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Influence of Environmental Stress on Enumeration of Indicator Bacteria from Natural Waters

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The problems associated with recovery of pure cultures of *Escherichia coli* and *Streptococcus faecalis* from stream environments were examined utilizing membrane filter chambers. It was observed that upon exposure to the aquatic environment a significant proportion of cells lost their ability to produce colonies on a selective medium, yet retained this capability on a nutritionally rich, nonselective medium. Discrepancies in colony-forming units between nonselective and selective media indicated that a substantial portion of bacterial cells may become physiologically injured due to the environmental stress imposed by the aquatic environment. The extent of injury was observed to vary considerably among the eight different stream environments, since the amount of injury was not uniform for all types of water environments examined. It was observed that the injury acquired by a population of *E. coli*, during exposure to the aquatic environment, could be rapidly repaired in a nutritionally rich, nonselective medium. As the injured population of cells was exposed to the rich, nonselective broth, increasing proportions of cells were able to repair themselves such that they became insensitive to inhibitory agents in selective media.

In evaluating the problem of detecting particular microorganisms from specific sources, proper consideration must be given to the influence of environmental factors upon detection methods. Data are available indicating that after exposure to freezing, heating, or freeze-drying some microorganisms are either physiologically debilitated or injured to such an extent that significant problems arise upon attempts at detection and enumeration (4, 17, 18, 19, 21). The detection of such stressed or injured microorganisms becomes more complicated by the use of selective media. Apparently, injured cells become sensitive to inhibitory agents in specific selective media and are unable to grow and produce colonies.

This report presents research directed toward determining whether aquatic environments similarly induce stress upon substantial proportions of cells such that these cells become physiologically debilitated and cannot be detected by direct selective procedures. Most sanitary indicator organisms, as well as the enteric water-borne pathogens, are bacteria whose natural habitat is the intestine of man and warm-blooded animals. When discharged in the feces, these microorganisms frequently gain entrance into a body of water. Once these bacteria are deposited into the water, they are in an environment that is not favorable to the maintenance of viability of most bacteria. Proper interpretation of sanitary water quality data relies partly on a basic understanding of survival characteristics of bacteria in water. In the majority of reported survival studies only two subpopulations of the total population of cells have been considered: those cells which can withstand the aquatic environment, as reflected by their detection and enumeration by standard laboratory procedures, and, conversely, those cells which cannot persist in the unfavorable environment, as reflected in death and nondetection.

There is a dearth of available literature concerning the possibility that substantial populations of injured bacteria exist in water as determined by comparison of detection and enumeration on nonselective versus selective media. With the aid of membrane filter chambers developed by McFeters and Stuart (14), it was possible to follow the survival, injury, and recovery characteristics of populations of indicator organisms as a function of time of exposure in various aquatic environments.

MATERIALS AND METHODS

Source of test microorganisms and physiological identification. Microorganisms used for pure culture

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studies consisted of indicator organisms that had been isolated from water samples or from cattle fecal droppings at the microbiology laboratory of Montana State University, Bozeman, Mont. The coliform organisms utilized in this study and their Montana State University laboratory cultural identification numbers were *Escherichia coli* C32OMP25, isolated from fresh cattle fecal droppings, and *E. coli* EC2075, isolated from water. These two strains produced gas from lactose and brilliant green lactose bile broth within 48 h and were characterized by an indole- and methyl red-positive, Voges-Proskauer- and citrate-negative pattern. The production of gas within 24 h from EC medium, when incubated at the elevated temperature of 44.5 C in a water bath, indicated that both strains could be considered to be of fecal origin. Both strains were able to ferment the carbohydrates glucose, mannitol, and arabinose when present in a broth medium at 0.5% concentrations. *E. coli* C32OMP25 was nonmotile whereas *E. coli* EC2075 was motile. Both strains were negative for production of hydrogen sulfide and urease. (All media used were Difco products unless otherwise specified).

The two strains of *Streptococcus faecalis* used in this study were isolated from water and were identified as Montana State University laboratory cultures *S. faecalis* RS1009 and *S. faecalis* ES1913. Physiological tests utilized in the identification of these organisms included the ability to grow in ethyl violet azide broth, 6.5% NaCl broth, and brain heart infusion broth incubated at 10 and 45 C. Also, the two strains of *S. faecalis* were negative for catalase activity, hydrolysis of starch, and liquefaction of gelatin. Both strains had the ability to ferment 0.5% concentrations of arabinose and melibiose.

Membrane filter chambers. Description, sterilization, and aseptic assembly of the membrane filter chambers have been previously described (14). Tear-resistant microweb membranes (WHWP 304 Fl, Millipore Corp.) with a porosity of 0.45 μm were used as the side walls for the chambers.

Preparation of cell suspensions. Pure cultures used in these experiments were grown in Trypticase soy broth (BBL) supplemented with 0.3% yeast extract and 0.5% glucose (TSY broth) for 20 to 24 h at 35 C. The cells were harvested by centrifugation (3,020 $\times$ *g*) for 10 min and washed twice with either standard phosphate buffer (1) or gelatin phosphate buffer (24) at refrigeration temperature. The standard phosphate buffer consisted of 3.4% KH_2PO_4, whereas the gelatin phosphate buffer consisted of 0.2% gelatin, 0.725% $NaH_2PO_4 \cdot H_2O$ and 0.37% Na_2HPO_4. After the final wash, the cells were suspended in one of the respective diluents and diluted to the desired population density. The total contact time of buffer with the cell suspension, including washing and diluting, was approximately 25 min. The final dilution used to fill the chamber was made in sterilized water obtained from one of the stream sites. The loaded chambers were then suspended in a plastic transport container filled with fresh water obtained from the same source as that used to make the final suspension in the chamber. These latter preparatory steps were taken to promote rapid equilibration between contents within the chamber and the natural aquatic environment.

The membrane filter chambers were transported to stream sites as rapidly as possible. The time in transit varied from a minimum of 15 min for those stream sites located near the University laboratory to a maximum of approximately 120 min for those stream sites located distant from the University laboratory. Stream sites for suspending the membrane filter chambers are shown in Fig. 1. Descriptions of these locations are given in Table 1.

Detection of death and injury. Immediately upon immersing the chamber in the stream, a sample was withdrawn with a sterile 1-ml syringe; this sample was designated as the 0-h sample. Subsequent sampling was done at various time intervals as dictated by the specific objectives of individual experiments. The sample removed from the chamber was placed in a sterile, screw-cap test tube and rapidly returned to the University laboratory for quantitative analyses.

Enumeration was accomplished by using two plating media in parallel, one nonselective and the other selective. Dilutions of samples, when necessary, were conducted by using gelatin phosphate buffer. Quantification of *E. coli* or *S. faecalis* strains with a nonselective medium was done with Trypticase soy agar (BBL) supplemented with 0.3% yeast extract and 0.5% glucose (TSY agar). The selective medium used to enumerate *E. coli* was deoxycholate lactose (DLA) agar, whereas KF Streptococcus (KF) agar (BBL) was used as the selective medium to enumerate *S. faecalis*.

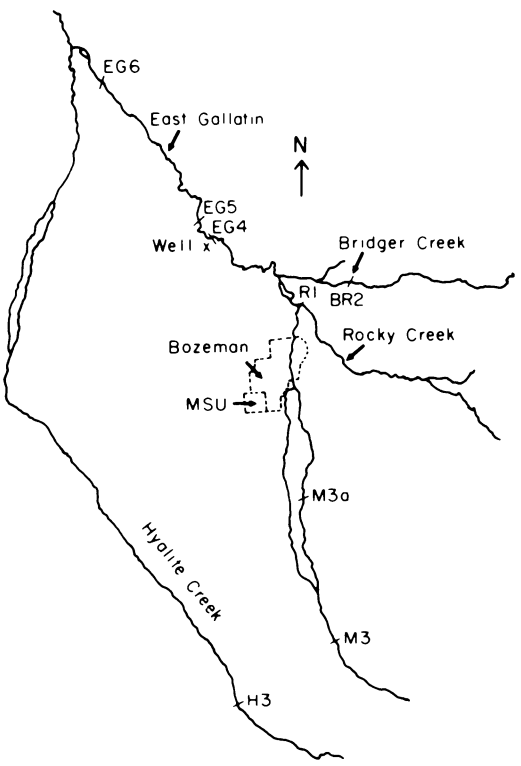

FIG. 1. *Sites for suspending membrane filter chambers. The direction of flow is toward the top of the figure.*

TABLE 1. *Description and location of sites for membrane filter chambers*

Site	Description and location
M3	Located on Bozeman Creek, approximately 7.0 miles (11.3 km) downstream from Mystic reservoir, a high mountain municipal impoundment.
M3A	Located on Bozeman Creek, approximately 3.7 miles (6.0 km) downstream from site M3, after flowing through agricultural land.
H3	Located on Middle Creek, approximately 7.0 miles (11.3 km) downstream from Hyalite reservoir, a high mountain municipal impoundment.
BR2	Located on Bridger Creek, after flowing through agricultural land, approximately 1.8 miles (2.9 km) upstream from its junction with the East Gallatin River.
R1	Located on Rocky Creek, after flowing through agricultural land, approximately 0.1 miles (0.2 km) upstream from its junction with Bozeman Creek.
EG4	Located on the East Gallatin River, approximately 0.2 miles (0.3 km) upstream from the outfall of the Bozeman Wastewater Treatment Plant.
EG5	Located on the East Gallatin River, approximately 0.3 miles (0.5 km) downstream from the outfall of the Bozeman Wastewater Treatment Plant.
EG6	Located on the East Gallatin River, approximately 5.0 miles (8.0 km) downstream from the outfall of the Bozeman Wastewater Treatment Plant.
Well	Nonpotable well located at the Bozeman Wastewater Treatment Plant as described in a previous publication (13).

Enumeration of *E. coli* with TSY and DLA agar was done either by the conventional pour plate with overlay procedure or by a surface overlay procedure described by Ray and Speck (20). Enumeration of *S. faecalis* with TSY and KF agar was performed solely with conventional pour-plate procedures. Plates for the enumeration of *E. coli* were incubated at 35 C for 24 h before counting. All platings with the respective media were done in duplicate.

Several calculations were made in order to construct tables of the observed data and were obtained as follows: (i) percentage of death at time t = (1 − nonselective medium count at time t/nonselective medium count at 0 h) × 100; (ii) percentage of survival at time t = 100% − percentage of death at time t; and (iii) percentage of injury at time t = (1 − selective medium count at time t/nonselective medium count at time t) × 100.

Repair experiment. An experiment was conducted to determine whether a population of *E. coli*, injured by environmental stress in natural water, had the capability to repair the nonlethal injury. Preparation of the membrane filter chamber and cell suspension followed procedures outlined in the previously described experiments. This experiment was conducted at site EG6 (Table 1 and Fig. 1).

In this experiment with *E. coli* C32OMP25, a 4-ml sample was withdrawn from the chamber immediately upon immersing the chamber in the stream water (0-h sample) in order to obtain control curves. The 4-ml sample was rapidly returned to the laboratory and inoculated into 36 ml of TSY broth. At 30-min intervals over a period of 6 h, 1-ml samples were withdrawn from the TSY broth, serially diluted, and enumerated by parallel platings on TSY and DLA agar. The surface overlay procedure (20) was used and all platings were done in duplicate. The TSY broth was incubated at 35 C during the course of sampling. After 2 days of exposure to the aquatic environment, another 4-ml sample was withdrawn from the chamber and brought back to the laboratory for inoculation into a new 36-ml flask of TSY broth. The TSY broth was sampled at various time intervals and surface-plated on TSY and DLA agar following the exact procedures as described for the original control sample. All plates were incubated at 35 C for 24 h, and the colonies were enumerated.

In this repair experiment, any increase in selective medium counts but not in nonselective medium counts was assumed to be due to repair of injured cells. Also, simultaneous increase in counts on nonselective and selective media was assumed to be due to cell multiplication.

RESULTS

Effects of wash solutions and diluents. Two suspensions of *S. faecalis* ES1913 were prepared for inoculation into two separate membrane filter chambers. The treatment of both suspensions was identical with the exception that the washing and diluting procedures for one suspension was done completely with standard phosphate buffer (1) while the other suspension was treated solely with gelatin phosphate buffer (24). The inoculated chambers were then immersed in water at the experimental well site (Fig. 1). The chambers were sampled simultaneously during a 48-h exposure period in the well water environment.

Table 2 summarizes recovery data obtained upon pour plating with nonselective TSY agar and selective KF agar for samples taken from each chamber. Comparison of counts obtained on the nonselective medium indicated that the die-away pattern for those cells which had undergone regular phosphate buffer preparation proceeded at a much faster rate than those cells which had been prepared by use of gelatin phosphate buffer. Furthermore, a greater degree of injury to surviving cells, as evidenced by differences in counts on TSY and KF agar, was observed for those cells which had been pre-

pared with regular phosphate buffer as compared to those cells which had undergone preparation with gelatin phosphate buffer.

Effects of preparation of separate cell suspensions of *E. coli* EC2075 with regular phosphate and gelatin phosphate buffer were similarly studied (Table 2). Fatality incurred by cells was found to be approximately equal for both treatment procedures at most sampling times. However, the injury observed as a result of preparation of cells with regular phosphate buffer was substantially greater than for cells prepared by use of gelatin phosphate buffer.

Detection of death and injury to cells exposed to different stream environments. Data from a number of preliminary experiments led to an experiment in which eight chambers, each containing a suspension of *E. coli* C32OMP25, were placed at eight different stream sites (Fig. 1) for an exposure period of 4 days. The eight sites were chosen to represent a variety of aquatic environments with differing physical and chemical characteristics.

TABLE 2. *Effect of regular phosphate and gelatin phosphate diluents upon death and injury to suspensions of S. faecalis ES1913 and E. coli EC2075 in membrane filter chambers suspended in well water over a 48-h exposure period*

Exposure time (h)	Death[a] (%)		Injury[b] (%)	
	Regular phosphate	Gelatin phosphate	Regular phosphate	Gelatin phosphate
S. faecalis 0	0.0	0.0	35.4	3.3
6	90.0	42.9	98.3	0.0
10	98.0	41.8	98.1	0.0
24	98.7	40.7	98.0	3.7
48	99.7	50.5	98.9	11.1
E. coli 0	0.0	0.0	14.3	6.3
6	7.1	12.5	43.1	0.0
10	14.3	25.0	60.8	16.7
24	28.6	31.3	51.0	10.9
48	40.7	41.9	56.6	1.1

[a] Determined from counts obtained with TSY pour plates.

[b] Determined by differences between counts obtained on TSY and KF pour plates for *S. faecalis* ES1913 and on TSY and DLA pour plates for *E. coli* EC2075.

Table 3 summarizes both the survival and injury characteristics for *E. coli* C32OMP25 at all eight stream sites as determined by counts obtained on TSY and DLA surface overlay plates. The ability of this population of cells to survive the aquatic environment was found to vary considerably among all the stream sites. Cells exposed to the environment of sites EG4, EG5, and R1 were found to be very susceptible to death as evidenced by the survival of only 0.2 to 1.6% of the original population after 4 days of exposure at the respective sites. In contrast, 37% of the original population survived the same exposure period when exposed to the environment of BR2. Nonlethal injury to survivors was also found to vary substantially among the sites. The extremes of injury after 4 days of exposure were from a low of less than 10% at site BR2 to a high of greater than 96% at site EG4. In general, it was observed at most sites that as the exposure time in the aquatic environment increased from 0 to 4 days an increasing proportion of the survivors exhibited nonlethal injury, as reflected by nondetection on selective DLA agar.

Table 4 summarizes both the survival and injury characteristics at the eight stream sites for a comparable experiment conducted with *S. faecalis* RS1009. As observed with the strain of *E. coli*, there was considerable variation of survivability as a function of the various expo-

TABLE 3. *Percentage of survival and injury for suspensions of E. coli C320MP25 in membrane filter chambers located at eight different sites over a 4-day exposure period*

Site location	Survival[a] (%)					Injury[b] (%)				
	0[c]	1	2	3	4	0[c]	1	2	3	4
M3	100.0	45.8	40.0	27.1	16.3	8.3	0.0	22.0	3.1	30.8
M3A	100.0	59.1	50.0	22.7	14.1	4.5	15.4	9.1	18.0	29.0
H3	100.0	21.3	10.0	6.1	5.2	4.3	24.5	17.4	63.6	90.0
BR2	100.0	60.9	52.2	38.3	37.0	0.0	7.1	8.3	18.2	9.4
R1	100.0	42.4	8.2	1.2	1.6	15.2	54.3	91.5	70.7	79.2
EG4	100.0	35.4	12.5	4.1	0.9	8.3	3.5	78.3	93.9	96.7
EG5	100.0	40.0	1.6	0.3	0.2	8.7	16.3	70.3	84.6	77.1
EG6	100.0	43.5	36.1	26.5	14.8	8.7	9.0	74.7	86.7	83.8

[a] Determined from counts obtained with TSY surface overlay plates.

[b] Determined by differences between counts obtained on TSY and DLA surface overlay plates.

[c] Day of exposure period (total of 4 days).

TABLE 4. *Percentages of survival and injury for suspensions of S. faecalis RS1009 in membrane filter chambers located at eight different sites over a 4-day exposure period*

Site location	Survival[a] (%)					Injury[b] (%)				
	0[c]	1	2	3	4	0[c]	1	2	3	4
M3	100.0	19.4	7.5	5.3	2.2	5.7	12.5	6.2	4.3	15.8
M3A	100.0	65.5	70.2	56.0	11.3	4.8	7.3	1.7	0.0	26.3
H3	100.0	85.2	71.6	40.7	14.8	1.2	7.2	10.3	12.1	95.0
BR2	100.0	85.4	70.8	13.5	3.8	4.5	11.8	3.2	8.3	2.9
R1	100.0	57.6	11.2	0.3	0.1	3.5	89.8	97.9	100.0	100.0
EG4	100.0	61.4	30.1	1.1	1.1	2.4	7.8	87.2	94.4	100.0
EG5	100.0	37.9	4.0	0.4	0.3	6.9	66.7	95.7	95.2	100.0
EG6	100.0	38.4	15.1	5.1	0.5	10.5	12.1	42.3	95.5	100.0

[a] Determined from counts obtained with TSY pour plates.
[b] Determined by differences between counts on TSY and KF pour plates.
[c] Day of exposure period (total of 4 days).

sure sites. The greatest degree of fatality was found to occur to those cells exposed to the aquatic environments of sites R1, EG4, EG5, and EG6 with less than 1% of the original population surviving after 4 days of exposure, while nearly 15% of the population was observed to survive the same exposure period at site H3. The variability of nonlethal injury to survivors extended from a low of approximately 3% at site BR2 to a high of 100% at sites R1, EG4, EG5, and EG6 after 4 days of exposure in the respective stream environments. Additionally, it was observed that increasing proportions of the survivors reflected nonlethal injury as the length of exposure to the various aquatic environments increased from 0 to 4 days.

Repair of injury. The data observed in the repair of injury of *E. coli* C32OMP25 are plotted in Fig. 2 as a function of time of exposure in rich TSY broth. The control or 0-h curves, using both TSY and DLA agar, represent growth curves for a population of cells which had not been exposed to the natural water environment. Relatively equal detection of viable organisms for the control population was observed over the 6-h growth period by using either the nonselective medium or the selective medium. The control curve illustrated approximately a 1-h lag period before initiation of multiplication and transition to the logarithmic phase of growth. The population of cells which had been exposed to the environmental stress of the stream for a period of 2 days was found to contain a substantial proportion of injured cells (95%) as evidenced by the lessened detection of injured cells on DLA agar as compared to TSY agar. As the injured population of cells was exposed to the rich TSY broth over the 6-h time period, an increasing proportion of cells was able to repair themselves such that they could

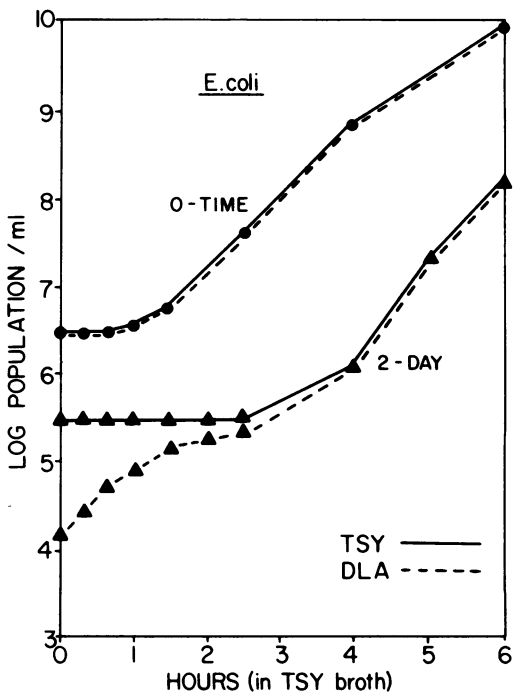

FIG. 2. *Repair of injury in TSY broth for E. coli C32OMP25 cells having been exposed to the stream environment for 2 days. Control, or 0-h, cells (●) and 2-day exposed cells (▲) were enumerated over a 6-h growth period in TSY broth with TSY (——) and DLA (- - -) surface overlay plates.*

produce colonies on the selective DLA agar. After 2.5 to 3 h of exposure to the TSY broth, counts obtained with both the nonselective and selective media were approximately equal. During the 2.5- to 3-h repair period, counts on TSY agar were observed to remain relatively constant, resulting in an extended lag period as compared to the control population. Upon com-

pletion of the repair period, multiplication was initiated as evidenced by concomitant increases in counts on both the TSY and DLA agar.

DISCUSSION

In general, most workers have observed that natural waters are unfavorable as a growth environment for bacteria, as evidenced by bacterial survival data exhibiting die-away characteristics. Essentially two subpopulations of the total population of cells have been considered in such survival studies. At one extreme of the possible physiological spectrum were those cells which could withstand the aquatic environment, as reflected by their detection and enumeration when using standard laboratory procedures. At the other extreme were those cells which could not persist in the unfavorable environment, resulting in death and nondetection.

The present study has indicated that upon exposure to certain aquatic environments a substantial proportion of the total population may not fit the distinctly extreme categories of death or survival. It was observed that a substantial proportion of the total population may become physiologically injured as a result of exposure to the stresses of certain aquatic environments. It should be emphasized that such damaged cells were not necessarily killed or destroyed; however, the detection and enumeration of these injured survivors was found to be substantially impeded by the presence of inhibitory compounds in specific selective media. Similarly, Wang et al. (25) observed that the recovery of *Shigella flexneri*, after 24 h of exposure in a flask of sterile sewage, was significantly decreased on selective Salmonella-Shigella agar as compared to a non-inhibitory Luria broth agar. Also, Andre et al. (2) followed the survival of *Enterobacter aerogenes* during exposure to farm pond water by parallel enumeration on nonselective and selective media and found that counts on nonselective tryptone glucose extract agar were significantly higher than that obtained on selective violet red bile agar. The authors reasoned that exposure to farm pond water had led to some alteration in physiology of the organism, rendering it less fit for growth on a specific selective medium.

The application of dialysis culture experiments for examination of survival of organisms in water was first utilized by Jordan et al. (7). Others (3, 6, 23) have similarly used dialysis techniques to study survival phenomena in water. Major modifications by McFeters and Stuart (14) resulted in the development of chambers with membrane filter side walls specifically designed for studies of survival of bacteria in natural and artificial waters. These chambers are so designed to allow for the suspended organisms to be in direct contact with many factors of the aquatic environment, including pH, temperature, diffusable nutrients, toxic substances, dissolved solids and other chemical constitutents. According to Schultz and Gerhardt (22), nutrient exchange cannot take place unless a concentration gradient exists between the inside and the outside of the growth chamber. The driving force for nutrient exchange in aquatic environments results from the constantly changing chemical make-up of the water environment during given time periods. The rapidly changing chemical and physical characteristics of water are especially notable at those stream sites located downstream from the introduction of sewage effluent.

The application of membrane filter chambers developed by McFeters and Stuart (14) uniquely allowed for detection of the presence of an injury phenomenon in the present study since it was possible to follow both the survival and injury characteristics of *E. coli* and *S. faecalis* as a function of exposure time in various stream environments. The parallel enumeration of samples withdrawn from the chamber with a rich, nonselective medium and a specific selective medium allowed for determination of both injured and noninjured proportions of the total viable population.

Several studies have been conducted dealing with the viability of populations of cells after exposures to specific types of environmental stress. In the food-processing industry, exposure of bacterial populations to such stresses as heating (4), freezing (19, 21), or freeze-drying (17, 18) has been shown to induce nonlethal injury to substantial proportions of the total surviving population. Such stressed or injured cells had become sensitive to inhibitory agents in specific selective media such that they did not have the ability to grow and produce colonies upon direct exposure to selective media, yet retained this capability when cultured on nonselective media. Such observations indicated that significant percentages of the population were physiologically injured or debilitated. In effect, the environmental conditions and/or added inhibitory agents in selective media utilized to allow for development of the specific types being sought had, in fact, exerted unsuspected inhibition.

Similarly, the data obtained in the present

research has indicated that large proportions of populations of *E. coli* or *S. faecalis* exposed to certain aquatic environments had become sensitive to selective media commonly used for their enumeration (Tables 3 and 4). In general, it was observed at most stream sites that as the exposure time in the aquatic environment increased from 0 to 4 days, an increasing proportion of the survivors exhibited nonlethal injury as reflected by nondetection on selective DLA or KF media. Since injured cells were found to be a large proportion of the total viable population when exposed to certain environments even within 24 to 48 h, it is important that this damaged population be recovered in order to correctly evaluate bacteriological water quality.

The pour-plate technique using DLA agar was the chosen enumeration method because of the ease of performing this technique with a minimum of equipment. Endo agar was used in preliminary experiments but it was observed that difficulty arose when attempting to count colonies in a pour plate of this medium. DLA agar is a selective medium used for the enumeration of coliforms in milk; however, there is no currently accepted plating method for enumeration of coliforms in water (1). Only most-probable-number and membrane filtration techniques are accepted methods for coliform enumeration according to Standard Methods (1).

McFeters and Stuart (14) have postulated that observed differences in survival data for a fecal coliform bacterium exposed to two different mountain streams may have been a reflection of differences of water chemistry between the two streams. It was not the objective of the present research to identify the chemical or physical factor(s) responsible for death and/or injury to bacterial cells. However, it should be noted that considerable variation in nonlethal injury, as well as survival, was observed among the eight stream environments. For example, the degree of injury observed at site BR2 for both organisms was very minimal in comparison to the extremes of injury observed upon exposure to the stream environments of sites EG4, EG5, and R1. Postgate (15) and Postgate and Hunter (16) have examined the effects of prolonged starvation upon viability of bacteria. Possibly starvation conditions at some of the stream sites may have accounted for death and/or injury to the exposed suspensions of indicator bacteria. Recently, Klein and Wu (9) have reported that microbial starvation in low-nutrient aquatic environments can lead to increased susceptibility to a secondary transient, warming stress as used in the Standard Methods (1) pour-plate procedure, causing significantly decreased recoveries in comparison with a spread-plate technique. In addition to starvation, the possibility of the existence of harmful chemical or physical factors in the stream cannot be excluded. It should be emphasized that these environmental stresses to indicator bacteria are related to high-quality mountain streams possibly containing some toxic levels of heavy metal ions or to chlorinated effluents discharging into high-quality receiving streams. Such environmental stresses may possibly be very minimal in poor-quality water polluted with nutritive wastes found in domestic raw sewage or certain industrial wastes.

To recover those cells which had undergone stress and resulting injury in the aquatic environment, it was necessary that such cells had the opportunity to repair themselves before they could multiply and divide. It was observed that the injury acquired by a population of *E. coli* C32OMP25, during 2 days of exposure to the aquatic environment, could be rapidly repaired in nutritionally rich, nonselective TSY broth (Fig. 2). As the injured population of cells was exposed to the rich TSY broth, an increasing proportion of cells was able to repair themselves such that equal counts were obtained on both the nonselective and selective media within 2.5 to 3 h. During the 2.5- to 3-h repair period, counts on TSY agar were observed to remain relatively constant, resulting in an extended lag period prior to multiplication, as compared to the control population which had not been exposed to the stresses of the aquatic environment. The extended lag period for the environmentally exposed cells lends further evidence to the hypothesis that the stressful situations of aquatic environments may in fact inflict injury upon substantial proportions of cells. Furthermore, the rapidly increasing counts on the selective DLA agar during the first 2.5 to 3 h in TSY broth indicated that these injured cells were capable of repair if exposed to a suitable nutritionally rich growth medium.

In an attempt to improve the recovery of coliforms from certain aquatic environments, several workers (5, 8, 10, 12) have developed enrichment techniques for the enumeration of coliforms with the membrane filtration method. Recently, Lin (11) observed that an enrichment LES two-step membrane filtration technique significantly improved recovery of total coliforms from chlorinated secondary effluents as compared to a one-step method. The observed repair of an indicator organism injured in the aquatic environment reported in this study

lends support to the use of an enrichment technique to improve the recovery of stressed cells from those stream environments where problems have arisen during attempts to isolate indicator organisms directly. Enrichment steps would likely not be necessary for detection of indicator organisms from those environments where stress was minimal. The observations documented in the present study have shown that as an injured population of *E. coli* was exposed to a nutritionally rich, nonselective broth, increasing proportions of cells were able to repair themselves such that they became insensitive to inhibitory agents in selective media. Perhaps application of a short enrichment period on a rich medium would allow for the repair of injury such that subsequent exposure to a specific selective medium would result in enhanced recovery of the total viable population of sanitary indicator organisms from stressful aquatic environments.

In addition to the death and injury that occurred in the aquatic environment, it was found that substantial amounts of laboratory-induced death and/or injury resulted from the use of standard phosphate diluent (1). Apparently, the exposure of bacteria to this buffer at refrigerator temperature, as in the washing procedure employed in this study, injured some bacteria and was reflected by decreased survival potential when exposed to aquatic environments. Treatment of a suspension of *S. faecalis* with regular phosphate buffer revealed a much faster die-away rate as well as a greater degree of injury to survivors as compared to treatment of an identical suspension of cells with gelatin phosphate buffer. Regular phosphate buffer was also found to contribute to nonlethal injury of survivors of a suspension of *E. coli* to a greater degree than gelatin phosphate buffer. In general, it appeared that *E. coli* was slightly more resistant to the toxic effects of regular phosphate buffer than was *S. faecalis*. Similar observations have been reported by McFeters et al. (13), whereby pure cultures of fecal streptococci washed with phosphate buffer died nearly 10 times faster than when buffer with 0.2% gelatin or sterilized well water was used. Since it was the objective of this research to determine whether bacteria were physiologically debilitated as a result of aquatic environmental stress, it was necessary to identify and minimize any apparent laboratory injury. The use of 0.2% gelatin phosphate buffer seemed to afford substantially greater protection from such laboratory-induced death and/or injury, thereby allowing a realistic evaluation of the effects of aquatic environments upon death and injury to indicator organisms.

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