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Seasonal Variation in Survival of *Escherichia coli* Exposed In Situ in Membrane Diffusion Chambers Containing Filtered and Nonfiltered Estuarine Water†

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Human fecal *Escherichia coli* isolates were exposed over a seasonal cycle to estuarine water in diffusion chambers filled with double-filtered (0.45 and 0.2 μm) and nonfiltered water. Laboratory manipulations of *E. coli* cultures before estuarine exposure were reduced to minimize sublethal stress, and nonselective or resuscitative enumeration techniques were employed to maximize recovery of stressed cells. *E. coli* was capable of extended survival during in situ exposure to estuarine water, provided eucaryotes were excluded from diffusion chambers. Survival was directly related to temperature in absence of the eucaryote component of the natural microbiota. Although it was not possible to prevent eventual bacterial contamination in double-filtered water, there was no direct evidence that such contamination affected *E. coli* survival. Conversely, *E. coli* disappearance was most pronounced at warmer temperatures in the presence of the natural microbiota, and decline coincided with increasing eucaryote densities. In contrast, the decline of *E. coli* during winter was similar in both filtered and nonfiltered seawater.

Public health decisions concerning the safety of estuarine waters for recreational use or for the harvesting of shellfish continue to be based primarily upon fecal coliform enumerations. Although the validity of the fecal coliform indicator system continues to be questioned (9, 10, 32), a suitable alternative has yet to be accepted. Selection of a reliable indicator requires information concerning the fate of the potential indicator in aquatic systems. Strategies to evaluate indicator survival in aquatic environments have included in vitro exposure of batch cultures to ambient water, simultaneous in situ release of coliforms with a conservative tracer, or in situ exposure within dialysis bags or diffusion chambers (7, 8, 11, 27, 41, 43). Enumeration of surviving organisms has most frequently employed selective plating procedures, which can be lethal to bacteria sublethally stressed by exposure to the aquatic environment (3, 4, 10, 16).

Diffusion chambers offer a methodology to study a confined population of microorganisms under relatively natural conditions. One of the most widely used chambers (27) consists of a central Plexiglas spacer covered with opposing membrane filters held by Plexiglas retainer rings. With such chambers, McFeters and Stuart (27) exposed pure cultures of *Escherichia coli* to river water and observed that bacterial survival was inversely proportional to temperature (5 to 15°C). Similar results have been noted in estuarine water (12, 41). In contrast, Soracco et al. (R. J. Soracco, D. L. Tison, and D. L. Pope, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N43, p. 170), using a modified McFeters-Stuart chamber, detected prolonged survival of pure *E. coli* cultures in a warm freshwater pond (>25°C). If T_{90} values (time required for an order of magnitude decrease in *E. coli* cell density) are used to compare results from coliform survival studies employing all types of exposure techniques, numerous inconsistencies become apparent. For example, at approximately similar temperatures, T_{90} values observed during seawater exposure in batch cultures (6, 11, 43) or dialysis bags (8, 39) exceeded those reported for diffusion chamber experiments (12, 41). Two factors which might account for discrepancies in experimental results are interactions with the autochthonous microbiota, including predation, and the development of sublethal stress. Incubation of coliforms in coarsely filtered seawater fractions containing autochthonous microbiota such as bacteria and protozoans resulted in a rapid decline of *E. coli* cell densities after a 2- to 4-day lag period (11, 23, 35).

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Sublethal stress can contribute to an apparent decline in *E. coli* viable count when selective enumeration procedures are used (3, 8). Using selective techniques, Verstraete and Voets (42) enumerated *E. coli* exposed in batch cultures to filter-fractionated river water sampled monthly. Since maximum die-off occurred during summer in both nonfiltered and 5-μm-filtered water and during winter in autoclaved and 0.45-μm-filtered water, they suggested that predation exerted its maximum influence on coliform densities during spring and summer but sublethal stress contributed to increased mortality after exposure to cold water temperatures.

Biological interactions between allochthonous bacteria and the natural microbiota are complex and poorly understood. Although predation, competition with autochthonous bacteria for substrates, and antibiosis due to products of autochthonous organisms may effect a reduction in the coliform population, stimulatory effects may also occur (5, 23, 24, 29).

In an attempt to resolve the discrepancies previously mentioned, experiments were performed to test the hypothesis that both seasonal temperature and the presence of natural microbiota would affect *E. coli* survival. Fecal *E. coli* mixed with nonfiltered or filtered water were exposed in diffusion chambers to estuarine waters during various seasons. Laboratory manipulation before exposure was reduced to minimize predisposition to sublethal stress, and nonselective or resuscitative enumeration techniques were employed to maximize recovery of stressed cells.

MATERIALS AND METHODS

Bacterial suspensions were prepared and inoculated into membrane diffusion chambers which were fabricated, deployed, and sampled in the estuary as previously described (34). Chambers were deployed in duplicate for each treatment.

Determination of solute diffusion rate. Solute diffusion rates were determined for chambers fitted with fresh polycarbonate membranes (0.2 μm; Nuclepore Corp., Pleasanton, Calif.) and for chambers that had been exposed in the environment for various lengths of time. Safranin was dissolved in estuarine water to give an optical density at 515 nm of approximately 1.0 before membrane filtration (0.2 μm). Chambers were flushed twice with this solution, filled, and immersed in the York River, Va., 75 m from shore at a depth of 1 m. At hourly intervals, 1.5-ml samples were taken for determination of optical density. Readings were corrected for dilution due to sampling and plotted versus time of immersion.

Enumeration techniques. (i) Enumeration of *E. coli* in mixed cultures. *E. coli* were enumerated by a modification of the violet red bile agar (BBL Microbiology Systems, Cockeysville, Md.) method for resuscitation of stressed fecal coliforms (19). Duplicate samples (0.1 ml) were spread plated onto Trypticase soy agar (BBL) plates and incubated at room temperature for 2 h, followed by addition of a 10-ml surface overlay of violet red bile agar. Plates were incubated at 44.5°C for 24 ± 2 h.

(ii) Enumeration of *E. coli* in pure culture. Duplicate volumes were spread plated onto Trypticase soy agar and incubated at 35°C for 24 ± 2 h.

(iii) Enumeration of indigenous estuarine bacteria. Duplicate samples (0.1 ml) were spread plated onto heterotroph medium (consisting of [g/liter of estuarine water based medium]: Bacto-Peptone, 1; yeast extract, 0.5; ferric citrate, 0.01; sodium glycerol phosphate, 0.1; agar, 15). Plates were incubated at room temperature for 5 days.

(iv) Enumeration of eucaryotic microorganisms by epifluorescence microscopy. A Zeiss standard microscope equipped for epifluorescence with a 63× oil immersion lens, a 450 to 490 nm excitation filter, an LP 520 barrier filter, and an FT 510 beam splitter was used. Samples were fixed immediately after collection by addition of gluteraldehyde (5% solution) in filtered (0.2 μm) estuarine water to a final concentration of 0.5%. Fixed samples were processed as soon as possible but held for no longer than 2 weeks. Nuclepore filters (0.2 μm, 25-mm diameter) were soaked for at least 5 min in 0.2% irgalan black BLG (Ciba-Geigy Corp., Greensboro, N.C.) in 2% acetic acid and rinsed in sterile distilled water. Each sample (0.5 to 1 ml), plus sufficient sterile distilled water to yield 2 ml, was added to the tower of a microanalysis filtration unit (Millipore Corp., Bedford, Mass.), and the cells were stained with 40 μl of a proflavine (hemisulfate; Sigma Chemical Co., St. Louis, Mo.) solution (0.033% proflavine in sterile distilled water). The mixture was swirled, filtered after 1 min under low vacuum (100 mm Hg) to dryness, and washed twice with successive 5-ml portions of sterile distilled water. While under suction, the filter was removed and placed over a drop of nonfluorescing immersion oil (type LF; Cargille, Cedar Grove, N.J.) on a clean slide. A drop of oil was placed on top of the filter followed by a cover slip. At least 100 fields of view or up to 400 eucaryotes were counted per slide. Since proflavine does not mask autofluorescence by chlorophyll, it is possible to distinguish between autotrophic and heterotrophic eucaryotes. However, a number of problems were encountered. In samples taken during winter, a proportion of heterotrophic microflagellates appeared to have lost flagella. In spring, high densities of autofluorescing autotrophs visually complicated enumeration of heterotrophs. We were not able to enumerate amoebae. For these reasons and because both autotrophic and heterotrophic eucaryotes are known to affect bacterial populations, eucaryote data reported herein are expressed in terms of total eucaryotes detectable by epifluorescence.

RESULTS

To minimized stress due to laboratory manipulation, *E. coli* grown to early-stationary phase in minimal M9 medium containing 5,000 ppm glycerol was introduced with culture medium into diffusion chambers for seawater exposure studies. The time necessary to reduce the initial glycerol concentration to 0.018 ppm was

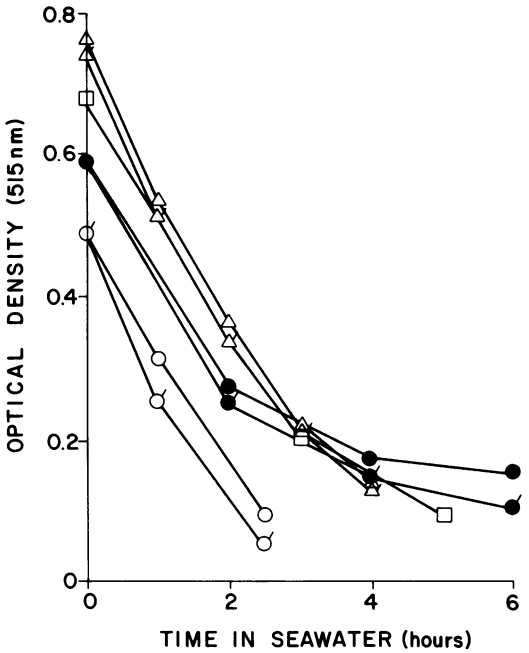

FIG. 1. Rates of safranin diffusion from chambers in seawater at 27°C. Chambers filled with sterile water were exposed to seawater for 0 (○), 7 (△), and 13 (□) days. One chamber (●) was inoculated with a bacterial suspension during 13-day exposure to seawater. After seawater exposure, all chambers were flushed with sterile seawater before addition of dye. Flag indicates replicate experiment.

derived from safranin diffusion experiments (Fig. 1). The growth rate of a steady state chemostat culture of *E. coli* (10^6 cells per ml) has been shown to be greatly reduced at glucose concentrations less than 0.018 ppm (37). Solute diffusion rates were calculated from the equation $C_t = C_0 e^{-kt}$, derived from Fick's law of diffusion (14), where C_0 is the concentration of safranin at zero time, C_t is the concentration at selected time t, and k is the rate of exchange. The time necessary to reduce the glycerol concentration to 0.018 ppm in seawater at 27°C varied from 17 h in fresh chambers to 26 h in chambers previously exposed to river water for 7 days. Since the Q_{10} for sugar diffusion is 1.37 (14), the diffusion rate will decrease at colder temperatures. For example, at 7°C, 24 h would be required to reduce the glycerol concentration to 0.018 ppm.

Seasonal survival of *E. coli* exposed in situ in filtered estuarine water. In situ survival of pure cultures of *E. coli* in filtered estuarine water was directly related to temperature over the seasonal range of 1 to 25°C (Fig. 2). Eucaryotes were not detected in any chambers from which survival data were collected, although contamination by autochthonous bacteria was observed after several days of exposure (open boxes, Fig. 4A and C). However, there was no direct evidence that these contaminants substantially affected *E. coli* survival in the diffusion chamber over the experimental period. At temperatures greater than 13°C, *E. coli* was capable of growth during a 5-day exposure period. To test the effect of the residual glycerol concentration and initial cell density on subsequent growth during in situ seawater exposure at 27°C, chambers were inoculated with a suspension containing 10^8 cells per ml in the growth medium containing 5,000 ppm glycerol and with a 100× dilution of this suspension. Growth and survival characteristics over the 5-day study period were similar (data not shown) for both suspensions.

Seasonal survival of *E. coli* exposed in situ in nonfiltered estuarine water. *E. coli* cultures in M9 medium were diluted twofold with either double-filtered (0.45 and 0.2 μm) estuarine water, 1-μm filtered water, or nonfiltered seawater before exposure in an attempt to separate the influence of autochthonous bacteria and eucaryotes on *E. coli* attrition. However, microflagellates were detected in most chambers containing *E. coli* in 1-μm filtered seawater. Therefore, it was not possible to examine the interaction of *E. coli* and autochthonous bacteria exclusive of eucaryotes and these data are not presented.

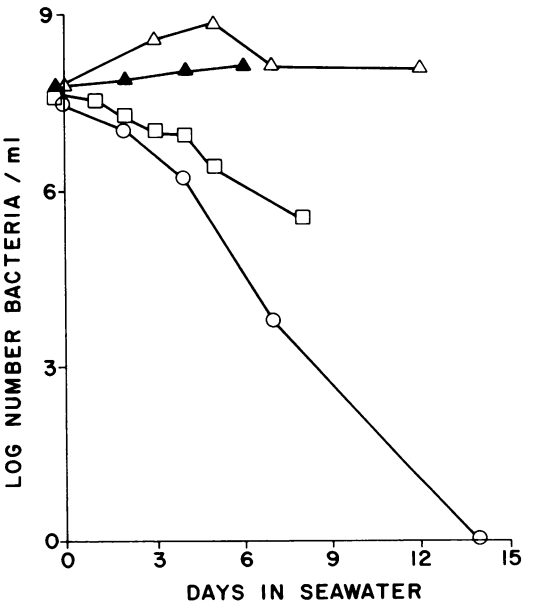

FIG. 2. Survival of *E. coli* during in situ exposure to seawater at various temperatures in the absence of eucaryotes. Bacteria pregrown in M9 medium at 35°C were introduced directly into diffusion chambers. Symbols: △, 23 to 25°C, 18 to 20 ppt salinity; ▲, 13.5°C, 16 ppt; □, 2.5 to 4°C, 24 ppt; ○, 1°C, 24 ppt.

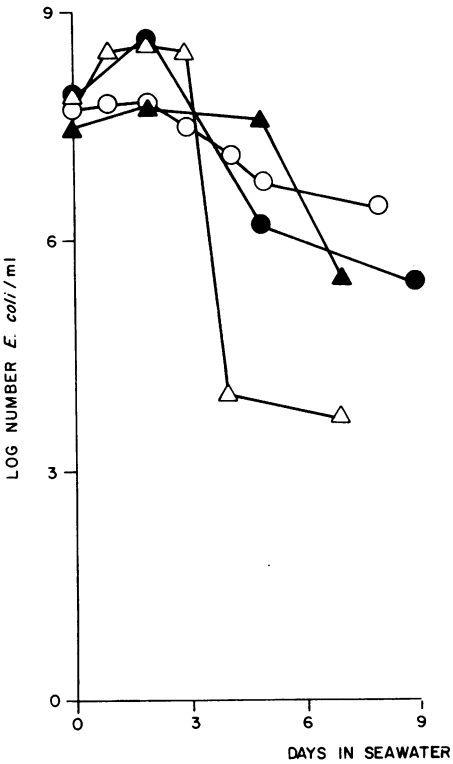

FIG. 3. Survival of *E. coli* during in situ exposure to seawater at various temperatures in the presence of eucaryotes. Bacteria pregrown in M9 medium at 35°C were diluted twofold with unfiltered seawater and introduced into diffusion chambers. Symbols: ○, 3°C, 23 ppt salinity; △, 13°C, 15 ppt; △, 21°C, 24 ppt; ●, 26°C, 22 ppt.

Other workers (2) have reported that microflagellates are capable of penetrating 1-μm membrane filters.

The direct relationship betwen *E. coli* survival and temperature observed in filtered seawater (Fig. 2) was altered by the presence of an indigenous estuarine microbiota (Fig. 3). In contrast to the multiplication and persistence of *E. coli* in filtered water at 13°C, and to a greater extent at 23 to 25°C, the multiplication phase in nonfiltered water was followed by marked decline in bacterial numbers.

During winter (3°C), *E. coli* disappeared at similar rates, regardless of the presence of eucaryotes (Fig. 4A). Autochthonous bacteria were detected in chambers filled with filtered water by 2 days. Although indigenous bacterial populations increased, increases in eucaryotic densities were not detected. A repeat experiment (performed at 1.5°C, 24 ppt salinity) produced similar results (data not shown). During the spring (13°C), *E. coli* decline in nonfiltered seawater coincided with an increase in densities of eucaryotes. In contrast, there were no decreases in *E. coli* in filtered water after a week of in situ exposure (Fig. 4B). In the fall (21°C), *E. coli* densities did not decline during 5 days of exposure in the absence of eucaryotes, although the number of autochthonous bacterial contaminants increased rapidly (Fig. 4C). In nonfiltered seawater, *E. coli* decline began after a 3-day lag period coincident with increasing population densities of both eucaryotes and autochthonous bacteria. In this particular experiment, although diatoms were abundant from 0 to 3 days, they were later displaced by heterotrophic microflagellates (<10 μm) whose appearance coincided with the disappearance of *E. coli*. Results of a summer experiment (26°C, 22 ppt salinity; data not shown) were similar to the fall observations except that microflagellates predominated during the 7-day study period.

DISCUSSION

Results reported in this paper are not in agreement with observations of other workers who employed diffusion chambers to study in situ survival of *E. coli* in seawater (12, 41). Whereas we have observed a direct relationship between *E. coli* survival and water temperature, others have noted an inverse relationship. We suggest that this disagreement was due to the occurrence of sublethal stress induced by laboratory manipulation and related to the pre-exposure history of the isolates used (1, 34), as well as to unrecognized contamination by indigenous microbiota.

Proper design of seawater exposure experiments requires an assessment of and compensation for the degree to which test bacteria have been sublethally stressed. Sublethally stressed organisms survive in the environment and may retain their pathogenicity (40), but they are sensitive to selective enumeration procedures and harsh sampling techniques (1, 3, 33, 34). Studies performed in this laboratory suggest that sublethal stress is related to the pre-exposure history, the laboratory manipulations, and physical factors encountered during exposure (1, 34). Recovery of sublethally stressed cells necessitates resuscitative techniques. Rates of bacterial disappearance exceeded those observed in our experiments when stressful laboratory manipulations were applied (41), when late-log-phase cultures pregrown in a rich medium were used (15), or when selective enumeration techniques were employed (12, 15, 26).

Initially, problems were encountered in preventing contamination of diffusion chambers of the design employed by other workers (12, 27, 28). Monitoring chamber contents by epifluorescence microscopy revealed that eucaryotic and

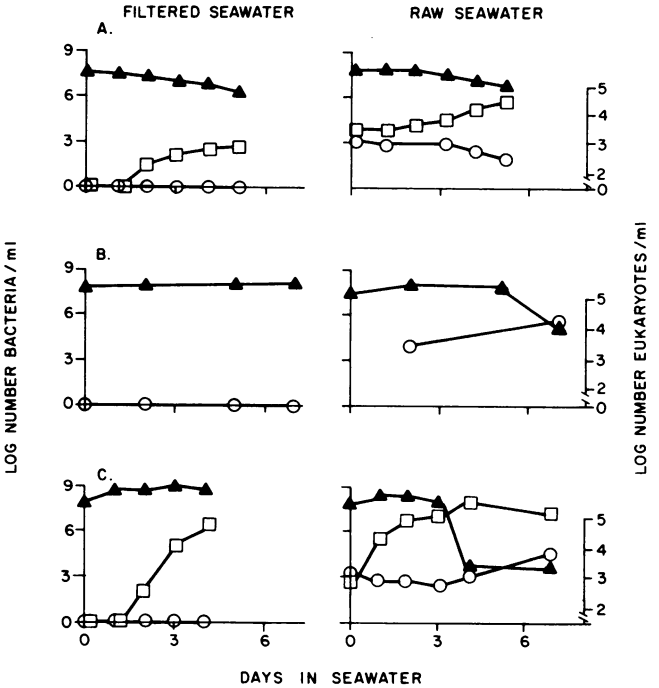

FIG. 4. Survival of *E. coli* during in situ exposure to seawater in the presence and absence of eucaryotes. Bacteria pregrown in M9 medium at 35°C were exposed to filtered (0.2 μm) or natural seawater at: (A) 3°C, 23 ppt salinity; (B) 13°C, 15 ppt; (C) 21°C, 24 ppt. Symbols: ▲, *E. coli*; □, autochthonous bacteria; ○, eucaryotes.

bacterial contamination regularly occurred and was most severe during warm weather. Although elimination of eucaryotic contamination was achieved using O-rings and cemented rubber sampling septa, contamination by autochthonous bacteria was not prevented with 0.2-μm membranes. MacDonell and Hood (22) have recently described estuarine ultramicrobacteria capable of passing through 0.2-μm filters. These bacteria resembled *Vibrio, Aeromonas, Pseudomonas*, and *Alcaligenes* species. Since most reports indicate diffusion chambers were not monitored for autochthonous microorganisms, the paucity of studies (36) reporting contamination after in situ exposure is not surprising.

Our results indicated that both the numbers and kinds of eucaryotes varied seasonally. Lag times before decline in coliform numbers were directly related to water temperature and eucaryote density at moderate and warm temperatures. At low temperatures, the rate of coliform disappearance was not increased in the presence of eucaryotes. Identification of those members of the natural microbiota or their products responsible for declines in coliform numbers was beyond the scope of these experiments. Microflagellates common to estuarine waters and belonging to the families *Bodonidae* and *Monadidae* phagocytize bacteria (17). Other microorganisms, difficult to detect by epifluorescence microscopy, such as amoebae, also graze on coliforms (30, 31, 35). Autotrophic eucaryotes such as *Skeletonema costatum* may be responsible for coliform death via an antibiotic effect (38). Autochthonous bacteria may cause increased coliform mortality through production of antibiotics or by competition for available nutrients (24).

Although diffusion chambers provide for in situ exposure of microorganisms, containment in an environment with a high surface area/volume ratio could enhance coliform survival or growth of coliforms or predators (or both). Thus, chamber effects may result in predator/prey ratios which are unlikely to occur in nature. Effective predation is dependent upon a critical predator/prey ratio (18, 24). For example, it has been demonstrated (23) that coliforms are capable of prolonged survival in estuarine water after addition of protozoans at a density of 2×10^3 cells per ml. In relatively unpolluted water, coliforms may survive or even multiply. Hoskins and Butterfield (20) pointed out many years ago that below the confluence of a polluted and a clean stream, coliform densities were higher than those in the polluted stream.

Whereas interactions with autochthonous microorganisms may exert primary control over

coliform survival in polluted streams, in batch culture or diffusion chamber experiments, physicochemical factors such as degree of insolation may exert a stronger influence in waters of low turbidity. A number of workers have reported a strong correlation between the degree of insolation and coliform mortality (13, 21, 25). In addition, sunlight has been shown to cause sublethal injury to *E. coli* and to act synergistically with predacious microorganisms in reducing coliform numbers (21, 25). Sublethal injury induced by sunlight may result from accumulation of peroxides, either endogenous or exogenous (21). Thus, the possibility exists that diffusion chambers or other closed containers may enhance lethal effects of sunlight through accumulation of peroxides or other toxic substances. Furthermore, sunlight may also react with exogenous photosensitizers such as chlorophylls or with quenching agents such as carotenoids (5) with corresponding increases or decreases in anticoliform activity. Diffusion chambers provide a unique system for the study of such phenomena in the natural environment.

In summary, this study has demonstrated that the relative importance of temperature and the autochthonous microbiota on *E. coli* survival in an estuary was seasonally dependent. Survival duration was directly related to temperature in the absence of the eucaryote component of the natural microbiota, and disappearance of *E. coli* was most pronounced at warmer temperatures in its presence. Although these investigations focused on the effects of temperature and natural microbiota on *E. coli* survival, it is recognized that persistence of these allochthonous bacteria in estuarine environments is affected by temporally variable, complex interactions of numerous biological and physicochemical factors. Diffusion chamber experiments, such as those described in this paper, contribute toward understanding these relationships.

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