

## Effect of Physical Parameters on the In Situ Survival of *Escherichia coli* MC-6 in an Estuarine Environment

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Received for publication 29 April 1975

Survival of *Escherichia coli* MC-6 of fecal origin in an estuarine environment as affected by time, water temperature, dissolved oxygen, salinity, and montmorillonite in diffusion chambers has been elucidated. Several in situ physical parameters were recorded simultaneously, and viable cell numbers were estimated. The survival of the bacteria varied seasonally. Montmorillonite addition extended the time needed for a 50% reduction of the viable cell population ( $t_{1/2}$ ) of cells by 40% over the  $t_{1/2}$  of cells in Rhode River water alone. The effect of this clay was not significantly greater between 50- to 1,000- $\mu\text{g/ml}$  montmorillonite concentrations. In all experiments, the relationships among pairs of variables were studied by regression and correlation analysis. The slope between viable cell numbers and water temperatures increased about 50% for each 10 C increment in temperature and gave a correlation coefficient  $r = 0.617$ , significant at 95% confidence level. A similar correlation coefficient,  $r = 0.670$ , was obtained between water temperature and  $t_{1/2}$  of the initial cell population. In all experiments regressions were performed considering all variables after bacteria had been in the Rhode River environment for 3 days. Coefficient of multiple determination was estimated as  $R^2 = 0.756$ . Approximately 75.6% of the variance of viable cell numbers can be explained by variation in water temperature, dissolved oxygen, and salinity. Simple correlation coefficients within the regression steps were also computed. Survival of bacteria was closely and negatively correlated with increasing water temperature ( $r = -0.717$ ). It is suggested that water temperature is the most important factor in predicting fecal coliform survival from point and nonpoint sources in assessing water quality in an estuarine ecosystem.

The quantity of coliform bacteria entering natural waters has increased dramatically in recent years (5, 15). Although natural waters have a strong inherent capacity to purify themselves, this process involves unknown biological and chemical antagonists in seawater and the protective action of biological colloids (15). The complex interactions between biological, physical, and chemical parameters of the water exert variations in the survival of coliform bacteria of different environments. Sanitary microbiologists to date have been concerned with the factors that affect the persistence of fecal coliform bacteria in recreational waters (5). The survival of coliform bacteria is affected by the complex interactions between dissolved nutrients (6, 15), organic matter (18), antibiotics (6, 11), lysis (17), heavy metals (9), competition for nutrients with marine bacteria (6, 15), predation by protozoa (6, 15, 19), algal toxins (17, 23), degradation of the bacterial cell wall by protozoa (15), seasonal variations (24), bactericidal action of seawater (3, 4, 19), temperature, and

the physicochemical nature of the marine environment (9, 18, 19).

Clay minerals exert a marked influence on the activity, ecology, and population dynamics of microorganisms in natural habitats, especially in soil (12, 14). Clay minerals are also present in large quantities in estuaries. Adsorption and sedimentation of bacteria to clays have been described (6, 18, 20, 25). Clay minerals, colloids, and organic matter may form a protective envelope around *Escherichia coli* cells (14). Montmorillonite protected *E. coli* cells from phage attack in marine sediments (21) and enhanced *E. coli* survival in seawater (1).

The Rhode River estuary consists of 3,200 hectares of rural watershed and 6.7 km of waterway. *E. coli* enters the Rhode River estuary from the watershed in great numbers. The seriousness of fecal pollution from rural sources greatly depends on the survival of the bacteria in the estuary. Therefore, the purpose of this investigation was to study the survival of *E. coli* MC-6 in diffusion chambers in situ and to

expose the bacteria to some natural physical and chemical conditions.

### MATERIALS AND METHODS

**Bacteria and media.** The bacterium used in this study was isolated from the Muddy Creek within the Rhode River estuary. It was identified as *E. coli* by growth on brilliant green lactose bile broth, characteristic colonies on eosin methylene blue agar, growth and gas production in 24 h in EC broth at 44.5 C and indole, methyl red, Voges-Proskauer, citrate reactions of  $^{++}---$ . This bacterium was designated as *E. coli* MC-6.

All cultures used in these experiments were grown in lactose broth (Difco) for 24 h at 37 C. Cells were harvested by centrifugation ( $3,000 \times g$ ) for 15 min and washed twice with sterile 0.01 M phosphate buffer, pH 7.0. After the final wash, cells were resuspended and diluted in sterile phosphate buffer (0.01 M) to the desired population density, using a Beckman DU spectrophotometer at 600-nm wavelength.

**Chambers.** Diffusion chambers were purchased from their designers, McFeters and Stuart (13). They are composed of three pieces of plexiglass held together with six nuts and bolts. Membrane filters (Millipore Corp.) of 0.45- $\mu$ m pore size were used to enclose the bacterial suspension, creating a chamber bordered by plexiglass and two filters. Before use, the plexiglass pieces were washed thoroughly. The filters were cut to 7.5-cm diameter. The plexiglass pieces and filters were then exposed to ultraviolet light (Ultra-Violet Products, Inc., San Gabriel, Calif.) for 30 min and the chambers were assembled aseptically. Each chamber was filled with 20 ml of cell suspension. Three to five chambers were used in each treatment.

**Survival experiments.** Each chamber was inoculated with 20 ml of the washed cell suspension ( $10^7$  to  $10^8$  cells/ml) in sterile phosphate buffer (0.01 M) and was placed in a plastic, polyethylene container punched with numerous holes to allow free movement of the river water and to provide some protection for the chamber. Each chamber was suspended from the lid of the container with a stainless-steel wire. Elastic clamps held the top and the bottom of each outer container together. The entire assembly was lowered into the Rhode River and incubated 1 m below the surface.

Samples were taken daily for the first 4 days and on the 6th and/or 7th day. Chambers were removed from the water and placed in a beaker of Rhode River water so that the chamber was filled to the original level. One-tenth milliliter of sample was removed from each chamber at each sampling after mixing the contents of the chamber with a sterile syringe. Samples were diluted with 0.01 M sterile phosphate buffer. Petri plates containing endo agar or eosin methylene blue agar and the spread plate inoculation technique were used for the enumeration of bacteria. Plates were incubated at 37 C for 18 h.

Survival rate of *E. coli* MC-6, computed as log number of viable cells versus time, water temperature, dissolved oxygen (DO), and salinity, and a loss

rate were obtained. Slopes were calculated by means of a linear least-squares regression model. Survival of *E. coli* MC-6 was monitored from May to November 1974.

**Temperature experiment.** Temperature was controlled in some experiments by using a Hotpack water bath (Hotpack Co., Philadelphia, Pa.). The water bath was placed on the dock and water was circulated through the tubing of a small Masterflex pump to bring the Rhode River water 10 ft (about 3 m) up into a container of the water bath that enclosed the dialysis chambers. Throughout the experiment the river water was pumped constantly (2 ml/min) into the bottom of the container of the water bath and overflowed through a hose at the top. The temperature of the water was cooled within the water bath to the desired temperature.

**Particulate experiment.** Dried, powdered montmorillonite (Wyoming bentonite) and illite (Illinois illite) were used to test whether the particulates that are abundant in the sediments of Rhode River affected the survival of *E. coli* MC-6. A known weight of the clay was thoroughly mixed with a known volume of cell suspension in sterile phosphate buffer (0.01 M) ( $10^7$  to  $10^8$  cells/ml), and the chambers were inoculated as described previously.

**Filters.** Millipore membrane sheets (HAWP 304 FO, 0.45- $\mu$ m pore size, Millipore Corp., Bedford, Mass.) were used in most experiments. The above filter has a tear-resistant microweb support. Nuclepore filters of 0.45- $\mu$ m porosity and 9.0-cm diameter were also tested (Nuclepore Corp., Pleasanton, Calif.). These filters have a smooth surface relative to the microweb Millipore membranes. It was indicated in the literature (2) that various filters affect the survival of bacteria differently. Since filters were an essential part of the diffusion chambers, the effects of Nuclepore and Millipore filters on the survival of *E. coli* MC-6 were compared. No difference was obtained in the viable cells remaining in the chambers containing either Nuclepore or Millipore filters after 7 days of incubation in the Rhode River.

**Data analysis.** In the present study we measured simultaneously three ecological variables in the Rhode River. Two statistical analyses were performed on all the data. (i) Two-variable linear regression, based on the relationship between two variables by using 7-day observations for each variable by a linear least-squares regression, and a correlation coefficient ( $r$ ) for each pair of variables were calculated. (ii) Multiple linear regression was also used to investigate the combined effects of several variables on *E. coli* MC-6 survival. The following independent variables were used: water temperature, DO, and salinity. The dependent variable ( $y$ ) was expressed as the logarithm of the number of viable bacteria estimated by plate counts. The multiple linear regression analysis was performed in a stepwise fashion after cells had been in the Rhode River for 3 days by using independent variables in the order of importance from most to least important. The multiple correlation coefficient ( $R$ ), which measures the overall degree of association between  $y$  and all the independent variables, was computed. The regression and correlation analyses were con-

ducted by using a Hewlett-Packard 9810 A calculator.

## RESULTS

**Seasonal effect.** *E. coli* MC-6 survival in the Rhode River in May, July, and November is illustrated in Fig. 1. The survival of *E. coli* MC-6 was about the same in November as in May, and the survival was the shortest in July. When washed cells of *E. coli* MC-6 were exposed to estuarine water and counts were made daily, the survival curves obtained showed no initial lag phase. In May a onefold increase in bacterial numbers occurred during the first 24-h period. The survival curves were sigmoid in shape in all experiments. The physical parameters of the water also changed with the seasons (Table 1). Temperature of water was highest in July, intermediate in May, and lowest in November. The DO, however, was highest in November and similar in May and July. The salinity of the water was highest in November and lowest in May. Because the physical characteristics of the water obviously had some effect on the survival of *E. coli* MC-6, these effects were assessed separately.

**Temperature.** The effect of temperature on *E. coli* MC-6 survival in situ was examined (Fig. 2). During the course of the study, the temperature ranged between 5 and 30 C. However, the water temperature on any one day varied only by 1 to 2 C. This variation remained about the same throughout a given 7-day experiment. The temperature of the water strongly affected the survival of *E. coli* MC-6. A linear relationship existed between survival rate of bacteria and water temperature. The slope of decline in viable cell numbers was calculated for all experiments, including those where the temperature of the water was controlled (Fig. 2a). Cell survival and water temperature values gave a correlation coefficient of  $r = 0.617$ . The value of the slope increased

about 50% for each 10 C water temperature increment, indicating that the rate of cell mortality was strongly affected by temperature.

The temperature effect on *E. coli* MC-6 survival was also expressed as the time (hours) needed for a 50% reduction of the viable cell population ( $t_{1/2}$ ) between temperature ranges of 5 and 30 C and was plotted in Fig. 2b. The  $t_{1/2}$  was estimated for each temperature value. The  $t_{1/2}$  at 5 C was 33.6 h, at 10 C 27.6 h, at 20 C 22.1

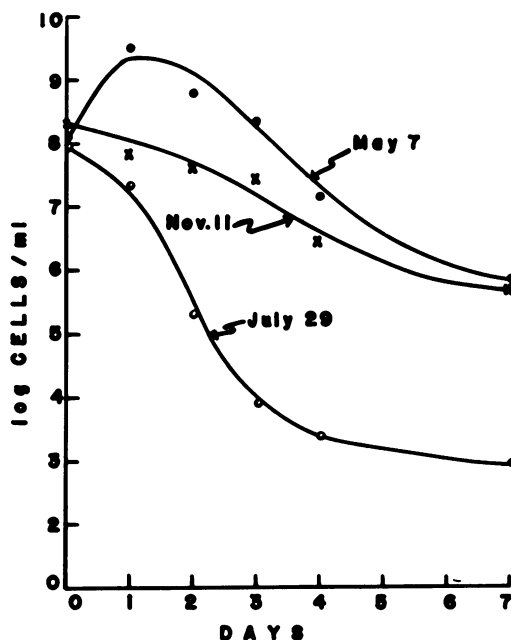


FIG. 1. Seasonal change in the survival rate of *E. coli* MC-6 plotted against time at the Rhode River sampling station at a depth of 1 m. Each point represents the arithmetic mean of log viable cell number of three to five chambers. Survival of bacterial numbers of selected experiments are: closed circles (7-13 May), crosses (11-18 November) and open circles (29 July-3 August).

TABLE 1. Physical parameters of the water at designated times of the year<sup>a</sup>

Date of 1974	Temp (C)		Dissolved oxygen				Salinity (‰)	
			mg/liter		Saturation (%)			
	Min-imum	Max-imum	Min-imum	Max-imum	Min-imum	Max-imum	Min-imum	Max-imum
7-13 May	16.0	17.5	4.8	6.1	51	66	5.2	5.6
29 July-3 Aug.	27.5	29.3	3.7	5.5	49	77	9.3	10.4
11-18 Nov.	8.0	9.7	10.3	10.8	95	104	13.8	14.1

<sup>a</sup> Data were collected by the Geological Survey's water quality monitor located in the Rhode River at the Smithsonian Institution's pier. Data as presented consist of daily maximum and minimum values summarized by week to give weekly averages and extremes.

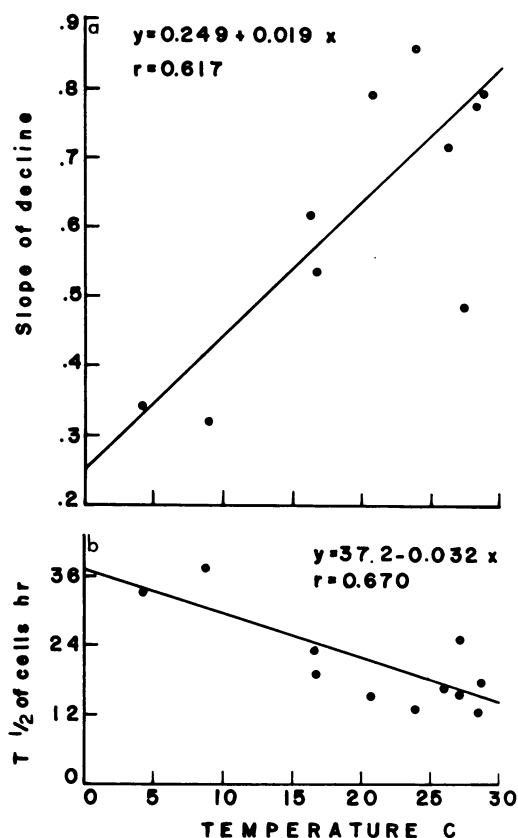


FIG. 2. Effect of temperature (5–30 C) on the survival of *E. coli* MC-6 cells. Correlation between average viable cell numbers and water temperatures throughout a 7-day period of all experiments was estimated by regression calculation. (a) The slope of decline in viable cell numbers affected by each water temperature value was estimated and plotted against the temperatures. A linear least-squares regression calculation gave  $y = 0.249 + 0.019x$  and a positive correlation of  $r = 0.617$ . (b)  $t_{1/2}$  at each temperature was estimated and plotted against the temperature. A linear least-squares regression calculation gave  $y = 37.2 - 0.032x$  and an inversely proportional correlation of  $r = 0.670$ . (Values were obtained for temperatures lower than 17 C and are from artificially cooled water samples.)

h, and at 30 C 14.4 h. An inversely proportional linear relationship existed between the  $t_{1/2}$  of bacteria and water temperature. The correlation coefficient between the  $t_{1/2}$  of bacterial survival and water temperatures was  $r = 0.670$ . The two correlation coefficients were very similar and both were significant at 95% confidence level.

**DO.** The effect of DO levels of the water on survival of *E. coli* MC-6 in situ was also examined. The DO levels of the water ranged between 1.4 and 10.8 mg/liter, with lower DO

values occurring during the summer months and higher values during November. The minimum and maximum DO concentrations of the water of selected experiments are presented in Table 1. DO levels of the water varied between 1 and 2 mg/liter throughout a 7-day experiment in the summer, and DO concentrations remained relatively stable in the fall, varying by only 0.5 mg/liter. Survival of cells was directly proportional to DO concentration of the water. In some experiments viable cell numbers declined from  $10^8$  to  $10^4$  cells/ml below 4 mg of DO/liter after 3 days in the Rhode River water, whereas the decline in viable cell numbers was very small at the higher DO concentrations during the same period of time.

**Salinity.** An apparent inverse relationship existed between survival of *E. coli* MC-6 and salinity concentrations of the water. At low salinity levels the rate of decline in cell numbers was low, and viable cell numbers of *E. coli* MC-6 changed little after 3 days in the chambers. At higher salinity the rate of decline was more pronounced, and the number of viable cells decreased from  $10^8$  to  $10^4$  cell/ml in 3 days. Salinity values of the Rhode River water of selected experiments are presented in Table 1. In these experiments the salinity levels were 5.2 to 5.6‰ in May, 9.3 to 10.4‰ in July, and 13.8 to 14.1‰ in November. The calculation of salinity effect on cell survival was obscured by changing water temperature, which had a strong effect on survival.

**Sediment.** The effect of montmorillonite on the survival of *E. coli* MC-6 in estuarine water was also tested, in the presence and absence of this clay, in chambers containing washed cell suspensions. Table 2 shows the effect of montmorillonite concentrations on the survival of these bacteria. Montmorillonite at 50  $\mu\text{g/ml}$  extended the  $t_{1/2}$  of these organisms by 40% over the  $t_{1/2}$  of the cells in Rhode River water alone. The effect of montmorillonite was not significantly better at 500- and 1,000- $\mu\text{g/ml}$  concentrations. Because the number of chambers availa-

TABLE 2. Effect of montmorillonite on survival of *E. coli* MC-6 in Rhode River estuarine water

Montmorillonite concn ( $\mu\text{g/ml}$ )	$t_{1/2}$ (days)	Increase over control $t_{1/2}$		Avg temp (C)
		Days	%	
50	0.87	0.25	40	20.5
0	0.62			
500	0.98	0.26	36	25.9
0	0.72			
1,000	1.05	0.38	56	27.1
0	0.67			

ble limited our experimental design, the various montmorillonite concentrations were run in consecutive weeks to allow three replications for each montmorillonite concentration and of the controls. The temperature, DO, and salinity levels of the Rhode River water were somewhat different during these experiments. The water temperature was 7 C lower (20.5 C) at the lowest montmorillonite concentration tested, as compared with high concentrations when water temperature was warmer (26 to 27 C) (Table 2). The DO levels also varied from 1.4 to 3.8 mg of DO/liter at 50  $\mu$ g of montmorillonite/ml and from 4.7 to 14.0 mg of DO/liter at the higher clay concentrations. The salinity of the water was similar in all three experiments, giving minimum (9.9%) and maximum (11.4%) salinity levels (not shown). The fact that no differences due to high montmorillonite were observed could be obscured by the changes in water temperature, DO, and salinity levels among these experiments. The average natural concentration of montmorillonite in the Rhode River is in fact about 50  $\mu$ g/ml (Jack W. Pierce, personnel communication), so the protection provided to the bacteria by the montmorillonite concentration used in the dialysis chamber experiments may be highly relevant.

**Data analysis.** All simultaneously collected data on *E. coli* MC-6 survival were statistically analyzed by using log viable cell numbers as the dependent variable and time, water temperature, DO, and salinity as independent variables. The relationship between two variables was estimated by using 7-day observations for each variable by the two-variable linear regression analysis of each experiment. Correlation coefficients between all pairs of variables were computed. The analysis revealed that approximately 70% of the variance in viable cell numbers was explained by the length of time ( $r^2 = 0.701$ ). Among the pairs of variables tested, time appeared to affect bacterial survival the most. The simple correlation coefficients between viable cell numbers and water temperature, DO, and salinity were relatively low.

The data were also analyzed by multiple linear regression and correlation to establish linear relations and correlations among more than two variables. The independent variables were selected in order of water temperature, DO, and salinity. Regressions were performed by using data points of physical parameters and viable cell numbers of *E. coli* MC-6 at the Rhode River after 3 days. By doing so, time was excluded as an independent variable affecting bacterial survival in order to assess the effects of the other three variables on the survival of

*E. coli* MC-6. Regressions were performed and multiple correlation coefficients were estimated by considering all three independent variables simultaneously (Table 3). Coefficient of multiple determination was  $R^2 = 0.756$ . This can be interpreted as meaning that 75.6% of the variance in the dependent variable can be explained with water temperature, DO, and salinity. Multiple correlation coefficients within the regression steps were estimated. Correlations were estimated between viable cell numbers and water temperature ( $R = -0.717$ ), with the combined effect of water temperature and DO ( $R = 0.748$ ), and with the addition of salinity to DO and water temperature ( $R = 0.756$ ). The water temperature among the independent variables appeared to affect *E. coli* MC-6 survival the most; DO affected bacterial survival to a much lesser degree, and addition of salinity showed very low correlation.

## DISCUSSION

Knowledge on the survival of fecal coliform bacteria in estuaries is essential in determining the degree of bacterial dispersal and the duration of their presence in the water. This information is needed in assessing the reliability of current water quality monitoring procedures. Most *E. coli* survival studies only extended over a short period (3, 4, 19) and were mostly performed in the laboratory. Those conducted in situ over extended periods include fresh and seawater conditions (10, 13). Few studies have dealt specifically with the estuarine system. In this work we have overcome some of the shortcomings of previous studies. We chose an estuarine environment to assess concomitantly the effect of physical parameters on *E. coli* survival over an extended period of time. We analyzed the collected data statistically in an attempt to evaluate the combined effects of time, water temperature, DO, and salinity on coliform survival.

The convenience of dialysis chambers, de-

TABLE 3. Results of the multiple correlation analysis of four variables of *E. coli* MC-6 survival after 3 days in the Rhode River

Determination	Step in regression		
	1	2	3
Variable	Temp	Temp Dissolved oxygen	Temp Dissolved oxygen Salinity
Multiple correlation coefficient	0.717	0.748	0.756

signed by McFeters and Stuart (13), led us to try their system in this bacterial survival study. These chambers allowed an in situ examination of the survival of *E. coli* MC-6 of fecal origin isolated from the study site. The bacteria remained within the chambers, yet the cells were exposed to the natural physicochemical properties of the estuarine environment, since the membranes allowed the water to flow through the chamber. The possibility of predation, however, was eliminated; other organisms could not enter the chambers, and thus the natural microflora was absent in the immediate vicinity of *E. coli* MC-6 cells.

The survival of *E. coli* MC-6 in the natural environment of the Rhode River is affected by several factors, as reflected in the seasonal variation of survival rates. The seasonal variation of 5 to 30 C in water temperature probably is the most marked fluctuation to which these bacteria are subjected. In early May, when the water temperature was around 17 C and the salinity was low (5 ‰), the number of viable bacteria increased during the first 24 h before their numbers declined. In November, when the temperature was around 9 C and the salinity was high (14 ‰), multiplication of bacteria did not occur and the rate of decline in viable cell numbers was slow. At the end of July, when the water temperature reached 28 C, the decline in viable cell numbers started immediately and the rate of decline was rapid. It appeared that temperature was an important parameter affecting the rate of decline of *E. coli* MC-6 numbers. This is also expressed as the  $t_{1/2}$ , which decreased proportionally with increasing temperature. The 50% reduction in viable cell number occurred for each 10 C increment in water temperature calculated from the slopes of bacterial survival (Fig. 2). Survival of *E. coli* expressed in  $t_{1/2}$  in a fresh-water simulated laboratory environment (13) is in close agreement with our values obtained in an estuary above 15 C but deviates from the values presented in this investigation below this temperature. Almost identical values were reported on *E. coli* survival in stored seawater samples by Carlucci and Pramer (4). Thus, water temperature in a variety of aquatic conditions appears to have a similar and predictable effect on *E. coli* survival.

Both phenomena, the beginning of a log phase before decline of coliform numbers in seawater and the effect of temperature on survival, have been reported previously. Numerous investigators (7, 10, 15, 18) have observed decreasing log phase with increasing temperature before the death of *E. coli*. Our data do not

concur with these findings. We observed no log phase in *E. coli* MC-6 survival experiments in estuarine water, which indicates the inconsistent and variable response of coliform bacteria to different sources of seawater under varying conditions. The fact remains, however, that temperature is inversely related to *E. coli* survival (3, 10, 11, 13, 18). Low temperature prolongs survival (3, 10, 13) because of the slow metabolic rate of bacteria at low temperature (10).

Microbial survival is affected by other physical parameters. Warm water usually contains smaller amounts of DO than colder water. The saturation level of DO at 5 C is 12.8 mg/liter, whereas at 30 C it is only 7.6 mg/liter. The Rhode River estuary during the summer was saturated only to 50% DO. The decline in *E. coli* MC-6 viable cell numbers was rapid, resulting in  $t_{1/2}$  of 7.2 h during the summer months (25 to 30 C) and at DO of 50% saturation. In natural conditions it is impossible to separate the various ecological factors affecting bacterial survival. However, when DO levels of the water of numerous experiments were plotted against the survival of *E. coli* MC-6 cells in the Rhode River for 3 days, this relationship was strongly positive. This relationship is the opposite of that reported for fresh-water laboratory cultures by Hanes et al. (8). They reported a longer survival time of *E. coli* when the average DO was low (0.4 mg/liter), and the decline in viable cell numbers was much more rapid at high DO levels (7.8 to 38.0 mg/liter), with little difference between survival of bacteria and the later two DO concentrations.

Sedimentation and flocculation may play an important role in the removal of bacteria from the water column, and attachment of bacteria to particulates can aid in their preservation (22). Montmorillonite and organic matter have been shown to provide protection to *E. coli* from phage attack at various salinity levels, and a physical protective mechanism has been indicated (21). Clays also appear beneficial to the physiology of selected bacteria (14). Montmorillonite within the chambers protected *E. coli* MC-6 survival in the Rhode River, which is similar to the report on *E. coli* K protection by montmorillonite in seawater (1). It is not presently known how montmorillonite protected *E. coli* MC-6, but it is possible that the mechanism is composed of an array of factors that influenced the succession and relative proliferation of specific microbial groups in soils (12). It is interesting to speculate on the possibility of prolonged survival of fecal coliform bacteria in an estuary containing a relatively high per-

centage of montmorillonite concentration of the sediment fraction of the water. From a practical point of view, the implication of this study relates to the utility of water temperature as an important predictor of coliform survival in an estuarine system. This information will be useful in estimating the resident time of fecal coliform bacteria from point and nonpoint sources in assessing water quality.

#### ACKNOWLEDGMENTS

We wish to express our gratitude to C. C. Crawford, Biology Department at the Johns Hopkins University, to James Lynch, and to John E. Mertens at the Smithsonian Institution for their valuable suggestions related to the manuscript. Thanks are due to Robert L. Cory, U.S. Geological Survey, for providing water quality data.

This research was supported in part by a grant from the Program for Research Applied to National Needs of the National Science Foundation, and by the Smithsonian Institution's Environmental Science Program and Fluid Research Fund. A.A.E. was a recipient by the Smithsonian Institution's Office of Academic Studies Summer Internship Program.

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