APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1981, p. 190-195
0099-2240/81/010190-06$02.00/0

Vol. 41, No. 1

In Situ and Laboratory Studies of Bacterial Survival Using a Microporous Membrane Sandwich†

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A new device and procedure for the study of bacterial survival in an aquatic environment are described. The device uses two appressed presterilized microporous membranes to expose a bacterial cell suspension to the environment at a cell concentration that closely resembles those levels found in natural aquatic ecosystems. The device has been used under laboratory controlled conditions and in situ to study and compare bacterial survival times. In laboratory studies, *Escherichia coli* and *Streptococcus faecalis* survived the longest at 12°C, pH 5, and in the presence of iron or calcium ions and cysteine. Cells in mid-stationary growth phase survived longer than those in mid- or late-logarithmic phase, whereas those maintained for a year or more as stock cultures survived for shorter periods of time than did recent environmental isolates. In situ studies indicate that 5% of the starting number of *E. coli* and *S. faecalis* cells may survive longer than 96 h at 16°C in potable lake water, whereas survival times in polluted lake water were approximately 12 h.

Detecting potable water supply contamination by enteric bacteria is the single most important aim in assessing water quality. To detect and differentiate pollution bacteria from bacteria considered autochthonous constitutes a significant challenge to microbiologists. To be detected, these pollution bacteria must survive in a hostile environment. A proper assessment of water quality requires knowledge of factors that influence survivability of indicator bacteria. Studies of this type have yielded contradictory results: either these bacteria do not survive for long periods of time (3, 15), they do survive for long periods of time (M. J. Allen, S. M. Morrison, and J. P. Waltz, Bacteriol. Proc., p. 32, 1971), or they not only survive, but multiply (4, 12). Thus, not only are published reports contradictory, but there is a lack of methodology that can be used without procedural modification and is specifically designed to assess aquatic survival, both in the laboratory and in the field. McFeters and Stuart (15) and Vasconcelos and Swartz (23) have recently sought a solution to this problem.

Early investigators concerned with survival of bacteria have used a variety of apparatus such as dialysis sacs or tubings (2, 27) and other approaches, as barriers to separate microorganisms (10^6 to 10^8/ml) from the external environment. For a review of dialysis cultures, see Schultz and Gerhardt (20). McFeters and Stuart (15) developed a dialysis chamber into which they placed a volume of approximately 20 ml of liquid containing 10^4 or 10^8 cells per ml, held between microporous membrane filters supported and separated by plastic retainer rings. This latter device was used in the study of survival of bacteria in freshwater (15). Vasconcelos and Swartz (23) developed a large-capacity autoclavable chamber equipped with a mixer, patterning their design after that proposed by Resi (17). Their device is capable of withstanding the stress of the marine environment.

In this study we describe and test a device that uses two appressed filter membranes to study bacterial survival in the laboratory and in situ.

MATERIALS AND METHODS

MS device design and construction. The basic design and construction of the membrane-sandwich (MS) device is shown in Fig. 1. A suitable sample of test isolate was grown to late log phase in tryptic soy broth and was diluted to an optical density of 0.15 at 550 nm with sterile tryptic soy broth, and then 1 ml of this suspension was further diluted in sterile distilled water to give a cell suspension containing 250 cells per ml. Finally 1 ml of the 10^{-6} dilution, along with 20 ml of sterile phosphate buffer (pH 7), was filtered through a sterile Millipore membrane (HAWG 047SO), grid-side up, supported on a sterile Millipore Sterifil apparatus (Millipore, Bedford, Mass.). A second dry membrane of the same type was placed grid-side down on top of the first membrane, thereby creating a membrane sandwich with the bacteria trapped in the pores of the membrane matrix (22). This membrane sandwich was then placed between two flat Plexiglas holders and secured by two plastic side-clamps. Once prepared, the membrane sandwich was held at 4°C on crushed ice. Six assemblies could be prepared in 25

† Vermont Agricultural Experiment Station Journal Article no. 453.

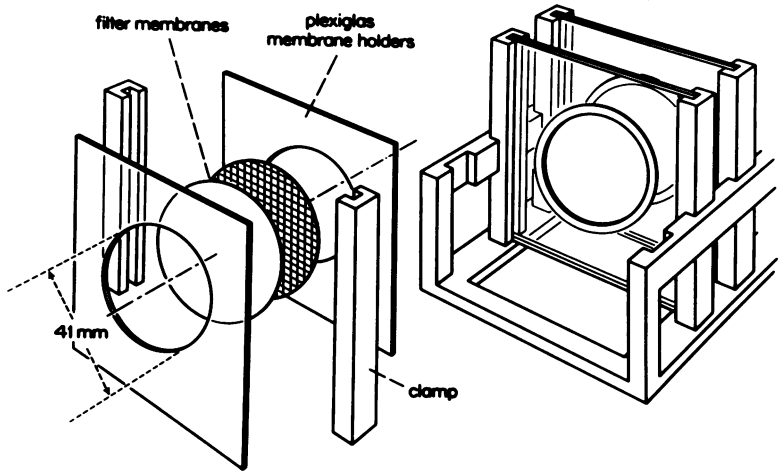

FIG. 1. *MS assemblies and rack.*

min. More membranes could be prepared in the same period of time by using a multi-holder filtration device. The third assembly in the series was used as the zero-time control.

Aquatic incubation of MS assemblies. For laboratory studies, the rack with assemblies was placed in 1 liter of water (distilled or lake) contained in a covered 2-liter glass beaker. Before incubation, the water was brought to the temperature selected for the experiment, which in our studies, unless otherwise noted, was 35°C. For in situ studies the rack with its assemblies was suspended 1 m below the lake surface at a preselected site. We selected a quiet bay from which water is drawn for potable purposes.

Preparation of MS assembly for planting on media. At the termination of aquatic incubation, the MS assembly was removed, and the two filter membranes were placed on the Sterifil filter holder. (Should the MS assembly be taken from a natural ecosystem, the assembly's exterior should be rinsed with sterile water before disassembly.) A vacuum was applied to draw off excess water. The two membranes were then peeled apart with sterile tweezers while maintaining the vacuum. The planted membranes were incubated at 35°C on the surface of prepoured media. Enumeration of *Escherichia coli* was on Endo agar and that of *Streptococcus faecalis* was on Enterococcus agar. After incubation, all colonies on the control membranes using duplicate plates were counted under low magnification (×10), and this starting number, numerically equal to 100%, was used to calculate surviving numbers of bacteria in the experiment. Semi-logarithmic transformation of the data gave die-away rates in hours.

Bacterial cultures. The bacteria used throughout this study either were obtained from freshwater aquatic environmental sources or were stock cultures of *E. coli* and *S. faecalis* maintained at 4°C on slants of Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) All working cultures were streaked on tryptic soy agar slants and incubated for 24 h at 32°C before use.

RESULTS

Laboratory experiments. (i) Effect of abiotic factors and phase of growth. (a) Temperature. The inverse relationship between aquatic temperature and aquatic survival has been documented (6, 15, 16). Examination of the effect of decreasing temperature on survival using the MS method confirmed this finding. The results of this study are given in Fig. 2. These data indicate that survival was prolonged as the temperature of the aquatic environment was decreased to 12°C.

(b) pH. Survival experiments conducted in the laboratory under controlled conditions were done using distilled water adjusted to the desired pH using sterile tris(hydroxymethyl)aminomethane and phosphate buffers. The optimum pH for survival was approximately pH 5.0 for *E. coli* and *S. faecalis* (Fig. 3). *E. coli* is apparently less sensitive to pH changes than *S. faecalis*. We judge this to be true because of the greater number of cells surviving at the extreme ends of the pH scale.

(c) Supplements. Following the approach of earlier investigators (13, 17), supplements were added to pH 7.0 distilled water to determine the effect of these components on survival. The data given in Table 1 were obtained by using the MS assemblies.

(d) Growth phase. Since bacteria exhibit distinct physiological attributes that can be correlated with phase of growth, it was considered necessary to examine the effect of growth phase on survival. Our results (Fig. 4) show that *E. coli* and *S. faecalis* cells in mid-stationary phase survived longer than those in mid- or late logarithmic phase.

(ii) Comparison of the MS method with a standard method. Numerous early studies (7–9) used a standard approach to study survival. Such an approach, as used in the work of Carlucci and Pramer (5), consisted of introducing a 1-ml suspension of cells (*E. coli*) in 100 ml of water contained in a sterile 250-ml Erlenmeyer flask. The flask was incubated without agitation, and at various intervals samples were removed and plated. We performed a similar experiment, and in our studies, identical suspensions of cells were prepared. Half of the suspension containing approximately 250 cells per ml was incubated in a 250-ml Erlenmeyer flask, and the other half was used to prepare MS assemblies. The two methods gave virtually identical survival curves (not shown). For *E. coli*, die-away rates were 45 h for the standard method and 58 h for the MS method at 12°C, whereas the die-away rates for *S. faecalis* under similar conditions were 58 h for the standard and 50 h for the MS method. Procedurally, the standard method, although simpler, lacks versatility and cannot be adapted to be used unmodified in both laboratory and in situ studies. Also, the standard method often uses high cell densities that result in adherence of the bacteria to the container walls (24).

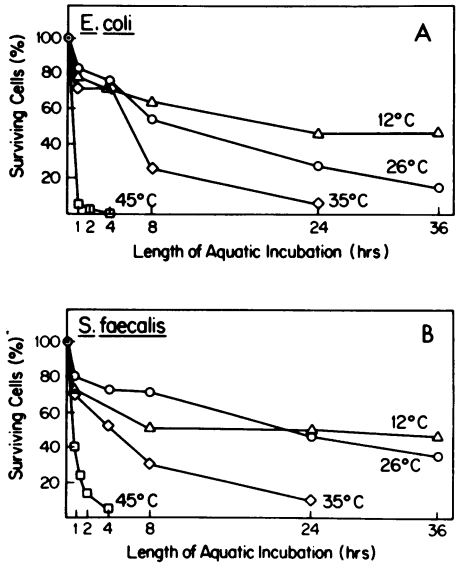

FIG. 2. *Effect of incubation temperature upon the aquatic survival of (A) E. coli and (B) S. faecalis in distilled water. E. coli die-away rate: 12°C, 340 h; 26°C, 100 h; 35°C, 45 h; 45°C, 4 h. S. faecalis die-away rate: 12°C, 417 h; 26°C, 210 h; 35°C, 84 h; 45°C, 5 h.*

(iii) Comparison of different strains of select bacteria. We thought that comparing a laboratory-maintained culture with one of the same genus and species recently derived from harbor lake water might produce interesting results. Such an experiment, using *E. coli* and *S. faecalis*, was done. For *S. faecalis*, the die-away rate for the wild-type strain was 290 h, whereas that for the stock strain was 238 h (results not shown). For *E. coli*, the die-away rate for the wild strain was 472 h, and that for the stock strain was 90 h. Thus, the wild strain survived five times longer than an old laboratory-maintained stock strain. It appears then that the previous biological history of a particular strain does affect survival and that this observation must be considered when interpreting results of experiments designed to ascertain survival of bacteria in an aquatic environment.

(iv) Effects of water source on survival. Since all of our previous experiments had been conducted using glass-distilled water, we decided that a study using water from a source known

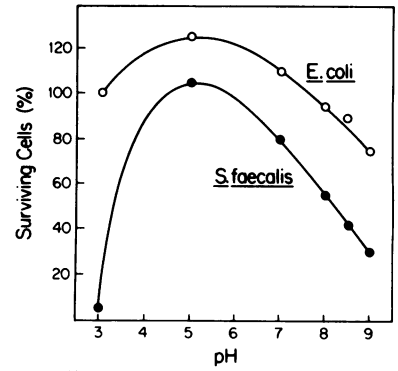

FIG. 3. *Effect of pH upon the aquatic survival of E. coli* (○) *and S. faecalis* (●).

TABLE 1. *Effect of supplements on survival of E. coli and S. faecalis*

Supplement	$T_{20\%}$ (h)[a]				Die-away rate (h)			
	E. coli		*S. faecalis*		*E. coli*		*S. faecalis*	
	Control	Test	Control	Test	Control	Test	Control	Test
$FeCl_2$ (100 μM)	2.5	5.5	3.5	5.0	42	52	28	34
$CaCl_2$ (100 μM)	7.5	13.0	4.5	16.5	52	90	40	54
Cysteine (170 μM)	8.0	18.0	4.0	30.0	52	72	28	138

[a] Time to reach 20% survival.

from previous work to be polluted by sewage treatment plant effluent might prove enlightening. This water was taken from a municipal harbor at a site approximately 135 m in-line from the outflow of a city's major sewage treatment plant. The water was handled in three ways: a control (no treatment), a first treatment (filtered through a 0.47-μm Millipore membrane), and a second treatment (autoclaving for 15 min at 121°C). The effects of these manipulations on the survival of *E. coli* and *S. faecalis* are shown in Fig. 5. It would appear that both filtration and autoclaving remove or tie up critical components necessary for the survival of *E. coli*. These same manipulations seemed to improve the survival of *S. faecalis*. These results are inconsistent with those reported by others (G. E. Jones and A. B. Cobet, Bacteriol. Proc., p. 20, 1966).

In situ experiments. One of the primary aims of this study was to develop a method designed to be used without modification, both in the laboratory and in situ. Therefore, we conducted experiments at McBride Bay in Lake Champlain, using the MS method. The bay is located 56 km north of Burlington, Vt., near South Hero, Vt. It is a secluded, quiet area, distant from large sources of pollution such as a major river or sewage treatment plant effluent, and contains water considered safe for drinking. The studies were conducted in the fall of the year when the lake temperature was between 14 and 19°C at a pH of 7 to 7.1. The results of this study and of those obtained in previous laboratory experiments are summarized in Table 2.

DISCUSSION

The device tested in this study of bacterial survival needed to meet the following criteria: be simple in concept, be easily prepared and assembled using commercially available sterile membranes, use bacterial concentrations similar to those encountered in natural aquatic ecosystems, eliminate responses due to cryptic cell growth, permit free permeability to water and solutes while providing an effective physical barrier to the test bacterium, and be used without procedural alterations for both laboratory and in situ experiments. We believe the device described in this study meets these criteria.

In studies that were conducted using the described device, we used cellulose ester membranes as a physical barrier. These membranes have a porosity of between 80 and 85% (Millipore

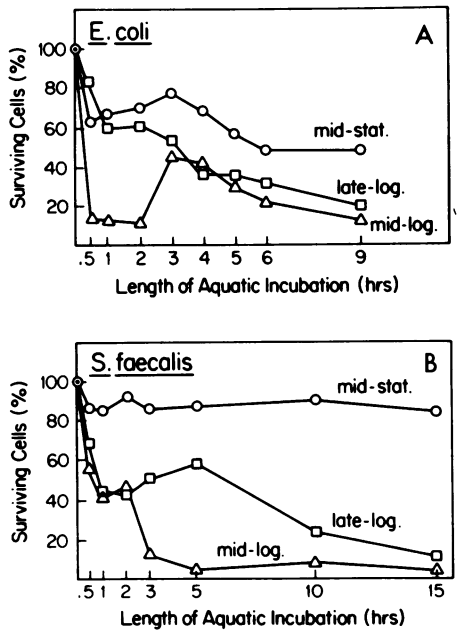

FIG. 4. *Effect of growth phase upon the aquatic survival of (A) E. coli and (B) S. faecalis in distilled water. E. coli die-away rate: mid-logarithmic phase (mid-log.), 20 h; late-logarithmic phase (late-log.), 28 h; mid-stationary phase (mid-stat.), 45 h. S. faecalis die-away rate: mid-log., 42 h; late-log., 34 h; mid-stat., 420 h.*

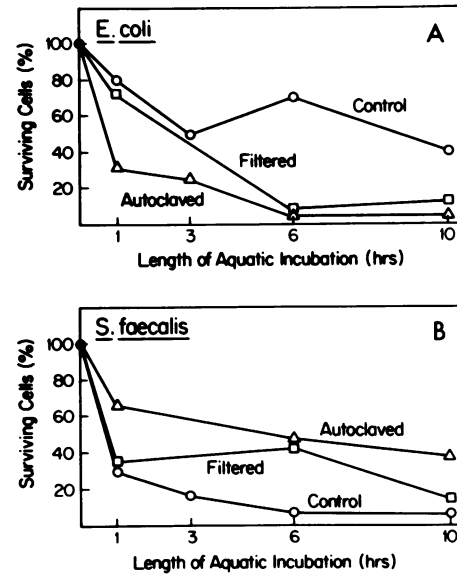

FIG. 5. *Survival of (A) E. coli and (B) S. faecalis in Burlington Harbor water after various water treatments: control (untreated) (○); filtered through 0.45-μm membrane filter (□); autoclaved (△). E. coli die-away rate: control, 60 h; filtered, 9 h; autoclaved, 24 h. S. faecalis die-away rate: control, 12 h; filtered, 34 h; autoclaved, 74 h.*

TABLE 2. *Survival times for E. coli and S. faecalis incubated in water taken from different sources*

Organism	Type of expt	Water type	$T_{5\%}$ (h)[a]	Die-away rate (h)	Temp (°C)
E. coli	Laboratory	Distilled water	24	620	12
E. coli	Laboratory	Municipal Harbor water, untreated	12	60	12
E. coli	In situ	McBride Bay	48	124	16
S. faecalis	Laboratory	Distilled water	96	500	12
S. faecalis	Laboratory	Municipal Harbor water, untreated	10	12	12
S. faecalis	In situ	McBride Bay	96	310	16

[a] $T_{5\%}$, Time at which 5% of total bacteria survived. These values represent average times.

Application Manual AM 302, 1972). Using such membranes permitted rapid exchange of solutes with the aqueous environment, as was convincingly demonstrated previously (15, 23).

Under controlled laboratory conditions we demonstrated that test cells in our device, when subjected to stress in an aqueous environment, were sensitive to changes in temperature and pH, the presence of selected supplements, phase of growth, and previous biological history. We noted that survival was highest at 12°C and decreased as the temperature rose. Others (6, 15, 16) have also noted this inverse relationship between survival and temperature. Such findings generate concern because one would then expect low temperatures to prolong survival in natural waters (19). Whitmore et al. (25) have noted elevated counts of indicator bacteria during colder months in temperature climates. Also, we demonstrated that pH 5 is nearly optimal for survival, a finding similar to data presented by McFeters and Stuart (15). Similar optima have been reported by others (5, 6, 16, 18). In addition, we demonstrated that the presence of iron, calcium, or cysteine enhances survival for *E. coli*. Postgate and Hunter (16) reported similar findings for *Enterobacter*, and Johannesson (13) found that cysteine extended survival of *E. coli*. Phase of growth apparently determines the outcome of bacterial response to stress. Several authors (10, 14, 26) agree that phase of growth does influence survival, whereas Postgate and Hunter (16) found it had little effect on susceptibility to death by starvation. Our studies indicate that cells in mid-stationary phase survive better than those from early growth phases. Strange et al. (21) have reported that stationary cells are less sensitive to starvation in an aquatic environment. This ability may be the result of mid-stationary-phase cells being better able to mobilize and use stored macromolecules. If this is so, then such cells would be expected to survive longer when released into a natural aquatic environment. Thus, one is forced to view a cell's response to stress in an aqueous environment as being a dynamic one involving mobilization of various internal macromolecules and the attraction of critical ions such as calcium and iron. Presumably these ions would be involved in permitting a cell to maintain its envelope as well as membrane function.

In other experiments we observed that our test bacteria survived best in distilled water, survived less well in potable water, and exhibited the shortest survival times when incubated in eutrophic water (Table 2). Others (1, 9, 11; D. M. Shilesky, P. V. Scarpino, and R. J. Fopma, Bacteriol. Proc., p. 21, 1966) reported a similar effect of water quality on survival. It is possible that eutrophic waters contain soluble and particulate organic and inorganic matter that could accelerate death similar to the substrate-accelerated death reported by Calcott and Postgate (4). The data in Table 2 and in Fig. 5 indicate that the explanation for variation in the survival response is complex.

From our studies, we are led to conclude that a bacterial cell present in an aqueous environment can exhibit prolonged survival if the physiological state of the cell and various abiotic factors are optimal. Temperatures near 12°C, pH levels around 5, and the presence of critical ions appear to permit survival of pollution bacteria in natural waters.

ACKNOWLEDGMENT

This investigation was supported through Hatch Project 297.

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