

In Situ Survival of Enteric Bacteria in Estuarine Environments

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TABLE OF CONTENTS

List of Figures	iv
List of Tables	v
Acknowledgments	vii
Abstract	1
Introduction	3
Materials and Methods.	5
I. Description of Study Area.	5
II. Chamber Design and Construction	5
III. Assembly of Chamber	6
IV. Organisms	6
V. Preparation of Inocula.	6
VI. Estuarine Exposure Studies.	7
VII. Sampling	8
VIII. Enumeration of Enteric Bacteria.	8
IX. Enumeration of Autochthonous Microorganisms	8
X. Determination of Sublethal Stress.	9
XI. Determination of Salinity, Dissolved Oxygen, and Temperature.	9
Results	10
I. Experiment Schedule	10
II. Bacterial Survival in Filtered Estuarine Water	10
III. Bacterial Survival in Nonfiltered Estuarine Water	11
IV. Sublethal Stress.	12
Discussion	13
References.	18
Figures	25
Table.	47

LIST OF FIGURES

1. Location of Ware River Subestuary	26
2. Location of Sewage Treatment Plant and Diffusion Chamber Deployment Sites in Fox Mill Run and Ware River	27
3. Design of Membrane Diffusion Chamber	28
4. Design of Moored Diffusion Chamber Array Buoy at Ware River Study Site	29
5. Survival of <i>E. coli</i> and <i>S. tennessee</i> in Diffusion Chambers Filled with Filtered Estuarine Water and Exposed In Situ at Fox Mill Run and Ware River	30
6. Seasonal Survival of <i>E. coli</i> and Salmonellae Exposed In Situ to the Estuarine Environment	32
7. In Situ Survival of <i>E. coli</i> and <i>S. tennessee</i> or <i>S. typhimurium</i> During April and May, Respectively, in Fox Mill Run	34
8. Survival of <i>E. coli</i> in Diffusion Chambers Filled with Nonfiltered Estuarine Water and Exposed In Situ	36
9. Survival of <i>S. tennessee</i> in Chambers Filled with Nonfiltered Estuarine Water and Exposed In Situ	38
10. Incidence of Sublethal Stress as Measured by an Electrochemical Detection Method for <i>E. coli</i> Exposed In Situ at Fox Mill Run and Ware River	40
11. Incidence of Sublethal Stress as Measured by Resuscitative Versus Nonresuscitative Plate Count Enumeration for <i>E. coli</i> and <i>S. tennessee</i> Exposed In Situ at Fox Mill Run	44

LIST OF TABLES

1. Mean Values of Salinity, Dissolved Oxygen, and Temperature Measurements During In Situ Bacterial Survival Experiments	48
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ABSTRACT

Survival and development of sublethal stress were determined for *Escherichia coli* and *Salmonella* spp. exposed in membrane diffusion chambers to estuarine waters of different chemical and physical characteristics. Sublethal stress was assessed using direct selective versus resuscitative enumeration procedures and an electrochemical detection method.

E. coli and *Salmonella* spp. were exposed in a shallow tidal creek 4.3 km downstream from a sewage outfall and at a point in the Ware River located an additional 7.1 km downstream. In general, survival rates were higher and stress was less at the shallow tidal creek site. Die-off and sublethal stress in filtered estuarine water were inversely related to water temperature. However, salmonella populations exhibited significantly less die-off and stress than *E. coli* at water temperatures $< 10^{\circ}$ C. While exposure to autochthonous microbiota reduced densities of both test bacteria, approximately 10^2 - 10^4 test cells/ml remained after two weeks of exposure at temperatures $> 15^{\circ}$ C. Survival differences in membrane-filtered versus nonfiltered estuarine water were most pronounced at warmer temperatures. Bacterial die-off at warm temperatures was associated with increases of microflagellates enumerated by epifluorescence microscopy and lytic microorganisms enumerated as plaque-forming units.

These studies demonstrate that indicator and pathogenic enteric bacteria can survive for extended periods in estuarine waters and that, under adverse conditions of low temperature, survival patterns of *E. coli* and salmonellae may differ significantly. The prolonged persistence of *Salmonella* spp. as compared to *E. coli* under cold water conditions suggests that fecal coliforms may be an inadequate indicator of pathogens during the prime months of shellfish harvesting.

Key Words: Enteric Bacteria, *Escherichia coli*, *Salmonella* spp., Water Quality, Estuaries, Environmental Microbiology, Sublethal Stress

INTRODUCTION

The presence of fecal coliform bacteria in environmental waters is the current index of potential contamination by pathogenic bacteria derived from sewage or other sources of feces. Two restrictions are inherent in this concept of a bacterial indicator system:

1. That the indicator (organism) not proliferate (or at least not to an extent greater than pathogens), and
2. That the indicator be more resistant to conditions of environmental exposure than pathogens are [Dutka, 1973].

When considering the diversity of environmental conditions in aquatic systems, one can see that a particular bacterial indicator may not be universally applicable.

Seawater has been considered an adverse environment for nonmarine microorganisms, and mechanisms relating to its self-purifying capacity have been discussed extensively [Greenberg, 1956; Carlucci and Pramer, 1959; Mitchell, 1968; Mitchell and Chamberlin, 1975]. Factors contributing to the disappearance or decline of enteric bacteria in seawater are thought to include adsorption and sedimentation, solar radiation, physico-chemical conditions, predation, bacteriophage, algal toxins, and nutrient deficiencies. With respect to the latter, numerous investigators have noted that the addition of nutrients in vitro to raw or sterilized seawater results in reduced toxicity [Vaccaro et al., 1950; Orlob, 1956; Carlucci and Pramer, 1960; Cook and Hamilton, 1971; Savage and Hanes, 1971; Won and Ross, 1973]. In vitro concentrations of organic nutrient amendments from one to several hundred ppm have produced effects such as prolonged lag times before onset of die-off, decreased rate of die-off, and bacterial multiplication.

In studies of the mechanisms of adaptation that permit growth of *Escherichia coli* over a range of glucose concentrations as low as 0.1 ppm, Koch [1971, 1979] noted that prolonged starvation results in a period prior to death when *E. coli* is nearly metabolically inert and yet still capable of rapid biosynthesis in the presence of glucose. Shehata and Marr [1971] reported on the ability of *E. coli* to grow at a steady state in a chemostat at nutrient concentrations as low as 0.018 ppm. Cook and Hamilton [1971] concluded that estuarine waters contain sufficient or-

ganic material to support fecal coliform growth. However, their observations were based on in vitro experiments using inocula consisting of washed cells derived from sewage and inoculated into filtered water.

A determination of the abilities of allochthonous bacteria, such as enterics, to multiply in seawater is vastly complicated by our lack of understanding of the ecological interactions between available inorganic and organic nutrients and mixed microbial populations. Carlucci and Pramer [1960] suggested that elimination of fecal bacteria in seawater is due in part to competition with the indigenous microbiota for available nutrients. Chemostat studies [Jannasch, 1968] have shown that under conditions of low dilution rates or low concentrations of a limiting substrate, enteric bacteria are replaced by other organisms having lower substrate saturation constants or growth rates. Conversely, *E. coli* was noted to compete successfully for nutrients in seawater at high dilution rates and substrate levels.

These findings demonstrate the following:

1. Under laboratory conditions, fecal coliforms such as *E. coli* can survive and/or multiply at low nutrient levels; and
2. Nutrient amendment can reduce or eliminate seawater toxicity.

However, the key problem remains concerning the fate of enterics in the estuarine environment where ambient nutrient conditions are temporally variable on tidal, diel, and seasonal bases and where nutrients from point sources decrease along a downstream gradient. The purpose of the investigation described herein was to compare the survival of *E. coli* and *Salmonella* spp. in filtered and nonfiltered estuarine water exposed in situ in membrane diffusion chambers at two study sites chosen on the basis of potentially contrasting physico-chemical regimes. One site was located in an area of an estuary known to be free of point sources of organic pollution. The second site was in a tidal creek that received secondary sewage effluent.

MATERIALS AND METHODS

I. Description of Study Area

The Ware River, formed by the confluence of two freshwater streams, Beaverdam Swamp and Fox Mill Run, flows 9.4 km downstream into Mobjack Bay on the southwestern shore of Chesapeake Bay (Figure 1). These streams are generally less than 1 m deep and 4 m wide. Fox Mill Run (Figure 2) receives approximately 5.7×10^5 l/day (5.7×10^2 m³/day) of secondary effluent from a sewage treatment plant situated 2.6 km above the confluence. From this point, Fox Mill Run meanders along a serpentine course through extensive marshland (2.1 km²) for approximately 4.3 km to the headwaters of the Ware River. Although stream nutrient concentrations have been observed to decrease markedly immediately downstream of the sewage treatment plant [Bosco et al., 1982], data analyses indicated that total phosphate and organic nitrogen levels were significantly higher at the mouth of Fox Mill Run compared to the downstream exposure site.

The Ware River is a subestuary characterized by weak gravitational circulation and minimal vertical stratification. Salinities generally range from 11 parts per thousand (‰) at the confluence of Beaverdam and Fox Mill Run to 16 ‰ near the river mouth under high-water slack and normal meteorological conditions. The amplitude of the tidal variation increases with distance from the river mouth with the range of variation as high as 6 ‰ at the stream confluence [Bosco et al., 1982].

Diffusion chambers were deployed near the mouth of Fox Mill Run at a high tide depth of 1-1.5 m. Extremely low water during spring tides necessitated the installation of a reservoir basin to ensure that the chambers remained submerged. A second site was selected 7.1 km further downstream in the Ware River (Figure 2). Chambers were initially deployed at this site close to mid-channel in 5-m water but were relocated nearer shore in 2-2.5-m water following vandalism of experimental gear.

II. Chamber Design and Construction

McFeters-Stuart [1972] diffusion chambers had been previously modified [Rhodes et al., 1982] to accommodate an increased volume 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 to prevent contamination via sampling ports (*Figure 3*). Threaded ports of Tuffak (Rohm and Haas Co., Philadelphia, Pennsylvania), a polycarbonate capable of withstanding autoclaving, were attached to the central spacer by female threads and were sealed using O-rings. Serum stoppers (7/32 in., No. 7116-00, Norcross, Inc.) were secured to ports with steel-filled epoxy and covered with threaded Tuffak caps to prevent fouling of the stopper site. Although modified chambers were routinely effective in excluding protozoa, contamination by autochthonous bacteria generally was observed by epifluorescence microscopy after three days of in situ exposure.

III. Assembly of Chamber

A silicone lubricant vacuum grease (Dow Corning, Midland, Michigan) was used to position membrane filters onto the central spacer, and port openings were fitted with thumb screws before autoclaving at 121° C for 15 min. After sterilization, retained plates were secured to the central spacer with stainless steel wing nuts and bolts.

IV. Organisms

E. coli was isolated from human feces by enrichment in lactose broth at 35° C for 24 hr, inoculated into EC broth, and incubated at 44.5° C for 24 hr. Isolates producing gas in EC broth were streaked on eosin methylene blue agar and incubated at 35° C for 24 hr. A typical green-sheen colony was streaked onto trypticase soy agar (TSA) and identified using an API 20E test strip (Analytab Products, Inc., Plainview, New York). Human salmonella isolates were obtained from either the Division of Consolidated Laboratory Services, Richmond, Virginia, or the Infectious Disease Division, Center for Disease Control, Atlanta, Georgia. With the exception of two occasions (February 10 and May 26, 1981) when isolates of *S. typhimurium* were used, *S. tennessee* isolates were used exclusively. Isolates maintained on TSA slants at 4° C were used for in situ exposure studies within eight weeks after initial isolation.

V. Preparation of Inocula

Isolates were cultured in M9 medium (pH 7.0) [Nakada and Magasanik, 1964] supplemented with 0.5 percent glycerol instead of glucose. Cultures were incubated at 35° C for 24 hr, diluted 1,000-fold into fresh

medium, and incubated an additional 24 hr, at which time cells were in the stationary growth phase. Optical densities were adjusted to yield approximately 10⁶-10⁷ cells/ml and were subsequently diluted 100-fold in autoclaved Ware River water that had been prefiltered (Whatman No. 1, Whatman Inc., Clifton, New Jersey) before autoclaving. Zero-hour analyses of bacterial suspensions were performed immediately after mixing with autoclaved river water in the laboratory. Bacterial suspensions were dispensed in 20-ml aliquots into sterile bottles and were transported to the study sites in insulated containers. Total elapsed time from laboratory preparation to inoculation of test organisms into diffusion chambers was 2-3 hr. Viable counts were adjusted for subsequent dilution with on-site water. Experiments revealed that the time lapse between laboratory preparation and inoculation into diffusion chambers was sufficient for one or two doublings equivalent to ≤0.5 log increase in bacterial densities.

VI. Estuarine Exposure Studies

Freshly collected estuarine water was always used to prepare bacterial suspensions for diffusion chamber experiments. Twenty ml of either non-filtered or double filtered (filtration through 0.4 μm followed by 0.2 μm polycarbonate membrane [Nuclepore Co., Pleasanton, California]) estuarine water collected on site were added to 20 ml of bacterial suspension, mixed, and introduced into the diffusion chamber through a port opening by means of a 50-ml syringe without needle. Sampling ports and caps were then installed. Duplicate chambers were provided for both treatments (nonfiltered versus filtered water) of each bacterial test suspension at the testing sites.

Chambers were placed in protective wire baskets closed with Plexiglas covers and secured to a flotation device approximately 0.5-1 m below the surface (*Figure 4*). Initially, breakage of chamber membranes at the downstream location appeared to be associated with squalls and accompanying turbulence. Breakage was reduced by installing cylindrical Plexiglas baffles within the basket housing.

Data from chambers that evidenced membrane damage or became contaminated by eukaryotic microorganisms (under filtered water conditions) were omitted.

VII. Sampling

Samples were removed at selected intervals from chambers by use of sterile hypodermic syringes with 20-gauge needles. Before sampling, chambers were shaken, membranes inspected for damage, and port stoppers swabbed with 95-percent alcohol and flamed.

VIII. Enumeration of Enteric Bacteria

E. coli densities were determined through a repair-detection procedure for stressed fecal coliforms [Hackney et al., 1979]. The procedure was modified to include spread plating (instead of pour plating) on TSA with incubation at 35° C for 2 hr followed by overlaying with 10 ml violet red bile agar (VRBA) and incubation at 44.5 ± 0.5° C for 22 ± 2 hr. Bacterial densities are expressed as log colony forming units (CFU) per ml.

IX. Enumeration of Autochthonous Microorganisms

Mesophilic, heterotrophic bacterial populations were enumerated by spread plating on a medium consisting of 1.0 g/l peptone, 0.5 g/l yeast extract, 0.01 g/l ferric citrate, 0.1 g/l sodium glycerol phosphate, and 1.0 l of estuarine water at pH 7.6. Plate counts were obtained after two weeks incubation at room temperature and were adjusted by subtraction for contributions by *E. coli* or salmonellae (which also were recovered on this medium). Densities of microorganisms lytic to test bacteria were assessed by counting plaques on dense lawns of *E. coli* or salmonellae in double layer agar after three weeks incubation at room temperature [Enzinger and Cooper, 1976].

A Zeiss epifluorescence microscope, equipped with a 63X oil immersion objective, a 450-490 nm excitation filter, LP 520 barrier filter, and FT 510 beam splitter, was used to count microflagellates (both heterotrophic and autotrophic forms). Samples were fixed by the addition of glutaraldehyde to a final concentration of 0.5 percent immediately after collection and were filtered at low vacuum (100 mm Hg) through the use of a microanalysis filtration tower (Millipore Corp., Bedford, Maine) onto a 0.2 µm Nuclepore filter previously stained overnight in 0.2 percent Irgalan Black (CIBA-Geigy Corp., Greensboro, North Carolina) in 2 percent acetic acid. Filtered cells were then washed in place by filtration to dryness with 10 ml of a 0.2 percent sodium metabisulfite solution in distilled water to remove excess glutaraldehyde and to decrease fading

of the excited fluorescent dye. Two ml of distilled water were placed on the filter without vacuum, and the filters were stained for 5 min by the addition of 0.2 ml of 0.033 percent proflavine hemisulfate (Sigma Chemical Co., St. Louis, Missouri), filtered, and rinsed by filtration with 10 ml of distilled water. While still under vacuum after the final wash, each filter was removed, placed on a slide, cleared using a nonfluorescing immersion oil (Cargille type LF, Cedar Grove, New Jersey), and covered with a No. 1 cover slip. Slides were stored at 4° C, and cells were counted (from a minimum of 50 fields of view chosen at random) within five days.

Chambers inoculated with enteric cultures in membrane-sterilized site water were evaluated for contamination by autochthonous microorganisms through epifluorescence microscopy, spread plating on a heterotroph medium, and plaque formation on double layer agar bacterial lawns.

X. Determination of Sublethal Stress

A quantitative index of sublethal stress was obtained through repair procedures by comparing the recovery of *E. coli* and *S. tennessee* to recovery obtained when selective media and temperatures were immediately applied with no resuscitation period. An electrochemical detection method [Anderson et al., 1979] was used to obtain a second stress index for *E. coli*. Stress was defined as the difference between a predicted electrochemical detection time (EDT) in EC broth at 44.5° C, calculated for non-exposed cells from a standard curve, and the observed EDT for cells following their exposure to estuarine water. A linear least-squares regression technique (coefficients of regression ranged from 0.996-0.987) was used to establish standard curves relating inoculum size to EDT for each isolate. Ninety-five percent confidence limits were determined by calculating the standard error of the estimated EDT from the linear regression [Sokal and Rohlf, 1981].

XI. Determination of Salinity, Dissolved Oxygen, and Temperature

A YSI Model 57 dissolved oxygen meter with a salinity compensator (Yellow Springs Instrument Co., Yellow Springs, Ohio) and a remote probe were used to measure temperature and dissolved oxygen. Salinities were determined through the use of a temperature-compensated refractometer (Model 10419, American Optical Corp., Keane, New Hampshire). Field determinations were made initially and again at each sampling interval during the experiments.

RESULTS

I. Experiment Schedule

In situ experiments were conducted during the months of February through May, July, and November 1981 and February 1982. During the first experiment in February 1981, limited data were acquired from the Ware River site because of equipment-deployment problems and unfavorable weather. Experiments comparing bacterial survival between sites were initiated in March, July, and November 1981 and February 1982. During April and May, experiments were conducted exclusively in Fox Mill Run to expand the data base on the comparative survival of *E. coli* and *Salmonella* spp. as water temperatures increased. Salinity, temperature, and dissolved oxygen data for each experiment are presented in *Table 1*.

II. Bacterial Survival in Filtered Estuarine Water

Bacterial die-off in the absence of predators was related inversely to water temperature. Viable counts for duplicate chambers are plotted in *Figure 5* with mean results summarized by season in *Figure 6*. During July, when the mean water temperature was 28° C, enteric bacterial densities increased by 1-1.5 logs during the first 3-day exposure and remained elevated above initial levels until termination of the experiment at 15 days. Although *E. coli*, *S. tennessee*, and *S. typhimurium* exhibited similar survival patterns at temperatures above 10° C (*Figures 5, 6, and 7*), salmonella populations were as much as three orders of magnitude higher than *E. coli* when mean water temperatures ranged from 4-9° C (February and March 1981 and February 1982). In March, when mean water temperatures were 8° and 9° C at the Ware River and Fox Mill sites, respectively, *E. coli* exhibited significant decline at both sites. Although the mean water temperature in November was 12° and 10° C at Ware River and Fox Mill sites, respectively, a decrease in bacterial numbers corresponding to that observed in March was not observed. In the November experiment, water temperatures declined during the 2-week period about 8° C, whereas the water temperature deviated by less than 1° C in March.

The influence of exposure site on bacterial survival was most pronounced under adverse conditions observed during cold weather (*Figure 5*). Salinity differences between study sites equivalent to 9.4 and 5.4 ‰ in February 1982 and March 1981, respectively, were greatest at these times.

During the February experiment, viable counts of salmonellae at Fox Mill Run were approximately 1 log greater than corresponding counts in Ware River after 11 days of exposure. In March the survival differential between these same sites was even greater, with 2-3 log greater recovery of salmonellae at Fox Mill Run after 15 days of exposure. Survival of *E. coli* was also favored at Fox Mill during February and March but to a lesser extent than for salmonellae, with only about 1 log greater recovery compared with the downstream site. During July, when warmer water temperatures would be expected to favor multiplication and survival, *E. coli* and salmonella densities after 15 days of exposure were 0.4 and 0.6 logs, respectively, greater at Fox Mill Run than at the downstream site.

III. Bacterial Survival in Nonfiltered Estuarine Water

Survival of *E. coli* and salmonellae decreased in the presence of autochthonous microorganisms when compared to survival in filtered water (*Figures 6 and 7*). These differences were most pronounced during warmer water temperatures in April, May, and July. At these times, bacterial densities in filtered water at 15 days were 3-4 logs greater compared with chambers filled with nonfiltered water. In February and March, there were no significant differences in survival in the absence and presence of the indigenous microbiota, with the exception of salmonella populations exposed in Fox Mill Run (*Figure 7*). Although the effects of the natural microbiota on bacterial densities were more pronounced at warmer temperatures, significant numbers of test bacteria remained after 15 days of exposure, e.g., 10³⁻⁴ bacteria/ml during the July experiment (*Figure 6*).

Population changes in densities of autochthonous microorganisms are shown in *Figures 7, 8, and 9*. During April and May, total microflagellates had increased by the third day. A similar response was delayed in the months of February, March, and November. Microflagellate densities remained elevated for the duration of all experiments, except in July when random fluctuations were observed. Increases in plaque-forming units (PFU) were more pronounced at warmer water temperatures and appeared earlier at Fox Mill Run than at the Ware River site. PFU densities increased at Fox Mill Run from 10² ml to 10⁴ ml by the third day in the July experiment. Little or no increase in PFU densities occurred in February and March. Observations of plaques by light microscopy routinely revealed the presence of amoebae. Lytic zones caused by bacteria were not detected. Indigenous estuarine bacteria increased 1-1.5 logs

above ambient levels by the third day, except in March when the response was slower, and remained elevated thereafter.

IV. Sublethal Stress

Sublethal stress, measured for *E. coli* by the electrochemical detection technique and resuscitative versus nonresuscitative enumeration, was inversely related to water temperature (Figures 10 and 11). Pronounced EDT delays for *E. coli*, observed in February and March, increased progressively with exposure. In November and April, minimum stress evident by three days did not intensify progressively. In May and July, EDT delays were observed only at 15 days. The VRBA overlay technique recovered 10^4 *E. coli*/ml from chambers exposed for three days in Fox Mill Run, while simultaneous enumeration using direct plating on VRBA failed to yield bacterial colonies. Contrastingly, maximum recovery differentials between selective versus resuscitative plating for *E. coli* was about 1 log after three days of exposure in the Ware.

Sublethal stress of *E. coli*, as determined by the relative enumeration efficiency, involves an all-or-none growth response under selective temperature and medium conditions. In contrast, the electrochemical detection technique measures a graded response under selective cultural conditions, i.e., the time required for a bacterial inoculum to produce a potential increase. Therefore, it is not possible to relate results of the two techniques directly. Under cold water conditions, continued die-off and failure of stressed cells to grow on VRBA precluded the usefulness of this technique.

Salmonella populations exhibited a similar pattern of greater stress at colder temperatures, although differences were not as large as those observed for *E. coli*. Selective plating procedures indicated that the most pronounced effect of environmental exposure on recovery of salmonellae was about a 0.5 log differential between resuscitative and nonresuscitative methods. As noted for *E. coli* populations, salmonellae were more stressed at Fox Mill Run during the February 1982 experiment than at the Ware River site. Reasons for this relationship between intensity of stress under cold water conditions and exposure site are not clear.

DISCUSSION

The results of this study demonstrate the potential of *E. coli* and salmonellae for prolonged survival in the estuarine environment. Under favorable temperature regimes, in situ increases in enteric bacterial densities were observed both in filtered and nonfiltered river water. In vitro studies using estuarine or marine waters with or without nutrient amendment demonstrated temporary increases in enteric bacterial populations typically followed by a decline or death phase [Vaccaro et al., 1950; Cook and Hamilton, 1971; Savage and Hanes, 1972; Won and Ross, 1973]. Organic nutrient amendments in the range of 100-200 ppm resulted in the onset-of-death phase during 1-3 days incubation at 20-25° C. In the present study, glycerol associated with the bacterial inoculum was present at an initial concentration of approximately 5-12 ppm in diffusion chambers. According to Shehata and Marr [1971], the growth rate of a steady-state culture of *E. coli* is sharply reduced at glucose concentrations of less than 0.018 ppm. Safranin diffusion experiments indicated the glycerol concentration would have been reduced to less than 0.018 ppm within 36 hr at 9° C or 19 hr at 29° C based on a Q_{10} (temperature coefficient) of 1.37 for sugar diffusion [Giese, 1966]. Thus, continued persistence of *E. coli* and salmonellae at densities elevated above initial levels suggests that this occurs at the expense of in situ nutrients.

The fouling of exterior surfaces of chamber membranes occurred and was particularly pronounced during spring and summer experiments. Previous observations [Anderson et al., 1982] have shown that fouling of membrane surfaces does not impede diffusion rates of safranin. Scanning electron microscopy of interior membrane surfaces from chambers filled with nonfiltered water showed the presence of attached microbial communities. Colonization by indigenous microbiota at the solid-liquid interface may have been due to nutrient adsorption and availability of membranes as attachment sites, conditions which may also have promoted growth and/or survival of *E. coli* and salmonellae.

It is interesting to note that heterotrophic bacterial populations within the chambers also evidenced substantial increases and remained elevated throughout the experiments. This type of response in closed containers is commonly known as a "bottle effect" and is considered to be the result of a number of possible causes, foremost of which is the availability of surface for concentration of organics and cells. Accumulation of inorganic and organic nutrients at solid interfaces in aquatic environments

results in colonization by microorganisms initially dominated by bacteria [Marshall et al., 1971; Paerl, 1975; Marshall, 1980]. Bacteria may become attached by fibrous polysaccharides [Fletcher and Floodgate, 1973; Marshall, 1979] that may further serve to trap and concentrate nutrients from the surrounding water [Corpe, 1980]. Primary bacterial colonization is succeeded by diatoms, unicellular algae, and cyanobacteria [Marshall, 1979], all of which may also provide nutrients to the chamber content.

It is possible that the survival of enteric bacteria observed in this study may have been enhanced not only by nutrient enrichment at membrane interfaces but also by a beneficial association with fouling organisms. Such an effect could have been exerted in chambers receiving filtered estuarine water where the succession of microbial types would have occurred on exterior membrane surfaces. Test bacteria would be exposed to diffusible extracellular products while protected from predation. McFeters et al. [1978a, b] demonstrated the potential of a symbiotic relationship between indicator and pathogenic bacteria and algal mat communities in oligotrophic freshwater. These investigators observed the emergence of benthic algal communities coincident with elevated coliforms in a remote alpine stream during late summer. Extracellular products produced by axenic cultures of an algal field isolate supported the laboratory growth of enteric bacteria. It also has been noted that certain algae produce antibacterial toxins [Sieburth, 1959; Saz et al., 1963; Aubert et al., 1975], and anticoliform activity of seawater has been associated with termination of phytoplankton blooms [Sieburth and Pratt, 1962]. The significance of algal toxins on survival of enteric bacteria remains to be elucidated [Mitchell and Chamberlin, 1975].

In vitro multiplication of fecal coliforms in sterile estuarine water with nutrients added has been shown to be temperature dependent [Won and Ross, 1973]. Hendricks [1971a, 1972] observed greater growth and respiration rates for indicator and pathogenic bacteria in autoclaved freshwater as incubation temperatures were increased. In our study bacterial increases in filtered estuarine water also were more pronounced at the warmer temperatures encountered during spring and summer experiments. Physiological indices of stress demonstrated that *E. coli* and salmonellae were minimally stressed at these water temperatures. These observations support previous findings in another estuary that *E. coli* die-off [Anderson et al., 1982] and sublethal stress [Rhodes et al., 1982] in filtered water are inversely related to temperature.

Conversely, other in situ survival studies have concluded that *E. coli* survival is inversely related to temperature [Faust et al., 1975; Vasconcelos and Swartz, 1976]. We believe that undetected contamination by indigenous microorganisms and the experimental methodology used may have influenced these earlier studies. The fact that commonly used membrane diffusion chambers become contaminated by bacteria and eukaryotes in the environment has been observed by us [Anderson et al., 1982] and others [Roper and Marshall, 1979]. Harsh laboratory manipulations of test bacterial inocula—e.g., centrifugation, washing, and resting at cold temperatures—stress the cells prior to exposure to environmental stressors, and recovery of debilitated cells through selective enumeration techniques will underestimate the surviving population [Rhodes et al., 1982].

Rather than attribute seasonal changes in enteric survival to temperature per se, the results of this study suggest that survival is also related to seasonal changes in the estuarine microbiota. When chambers containing filtered and nonfiltered estuarine water were compared, reductions associated with the presence of indigenous microorganisms were most pronounced at warmer temperatures. However, substantial numbers of test organisms persisted, e.g., 10^2 - 10^4 ml after two weeks exposure during April-July experiments when water temperatures ranged from 18-28° C. In vitro studies using nonfiltered seawater have shown more dramatic reductions in *E. coli*: 10^6 ml to $<10^1$ ml by 7 days [Mitchell et al., 1967]; 10^8 ml to $<10^3$ ml by 5 days [Roper and Marshall, 1978]; and 10^8 ml to $<10^1$ ml by 10 days [McCambridge and McMeekin, 1980b]. Differences in the magnitude of bacterial decline observed by these investigators and in the present study may be due to natural variation in composition and quantity of microbiota as well as to initial concentrations of prey organisms. It has been shown in vitro that 10^6 - 10^7 bacteria/ml are required to support bacteriovores such as bdellovibrios [Fry and Staples, 1974; Varon and Zeigler, 1978], ciliates [Berk et al., 1976; Habte and Alexander, 1978], and amoebae [Danso and Alexander, 1975]. Below critical prey densities, predators and prey can coexist. The continued decline of bacteria beyond the critical level 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 at the membrane interface or by diffusion.

It generally has been agreed that *E. coli* and salmonellae have comparable death rates in the aquatic environment [Beard and Meadowcroft, 1935; Smith et al., 1973; McCambridge and McMeekin, 1980a]. The results pre-

sented here demonstrate that while *E. coli* and salmonellae show similar survival in the absence or presence of natural microbiota at warm temperatures, responses of the indicator and pathogen at temperatures below 10° C are quite different. These results may explain the findings of Brezenski and Russomanno [1969], who reported the isolation of salmonellae from individual clams with fecal coliform most-probable-number values of <20/100 g. These authors noted that the water temperature was 5° C and speculated that salmonellae may exhibit increased survival over fecal coliforms within the particular clam species. According to our results, salmonella survival in the aquatic environment is favored at warmer temperatures, but at colder water conditions it is more resistant than *E. coli*. Finally, it should be mentioned that our estimates of salmonella survival may be conservative. Kaper et al. [1977] reported salmonellae may remain undetected in an estuarine environment unless specialized resuscitative procedures are employed for recovery.

Differences in survival between the study sites were manifested primarily under filtered water conditions at the colder water temperatures. The protective effects of nutrients on *E. coli* survival at water temperatures of 3-5° C have been demonstrated [Won and Ross, 1973]. Based on the data obtained, however, it is not possible to isolate a nutrient effect from other influencing factors, such as greater fluctuations in salinity and temperature at Fox Mill Run or variations in penetration of solar irradiation or composition of microbiota between the two study sites.

Although various nutrients in Fox Mill Run statistically appeared to be elevated above Ware River levels, concentrations in the tidal creek were relatively low when compared to upstream sites [Bosco et al., 1982]. The relative significance of hydrological processes (e.g., dilution) and biological processes is not known. Nutrient transformation processes can occur during water passage through wetlands and has led to the use of marshes for assimilation of sewage nutrients [Sloey et al., 1978]. In a mass balance study of a tidal marsh receiving sewage effluent, Wetzel et al. [1977] noted that the export of nutrients from the creek equaled or exceeded input by the sewage plant. In order to understand the performance of a wetland in altering water quality, a knowledge of hydrology of the system, nutrient concentrations, mass flow, and nutrient storage is required [Kadlec and Kadlec, 1978].

The ability of *E. coli* and salmonellae to survive in the estuarine environment has been demonstrated. Seasonal factors and physico-chemical

characteristics of the exposure site were shown to influence bacterial survival and sublethal stress. The results obtained within the microcosm of membrane diffusion chambers may parallel natural events, such as suspended particles or sediments, at submerged surfaces. This agrees with observations that *E. coli* [Erkenbrecher, 1981] and salmonellae [Hendricks, 1971b; Van Donsel and Geldreich, 1971] densities are greater in sediments than overlying waters. The prolonged persistence of *Salmonella* spp. as compared to *E. coli* under cold water conditions suggests that fecal coliforms may be an inadequate indicator of pathogens during the prime months of shellfish harvesting.

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FIGURES

FIGURE 1
Location of Ware River Subestuary

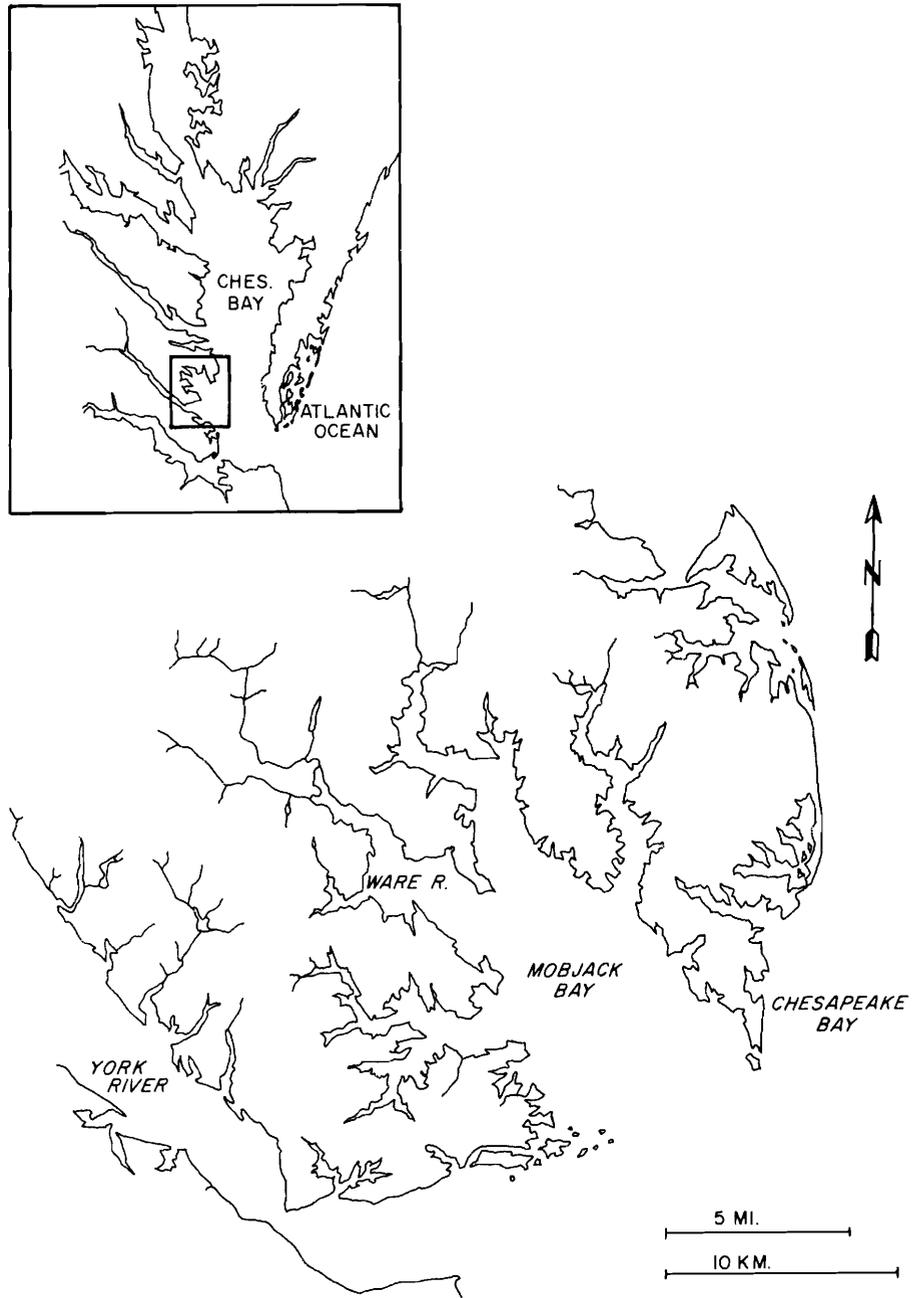


FIGURE 2
Location of Sewage Treatment Plant (STP) and Diffusion Chamber Deployment Sites in Fox Mill Run (FMR) and Ware River (WR)

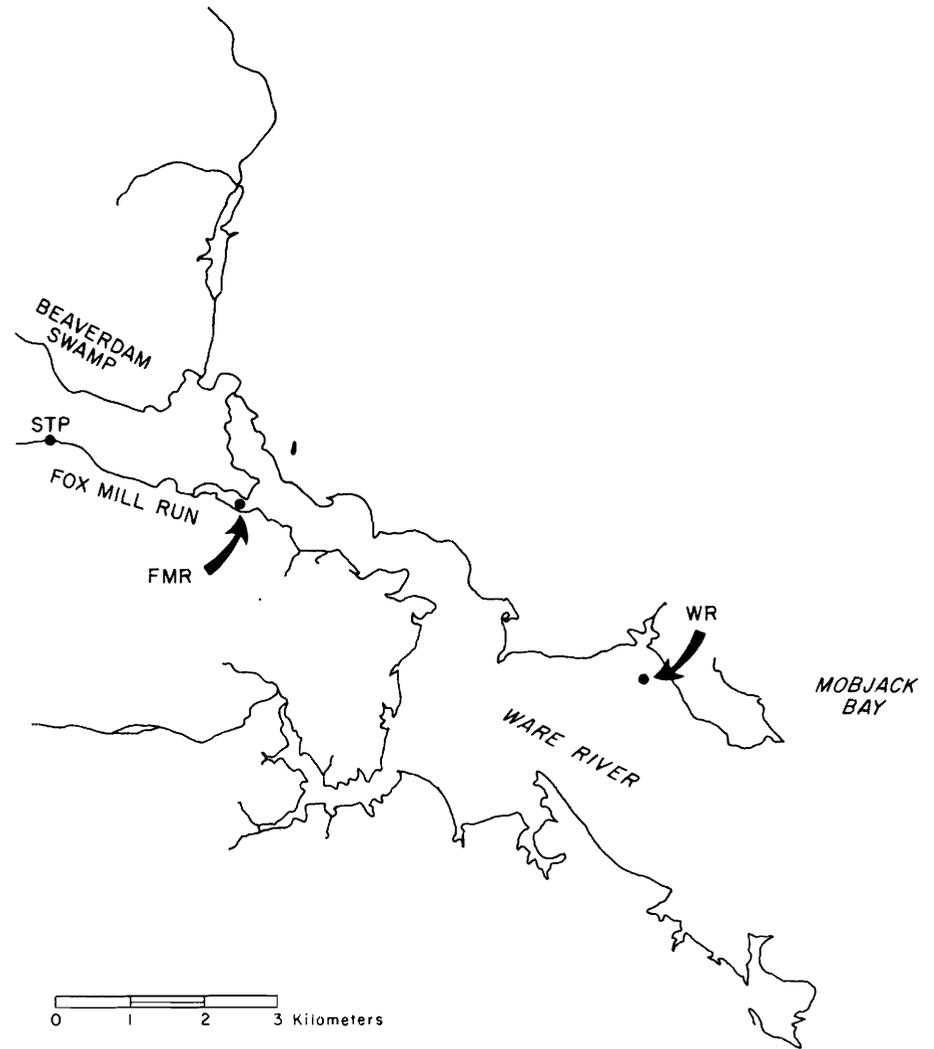


FIGURE 3
Design of Membrane Diffusion Chamber

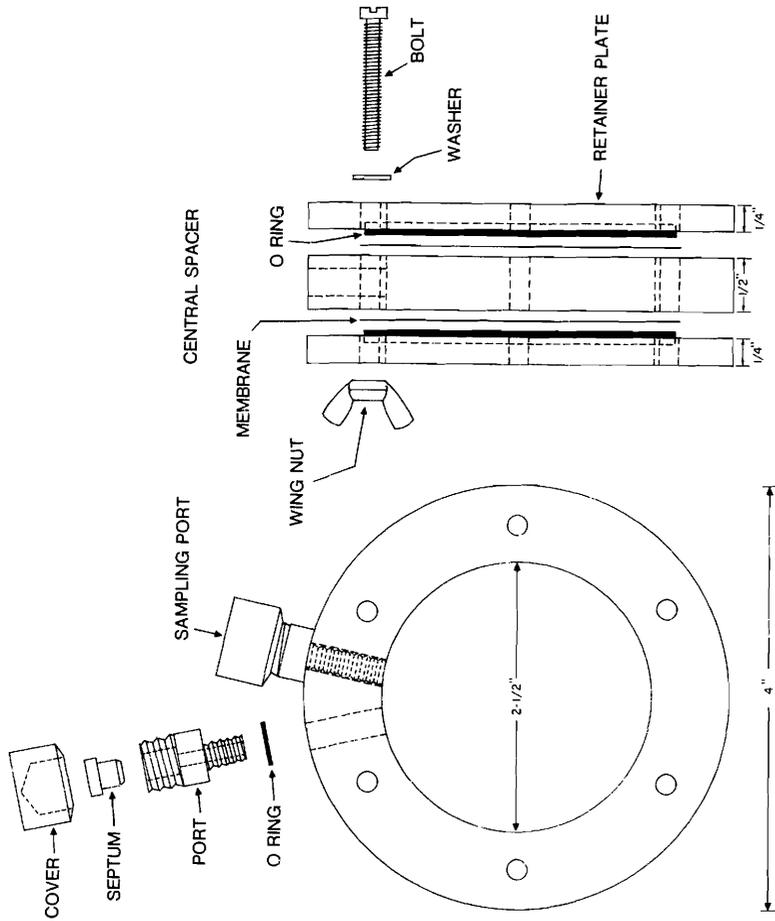


FIGURE 4
Design of Moored Diffusion Chamber Array Buoy
at Ware River Study Site

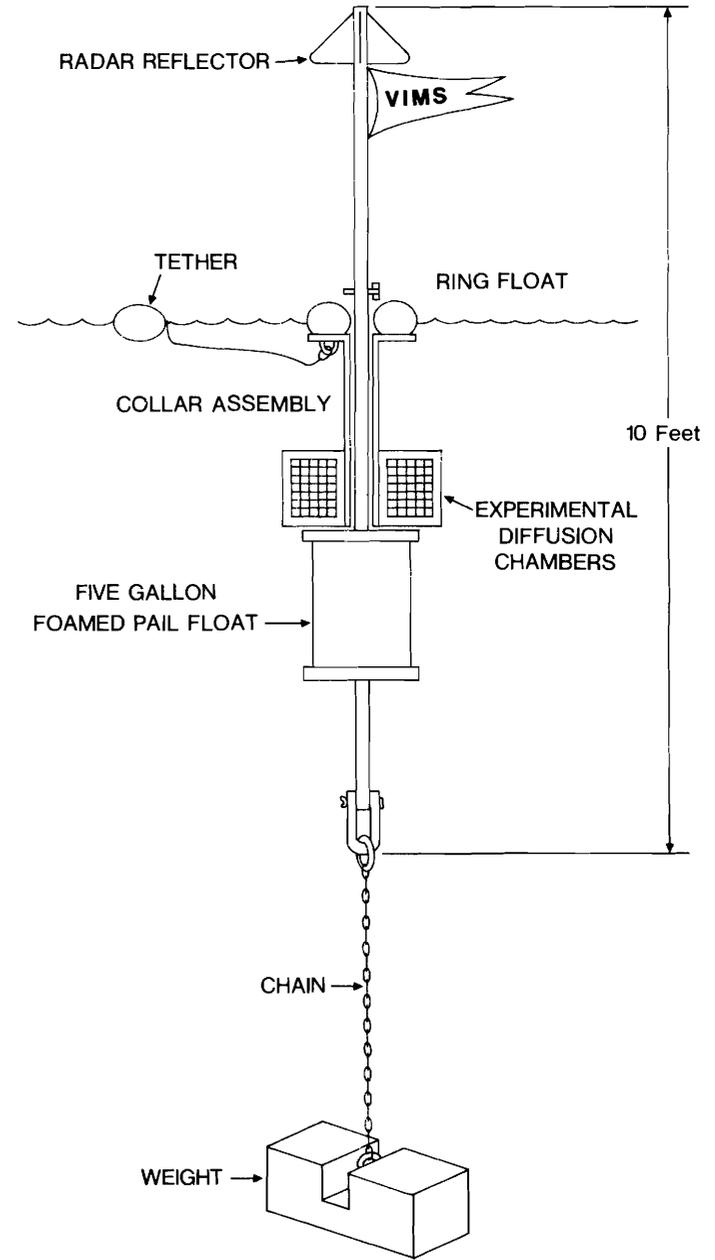
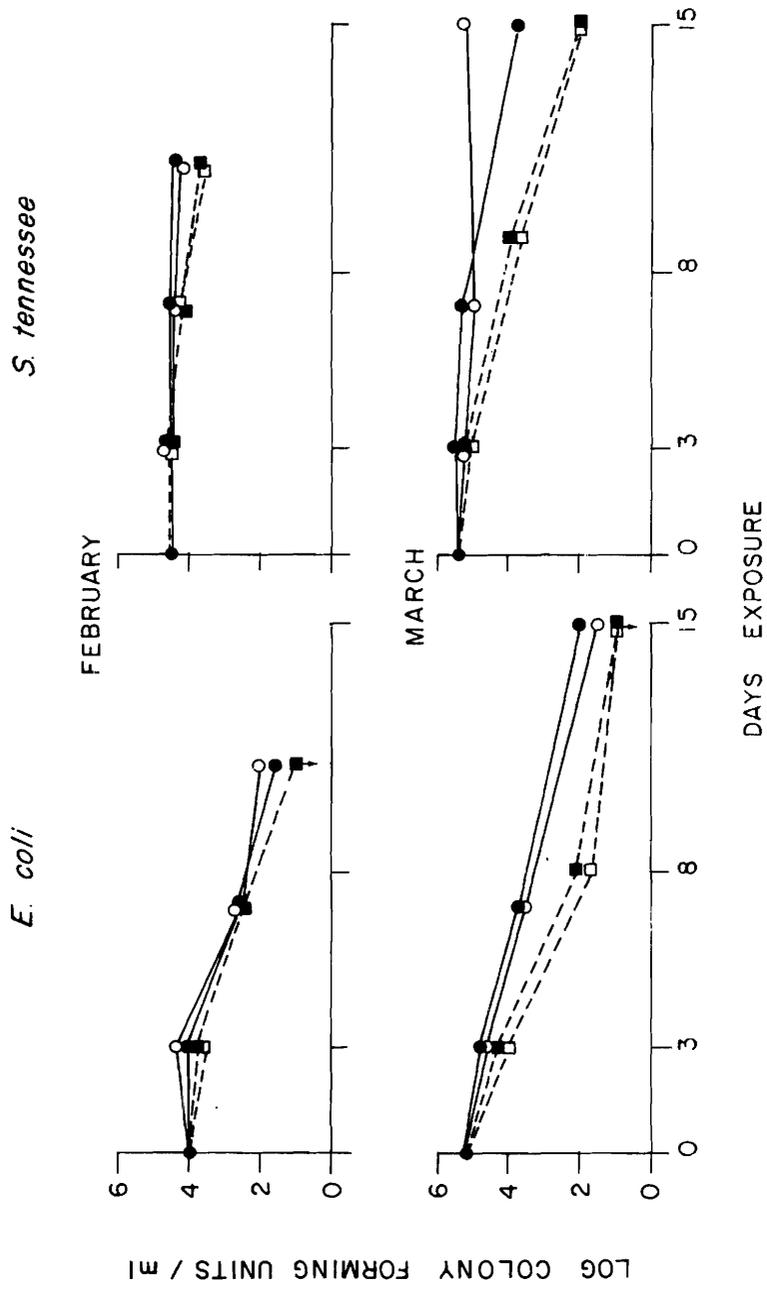


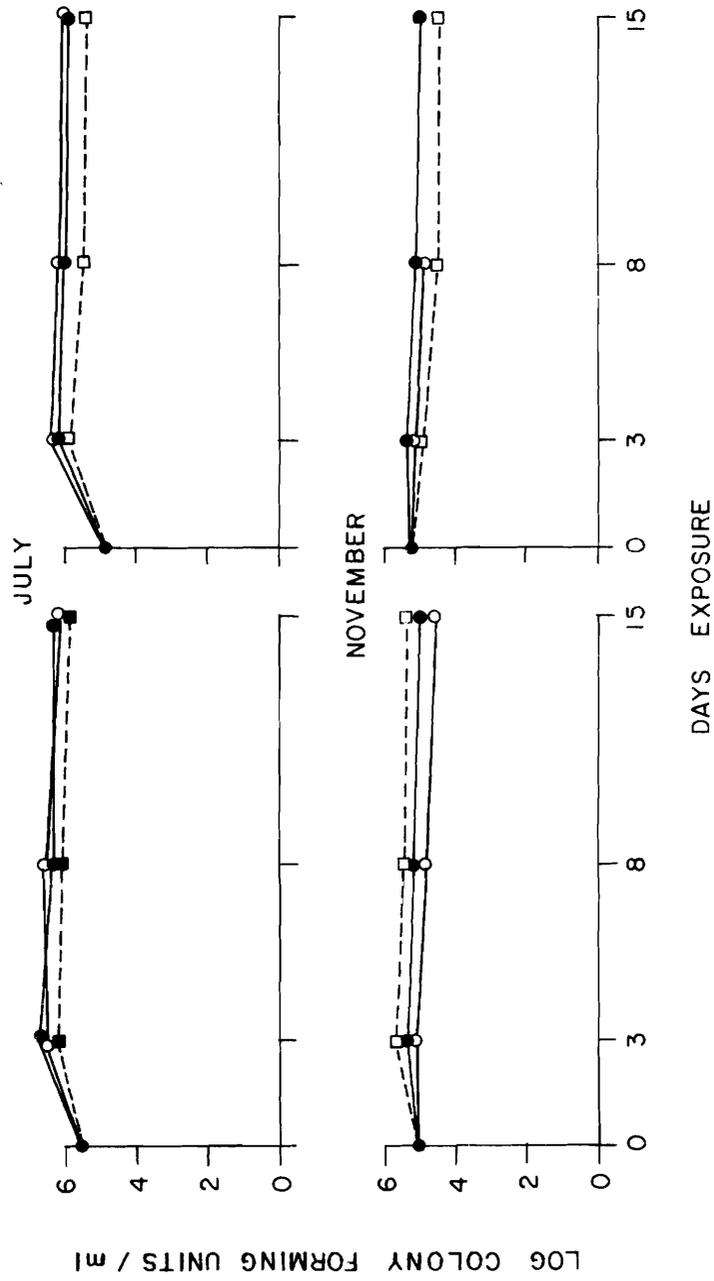
FIGURE 5
Survival of *E. coli* and *S. tennessee* in Diffusion Chambers Filled with Filtered Estuarine Water and Exposed In Situ at Fox Mill Run and Ware River*



E. coli

S. tennessee

FIGURE 5 (continued)



*Symbols: ○ ● Fox Mill Run; □ ■ Ware River. (Replicates indicated by open and closed symbols.)

FIGURE 6
 Seasonal Survival of *E. coli* and Salmonellae Exposed In Situ to the Estuarine Environment*

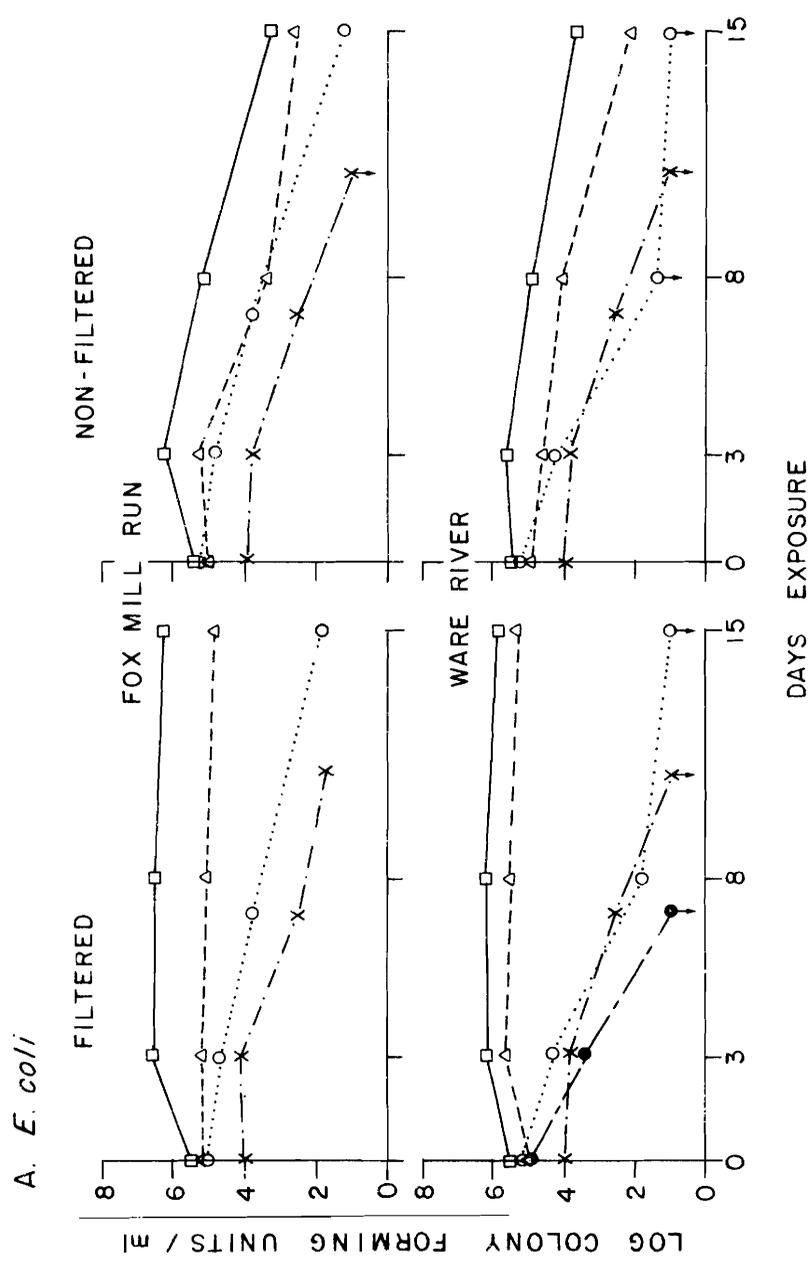
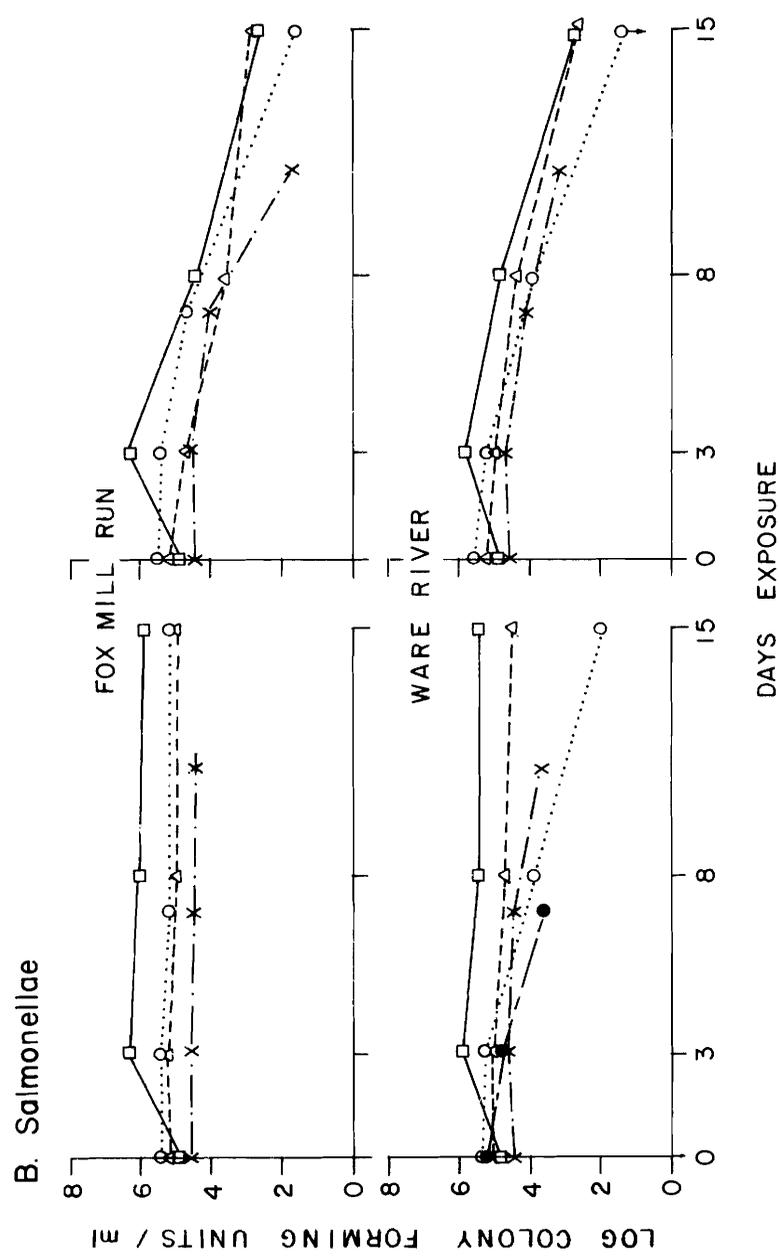


FIGURE 6 (continued)



*Symbols: ● February 1981; ○ March 1981; □ July 1981; △ November 1981; x February 1982. Data from duplicate chambers averaged.

FIGURE 7
In Situ Survival of *E. coli* and *S. tennessee* or *S. typhimurium*
During April and May, Respectively, in Fox Mill Run*

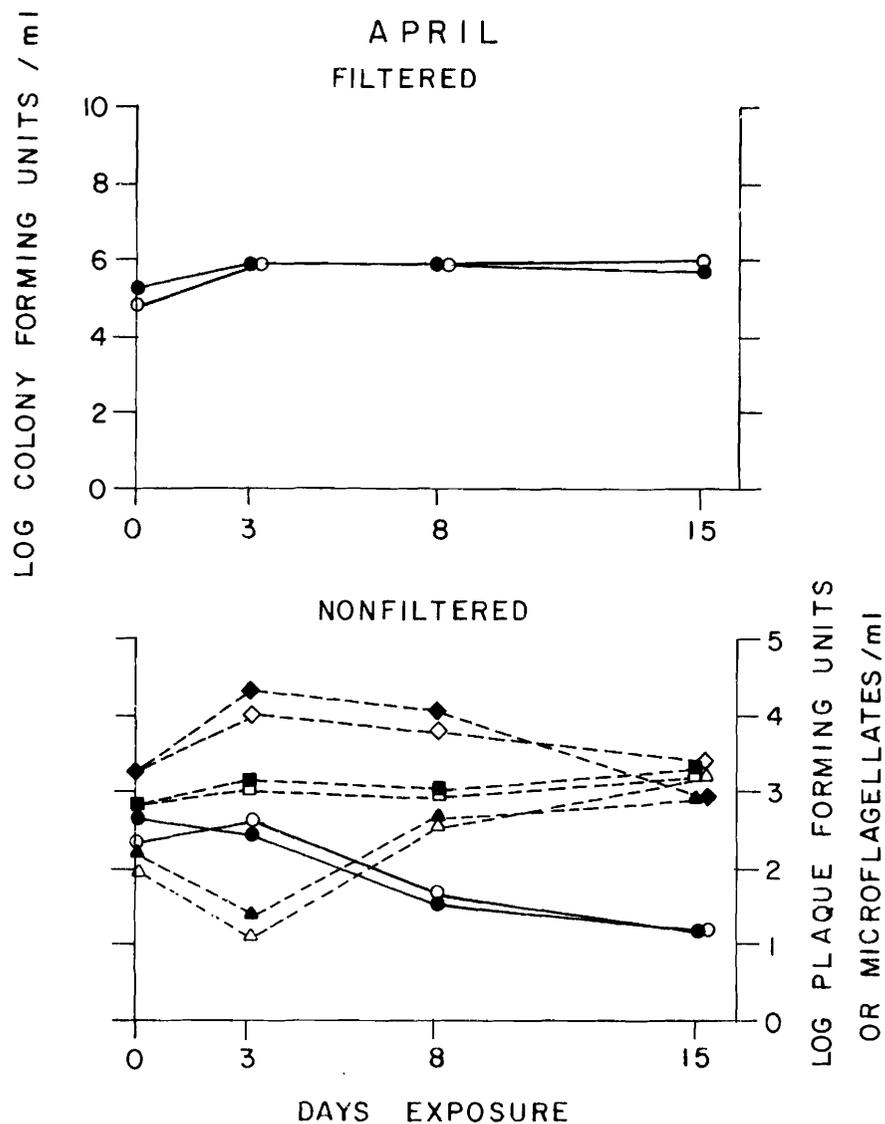
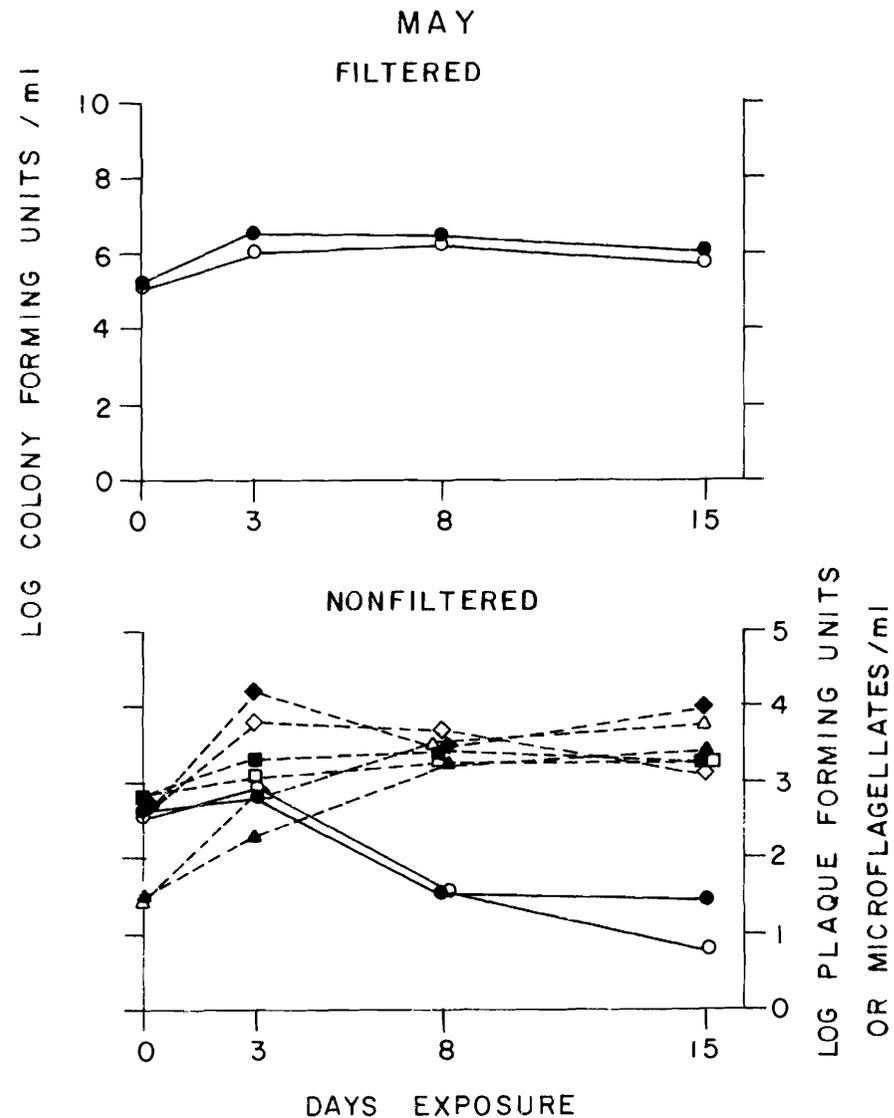


FIGURE 7 (continued)



*Symbols: ○ *E. coli*; ● salmonellae; ■ autochthonous bacteria; △ ▲ plaque forming units; ◇ ◆ microflagellates. Open and closed symbols represent indigenous microbiota in chambers filled with *E. coli* and salmonella suspensions, respectively, and non-filtered water. Data from duplicate chambers averaged.

FIGURE 8
Survival of *E. coli* in Diffusion Chambers Filled with Nonfiltered Estuarine Water and Exposed In Situ*

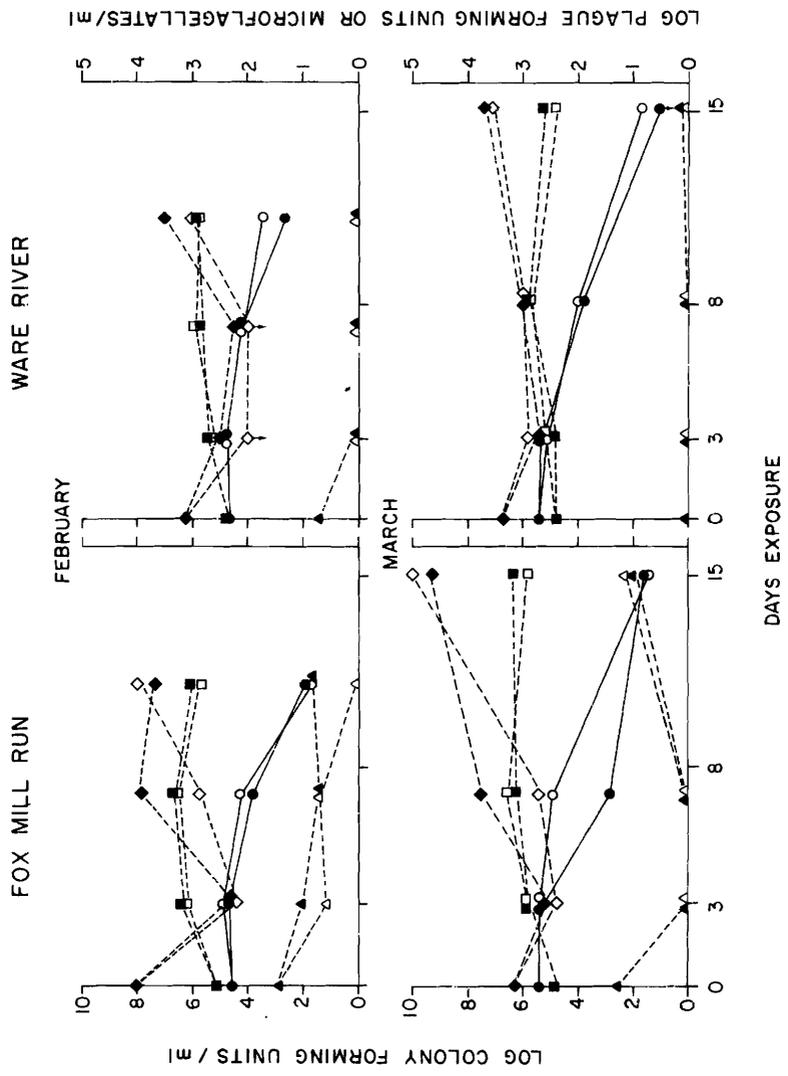
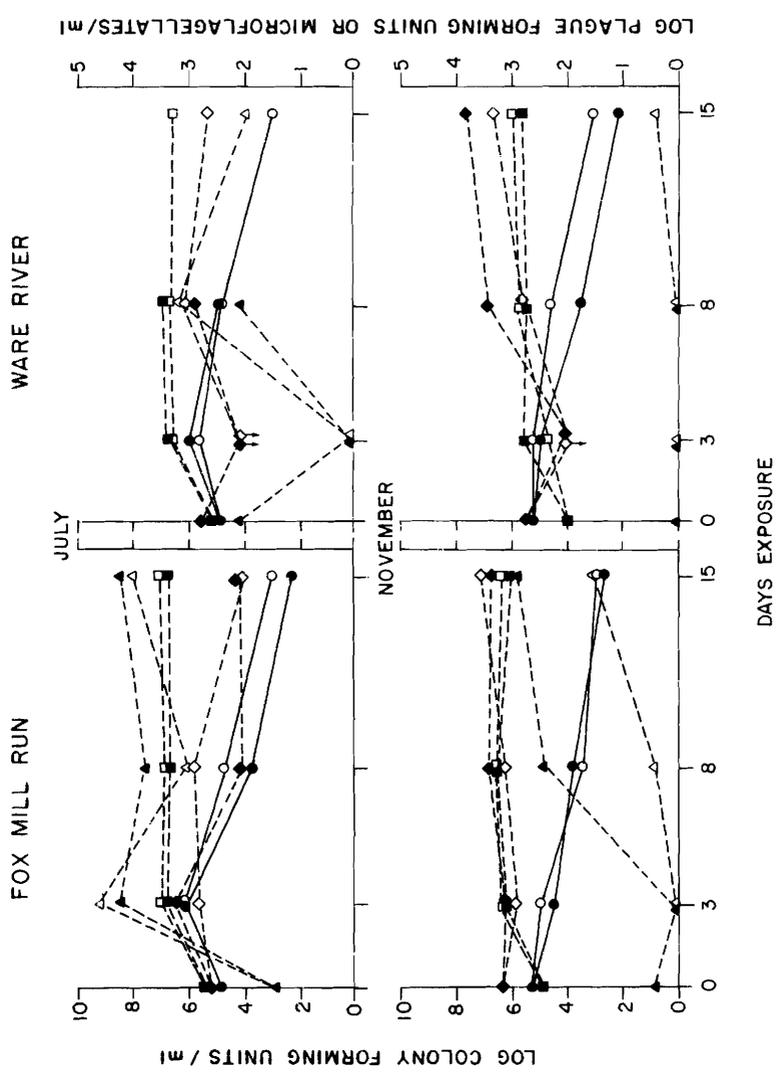


FIGURE 8 (continued)



*Symbols: ● *E. coli*; ■ autochthonous bacteria; ▲ plaque forming units; ◇ microflagellates. Replicates indicated by open and closed symbols.

FIGURE 9
Survival of *S. tennessee* in Chambers Filled with Nonfiltered Estuarine Water and Exposed In Situ*

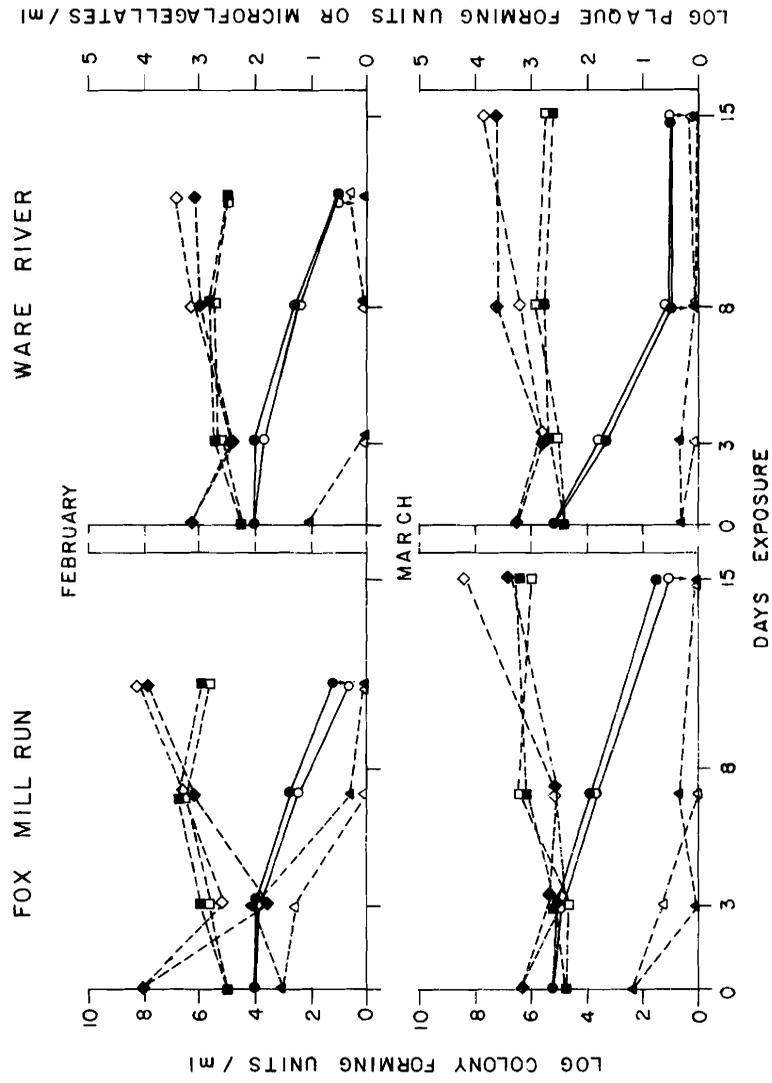
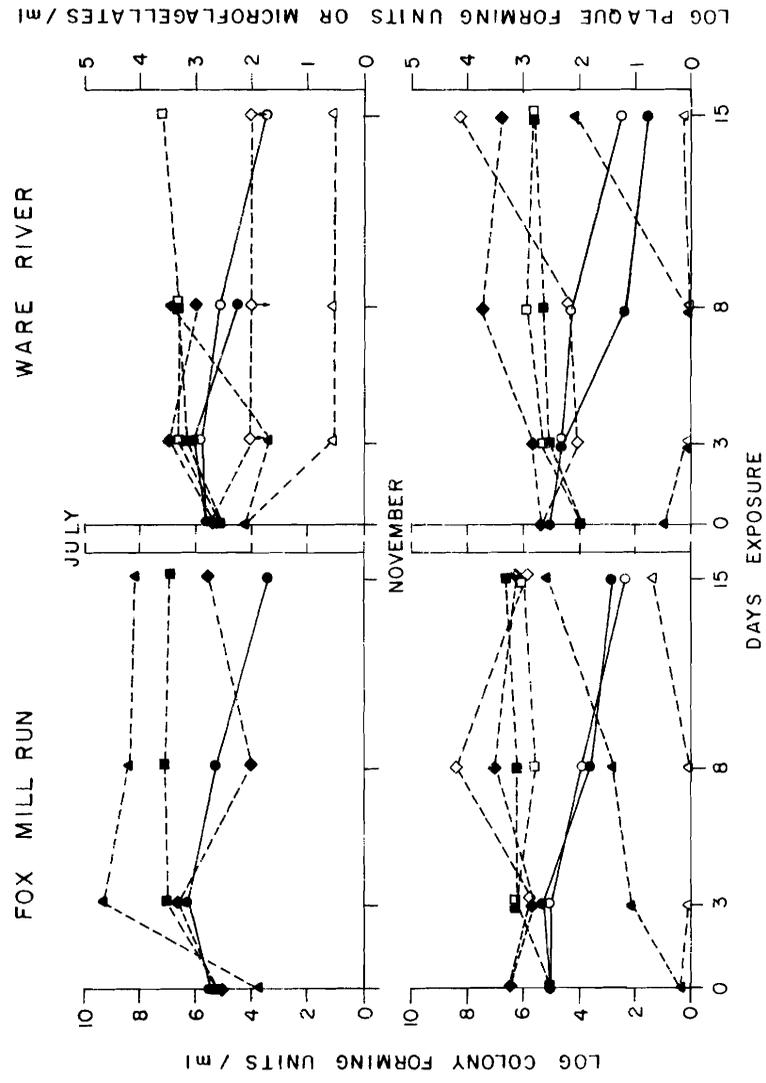


FIGURE 9 (continued)



*Symbols: ○ ● *Salmonella*; □ ■ autochthonous bacteria; △ ▲ plaque forming units; ◇ ◆ microflagellates. Replicates indicated by open and closed symbols.

FIGURE 10
Incidence of Sublethal Stress
as Measured by an Electrochemical Detection Method
for *E. coli* Exposed In Situ at Fox Mill Run and Ware River*

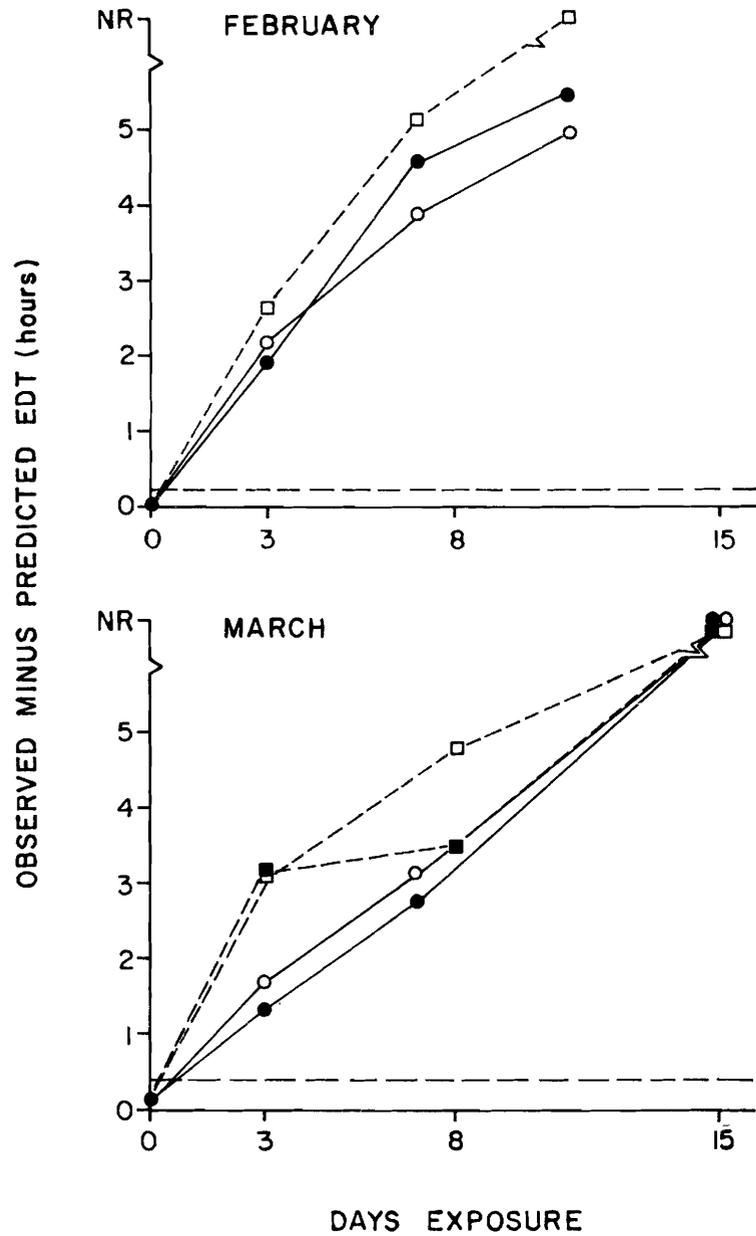
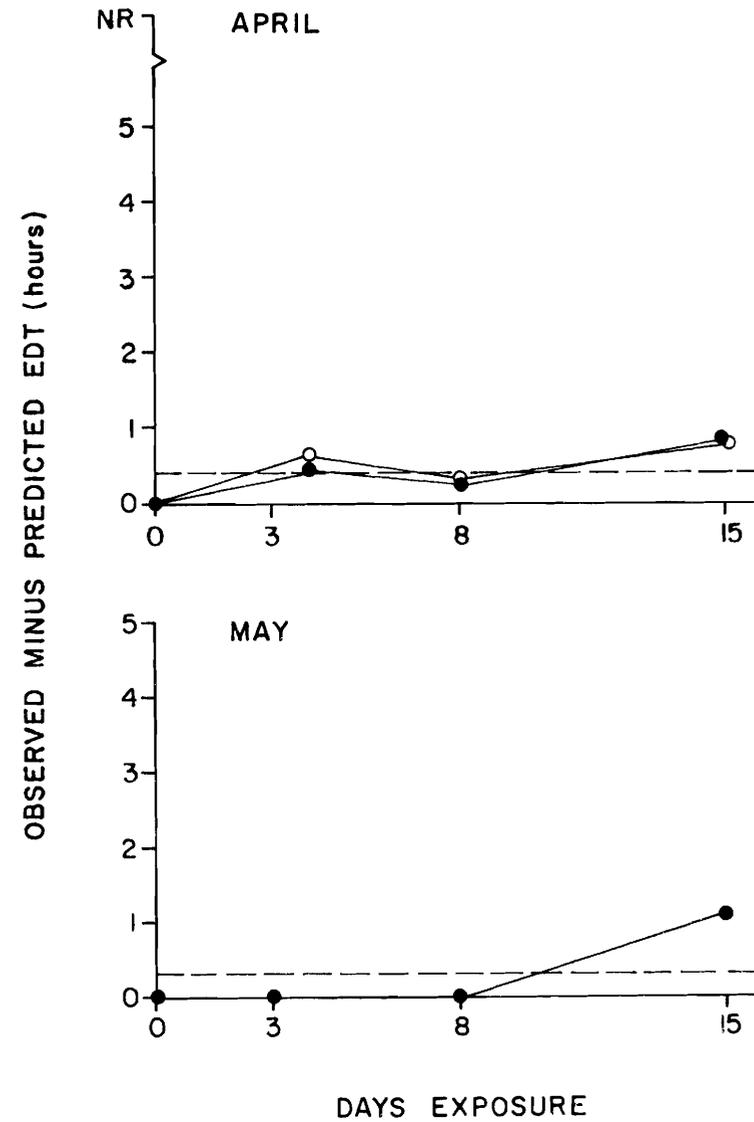
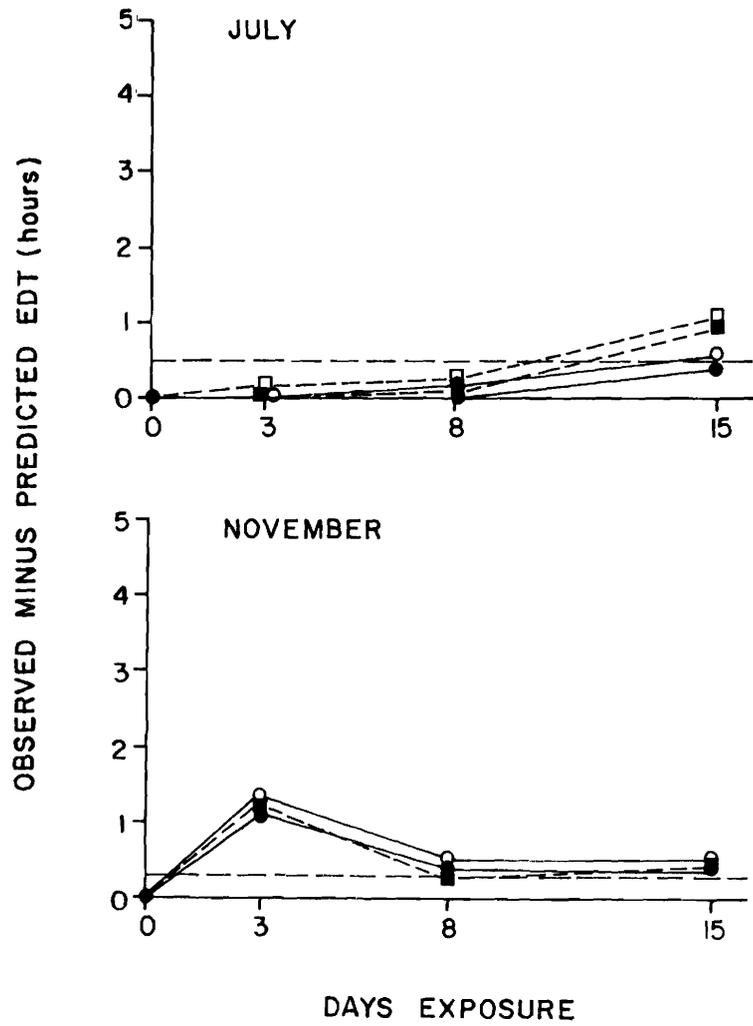


FIGURE 10 (continued)



(continued)

FIGURE 10 (continued)



*Symbols: ○ ● Fox Mill Run; □ ■ Ware River. Replicates indicated by open and closed symbols. Upper 95 percent confidence limit for predicted EDT indicated as (---). Lack of a detectable electrochemical response is represented by NR.

FIGURE 11
Incidence of Sublethal Stress as Measured by Resuscitative Versus Nonresuscitative Plate Count Enumeration for *E. coli* and *S. tennessee* Exposed In Situ at Fox Mill Run and Ware River*

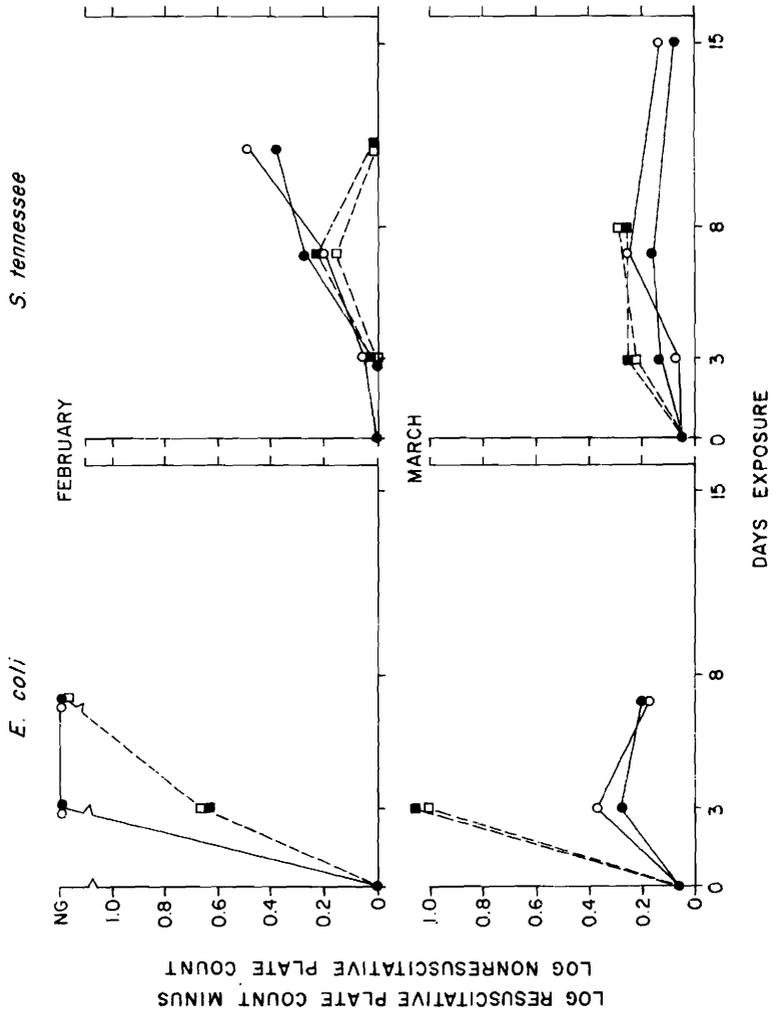
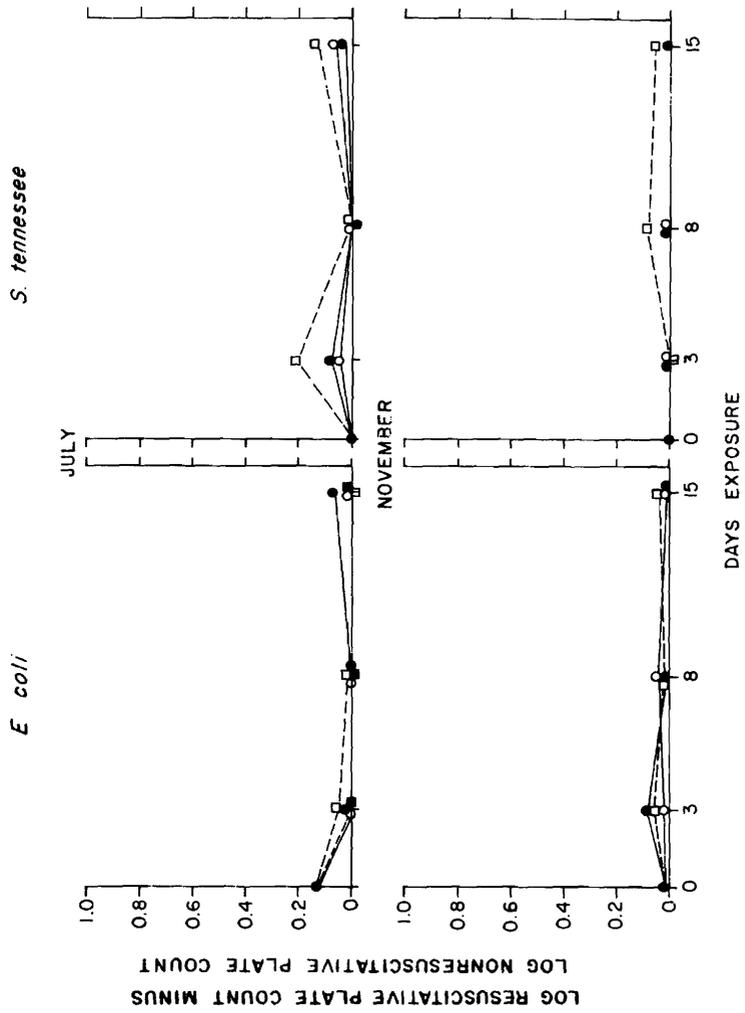


FIGURE 11 (continued)



*Symbols: ○ Fox Mill Run; □ Ware River. Replicates indicated by open and closed symbols. No growth using the nonresuscitative technique is represented by NG. Plate counts ≤ 10 colonies per 0.1 ml sample plated are not represented.

TABLE

TABLE 1
Mean Values of Salinity, Dissolved Oxygen, and Temperature Measurements
During In Situ Bacterial Survival Experiments

Date	Fox Mill Run			Ware River		
	Salinity (‰)	Dissolved Oxygen (mg/l)	Temperature (°C)	Salinity (‰)	Dissolved Oxygen (mg/l)	Temperature (°C)
2/10/81-						
2/17/81				23.3	11.2	3.7
3/10/81-						
3/25/81	18.2	9.0	8.8	23.6	10.0	7.7
4/13/81-						
4/28/81	14.3	7.2	17.8			
5/26/81-						
6/10/81	18.2	7.4	25.1			
7/6/81-						
7/21/81	19.9	5.9	28.2	21.7	6.3	27.9
11/9/81-						
11/24/81	22.1	8.6	10.3	24.4	7.9	11.7
2/22/82-						
3/5/82	11.7	10.2	6.7	21.1	10.1	5.9

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