In contrast, it has been shown that fewer cells of *E. coli* are recovered on selective media than on nonselective media when the cells were ozonated in phosphate buffer (6). Also, it has been shown that there is no significant difference in the sensitivity to ozone of an encapsulated or a nonencapsulated *Klebsiella aerogenes* (4).

The purpose of this study was to compare a method with a medium designed to maximize the recovery of chlorinated coliforms in drinking water (10) with the standard membrane filter method (1) for their efficiencies in recovering ozonated cells of *E. coli* and *Citrobacter freundii*. Also, different water sources, different points in the water treatment plant, and heterotrophic bacteria were studied.

MATERIALS AND METHODS

E. coli and *C. freundii* were obtained from the stock culture collection of the Department of Molecular Biology, University of Wyoming. These bacteria were grown overnight in nutrient broth at 35°C, harvested by centrifugation at 5,000 × *g*, washed twice, and suspended to 10 Klett units (Klett-Summerson photoelectric colorimeter; number 66 filter) in sterile distilled water. A total of 120 ml of the cell suspension was added to 12 liters of the appropriate water to be ozonated. This resulted in water which had approximately 100 to 1,000 cells of the desired organism per ml and was chosen to simulate conditions that are more representative of those of a variety of natural raw waters. Waters which were studied were spring water with a temperature of 9°C, raw water with temperatures of 2 to 5°C and 8 to 9°C, settled water, and filtered water at the Laramie, Wyo., surface water treatment plant. Surviving cells were enumerated by standard membrane filter coliform analysis procedures by using m-Endo and mT7 media (1, 10). We used Millipore HC type filters (Millipore Corp., Bedford, Mass.). Incubation was at 35°C for 24 h. Each experiment was repeated twice.

Twelve liters of raw water was ozonated, to determine the effect of medium formulation on the recovery of heterotrophic bacteria. Samples were plated onto modified Henrici agar and R2A agar (13, 15). Samples were processed by spread plate and membrane filter methodologies. Millipore HC filters were used. Incubation was at 20°C for 7 days. Each experiment was repeated 3 times.

Ozonation was carried out in batch culture, with stirring done with a magnetic stirrer and circulation done through a pump. Samples were taken at various times, and the residual ozone was removed by the presence of excess sodium thiosulfate. Ozone was generated by using an ozonator (model 035P19-0; Ozone Research and Equipment Corporation, Phoenix, Ariz.). Ozonation was done at a constant gas flow rate and ampere setting. Residual ozone was determined by the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) method of the Hach Co. (Procedures for water and wastewater analysis, p. 2–85, Hach Co., Loveland, Colo.).

Data were analyzed by analysis of variance and the Duncan new multiple range tests (16).

RESULTS AND DISCUSSION

The results of ozonation of raw water samples on the plate counts of heterotrophic bacteria are presented in Fig. 1. There was no statistically significant difference between the results obtained with the two media. Modified Henrici agar is a general all-purpose medium which has been shown to yield higher counts than plate count agar in water samples taken from mountain streams around Laramie, Wyo. (14). R2A medium was developed to determine the plate count of heterotrophic bacteria in water (13). There was a 99.9% reduction in the number of viable cells of heterotrophic bacteria after 20 min of ozonation.

The results of ozonating *E. coli* in different water samples

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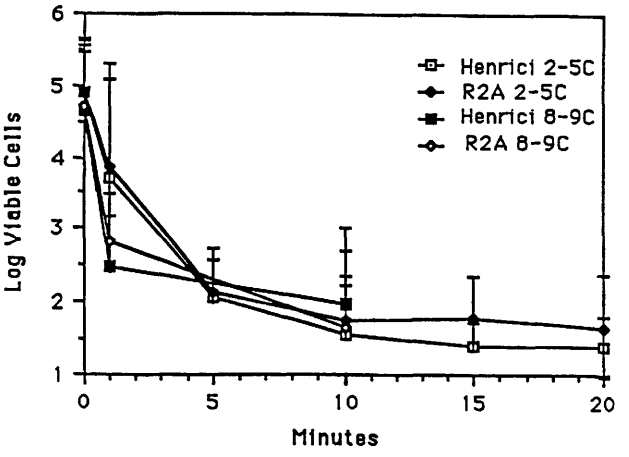

FIG. 1. Comparison of modified Henrici agar with R2A agar for the recovery of ozonated heterotrophic bacteria by the plate count method. Numbers in the legend indicate the temperature, in degrees Celsius.

are given in Fig. 2. There were no statistically significant differences between the results obtained with the two media. *E. coli* survived ozonation better in settled water than it did in any of the other water types ($P = 0.05$). There was at least a 98% reduction in viable cells after 20 min of exposure to ozone.

The results of exposing *C. freundii* to ozone in various water samples are given in Fig. 3. Again, there were no

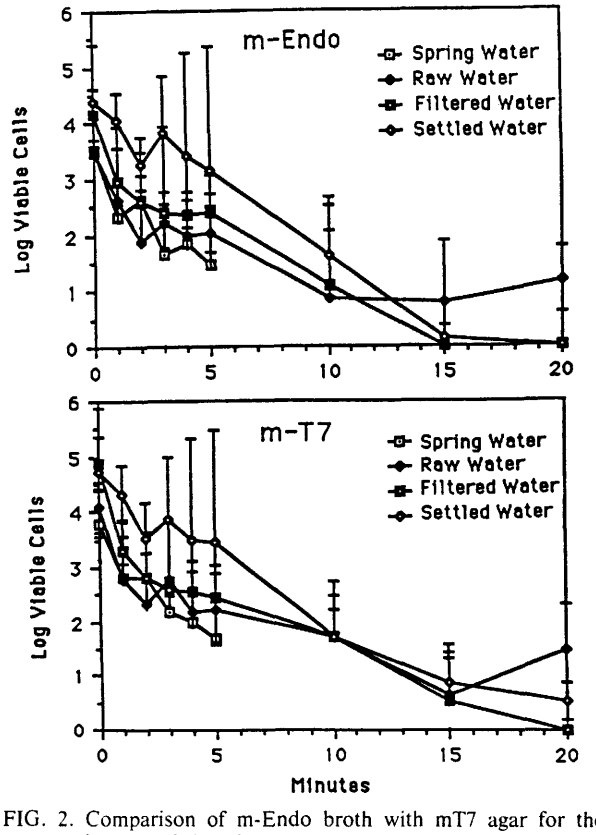

FIG. 2. Comparison of m-Endo broth with mT7 agar for the recovery of ozonated *E. coli*.

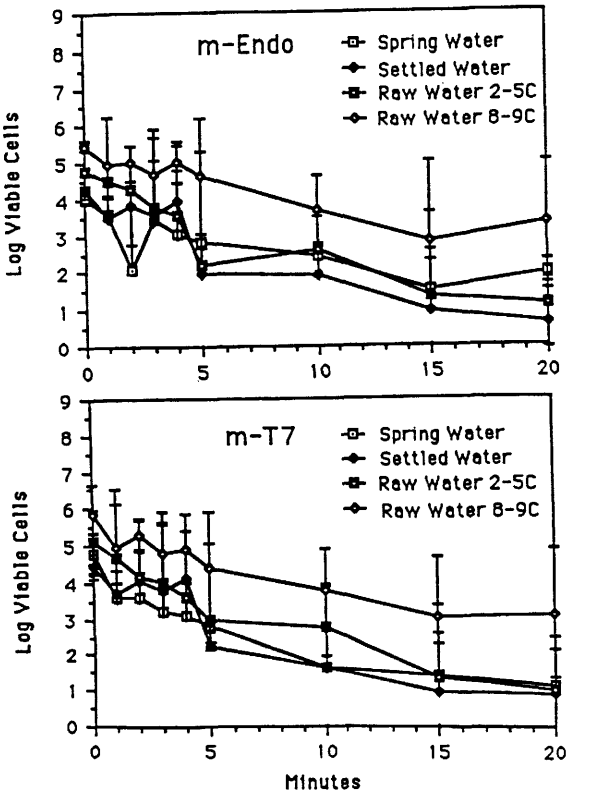

FIG. 3. Comparison of m-Endo broth with mT7 agar for the recovery of ozonated *C. freundii*. Numbers in the legend indicate the temperature, in degrees Celsius.

significant differences between the results obtained with the two media in any water sample or under any condition. *C. freundii* survived ozonation better in raw water than it did in spring or settled water (alpha = 0.05). Also, the survival in raw water was better at 8 to 9°C than it was at 2 to 5°C (alpha = 0.05). There was at least a 99% reduction in viable cells after 20 min of exposure to ozone.

When comparisons between the two organisms were made, it was found that *C. freundii* survived ozonation for 5 min in spring water better than did *E. coli* (alpha = 0.01). The comparison of the two bacteria in raw and settled water samples that were ozonated for 20 min indicated that there was no difference in results between *C. freundii* in raw water and *E. coli* in settled water, both of which were significantly different from those of *C. freundii* in settled water or *E. coli* in raw water (alpha = 0.01). Results for *C. freundii* in settled water and *E. coli* in raw water were not different from each other. Thus, it appears that the effectiveness of ozone in the killing of coliforms in drinking water depends on the specific identity of the coliforms and the location(s) of ozone application in a water treatment plant.

A slight increase in the numbers of viable cells was seen for both organisms between 2 and 4 min of exposure to ozone. The reason for this is unclear; however, it could be speculated that clumps of cells that formed during cell suspension preparation were broken apart during this time. No work was done to try to explain this observation.

The residual ozone in these experiments ranged from 0.1 to 1.0 mg/liter, with most exposure being in the 0.3- to 0.6-mg/liter range. Generally, increasing ozone concentra-

tions were seen near the end of the experiments, when the ozone demand was met.

These results indicate that mT7 medium is no better than m-Endo medium for enumerating coliforms in ozonated water. Additional work is needed to compare other methods that have been designed to recover injured cells.

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