APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1988, p. 2902–2907
0099-2240/88/122902-06$02.00/0

Vol. 54, No. 12

Survival of *Escherichia coli* and *Salmonella* spp. in Estuarine Environments†

MARTHA W. RHODES* AND HOWARD KATOR

Department of Biological and Fisheries Sciences, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062

Received 12 May 1988/Accepted 31 August 1988

Survival of *Escherichia coli* and *Salmonella* spp. in estuarine waters was compared over a variety of seasonal temperatures during in situ exposure in diffusion chambers. Sublethal stress was measured by both selective-versus-resuscitative enumeration procedures and an electrochemical detection method. *E. coli* and *Salmonella* spp. test suspensions, prepared to minimize sublethal injury, were exposed in a shallow tidal creek and at a site 7.1 km further downriver. Bacterial die-off and sublethal stress in filtered estuarine water were inversely related to water temperature. *Salmonella* spp. populations exhibited significantly less die-off and stress than did *E. coli* at water temperatures of <10°C. Although the most pronounced reductions (ca. 3 log units) in test bacteria occurred during seasonally warm temperatures in the presence of the autochthonous microbiota, 10^2 to 10^4 test cells per ml remained after 2 weeks of exposure to temperatures of >15°C. Reductions in test bacteria were associated with increases in the densities of microflagellates and plaque-forming microorganisms. These studies demonstrated the survival potential of enteric bacteria in estuarine waters and showed that survival was a function of interacting biological and physical factors.

Fecal coliforms are an operationally defined grouping of enteric bacteria whose presence in natural waters is used as an indicator of fecal contamination and therefore the possible presence of pathogenic microorganisms. An assumption fundamental to the indicator concept is that of parity in the survival of the indicator and enteric pathogens (6) over a range of aqueous environments and varied physicochemical conditions. This report considers an aspect of this parity paradigm as it applies to estuarine waters.

Studies comparing survival of fecal coliforms and pathogenic bacteria in estuarine and coastal seawaters have frequently yielded conflicting results. Beard and Meadowcraft (3) observed that *Escherichia coli* survived longer than did *Salmonella typhosa* in sterile seawater. In contrast, other observations for sterile seawater have ranged from slightly slower die-off of *Salmonella* spp. than of *E. coli* (38) to prolonged survival of *Salmonella enteritidis* compared with *E. coli* (41). Recent in vitro studies (9, 37) using sterile estuarine water have reported grossly dissimilar survival (and culturability) between *S. enteritidis* and *E. coli*. In opposition, McCambridge and McMeekin (25) observed that populations of *E. coli* and *Salmonella typhimurium* remained virtually unaltered after 10 days of exposure to sterile estuarine water in vitro.

To some degree, the disparities in these reports may be attributed to differences in experimental methods and related factors which can influence bacterial survival and recovery. There is evidence that laboratory manipulations (34) and isolate history (1, 16, 39) affect cell viability and can yield test suspensions of sublethally stressed cells whose physiological status may be undetected by common selective enumeration procedures (5, 19, 21, 33, 34). Similarly, failure to recover stressed cells after exposure to estuarine environments can lead to misinterpretation of the fate of the cells and underestimation of the associated health risk.

Of possible greater concern than the influence of these factors in comparative survival experiments is the questionable validity of an approach whereby enteric cells are exposed, either in vitro or in situ, to sterile estuarine or coastal seawater in order to assess their survivability. Survival of allochthonous bacteria under estuarine conditions is known to be affected by physical factors as well as by a variety of interactions with native microbial populations. Enteric bacteria may compete poorly with autochthonous microbes at low natural levels of nutrients (8, 20). Predation and antagonism have been demonstrated as processes contributing to the demise of enteric bacteria in vitro (23–26, 31, 35, 42). Unfortunately, data describing these processes are limited because there have been few in situ studies of enteric survival in estuarine environments and most involved test cultures suspended in sterilized seawater.

This report describes the results of in situ experiments designed to examine the effect of the autochthonous estuarine microbiota on enteric bacteria. Specifically, survival of *E. coli* and *Salmonella* spp. was measured in the presence and in the absence of the natural microbiota at two estuarine sites of contrasting physicochemical characteristics. In addition, the effect of sublethal stress on bacterial survival was measured by using procedures for the detection and recovery of sublethally injured cells.

MATERIALS AND METHODS

Description of study area. The Ware River, a subestuary formed by the confluence of two tidal freshwater streams, flows 9.4 km to Mobjack Bay on the southwestern shore of Chesapeake Bay (Fig. 1). One of the streams, Fox Mill Run, receives approximately 0.6 million liters of secondary effluent per day from a sewage treatment plant located 4.3 km upstream of the Fox Mill Run exposure site. Concentrations of total phosphate and organic nitrogen were generally higher at Fox Mill Run than at the second exposure site, located 7.1 km further downriver on the main stem of the Ware River. Mean salinities during survival experiments ranged from 12 to 22‰ at the Fox Mill Run site and from 21 to 24‰ at the Ware River site.

* Corresponding author.
† Contribution 1484 from the Virginia Institute of Marine Science.

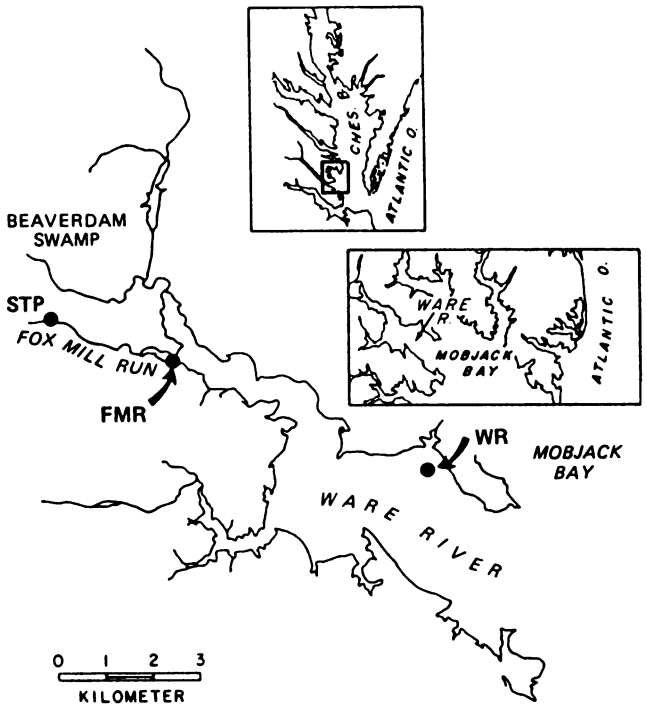

FIG. 1. Location of study area and diffusion chamber deployment sites in Fox Mill Run (FMR) and the Ware River (WR). STP, Sewage treatment plant.

Chamber design and construction. McFeters-Stuart (27) diffusion chambers were modified (34) to accommodate 50 ml of test suspension and to minimize contamination by indigenous microorganisms through leakage, presumably between membrane-chamber interfaces. Additional changes were made during the present study to facilitate sampling and prevent entry of contaminating microorganisms through the sampling ports. Threaded ports of Tuffak (Rohm and Haas Co., Philadelphia, Pa.), an autoclavable polycarbonate, were attached to the central spacer by female threads and sealed with O rings. Ports were sealed with serum stoppers with steel-filled epoxy and covered with threaded Tuffak caps to prevent fouling of the stopper.

Before being autoclaved at 121°C for 15 min, polycarbonate membranes (pore size, 0.2 μm; Nuclepore Corp., Pleasanton, Calif.) were attached to the central spacer with a silicone lubricant vacuum grease (Dow Corning Corp., Midland, Mich.) and port openings were fitted with thumb screws. After sterilization, retainer plates were secured to the central spacer with stainless-steel wing nuts and bolts.

Organisms. *E. coli* was isolated from human feces and identified as previously described (34). Human salmonella isolates were obtained from either the Virginia Department of General Services, Richmond, or the Infectious Disease Division, Centers for Disease Control, Atlanta, Ga. *Salmonella tennessee* isolates were used, except in the May experiment, when *S. typhimurium* was used. Isolates were maintained on tryptic soy agar slants at 4°C and used within 8 weeks.

Preparation of inocula. Isolates were cultured in M9 minimal medium (32) (pH 7.0) modified to contain 5% glycerol instead of glucose. Cells were incubated at 35°C for 24 h, diluted 1,000-fold into fresh medium, and incubated for an additional 24 h, at which time they were in stationary phase. A cell suspension was prepared in sterile river water to yield 10^4 to 10^5 cells per ml. Portions of 20 ml were dispensed into sterile bottles and transported to the field for inoculation of diffusion chambers. Total elapsed time from preparation to chamber inoculation allowed for one to two cell divisions (<0.5 log increase in density).

Chamber exposure protocol. Cells were suspended in water collected from each exposure site immediately before introduction into the diffusion chambers. Each chamber was filled with 20 ml of either nonfiltered or double-filtered water (0.45- and 0.2-μm-pore-size filters) and 20 ml of an appropriate bacterial suspension. Duplicate chambers were prepared at each exposure site for filtered and nonfiltered treatments. Chambers were deployed 0.5 to 1 m below the surface in flotation devices tailored for each exposure site. At selected exposure intervals, samples were removed aseptically by use of sterile hypodermic syringes with 20-gauge needles. Before samples were taken, chambers were shaken, membranes were inspected for damage, and port stoppers were swabbed with alcohol and flamed.

Enumeration. *E. coli* densities were measured by a repair detection procedure for stressed fecal coliforms (18). This procedure was modified to include spread plating on tryptic soy agar with incubation at 35°C for 2 h, followed by overlaying with 10 ml of violet red bile agar (GIBCO Diagnostics, Madison, Wis.) and incubation at 44.5 ± 0.5°C for 22 ± 2 h. We adapted the repair detection procedure for *Salmonella* spp. enumeration by overlaying the plates with 10 ml of brilliant green sulfa agar (Difco Laboratories, Detroit, Mich.) and incubating the samples at 42 ± 0.5°C. Test bacteria were also enumerated by nonresuscitative procedures. *E. coli* was recovered by being spread plated directly onto violet red bile agar and incubation at 44.5°C for 22 ± 2 h. *Salmonella* spp. were enumerated by being spread plated onto brilliant green sulfa agar and incubated at 42.5°C for 22 ± 2 h.

Heterotrophic bacterial populations were enumerated by being spread plated on a heterotroph medium (2). Colonies were counted after 2 weeks of incubation at room temperature and corrected for growth of enteric test cells by subtraction. Bacteriovorous and lytic microorganisms were counted by plaque assay (12). Total heterotrophic and autotrophic microflagellates were enumerated by epifluorescence microscopy (2). Chambers containing enteric bacteria in filter-sterilized site water were routinely examined for contamination by autochthonous microorganisms, using methods discussed above.

Determination of sublethal stress. A quantitative index of sublethal stress was obtained by comparing densities of organisms recovered by the repair procedures described above with those obtained when selective media and temperatures were applied. A second method, based on electrochemical detection (1), was used to quantify stress in *E. coli*. Stress was defined as the difference between a predicted electrochemical detection time (EDT) in EC medium (Difco) at 44.5°C for nonexposed cells and the observed EDT for cells exposed to a selected treatment.

RESULTS

Changes in densities of *E. coli* and *Salmonella* spp. were observed in filtered and nonfiltered diffusion chambers at each exposure site. These changes are illustrated with survival curves from the Fox Mill Run site (Fig. 2 and 3), which were generally nonlinear; the curves often manifested a multiplication phase during the first 3 days, followed by a second period characterized by stable or declining densities.

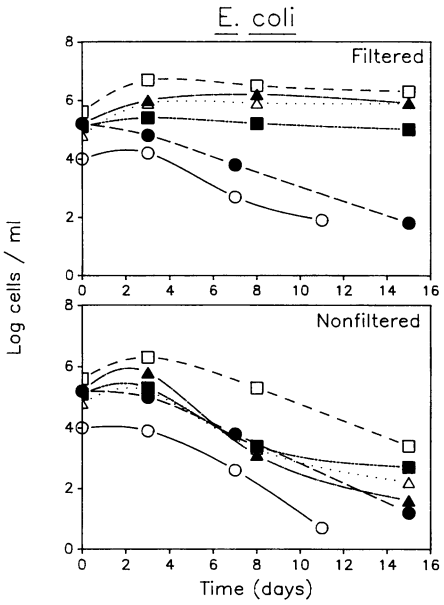

FIG. 2. Survival of *E. coli* in diffusion chambers containing filtered and nonfiltered Fox Mill Run water after in situ exposure at Fox Mill Run during various months. Symbols: ○, February; ●, March; △, April; ▲, May; □, July; ■, November.

To clearly focus this pattern, the exponential die-off coefficient, k (11, 22) (Table 1), was calculated for the first 3 days of exposure as well as the remaining exposure period at each site.

Effect of filtration and seasonal water temperature. When water temperatures were 18°C or higher, large negative values of k, reflecting multiplication, occurred during the initial exposure phase in both filtered and nonfiltered water. Subsequently, reductions in densities of *E. coli* and *Salmonella* spp. were significantly greater in nonfiltered than in filtered water (Wilcoxon signed-ranks test, $P \leq 0.002$). Below 10°C, survival differed between *E. coli* and *Salmonella* spp. *E. coli* survival during the first 3 days was variable, with small differences between filtered and nonfiltered water conditions. During the remaining period, *E. coli* mortality rates were inversely correlated with temperature (Kendall's tau, $P \leq 0.02$) in both filtered and nonfiltered treatments. In contrast, at low temperatures small absolute values of k for *Salmonella* spp. reflected low rates of aftergrowth or die-off during the initial exposure period. After 3 days, *Salmonella* spp. die-off was greater in nonfiltered than in filtered water. There was no significant correlation between mortality of *Salmonella* spp. and temperature (Kendall's tau, $P \geq 0.05$) in either filtered or nonfiltered water.

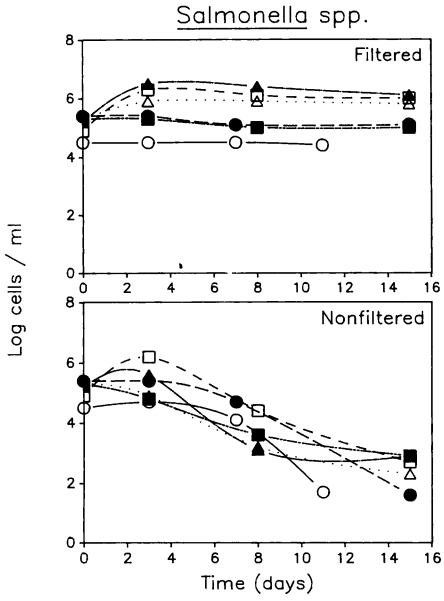

FIG. 3. Survival of *Salmonella* spp. in diffusion chambers containing filtered and nonfiltered Fox Mill Run water after in situ exposure at Fox Mill Run during various months. Symbols: ○, February; ●, March; △, April; ▲, May; □, July; ■, November.

Microflagellate densities reached mean maximum values during the initial 3-day exposure period (Fig. 4) in April, May, and July. These times corresponded with initiation of the spring bloom and increasing seasonal water temperatures. After 3 days, densities declined over the remainder of the exposure period. In contrast, at seasonal temperatures of ca. 10°C or less, microflagellate numbers decreased and then increased, reaching maximum values at 8 days (November) or later (February and March). Plaque densities reached maximum values at the warmest temperatures and declined at temperatures below 10°C. Maximum decreases in enteric bacterial densities (3 to 8 days) generally corresponded with the times at which components of the autochthonous microbiota were increasing or had reached maximum densities. Heterotrophic bacteria also reached maximum densities within 3 to 8 days of exposure (data not shown).

Effect of exposure site. *Salmonella* spp. survival in filtered water was favored at the Fox Mill Run site (Wilcoxon signed-ranks test, $P = 0.03$) relative to that at the Ware River location. Differences in die-off coefficients between sites were greatest during February and March. There was no significant difference between sites in *E. coli* survival in filtered water or under nonfiltered conditions for either type of enteric bacteria (Wilcoxon signed-ranks test, $P > 0.05$).

Survival of *E. coli* versus *Salmonella* spp. Survival of *Salmonella* spp. was significantly greater than that of *E. coli* (Wilcoxon signed-ranks test, $P = 0.025$) in filtered water at Fox Mill Run. If the entire data set is considered, there was no significant difference (Wilcoxon signed-ranks test, $P > 0.05$) in survival between species of test bacteria in filtered water at the Ware River site. However, at low water temperatures, survival of *Salmonella* spp. was favored at both sites; 100 and 83% of salmonellae survived after 1 week of exposure in Fox Mill Run and the Ware River site, respectively, compared with ≤6% of *E. coli* during the same period. Similar differences were not observed in nonfiltered water.

Sublethal stress. Sublethal stress in *E. coli* was inversely related to water temperature, as demonstrated by representative experiments shown in Fig. 5. Pronounced EDT delays observed in February and March increased progressively with exposure. During November and April, the minimum stress evident at 3 days did not progressively intensify. EDT delays were observed only after 15 days in May and July, periods of elevated temperature.

Enumeration of *E. coli* and *Salmonella* spp. by resuscitative and nonresuscitative methods showed the development of sublethal stress in the February and March experiments (Table 2). In contrast, differences in recovery as determined by resuscitative and nonresuscitative methods

TABLE 1. Survival of *E. coli* and *Salmonella* spp. in filtered and nonfiltered estuarine water during two exposure intervals at each site

Mo of exposure	Mean water temp (°C)	Mean salinity (‰)	Die-off coefficient (k [day^{-1}])[a]							
			E. coli				*Salmonella* spp.			
			Exposure interval (days)				Exposure interval (days)			
			0–3		3–15		0–3		3–15	
			F	NF	F	NF	F	NF	F	NF
Fox Mill Run										
Feb.	6.7	11.7	−0.15	0.03	0.68	0.92	−0.03	−0.16	0.04	0.87
March	8.8	18.2	0.36	0.23	0.58	0.72	0.02	0.01	0.07	0.74
April	17.8	14.3	−0.84	−0.30	0.01	0.58	−0.44	0.36	0.03	0.49
May	25.1	18.2	−0.65	−0.48	0.03	0.80	−0.99	0.32	0.07	0.45
July	28.2	19.9	−0.86	−0.56	0.07	0.57	−1.06	−0.96	0.06	0.68
Nov.	10.3	22.1	−0.17	−0.08	0.09	0.51	0.04	0.47	0.07	0.37
Ware River										
Feb.	5.9	21.1	0.05	0.07	0.92	0.83	−0.09	−0.20	0.25	0.51
March	7.7	23.6	0.72	0.77	0.64	0.62	0.18	0.16	0.61	0.75
July	27.9	21.7	−0.51	−0.38	0.07	0.43	−0.78	0.67	0.10	0.55
Nov.	11.7	24.4	−0.50	0.33	0.08	0.53	0.22	0.11	0.10	0.48

[a] $-k = \ln(C_2/C_1)/(T_2 - T_1)$, where C_2 and C_1 are the bacterial densities at final (T_2) and initial (T_1) times of exposure. F, Filtered estuarine water; NF, nonfiltered estuarine water.

were generally less than 0.1 log unit for both organisms during July and November.

DISCUSSION

Die-off of enteric bacteria in estuarine environments has been broadly attributed to a variety of interacting physical, chemical, and biological factors and processes. Although understanding these factors and their relative importance is justifiable purely on an ecological basis, in practical terms such studies are necessary to assess the validity and suitability of the fecal coliform group or other microbial indicators which may be used as quantitative measures of fecal pollution in shellfish-growing waters.

Results of this study demonstrated the prolonged survival potential of *E. coli* and salmonellae in the estuarine environment. At warm temperatures, pronounced multiplication of the enteric bacteria examined occurred initially in both filtered and nonfiltered estuarine water. Our experiments did not determine whether this multiplication occurred at the expense of ambient nutrients or was due to utilization of endogenous reserves. Physiological indices of stress demonstrated that *E. coli* and salmonellae were also minimally stressed at warm water temperatures. These observations support previous findings in another estuary that *E. coli* die-off (2) and sublethal stress (34) in filtered water are inversely related to temperature.

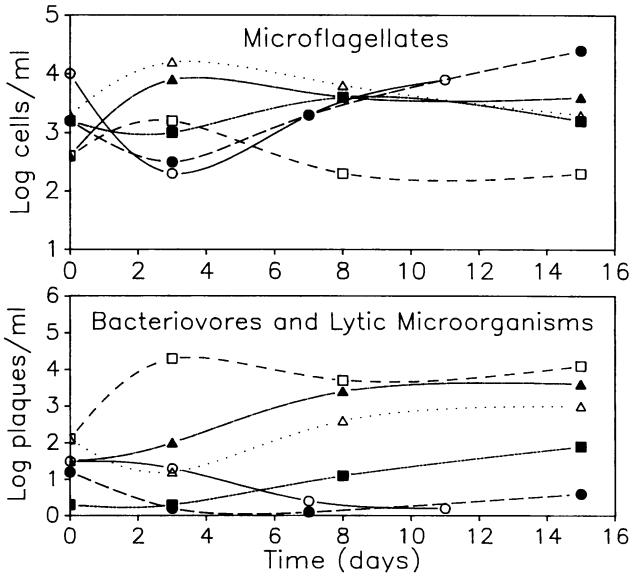

FIG. 4. Changes in densities of microflagellates and plaque-producing microorganisms during various months in diffusion chambers containing nonfiltered estuarine water inoculated with enteric bacteria. Symbols: ○, February; ●, March; △, April; ▲, May; □, July; ■, November.

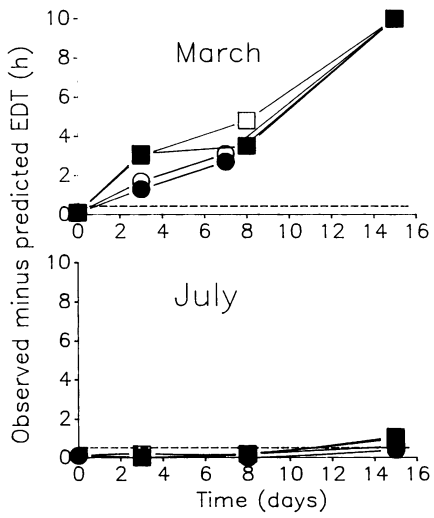

FIG. 5. Incidence of sublethal stress in *E. coli* exposed in diffusion chambers at Fox Mill Run (○, ● [replicates]) and Ware River (□, ■ [replicates]) as measured by an electrochemical detection method. Values of 10 indicate no electrochemical response after at least 18 h of incubation. ---, Upper 95% confidence limit for the predicted EDT.

TABLE 2. Sublethal stress as measured by percent culturability, using nonresuscitative and resuscitative enumeration methods

Mo of exposure	% Recovery after 1 wk of exposure[a]	
	E. coli	*Salmonella* spp.
Fox Mill Run		
Feb.	NG[b]	58
March	65	62
April	107	96
May	105	94
July	106	95
Nov.	98	119
Ware River		
Feb.	NG	65
March	37	53
July	102	120
Nov.	107	81

[a] Percent recovery calculated as (bacterial density as nonresuscitated cells/bacterial density as resuscitated cells) × 100.
[b] NG, No growth on nonresuscitative medium.

In contrast, a conclusion of other in situ survival studies (13, 41) was that *E. coli* die-off was directly related to temperature. We believe that undetected contamination by indigenous microorganisms and the experimental methodology used may have contributed to this conclusion. It has been observed by us (2) and others (36) that commonly used membrane-diffusion chambers become contaminated by bacteria and eucaryotes in the environment. Harsh laboratory manipulations of test bacterial inocula (e.g., centrifugation, washing, and resting at cold temperatures) stress the cells before exposure to environmental stressors, and recovery of debilitated cells by selective enumeration techniques will underestimate the surviving population (34).

Differences in survival as a function of season were also related to the autochthonous estuarine microbiota. Pronounced differences in mortalities under filtered versus nonfiltered conditions during the warmest temperatures were associated with the presence of maximum densities of indigenous microorganisms. Bacterial densities at elevated temperatures were the net result of multiplication (during the initial 3 days) and predation-antagonism and death. Because of greater multiplication at warm temperatures, die-off in nonfiltered water was less apparent than at cold temperatures.

Although enteric attrition was significant in nonfiltered water, substantial numbers of test organisms persisted (e.g., 10^2 to 10^4 bacteria per ml) after 2 weeks of exposure. In vitro studies using nonfiltered seawater have shown more dramatic reductions in *E. coli* (25, 31). It is possible that below critical prey densities of 10^6 to 10^7 bacteria per ml (4, 10, 14, 15, 17, 40), predators and prey may coexist. The continued decline of bacteria beyond the level critical to support predation in vitro may be due to unsuccessful competition with indigenous microorganisms for nutrients. In diffusion chambers, nutrients may continue to be replenished by diffusion at the membrane interface.

Comparative in situ survival studies of *E. coli* and *Salmonella* spp. are few and have neither addressed the significance of temperature nor been conducted at temperatures below 10°C. Although comparable initial death rates of *E. coli* and salmonellae, followed by prolonged survival of the former, were reported (3), others (30, 41) noted that *Salmonella* spp. exhibited somewhat greater viability than did *E. coli*. Our results demonstrated that although *E. coli* and salmonellae showed similar survival both in the absence and in the presence of natural microbiota at warm temperatures, die-off results for the indicator and pathogen in filtered water at temperatures below 10°C were quite different. These results may explain the findings of Brezenski and Russomanno (7), who reported the isolation of salmonellae from individual clams with fecal coliform most-probable-number values of <20/100 g. The authors noted that the water temperature was 5°C and speculated that salmonellae may exhibit greater survival than do fecal coliforms in the clam species studied. Finally, it is of particular interest that even at environmental temperatures below 10°C, the presence of the microbiota was associated with reduced survival of salmonellae.

Recent in vitro studies of survival of *E. coli* and *Salmonellae* spp. in sterile estuarine water have demonstrated both virtually unaltered bacterial densities over a 10-day period (24, 25) and an immediate decline in culturable bacteria (9, 37, 43). It is possible that the survival of enteric bacteria observed in this study may have been enhanced not only by nutrient enrichment across the membrane interfaces but also by a beneficial association with fouling organisms. McFeters et al. (28, 29) demonstrated the potential of a symbiotic relationship between indicator and pathogenic bacteria and algal mat communities in oligotrophic fresh water.

In the presence of the indigenous microbiota, there were no discernible differences in bacterial survival between the study sites.

This investigation has demonstrated that *E. coli* and *Salmonella* spp. can multiply and survive in the estuarine environment. Their demise was attributed primarily to either low water temperatures or predation-antagonism by the natural microbiota at warmer temperatures. Although results obtained within the microcosm of membrane-diffusion chambers may be subject to bottle effects, these findings potentially parallel natural events on suspended particles or within sediments. The most important findings of this study were (i) the persistence of *Salmonella* spp. compared with *E. coli* in filtered water below 10°C and (ii) the potential of these organisms for aftergrowth and survival during warm temperature conditions. The former suggests the need for studies to examine the role of the indigenous microbiota in enteric survival at low water temperatures. The latter questions the universal validity of fecal coliforms as a numerical standard in shellfish-growing areas.

ACKNOWLEDGMENTS

We thank Jane Wingrove, Joseph Boyer, and Kenneth Worrell for their valuable technical and field assistance. Appreciation is expressed to Sally Henderson, Virginia Department of General Services, and Alma Murlin, Centers for Disease Control, for supplying bacterial cultures and to the Ware River Yacht Club for use of their facilities. We also thank Eric T. Koepfler and Michael E. Sieracki for critical review of the manuscript.

This work was supported in part by funds provided by the Virginia Water Resources Research Center (grant A-093-VA).

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