



Recovery characteristics of bacteria injured in the natural aquatic environment
by Gary Kent Bissonnette

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Microbiology
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Abstract:

The recovery characteristics of indicator organisms exposed to the aquatic environment were examined using membrane filter Chambers.

The membrane filter chambers, containing pure culture suspensions of indicator organisms or natural suspensions of raw sewage, were immersed in the stream environment at a number of different sites having water of varying physical and chemical composition. Periodically, samples were withdrawn from the chamber and enumerated using both non-selective and selective media.

It was observed that upon exposure to some aquatic environments significant proportions of bacterial populations lost their ability to produce colonies on the selective medium yet retained this capability on the nutritionally rich non-selective medium. Discrepancies in colony-forming units between non-selective and selective media indicated that a substantial portion of bacterial cells may become physiologically injured due to stress imposed by the aquatic environment.

The extent of injury was observed to be a function of the chemical and physical characteristics associated with individual stream environments, since the amount of injury was not uniform for all types of water environments examined.

Comparison of the recovery efficiency of various methods used to enumerate bacteria from water yielded information indicating that, in general, multiple tube fermentation techniques gave superior recovery of coliforms than did plating or membrane filtration procedures. The least efficient method of recovery of coliforms, fecal coliforms, or fecal streptococci was by membrane filtration procedures.

The injury inflicted upon *E. coli* and *S. faecalis* by the aquatic environment was found to be reversible and could be repaired rapidly in a medium such as Trypticase soy yeast extract broth. The observed rapid repair of injured cells in a nutritionally rich, non-selective broth lends support to the use of an enrichment technique to improve recovery of stressed cells. It was observed that a short 2 h enrichment on a rich non-selective medium prior to exposure to selective media substantially enhanced recovery of coliforms and fecal coliforms from raw sewage relative to recovery by direct primary exposure to, selective media. Such an enrichment period appears to provide a non-toxic environment for the gradual adjustment and repair of the injured microorganisms. The enrichment technique appeared to be especially applicable to the membrane filtration procedure.

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IN THE NATURAL AQUATIC ENVIRONMENT

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GARY KENT BISSONNETTE

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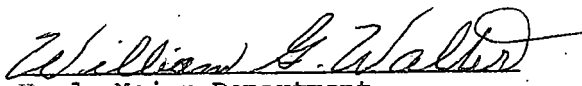
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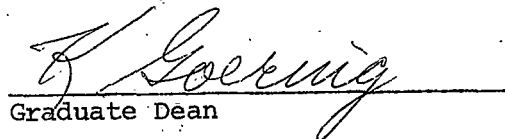
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ABSTRACT

The recovery characteristics of indicator organisms exposed to the aquatic environment were examined using membrane filter chambers. The membrane filter chambers, containing pure culture suspensions of indicator organisms or natural suspensions of raw sewage, were immersed in the stream environment at a number of different sites having water of varying physical and chemical composition. Periodically, samples were withdrawn from the chamber and enumerated using both non-selective and selective media.

It was observed that upon exposure to some aquatic environments significant proportions of bacterial populations lost their ability to produce colonies on the selective medium yet retained this capability on the nutritionally rich non-selective medium. Discrepancies in colony-forming units between non-selective and selective media indicated that a substantial portion of bacterial cells may become physiologically injured due to stress imposed by the aquatic environment. The extent of injury was observed to be a function of the chemical and physical characteristics associated with individual stream environments, since the amount of injury was not uniform for all types of water environments examined.

Comparison of the recovery efficiency of various methods used to enumerate bacteria from water yielded information indicating that, in general, multiple tube fermentation techniques gave superior recovery of coliforms than did plating or membrane filtration procedures. The least efficient method of recovery of coliforms, fecal coliforms, or fecal streptococci was by membrane filtration procedures.

The injury inflicted upon *E. coli* and *S. faecalis* by the aquatic environment was found to be reversible and could be repaired rapidly in a medium such as Trypticase soy yeast extract broth. The observed rapid repair of injured cells in a nutritionally rich, non-selective broth lends support to the use of an enrichment technique to improve recovery of stressed cells. It was observed that a short 2 h enrichment on a rich non-selective medium prior to exposure to selective media substantially enhanced recovery of coliforms and fecal coliforms from raw sewage relative to recovery by direct primary exposure to selective media. Such an enrichment period appears to provide a non-toxic environment for the gradual adjustment and repair of the injured microorganisms. The

enrichment technique appeared to be especially applicable to the membrane filtration procedure.

Chapter 1

INTRODUCTION

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. Considerable data are available indicating that after certain exposures to environmental stress, some microorganisms are either physiologically debilitated or injured to such an extent that significant problems arise upon attempts at detection and enumeration. It should be emphasized that this environmental stress may merely injure the cell but not necessarily kill or destroy it.

The detection of injured microorganisms becomes more complicated by the use of selective media. The environmental conditions and/or added inhibitory agents utilized to allow for development of the specific types being sought may, in fact, exert unsuspected inhibition. This shortcoming in methodology occurs during the use of selective media for quantitative determinations, as well as through the use of enrichment media that are designed to encourage qualitative detection of specific pathogens or indicator organisms.

The combination of environmental stress with subsequent utilization of specific selective media may result in significantly lowered recoveries of injured cells. This phenomenon is frequently encountered

under conditions where organisms are exposed to highly oxidizing environments or the action of some chemical disinfectants as well as in certain types of processed foods. There have been a number of studies on microbial survival and recovery concerned with the viability of populations after they have been exposed to a specific kind of stress: a population is examined after it has been heated, frozen, dried; or subjected to adverse pH, pressure, toxic chemicals, or radiation. Attempts to directly enumerate such stressed populations with conventional selective media often indicate that a substantial portion of cells is injured or debilitated to such an extent that they cannot produce detectable colonies on the selective media. Only after first becoming aware of various environmental factors and limitations of conventional methodology will the microbiologist have the ability to achieve some degree of maximal recovery of the entire viable population.

Statement of Purpose

The survival characteristics of bacteria in aquatic environments are of utmost importance in the determination of bacteriological water quality and its implications concerning public health significance. The purpose of the present research is to determine whether the aquatic environment has the ability to induce stress upon a significant proportion of cells such that these cells become

physiologically injured and cannot be detected by direct, conventional selective procedures. The specific objectives are to establish and document the extent of inadequacies and limitations of standard procedures used in the bacteriological analyses of sanitary indicator organisms in natural waters and wastewaters and to consequently provide improved methods for greater efficiency of recovery of those organisms which have been exposed to such environmental stresses.

Chapter 2

LITERATURE REVIEW

Survival of Organisms in Water

Long before the establishment of bacteriology as a science, water was suspected of transmitting disease-producing microorganisms. Yet, not until 1854 in England was it conclusively established that an epidemic of cholera had its origin in polluted water. Since that time, many studies and advances in water research have been made to establish the sources of organisms of public health importance and to develop methods and procedures of detecting, enumerating and identifying them. These diseases include typhoid fever, bacillary dysentery, cholera, leptospirosis, and many others. The causative organisms of these diseases are present in the feces or urine of infected persons or animals. When discharged, they may gain entrance into a body of water that ultimately serves as a source of drinking water.

Routine analysis of water for its bacteriological quality does not include examination for water-borne pathogens. Pathogens are likely to gain entrance to water sporadically and are therefore likely to be missed in a sample submitted for analysis. Additionally, disease-producing organisms are usually present in very small numbers in comparison to other microorganisms making detection methods difficult and complicated. Until methods for the detection of pathogens

are improved, it is necessary to rely on groups of indicator organisms to determine bacteriological water quality.

It would be desirable to find one bacterium in contaminated water that would indicate the presence or absence of not only bacterial enteric pathogens but all enteric disease-producing microbes. With regard to selecting an ideal indicator organism, there are several requirements that should be met. Among these is the indicator organism should have the capability of surviving slightly longer than the hardiest pathogen in water (9,86). Much of the validity of the bacterial indicator system largely depends upon the relative persistences of the indicator organisms and the disease-producing organisms in the water environment. If indicator bacteria survived or persisted at the same rate as the pathogens, then waters showing the absence of the indicator group could be considered to be essentially free of pathogenic microorganisms.

The earliest studies concerning survival of indicator organisms and pathogens were initiated in the late 1800's. Most of these studies utilized a closed environmental test system whereby a sample of water was collected and incubated in a bottle. The bottle was periodically sampled in order to construct survival curves. A limitation of this technique was the fact that the system was closed to the environment, not allowing for the natural processes in the stream and their resulting effects on survival to occur.

One of the earliest laboratory experiments concerning survival of the typhoid bacillus was by de Giaksa in 1889, as cited by Greenberg (38). These studies showed that the organism persisted for 25 days in heat-sterilized sea water but only for 9 days in raw sea water.

Jordan et al. (45) also examined the viability of the typhoid bacterium in water. They observed that the survival time was in inverse relationship to the degree of contamination: the organism survived for 15 to 25 days in sterile tap water, but only 4 to 7 days in unsterilized tap water. Also, they found that survival was usually less than 4 days in raw river water.

On the other hand, Wheeler (112) observed opposite results. He found that the greater the pollution, or presence of dissolved material, the longer the survival. Also, Won and Ross (115) observed that at a temperature of 3 - 5 C survival of *Escherichia coli* (*E. coli*) was significantly enhanced in sea water deliberately "polluted" with small concentrations of organic materials including peptone, heart infusion, casamino acid, yeast extract, trypticase soy, and human feces.

As research continued on survival of bacteria in water, attempts were made to determine the influence that various individual factors had on persistence. Such factors as seasonal variation, temperature, pH, oxygen, sunlight, toxic chemicals, and predation were examined. A review of the early literature concerning the importance of these

factors upon survival of bacteria in soil, water, sewage, and on vegetation was reported by Rudolfs et al. (84). Similar reviews of early literature on the survival of microorganisms in sea water were also written (8,13,38,69,85,93).

A considerable amount of literature has accumulated concerning the relative rates of survival of the coliform and fecal streptococcal groups. Persistence of these indicator groups was then compared to survival patterns of water-borne pathogens in order to assess the significance of using indicator organisms as an index of sanitary water quality. Allen et al. (1) examined several factors affecting the viability of indicator organisms. *Streptococcus faecalis* (*S. faecalis*) was not affected by the degree of oxygenation of the water, but *E. coli* died much more rapidly under anaerobic than under aerobic conditions. Also, a much higher concentration of nutrients was required for the growth of *S. faecalis* in comparison to that of *E. coli*. The effects of certain environmental factors (temperature, pH, turbidity, and nutrients) on survival of coliforms were similarly examined by Kittrell and Furfari (48).

Observations by Ballentine and Kittrell (5) indicated that the fecal coliform group rather than the total coliform group would be of more value as indicator organisms since the fecal coliforms exhibited a more rapid rate of die-away than total coliforms. They also observed

that death curves for fecal coliforms illustrated a much slower die-away rate in winter than in summer indicating an inverse relationship between survival and temperature. Similarly, Platt (71) found that *E. coli* and *Aerobacter aerogenes* (*A. aerogenes*)* persisted for a much longer time at 0 C than at 37 C. Survival studies by Halton and Nehlsen (41) indicated that very low temperatures in sea water tended to favor the persistence of large numbers of *E. coli*.

Survival studies of pathogens in well water by Masharipov (59) revealed that survival of *Shigella flexneri* (*S. flexneri*) and *Shigella sonnei* (*S. sonnei*) was dependent upon the temperature of the water and the degree of contamination. In polluted water, *S. sonnei* survived for 8 days and *S. flexneri* survived for 7 days while their survival times increased to approximately 15 days in relatively pure well water and to 43-45 days in boiled water. Examination of stored samples of well water for coliforms and fecal streptococci by Morris and Weaver (64) indicated that the test for fecal streptococci was slightly superior, since the numbers of streptococci never increased in the sample, while the numbers of coliforms might either increase or decrease.

* Nomenclature for organisms are cited directly as used by the author although changes in nomenclature have taken place since the cited publications.

Geldreich et al. (32) examined stored samples of stormwater at incubation temperatures of 10 and 20 C which were inoculated with a fecal coliform, *A. aerogenes*, *S. faecalis*, and *Salmonella typhimurium* (*S. typhimurium*). Survival studies showed that the organisms persisted at higher levels at the lower temperature. Also, the die-away of *S. typhimurium* was observed to resemble that of the fecal coliform strain more closely than that of *S. faecalis*.

Studies by Van Donsel et al. (105) on survival of indicator bacteria in soil and its resulting contribution to stormwater pollution indicated that survival in the colder months was considerably greater than that in the warmer summer months and that in spring and winter the fecal streptococci survived much longer than did fecal coliforms. Mallman and Litsky (57) also examined survivability of microorganisms in soil. They observed that coliforms persisted in soil for long periods while enterococci died out much more rapidly. Additionally, virulent typhoid bacilli died out in soil even more rapidly than the enterococci.

Boyd et al. (10,11) conducted viability studies of bacteria in relation to cold arctic soils and waters. When pure cultures of coliform bacteria and fecal streptococci were employed *in situ*, rapid death was observed for both, while an indigenous *Pseudomonas* species tested in parallel with the others, showed greater stability to the

lagoon environment of a polar region. Survival of fecal indicator bacteria in a subarctic Alaskan river during winter months when there was total ice cover and the water temperature was 0 C showed that significant numbers of fecal indicator bacteria survived for extended periods of longer than 7 days (37). The authors believed that this high survival rate may have been caused by one or more factors such as lower water temperature, total ice cover, or physical and chemical characteristics of the water.

Andre et al. (3) studied the longevity of representative species of bacterial enteric pathogens in farm pond water. It was observed that *Salmonella* species survived about 16 days, *Shigella* species survived approximately 12 days, while *A. aerogenes* persisted for a considerably longer time under the same conditions. Joyce et al. (46) examined the survival characteristics of enteroviruses and bacteriophages in farm pond waters and found that enteroviruses were capable of surviving long periods of time at temperatures simulating the yearly extremes of 4 and 20 C. Bacteriophages did not appear to survive for any significant length of time in the absence of their parent bacterium.

Gallagher and Spino (29) stored samples of filter-sterilized nutrient-rich wastes from beet sugar lagoons at 10 and 20 C and observed the survival characteristics of fecal coliform bacteria and

S. typhimurium. The data indicated that the rates of growth and die-off of both species were different with regard to changes in environmental conditions. *S. typhimurium* was much more persistent than the fecal coliform at low temperatures indicating that large reductions in fecal coliform concentrations may not necessarily be indicative of comparative reductions in salmonellae.

A number of studies have been conducted concerning the survival characteristics of indicator organisms as well as pathogens in raw sewage, treated sewage and effluents, and wastewaters. Studies by Wiley and Westerberg (113) on survival of human pathogens in composted sewage revealed that 43 h of composting will result in the absence of *Salmonella newport* (*S. newport*) and other specific pathogens. Klock (50) used mathematical and statistical multiple regression procedures to explain coliform survival in wastewater treatment lagoons. In wastewater and irrigation water, Wang et al. (109) found that survival of *S. flexneri* was enhanced by the presence of lower total bacterial counts as well as temperatures lower than 15 C. Similarly, Butler and Ludovici (18) found that organisms indigenous to wastewater significantly interfere with survival and recovery of salmonellae. Removal of indigenous populations by membrane filtration greatly enhanced survival and recovery of *Salmonella* species.

Studies dealing with persistence of bacteria in chlorinated waters as a function of relative dosage and contact time have been conducted by a number of investigators. Brezenski et al. (14) found that chlorination of sewage effluent provided an effective means of reducing survival of *Salmonella* and *Shigella* populations. In contrast, studies by Ballentine and Kittrell (5) indicated persistence of pathogens at least as long as fecal coliforms, as evidenced by isolation of *Salmonella* species downstream from sewage discharges. Deaner et al. (24) reported that fecal coliforms were incapable of regrowth below the outfall of a highly treated wastewater effluent. However, Shuval et al. (91) observed that coliforms and fecal coliforms are capable of regrowth in chlorinated wastewater both in laboratory and field experiments. Additionally, it was noted that fecal coliforms did not generally show regrowth to the same extent as the total coliform group.

The significance of survival characteristics of enteric bacteria in wild animal feces has been examined by Goodrich et al. (36) where it was found that bacteria of fecal origin are capable of survival in elk droppings for a period of at least one year under natural conditions. Since Fair and Morrison (28) have shown that pathogens are capable of surviving in high quality mountain waters, proper consideration should be given to the possibility that aged fecal droppings

may contribute substantially to mountain stream contamination.

Will et al. (114) studied the persistence of *S. typhimurium* in manure disposal ditches under laboratory conditions of a model oxidation ditch. It was observed that the pathogens survived for 17 days in the model oxidation ditch at summer temperatures of 20 C while persisting for 47 days under winter conditions of 2 C.

Gyllenberg et al. (39) studied the survival of bifid bacteria from fecal matter as compared with that of coliform bacteria and enterococci in water. Once the coliform bacteria had entered the phase of reduction in their numbers, there seemed to be no distinct difference in the relative survival of coliforms and bifid bacteria at room temperature while enterococci exhibited a greater degree of persistence. At refrigerator temperatures the relative reduction rates were bifid bacteria > coliform bacteria > enterococci.

Cohen and Shuval (22) studied the survival of coliforms, fecal coliforms, and fecal streptococci in sewage plants, heavily polluted rivers, a lake, and drinking water sources. They found that the fecal streptococci were generally more resistant to the natural water environment and to purification processes than the other indicator organisms. Also, the survival of fecal streptococci better paralleled the persistence of enteric viruses than did coliforms. Evidence is also presented by Burman (17) that indicated relatively greater ability of

of fecal streptococci than *E. coli* to survive in various natural and antagonistic environments.

Geldreich et al. (30,31) strongly suggested that the presence of fecal coliforms was the most accurate and reliable indicator of fecal contamination by all warm-blooded animals. It was found that the survival pattern of the fecal coliform more closely resembled that of the pathogen *S. typhimurium* than that of *S. faecalis* (32). Geldreich and Kenner (35) found that the enterococcal group of the fecal streptococci were considerably more resistant to die-away than fecal coliforms in natural waters and that the species *Streptococcus bovis* (*S. bovis*) and *Streptococcus equinis* (*S. equinis*) were even more sensitive to rapid die-away than fecal coliforms outside the animal intestinal tract. Others (7,93) have similarly shown that the survival time for *S. bovis* to be extremely brief in fresh water as well as salt water.

Application of Dialysis Sacs and Membrane Filter Chambers to Survival Studies

A method utilized in the early 1900's to study survival of microorganisms in aquatic environments involved immersing permeable dialysis sacs or cells into the natural water habitat. The earliest application of this technique was in 1904 by Jordan et al. (45) whereby permeable collodion sacs containing typhoid organisms were immersed

in a Chicago drainage canal. The collodion sacs were permeable to water allowing the organisms to be influenced directly by the natural conditions in the environment. The use of semi-permeable cells or sacs introduces certain limitations that must be properly considered. These include the fact that bacteria are confined in a localized area of water and are not subject to dispersion by dilution, adsorption, and settling. Additionally, the natural effects of predation by protozoa upon bacterial populations are not reflected by this technique. However, this system does permit the suspended organisms to be in direct contact with many factors of the aquatic environment, including pH, temperature, diffusible nutrients, toxic substances, dissolved solids, and other chemical constituents.

Since the pioneering work of Jordan et al. in the early 1900's (45), dialyzer materials have undergone improvements in quality allowing for a wide range of applications. One of these improvements resulted in the membrane filter which is widely used in various bacteriological investigations. The membrane filter, a semi-permeable membrane usually made of cellulose-acetate, retains bacteria separate from media with nutritional requirements being satisfied by the diffusion of nutrients through the membrane. An early application of the membrane filter was made by Beard and Meadowcraft (8) in 1935 which involved the development of a cell whose sides were enclosed by

membrane filters. It was observed that populations of *S. typhosa* and *E. coli* could survive over a period of at least one month in sea water.

Bartley and Slanetz (7) placed permeable sacs containing organisms in vessels of natural waters in the laboratory and studied the survival of coliforms, fecal coliforms, and fecal streptococci.

Slanetz and Bartley (93) expanded the survival studies by suspending cellophane dialysis sacs under natural field conditions in sea water. Coliforms and fecal coliforms were found to increase in number during the first 4 days of exposure to sea water, followed by a rapid decline during the next 6 days. Fecal streptococci did not increase in number under the same conditions. Of the members of the fecal streptococcal group, *S. bovis* appeared to be the most sensitive to environmental conditions as reflected by a very rapid die-away pattern. The survival curves of suspensions of cells from pure cultures of *Salmonella* resembled those obtained for *E. coli*, although the die-off rate was somewhat slower.

Hendricks and Morrison (42) utilized dialysis sac techniques to study the growth rates of various organisms in mountain stream water. Results showed that *A. aerogenes* and *Paracolonobacterium arizonae* (*P. arizonae*) were able to multiply at a site in the stream above the entrance of sewage effluent while *A. aerogenes*, *P. arizonae*, *Salmonella senftenberg* (*S. senftenberg*), and *S. flexneri* could multiply at a site

downstream from the entrance of the sewage effluent. The results suggested that the environment of mountain streams not only can maintain populations of enteric bacteria but also supply sufficient nutrients to initiate multiplication.

André et al. (3) used filter-sterilized pond water inoculated with *S. typhimurium* and *A. aerogenes* in collodion dialysis bags suspended in glass jars filled with raw pond water. At ambient temperatures, the *Salmonella* species persisted for 14-16 days while the *A. aerogenes* population was detectable for a much longer period.

O'Connor (67) placed dialysis cells containing *S. typhimurium* and *E. coli* at locations above and below the outfall of a sewage treatment plant. In all cases, the fecal coliform survived longer than the *Salmonella* species as reflected by faster die-away rates for the *Salmonella* as compared to the fecal coliform. Both the fecal coliform bacteria and *Salmonella* species showed increased persistences at the site located downstream from the outfall. The author attributed this increased survival to the influence of increased levels of bacterial nutrients as a result of the sewage effluent contribution to the water. Smith et al. (94) studied the survival of freshly isolated *Salmonella*, fecal coliform, and fecal streptococcus strains suspended within dialysis tubing at several river sites. It was observed that the survival characteristics of *Salmonella* more closely resembled

those of the fecal coliform than those of fecal streptococcus organisms.

Major modifications by McFeters and Stuart (56) resulted in the development of chambers with membrane filter side walls specifically designed for studies of survival of bacteria in natural and artificial waters. Comparison uptake rates for inorganic ions, total carbon, and glucose between standard viscose dialysis tubing sacs and the newly designed membrane filter chambers indicated that the membrane filter chamber provided a much faster equilibration rate. Laboratory experiments using controlled pH indicated that optimum survival for a fecal coliform strain was between pH 5.5 and 7.5. Also, laboratory experiments with a fecal coliform showed that response of survival to temperature was inversely proportional between 5 and 15 C but above 15 C this relationship was less critical. Field studies with the same organism indicated that survival characteristics varied according to different sites which yielded water having differing chemical and physical characteristics. Using multiple linear regression statistics, Brasfield (12) found significant correlations between populations of coliforms or fecal streptococci with a number of chemical variables in the water.

Recovery of Injured Cells

Bacteria which are exposed to the influences of a variety of environmental conditions are commonly subjected to stress. As a result, some of these stressed cells are apparently damaged or injured. These injured cells are not necessarily killed or destroyed; however, recovery of this portion of the population may require application of less restrictive techniques than presently utilized with selective media. In effect, the detection or enumeration of non-lethally injured survivors of different environmental stresses may be somewhat impeded by the presence of inhibitory compounds in specific selective media. The detection of such injured populations of cells require an improvement of the recovery efficiency of media and methods. That is, improved media and methods must be developed in order to manifest a greater percentage of recovery of environmentally stressed bacterial populations.

Most studies concerned with the viability of populations after exposure to stress have dealt in areas of the food industry. Populations of bacteria in food are examined after exposure to such stresses as heating, freezing, drying, freeze-drying, and other processes. Research in this area has shown that a substantial population of cells become debilitated or injured to such an extent that they do not have the ability to grow and produce colonies on conventional selective media directly.

Clark and Ordal (19) observed that sub-lethal heat treatment of 48 C for 30 min to *S. typhimurium* resulted in 90 per cent of the viable population being incapable of producing colonies on selective eosin methylene blue agar containing 2 per cent sodium chloride, yet remained capable of producing colonies on a rich, non-selective medium. Similar losses in recovery were observed with Brilliant Green agar, SS agar, and desoxycholate citrate agar. Comparable reductions in tolerance to selective agents in recovery media as a result of heat injury have been reported by Tomlins and Ordal (104) for *S. typhimurium* and by Maxcy (60) for *E. coli*. Ordal (68) also observed that substantial heat injury to *Staphylococcus aureus* (*S. aureus*) resulted in reduced recoveries on selective media when compared to a rich, non-selective medium.

Roth et al. (83) observed a communal growth response of a heat-injured strain of *E. coli* whereby sub-lethal heat treatment resulted in two types of injured cells; those able to grow on selective media when present in excess of about 400 colonies/Petri dish and those unable to grow on selective media when present in concentrations below the threshold level of 400 colonies/Petri dish. The authors theorized that an exaggerated communal activity phenomenon occurred whereby it was necessary to have a threshold number of organisms present (400/plate) in order to initiate growth in a selective medium,

indicating that the normally accepted 30-300 colonies per Petri dish was not sufficient to detect injured cells.

Scheusner et al. (87,88) observed that the action of sanitizing agents could induce substantial injury to various microorganisms.

S. aureus, *S. faecalis*, and several strains of *E. coli* were damaged when exposed to a quaternary ammonium compound or representative hypochlorite sanitizers as evidenced by differences in colony counts obtained with a complete medium and those obtained on conventional selective media. Stersky and Hedrick (99) observed that the growth of airborne coliforms and *Salmonella new brunswick* (*S. new brunswick*) was reduced or inhibited by selective media when compared to growth on a rich standard plate count medium.

Cold stress resulting from either sudden exposure or prolonged exposure below growth temperatures has been shown to reduce the ability of cells to recover on selective media. Metabolic injury to bacteria at sub-zero temperatures has been demonstrated by Straka and Stokes (101) with several species of *Pseudomonas* and with *E. coli*. Similarly, Strange and Dark (102) observed losses in recovery of *A. aerogenes* as a result of cold shock. Ray and Speck (81) found that more than 90% of the surviving cells of *E. coli* were injured following freezing in water at -78 C. Also, Warseck et al. (110) observed that injury to *E. coli* was substantial after freezing the organisms in

water or sterile foods since injured survivors were inhibited from forming colonies on violet red bile agar or deoxycholate lactose agar.

Maxcy (60) found that repeated cycles of freezing and thawing were accompanied by an increase in the extent of non-lethal injury which prevented recovery on selective media for a strain of *E. coli*. Similarly, Ray and Speck (78) determined that freezing an aqueous suspension of *E. coli* at -78 C for 10 min followed by thawing in water at 8 C for 30 min resulted in 90% of the surviving population being injured as they failed to form colonies on a medium containing 0.1% deoxycholate.

Sorrells et al. (97) observed that *Salmonella gallinarum* (*S. gallinarum*) showed metabolic injury after freezing. Chicks were then inoculated to evaluate the pathogenicity of such injured cells. The study showed no significant differences at the 95% confidence limit between the pathogenicity of injured and uninjured cells.

Ray et al. (76,77) examined the effects of freeze-drying on *Salmonella anatum* (*S. anatum*) and found that 70-90% of the surviving cells were injured. After rehydration, these injured survivors failed to grow on a selective plating medium containing deoxycholate, but could form colonies on a non-selective medium.

Repair of Injured Cells

In order to recover those cells which have undergone stress and resulting injury, it is necessary that such cells have the opportunity to repair themselves before they can multiply and divide. The conditions under which damaged cells can repair themselves varies with strain and species. In general, appreciable repair from injury is obtained by first exposing the injured cells to a rich, non-selective medium for a short time to permit recovery prior to use of selective media. In effect, this results in a pre-enrichment period in order to allow repair of damaged cells rather than direct primary exposure to conventional selective media.

Clark et al. (19) showed that when heat-injured *S. typhimurium* cells were placed in a suitable medium such as non-selective tryptic case soy broth, the cells recovered rapidly, restoring their ability to grow on selective media. Similar repair of heat-injured organisms has been demonstrated by several workers (61,68,70,98,104). Also, those microorganisms damaged by freeze-drying (76,77) or freeze-injury (63,78,79,81,110) have proven to be capable of repair of injury upon exposure to rich media prior to the use of specific selective media.

Injury as a Result of Laboratory Manipulation

In addition to the effects of using selective media upon recovery of injured cells, a considerable degree of injury can be

initiated through common laboratory manipulations. The possibility of laboratory injury must be recognized in order to correctly evaluate the cause of death or injury to bacterial populations.

The physiological age of pure cultures used for survival studies has been shown to have definite influence upon resulting persistence patterns. Allen et al. (1) observed that young cultures of *E. coli* and *A. aerogenes* were more sensitive to environmental stress in water and that the survival of both organisms increased as older cultures of 24 and 96 h were utilized. Postgate and Hunter (73) similarly observed that populations of *A. aerogenes* harvested from the stationary phase of growth were often less fragile than those from the exponential phase. Strange and Dark (102) found comparable results for another strain of *A. aerogenes*. Cultures of *Pseudomonas aeruginosa* (*P. aeruginosa*) incubated for 7 days were found by Skaliy and Eagon (92) to be more resistant to dessication than 6 or 24 h cultures.

In preparing suspensions of cells for enumeration, diluents are frequently needed. Straka and Stokes (100) have established that rapid and extensive destruction of bacteria occurs in many commonly used diluting fluids, namely distilled, tap, and phosphate water, and saline. A large number of other investigators (40,60,72,80,102,107, 111) have extensively examined the influence of a variety of diluents

upon recovery of microorganisms from different environments. Hall (40) and Straka and Stokes (100) advocate the use of 0.1% peptone water for routine laboratory diluents. Weiler and Hartsell (111) preferred the use of 0.1% trypticase for the recovery of freeze-injured *E. coli* cells. Wagenaar and Jezeski (107) established the applicability of a gelatin phosphate buffer to maximize recovery of *Pseudomonas putrefaciens* (*P. putrefaciens*).

The specific technique utilized to enumerate microorganisms also has considerable influence upon death and injury to cells. Comparison of the spread or surface plate technique with conventional pour plate procedures by Buck and Cleverdon (16) showed marked superiority of recovery using spread plate techniques. Apparently, pouring 46 C agar over cells resulted in substantial loss of recovery in comparison to spread plate procedures. Van Soestbergen and Lee (106) also observed that surface plate methods gave greater recovery efficiency than that of pour plates. Recently, Klein and Wu (49) observed that heterotrophic microorganisms in water samples are susceptible to the transient stress of warmed agar used in the standard methods pour plate procedure, causing significantly decreased recoveries in comparison with a surface plate technique.

Ray and Speck (80) enumerated stationary phase cells of *E. coli* by the pour plate method on Trypticase soy agar containing 0.3% yeast extract, violet red bile agar, and deoxycholate lactose

agar, and by the most probable number method in Brilliant Green bile broth and lauryl sulfate broth. In general, numbers detected were lower with the selective solid media and higher with the selective liquid media. The lower detection on selective solid media was thought to be partly due to the stress induced in some cells by the temperatures of the melted media used in the pour plate method. Also, it was observed that higher detection on selective agar media was effected by surface plating or by surface-overlay plating of cells.

Warseck et al. (110) examined enumeration procedures for injured cells of *E. coli* which had been damaged by being frozen in water or sterile foods at -20 C. The injured survivors were inhibited from forming colonies on selective solid media, and this inhibition was greater when enumeration was done by the pour plate method as compared with the surface or surface-overlay method. Additionally, it was observed that plating procedures gave more reproducible recovery of coliforms than did the most probable number method using selective liquid broth. The greater degree of variability with enumeration by selective liquid media was considered to be a serious limitation of most probable number methods.

Injury of Cells in the Aquatic Environment

There is a dearth of available literature concerning the possibility that substantial populations of injured bacteria exist in

water, wastewater, or sewage as determined by comparison of detection and enumeration on non-selective versus selective media. In contrast, a considerable amount of published research is available dealing with comparisons of recovery efficiency between multiple tube methods and the later developed membrane filtration techniques.

Wang et al. (108) were among the first to discover that substantial loss in recovery occurred when using selective media directly in attempting to enumerate microorganisms in sewage. The results showed that *S. flexneri* added to sterile sewage would not grow on selective SS agar when transferred after 6 to 24 hours exposure. On the other hand, transfers to LB agar, which did not contain any inhibitory agents, indicated that the organisms were actually viable and capable of producing detectable colonies. This phenomenon was also found to be true but to a lesser extent when desoxycholate citrate or MacConkey's agar were tested. Although the authors did not establish the concept that a population of injured cells was responsible for this phenomenon, they stated that awareness should be made that sewage negative for *Shigella* when cultured on selective media may, in fact, contain viable cells if a less inhibitive medium were used.

Andre et al. (3) similarly observed that losses in recovery of bacteria from farm pond waters occurred upon use of selective media

directly. A suspension of *S. newport* was exposed to farm pond water using the dialysis sac technique. A suspension of *A. aerogenes* was exposed in a second dialysis sac immersed in the same water. Samples were withdrawn as a function of time and parallel enumeration of *S. newport* was made using both SS agar and the less inhibitory MacConkey agar. It was observed that the *Salmonella* count obtained with MacConkey agar was considerably higher than that obtained with the more selective SS agar. Parallel counts of *A. aerogenes* on rich tryptone glucose extract agar and selective violet red bile agar also showed that counts on the rich, non-selective medium were significantly higher than on violet red bile agar. The authors state such results suggest that exposure to the farm pond water led to some alteration in physiology of *S. newport* and *A. aerogenes* rendering them less fit for growth on specific selective media.

Enrichment Methods to Improve Recovery of Bacteria From Aquatic Environments

In an attempt to improve the recovery of coliforms from aquatic environments, several workers have developed enrichment techniques for use with the membrane filtration method. In general, a non-selective nutrient medium has been recommended for the preliminary enrichment of organisms on the membrane filter prior to transfer to specific selective media. Laubausch et al. (51) and Levin and Laubausch (53)

observed improved coliform recovery when incubating membrane filters for 2 h on a rich enrichment broth prior to transfer to selective EHC Endo medium for final incubation of 16 to 18 h. Similarly, Clark et al. (21) examined the recovery of coliform organisms from water employing selective EHC broth following preliminary enrichment on a non-selective medium.

Clark et al. (20) described the use of an enrichment medium, Albimi M lactose broth, in conjunction with a modified Endo broth for the detection of coliforms by membrane filtration procedures. They compared counts obtained by the membrane filtration procedure to standard plate counts made with nutrient agar and found that recovery of coliforms by the enrichment membrane filtration technique was at least equivalent to that obtained by the nutrient agar plate counts. Kabler (47) and Shipe and Cameron (90) reported Bacto- m Enrichment broth (Difco) as a satisfactory medium for the enrichment incubation period prior to transferring the membrane to selective Endo medium. McCarthy et al. (55) confirmed the improved recovery efficiency of a two step membrane filter procedure using lauryl tryptose broth as the preliminary enrichment medium prior to transfer to selective M-Endo agar LES.

Recognition of the fact that losses in recovery of coliforms may occur when using selective media directly is found in the 13th

edition of Standard Methods (2). As an alternative to the direct application of selective M-Endo medium when using membrane filtration procedures, an enrichment method is presented. The enrichment technique consists of placing the membrane filter through which the sample has passed in a Petri dish containing a sterile absorbent pad saturated with selective lauryl tryptose enrichment broth. The membrane filter is then incubated on this enrichment medium for $1\frac{1}{2}$ - 2 h at 35 ± 0.5 C, after which the filter is stripped from the enrichment pad and rolled onto the surface of selective M-Endo medium. Standard Methods (2) states the following when attempting to determine whether enrichment techniques should be utilized:

Generally speaking, an enrichment procedure will give the best assessment of the quality of drinking waters. However, this step may be eliminated in the routine examination of drinking water where repeated determinations have shown that adequate results are obtained by a single-step technic. Enrichment is generally not necessary in the examination of non-potable waters or sewages.

Rose and Litsky (82) advocated the use of an enrichment procedure for use with the membrane filter technique for the detection and enumeration of fecal streptococci in water. By the use of peptone-yeast extract-Casitone (PYC) enrichment medium, the recovery of fecal streptococci from river water was increased more than twofold over that of direct application of M-Enterococcus agar. Two enrichment incubation times were examined, 3 and 18 h. It was the author's

opinion that a 3 h enrichment incubation was sufficient since there was no appreciable difference in counts between 3 and 18 h enrichment incubation. This enrichment technique has been adopted in an application manual for analysis of water and wastewater by the Millipore Corporation (62)..

Chapter 3

MATERIALS AND METHODS

Source of Test Microorganisms and Physiological Identification

Microorganisms used for pure culture 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, Montana. All coliform organisms were reisolated on eosin methylene blue (EMB) agar (Difco) to ensure purity of the strains. Individual colonies were inoculated to fresh stock culture agar (Difco) slants, incubated for 24 h at 35 C, then stored at 7 C until use. Fecal streptococcus strains were reisolated on KF streptococcal (KF) agar (BBL) and individual colonies were inoculated as deep stabs into Trypticase soy agar (Difco) containing 0.5% yeast extract (Difco) and incubated at 35 C for 48 h. Cultures were stored at 7 C until needed. Transfers of coliform and fecal streptococcal strains to respective fresh stock culture media were made at 4 week intervals.

Fecal coliform organisms utilized in the study and their Montana State University laboratory culture identification numbers were as follows: *E. coli* C320MP25, isolated from fresh cattle fecal droppings; *E. coli* WC959, *E. coli* RC1435, and *E. coli* EC2075 all isolated from water. Fecal coliforms were considered to be those

organisms giving a ++-- IMViC pattern in combination with the production of gas within 24 h from EC medium (Difco) when incubated at the elevated temperature of 44.5 ± 0.1 C in a water bath.

Non-fecal coliform organisms utilized were those displaying a ---+ IMViC pattern without production of gas from EC medium at the elevated temperature of 44.5 ± 0.1 C. These organisms were classified as *Enterobacter aerogenes* (*E. aerogenes*) and were identified with the following Montana State University laboratory culture identification numbers: *E. aerogenes* RC1282 and *E. aerogenes* EC2072, both isolated from water.

In addition to the classical IMViC and elevated incubation temperature determinations, these cultures were also subjected to an examination for motility and a series of biochemical tests. These included the ability to produce hydrogen sulfide in triple sugar iron (TSI) agar, urease, lysine decarboxylase, and ornithine decarboxylase, and to ferment carbohydrates such as glucose, lactose, mannitol, dulcitol, sorbitol, and arabinose when present in a broth medium at 0.5% concentrations. Preparation of these media (Difco) was according to manufacturer's directions. Interpretations of results and classification of the cultures was according to Edwards and Ewing (25).

A number of fecal streptococcus strains were used for pure culture studies and were given the following Montana State University

laboratory culture identification numbers: *S. faecalis* ES1913, *S. faecalis* BS692, *S. faecalis* BS905, *S. faecalis* MS304, and *S. faecalis* RS1009 all isolated from water; *S. faecium* BS895, isolated from water and *S. faecium* ES2017 obtained from raw sewage; *S. faecalis* var. *liquefaciens* ES1730 and *S. durans* GS255 both isolated from water; and *S. bovis* CKF20 and *S. bovis* C3SE17 both isolated from fresh cattle fecal droppings.

A series of physiological tests were utilized in the identification of these microorganisms. These included the ability to grow in ethyl violet azide (EVA) broth, 6.5% NaCl, and brain heart infusion (BHI) broth incubated at 10 and 45 C. Also, the organisms were examined for the ability to form ammonia from peptone broth, liquefy gelatin, hydrolyze starch, reduce potassium tellurite, and produce a catalase enzyme. Fermentations of glucose, lactose, mannitol, dulcitol, sorbitol, arabinose, melezitose, and melibiose were determined in a broth medium containing 0.5% concentrations of the respective carbohydrate. Preparation of media (Difco) was according to the manufacturer's directions. Classification according to species was based on Ayres et al. (4) and Mundt (66).

A strain of *Klebsiella pneumoniae* (*K. pneumoniae*) was also included in the pure culture study. This culture was obtained from Dr. D. W. Duncan of British Columbia Research and was designated as *K. pneumoniae* 1881. The organism had been isolated from water.

Complete cultural identification was based on those biochemical tests previously described for the coliform group.

Membrane Filter Chambers

Membrane filter chambers were utilized for studying survival and recovery characteristics of bacteria in stream environments. The chambers employed in the study were those developed by McFeters and Stuart (56). Chambers were constructed of 6.5 mm Plexiglass (Figures 1 and 2). The lumen in the central spacer was 6 cm in diameter, which accommodated a 20 ml sample when the chamber was assembled. Tear-resistant microweb membrane sheets (WHWP 304 F1, Millipore Corp.) with a porosity of 0.45 micrometer were cut into circular pieces with a diameter of 7.5 cm. These were inserted on either side of the central spacer and held in place by the two 6.5 mm Plexiglas retainers. The total surface area of the membranes in the chamber was 56.8 cm²; and the surface area to volume ratio was 2.84.

Two 18 gauge hypodermic needles were fitted into the top of the central spacer to allow filling and withdrawal of samples. Tygon tubing was attached to one of the needles to allow sampling near the bottom of the chamber and to help promote adequate mixing when sampled with a 1 ml syringe. Removable dust caps, made by filling the ends of plastic syringes with plastic cement, were placed in the top opening of needles to prevent contamination.

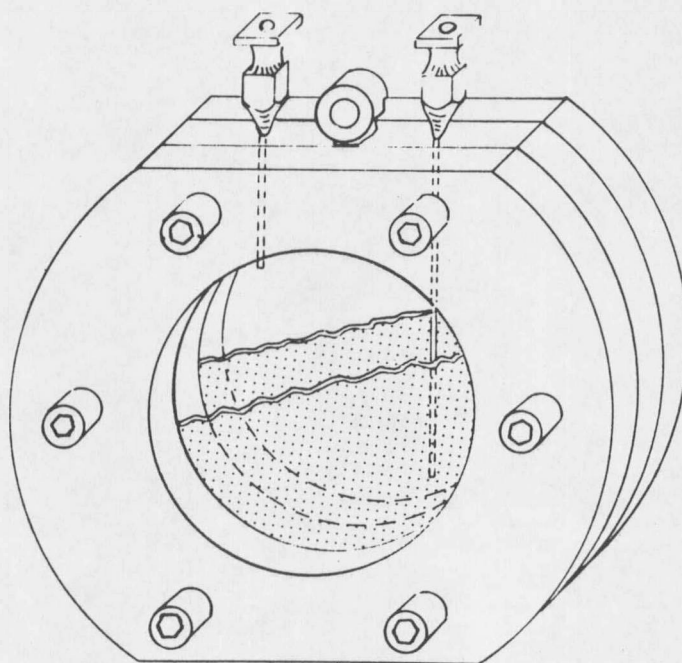


Figure 1. Schematic drawing of the membrane filter chamber.

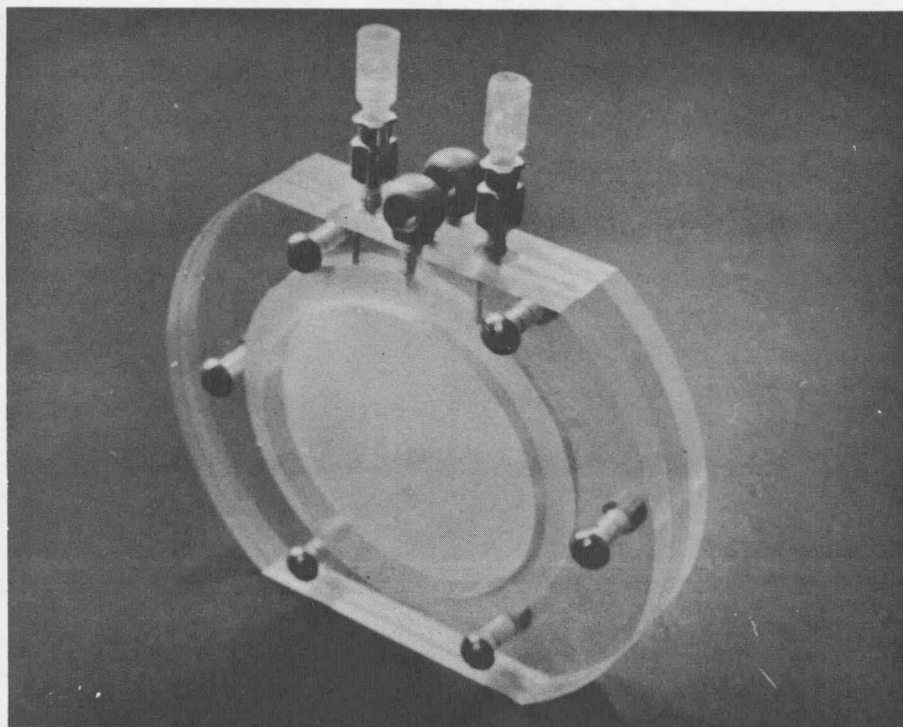


Figure 2. Photograph of a membrane filter chamber that is assembled and ready for use.

The Plexiglass parts of the chambers and the dust caps were sterilized by autoclaving for 10 min at 121 C and 15 lb pressure. Membranes were sterilized on each side for 15 min using ultraviolet light. In early experiments, the membranes were soaked in sterile phosphate buffered water (2) for 15 min prior to assembly of the chambers. However, removal of the membranes from the buffered water with sterilized forceps often resulted in curling of the membranes making assembly procedures extremely difficult. With the exception of a few preliminary experiments, the pre-soaking of the membranes was eliminated. Although dry membranes were somewhat brittle and subject to cracking, this problem was found to be minor compared to that of curling of wet membranes. The chambers were assembled using aseptic techniques. The membranes were placed between the central spacer and the retainers and secured with stainless steel bolts and nuts.

Preparation of Cell Suspensions

Pure cultures used in these experiments were grown in Trypticase soy broth (Difco) supplemented with 0.3% yeast extract (Difco) and sufficient glucose (Difco) to make the final concentration 0.5% glucose (TSY broth). Cells were incubated at 35 C for 20 to 24 h. The cells were harvested by centrifugation (3,020 x g) for 10 min and washed twice with one of three sterile wash solutions. The three wash solutions employed were a standard phosphate buffer (2), gelatin

phosphate buffer (107), and sterile water obtained from a ground water well. Early experiments were conducted using the phosphate buffer (2); however, this solution was found to be toxic to several strains of microorganisms. Thereafter, most washing procedures utilized the gelatin phosphate buffer (107). (Evidence of the harmful effects of the phosphate buffer will be presented in the results section).

After the final wash, the cells were suspended in sterile gelatin phosphate buffer and diluted to the desired population density. The final dilution used to fill the chambers was made in sterilized water obtained from one of the site locations. A sterile 20 ml syringe was used to fill the membrane filter chambers. The most frequently used initial population density was approximately 10^4 organisms/ml; however, the range of population densities used for specific experiments was from 10^3 to 10^8 organisms/ml. The loaded chambers were then suspended in a large plastic transport container filled with fresh water obtained from one of the site locations. In each experiment, the water in the transport container and the water used to make the final suspension of bacteria within the chamber was obtained from the same source.

In some experiments, raw sewage suspensions were utilized in the chamber rather than pure cultures. The raw sewage was obtained at the Bozeman Wastewater Treatment Plant. A 20 ml sample of raw sewage

was loaded into the chamber without subjecting the sample to any of the forementioned treatments.

Locations for Membrane Filter Chambers
and Sampling Procedure

Upon assembly and filling, the membrane filter chambers were transported to site locations as rapidly as possible. The chambers were immersed in relatively quiescent areas of the stream. Heavy string was used to suspend the chambers from an overhead support, allowing for some rotational movement within the stream. 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 for each experiment. Subsequent sampling was done at various time intervals as dictated by the specific objectives of individual experiments. Before a sample was withdrawn from the chamber, the syringe was pumped approximately 10-15 times to resuspend cells that may have settled to the bottom of the chamber or adhered to the walls or membranes.

Samples taken from the chamber were then deposited into a 9 ml dilution blank consisting of either standard phosphate buffer (2) or gelatin phosphate buffer (107) for transport back to the laboratory. As indicated previously, the phosphate buffer was found to be harmful to several bacterial species; therefore, for the majority of experiments gelatin phosphate buffer was utilized as a transport medium.

Site locations for suspending the membrane filter chambers were chosen so as to represent a variety of different types of water in regard to their physical and chemical characteristics. Figure 3 shows the locations, and descriptions of these field sites are given in Table 1.

Additional experiments were conducted with chambers suspended in a relatively uniform source of ground water from a non-potable well located at the Bozeman Wastewater Treatment Plant. A large 40 liter vessel for suspension of the chambers was so designed as to allow a constant flow of water at 5 liters per minute with adequate opportunity for overflow from the vessel to insure a constant introduction of fresh water.

Enumeration Procedures for Pure Cultures

Transportation of samples back to the laboratory was done as rapidly as possible. The sample was then appropriately diluted in gelatin phosphate buffer to obtain acceptable counts (2) on the various enumeration media used. All experiments involved the use of both selective and non-selective media in order to obtain parallel counts on the same sample. Generally, the chambers were sampled daily and examined for recovery on selective and non-selective media simultaneously. Termination of sampling varied from experiment to experiment

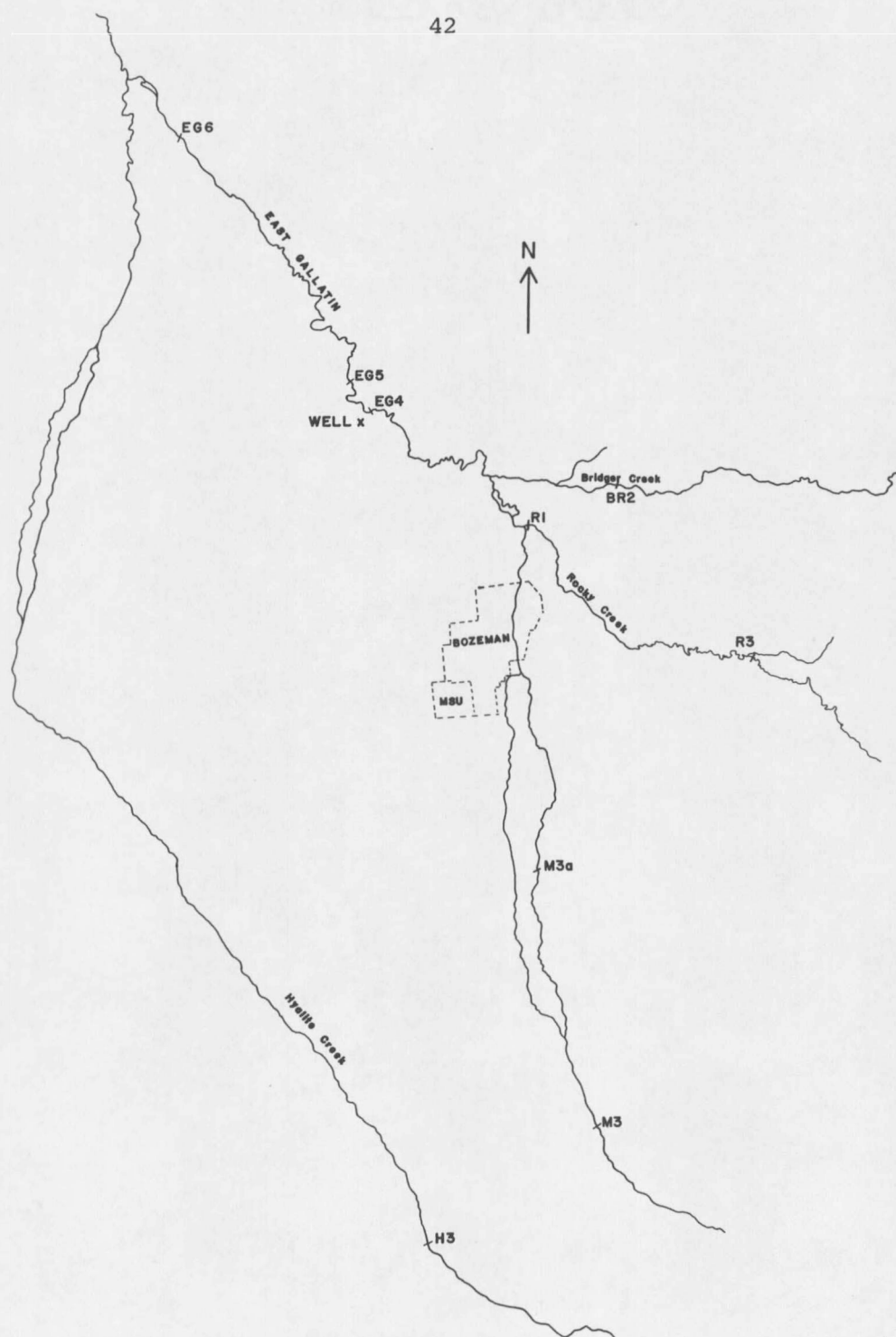


Figure 3. Site locations for membrane filter chambers.

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 the East Gallatin River
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	Non-potable well located at the Bozeman Wastewater Treatment Plant

but most experiments were ended after 3 or 4 days exposure to the water.

Coliforms. Coliform organisms were quantified using three general enumeration procedures in conjunction with a variety of media. The procedure utilized was dictated by the objectives of each individual experiment; however, quantification in each experiment was done utilizing at least one non-selective medium in parallel with at least one selective medium. The three general methods employed were enumeration by plating methods using solid media, multiple tube dilution extinction methods using liquid media, and membrane filtration procedures.

Quantification of coliform organisms with a non-selective medium by plating procedures was done with Trypticase soy agar supplemented with 0.3% yeast extract and 0.5% glucose (TSY agar). (Unless specifically indicated otherwise, all media employed were Difco products). Violet red bile (VRB) agar and desoxycholate lactose (DLA) agar were used as the selective solid media. Pour plate as well as surface plate techniques were used in various experiments. All media were sterilized by autoclaving. For the pour plate method, media were stored in 100 ml portions for no longer than 7 days. Plates for surface plating were poured with 12 to 15 ml of medium per plate and

dried at room temperature for 24 h prior to use. The appropriately diluted sample in the pour plate procedure was mixed with approximately 15 ml of the tempered (46 ± 1 C) medium. In the surface plate method, 0.1 ml of diluted sample was placed onto the surface of a pre-poured plate and distributed with a sterile bent glass rod. Duplicate sets of plates were made for each dilution and medium used. All plates were overlayed 15 min after plating with an additional 5 ml of the same medium. The plates were inverted and incubated at 35 C for 24 h before counting.

Liquid media enumeration procedures using the most probable number (MPN) method involved the use of three different broths. The rich, non-selective broth used was Trypticase soy broth supplemented with 0.3% yeast extract and sufficient glucose to make the final concentration 0.5% glucose (TSY broth). Selective media used were lactose (LAC) broth and Brilliant Green lactose bile (BGB) broth. All media were sterilized by autoclaving and were used within 10 days after preparation. A total of five tubes in each of five successive decimal dilutions were used in the MPN procedure. A 1 ml amount of the appropriate serially diluted sample was inoculated into a tube containing 9 ml of medium. The tubes were incubated at 35 C for 48 h. MPN was estimated based on the tubes showing gas production for the selective media and those showing growth for the non-selective TSY broth (2).

Membrane filtration procedures for enumeration of coliforms employed three different media. Autoclaved TSY agar was used as the non-selective medium. Selective M-Endo broth MF supplemented with 1.4% agar was used. This medium was not autoclaved. Ten ml amounts of melted TSY or M-Endo MF agar were poured into sterile 60 x 15 mm glass Petri dishes. Petri dishes were stored at 8 C until used and were discarded if not used within 96 h of preparation. M-FC broth base containing a final concentration of 0.01% rosolic acid (M-FC medium) was used as the other selective medium when enumeration of fecal coliforms was desired. The medium was heated to the boiling point but was not autoclaved. Approximately 2 ml of M-FC medium was distributed in a sterile tight-fitting 50 x 12 mm plastic Petri plate (Falcon Plastics) containing a sterile absorbent pad. Plates containing M-FC medium were discarded if not used within 5 h of preparation.

The procedure used for membrane filtration with the three different media followed Standard Methods for the Examination of Water and Wastewater (2). Membrane filters used were Millipore filters, type HAWG 047 SO with a pore size of 0.45 micrometer. Duplicate plates of all samples were run in each experiment. TSY and M-Endo MF plates were sealed with waterproof tape (Millipore Corp.), inserted in waterproof plastic bags, and incubated in a water bath for 24 h at the elevated temperature of 44.5 ± 0.1 C.

Fecal streptococci. Fecal streptococci were also enumerated using the same three general procedures: plating, MPN, and membrane filtration. Both non-selective and selective media were employed.

Quantification of fecal streptococci with a non-selective medium by plating procedures was done with TSY agar. Pfizer selective enterococcus (PSE) agar (Pfizer Diagnostics Div.), M-enterococcus agar (M-E), and KF streptococcus (KF) agar (BBL) without bromo cresol purple were used as the selective solid media. Only pour plate techniques were used. PSE agar was sterilized in the autoclave at 121 C under 15 lb pressure for 15 min. KF agar was sterilized under the same conditions for 10 min after which a 1% solution of 2,3,5 tri-phenyl tetrazolium chloride (TTC) was added to make the final concentration 0.01%. M-E agar was brought to boiling to dissolve the medium but was not autoclaved. The appropriately diluted sample was mixed with approximately 15 ml of the tempered (46 ± 1 C) medium. Duplicate sets of plates were done for each dilution and medium used. The plates were inverted and incubated at 35 C for 48 h before counting.

Liquid media enumeration procedures using the MPN method involved the use of four different broths. TSY broth was used as the rich, non-selective medium while selective media utilized were azide dextrose (AD) broth (BBL), Pfizer selective enterococcus (PSE) broth (Pfizer Diagnostics Div.), and ethyl violet azide (EVA) broth. All

broth media were sterilized by autoclaving at 121 C under 15 lb pressure for 15 min and were used within 10 days after preparation. A total of five tubes in each of five successive decimal dilutions were used. The MPN determination was made according to Standard Methods (2) after 48 h incubation at 35 C.

Membrane filtration procedures for enumeration of fecal streptococci employed the same four media used for the pour plate method: TSY agar, PSE agar, M-E agar, and KF agar. Ten ml amounts of the respective media were poured into sterile 60 x 15 mm glass Petri plates. Media were stored at 8 C and were discarded if not used within 7 days of preparation. Standard procedures (2) were used during the filtration operations. All plates were incubated at 35 C for 48 h prior to counting.

Repair Experiments

Experiments were conducted to determine whether cells injured by environmental stress in natural waters had the capability to repair themselves when exposed to a suitable environment. Two repair experiments were done with *E. coli* while one comparable repair experiment was conducted with *S. faecalis*.

Preparation of cells and membrane filter chambers followed previously described procedures. In the first experiment with *E. coli*, a 4 ml sample was withdrawn from the chamber at 0-h in order to obtain

control curves. The 4 ml sample was immediately returned to the laboratory and planted into 36 ml of TSY broth. Generally, at 30 min intervals over a period of 6 h, 1 ml samples were withdrawn from the TSY broth, serially diluted, and enumerated by surface-overlay procedures. The TSY broth was incubated at 35 C during the course of sampling. Parallel platings of both TSY and DLA agars, in duplicate, were used. After two days of exposure to the aquatic environment, another 4 ml sample was withdrawn from the membrane filter 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 agars 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.

The second repair experiment was performed using a different strain of *E. coli*. Exactly the same experimental procedure as used in the first experiment was utilized with two exceptions. First, the exposure period used in this experiment was 7 days rather than two days. Secondly, enumeration was conducted using membrane filtration methods rather than surface-overlay plating methods. The media used for membrane filtration were TSY agar, M-Endo MF agar, and M-FC broth. The TSY and M-Endo MF plates were incubated at 35 C for 24 h while the M-FC plates were incubated at 44.5 ± 0.1 C for 24 h, as previously described.

A similar repair experiment was done with *S. faecalis*. At 0-h, a 4 ml sample was withdrawn from a membrane filter chamber immersed in a stream, brought back to the laboratory, and inoculated into 36 ml of TSY broth. This flask was incubated at 35 C for 8 h. At various time intervals during this 8 h period, 1 ml samples were withdrawn from the TSY broth, serially diluted, and enumerated by membrane filtration methods. Parallel filtrations using TSY and KF agar, in duplicate, were used. After 2 days and 4 days exposure to the water, additional 4 ml samples were obtained from the chamber and analyzed in the exact manner as the control 0-h sample. Plates were enumerated after 48 h incubation at 35 C.

Enumeration Procedures for Raw Sewage

Experiments were performed to compare recovery of coliforms, fecal coliforms, and fecal streptococci from samples of raw sewage utilizing various conventional enumeration procedures. Membrane filter chambers containing recently obtained suspensions of raw sewage were immersed in natural water. A 3 ml sample was removed from the chamber at 24 h intervals for a period of 3 to 4 days. Analysis for coliform densities was accomplished using pour and surface-overlay plating procedures with DLA and VRB agars. MPN determinations were made using lactose broth while M-Endo MF agar and M-FC broth were used for enumeration by membrane filtration procedures. Fecal streptococci

were enumerated by pour plate methods using KF and M-E agar as well as by membrane filtration procedures using the same two media. Specific procedures, incubation time, and incubation temperatures have previously been described.

Confirmation of probable coliform colonies was accomplished by transferring isolated colonies from the selective solid media and membrane filters to BGB broth. Plates were divided into quarters and all suspect coliform colonies from a single quarter were picked into BGB broth. Also, BGB broth was inoculated from those tubes of presumptive lactose broth showing the presence of gas. All BGB tubes containing gas within 48 h at 35 C were considered to be confirmatory evidence that coliform organisms were present. Additionally, all positive BGB tubes were further analyzed for the presence of coliforms of fecal origin by transferring a loopful of culture to EC medium. The EC medium was incubated at 44.5 ± 0.1 C in a waterbath. Production of gas within 24 h in EC medium indicated the probable presence of coliforms of fecal origin.

Parallel with analyses for coliforms and fecal coliforms, most samples were quantitatively examined for fecal streptococci. Enumeration was conducted by using KF agar in both the pour plate and membrane filtration procedure. Confirmation of the presence of fecal streptococci was accomplished by picking all suspected colonies from

a quarter section of the plate into EVA broth. Confirmation was evidenced by growth in the tube within 48 h at 35 C.

Enrichment Procedures

An enrichment procedure was used in order to determine whether improved recovery of coliforms and fecal coliforms could be attained by exposing cells to a rich, non-selective medium prior to transfer to a specific selective medium. The enrichment technique was utilized both with a pure culture of *E. coli* and a natural suspension of raw sewage.

After immersion of a membrane filter chamber containing a suspension of *E. coli* in the stream environment, 1 ml samples were withdrawn at 0-h, 1 day, 2 day, and 3 day intervals. Enumeration was done by membrane filtration methods using both the conventional direct technique as well as an enrichment technique. Duplicate filtrations for all dilutions were performed such that one set could be enumerated by the direct method and the other by an enrichment technique. The direct technique was done by directly transferring one set of the membrane filters to TSY agar, M-Endo MF agar, and M-FC broth. The enrichment technique consisted of first transferring the other set of membrane filters to 60 x 15 mm Petri plates containing pads soaked with 2 ml of TSY enrichment broth. These enrichment plates were then incubated in the upright position for 2 h at 35 C. After the 2 h

enrichment period, the filters were transferred from the TSY broth pads to the respective media used in the direct technique; i.e., TSY agar, M-Endo MF agar, and M-FC broth. Incubation temperatures and times have been previously described.

A suspension of raw sewage was analyzed in exactly the same manner as the pure culture of *E. coli* using both direct and enrichment methods. In addition, probable coliform colonies from a quarter section of the membrane filters were picked into BGB broth for confirmatory purposes. Those tubes showing the presence of gas within 48 h at 35 C were then further analyzed by transferring a loopful of culture to EC medium. The EC medium was incubated at 44.5 ± 0.1 C for 24 h and thereby allowed calculations for fecal coliform densities obtained by the respective media and methods.

Effects of Wash Solutions and Diluents

Several experiments were performed in order to determine the most suitable solutions to be used for washing cell preparations during centrifugation, for transport solutions for membrane filter chambers to site locations, and for laboratory diluents during enumeration procedures. Test organisms included strains of *E. coli* and *S. faecalis*.

The wash procedure for cell preparations, transport of prepared chambers, transport of samples, and laboratory dilution procedures

were done exactly as previously described except that three different test solutions were utilized. The complete operation of washing, transporting, and enumerating was done simultaneously using three separate chambers treated with either gelatin phosphate buffer (107), phosphate buffer (2), or sterilized natural well water, respectively. The chambers were sampled periodically over a period of 2 to 3 days and enumerated with non-selective and selective media. When *E. coli* was the test organism, TSY and DLA agar were used for enumeration by the pour plate method. Pour plate procedures, using TSY and KF agar, were used when *S. faecalis* was the test organism.

Calculations and Assumptions

Several calculations were made in order to plot graphs of the observed data and were obtained as follows: % death at time t = $(1 - \text{non-selective medium count at time } t / \text{non-selective medium count at } 0\text{-h}) \times 100$; % survival at time t = $100\% - \% \text{ death at time } t$; and % injury at time t = $(1 - \text{selective medium count at time } t / \text{non-selective medium count at time } t) \times 100$.

Definitions of repair and multiplication were determined as follows: any increase in selective medium counts but not in non-selective medium counts were assumed to be due to repair of injured cells; and, simultaneous increase in counts on non-selective and selective media was assumed to be due to cell multiplication.

Chemical and Physical Procedures

Periodically throughout the course of the experiments, water samples were obtained at those sites where membrane filter chambers were located and subsequently analyzed for their chemical and physical characteristics. At the time of collection, water temperature was recorded. At each site, a sample was collected in a 1 liter polyethylene bottle as well as in a 250 ml glass-stoppered Pyrex bottle.

Water in the glass bottle was filtered and used to determine orthophosphate, ammonia, and nitrate concentrations. Orthophosphate was determined by the ascorbic acid method as described by the Environmental Protection Agency (26). Ammonia determinations were done according to Solorzano (96) using the phenohypochlorite method, while nitrate concentrations were determined by the Mullin and Riley reduction method (6). Total phosphate concentrations of unfiltered water were determined following procedures advocated by the Environmental Protection Agency (26). A Bausch and Lomb "Spectronic 20" was used for these colormetric analyses.

Alkalinity, sulfate, chloride, total hardness, calcium, magnesium, sodium, potassium, and residual chlorine were determined by standard methods (2). Non-filtered water from the polyethylene bottles was used to determine total alkalinity by potentiometric methods. Sulfate concentrations were analyzed by the turbidimetric method; and

chloride concentrations were done by the mercuric nitrate method. Total hardness, calcium, and magnesium were determined by the EDTA titrimetric method. Sodium and potassium were determined by flame emission with a Beckman DU Flame Spectrophotometer. Residual chlorine concentrations were determined by the iodometric method.

Total and soluble carbon fractions were determined by employing a Beckman Laboratory Carbonaceous Analyzer. Measurement of hydrogen ion concentrations were made with a Beckman Zeromatic SS-3 pH meter in the laboratory. The specific conductance of the water at 25 C was determined using a Labline Lectro MHO - meter (Model MC-1, Mark IV).

Statistical Methods

Statistical analyses for the comparison of data obtained upon parallel enumeration with non-selective and selective media were based upon computation of 95% confidence limits for proportions following the normal approximation for the binomial distribution, as described by Snedecor and Cochran (95). Assuming the colony counts follow a Poisson distribution, optimum statistical inference concerning the ratio of the mean counts is based on the binomial distribution (52).

Chapter 4

RESULTS

Physiological Identification of Pure Cultures

The cultural and biochemical characteristics of the strains of *E. coli*, *E. aerogenes*, and *K. pneumoniae* are given in the appendix. Similarly, physiological characteristics of strains of *S. faecalis*, *S. faecium*, *S. durans*, *S. faecalis* var. *liquefaciens*, and *S. bovis* are included in the appendix.

Effects of Wash Solutions and Diluents

Several experiments were conducted to determine whether substantial death or injury could be induced during centrifugation-washing procedures used in the harvesting of cells for the preparation of cell suspensions, transport of chambers to site locations, transport of samples back to the laboratory, and laboratory dilution procedures. In one such experiment two separate chambers, located at the experimental well site, containing suspensions of *S. faecalis* ES1913, were prepared and sampled simultaneously over a period of 48 h. The treatment of both suspensions was identical with the exception that all washing, transporting, and diluting for one suspension was done completely with standard phosphate buffer (2) while the other suspension was treated solely with gelatin phosphate buffer (107). Figure 4

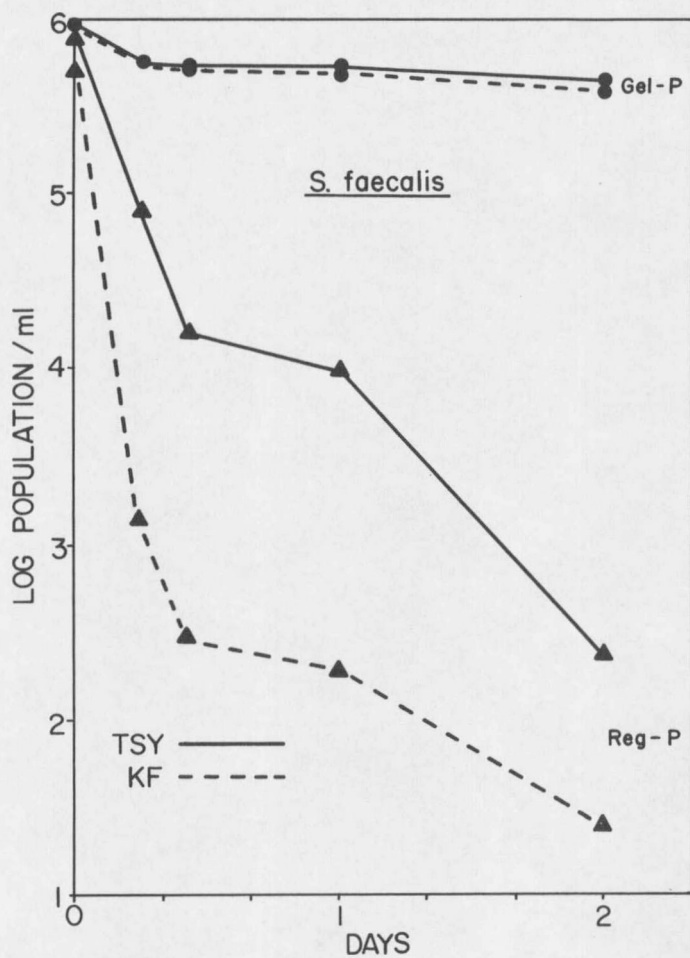


Figure 4. Effect of regular phosphate (▲) and gelatin phosphate (●) diluents upon recovery of suspensions of *S. faecalis* ES1913 in membrane filter chambers located in well water during a 48 h exposure period. Samples were pour plated using TSY (——) and KF (---) agar.

shows the results obtained upon pour plating with non-selective (TSY agar) and selective (KF agar) media for samples taken from each chamber. Comparison of counts obtained on the non-selective medium over the 48 h exposure period indicates that the die-away pattern for those cells treated with the regular phosphate buffer proceeds at a much faster rate than those treated with 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 treated with the regular phosphate buffer as compared to treatment with gelatin phosphate buffer. Summarization of data from this experiment in Table 2 provides indication of the deleterious effects of treatment with regular phosphate buffer in terms of resulting recovery on both non-selective and selective media. The loss in recovery on the selective medium indicates that a large proportion of the survivors had become injured. In comparison, injury to surviving cells treated with gelatin phosphate buffer was relatively minimal, ranging from 0 - 11% injury over the 48 h exposure period.

A similar experiment was done with *S. faecalis* MS304. In addition to treatment of cells with regular phosphate and gelatin phosphate buffer, a third treatment using sterilized well water was included. The three chambers were immersed in the well water, sampled over a period of 24 h, and analyzed using the same techniques and

Table 2. Effect of regular phosphate and gelatin phosphate diluents upon death and injury to suspensions of *S. faecalis* ES1913 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
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

a = Determined from counts obtained with TSY pour plates

b = Determined by differences between counts obtained on TSY and KF pour plates

media as in the preceding experiment. Both in terms of death and non-lethal injury, the gelatin phosphate buffer was found to be considerably less harmful than the regular phosphate buffer (Table 3). The treatment with sterilized well water was observed to contribute to fatality of cells to approximately the same extent as gelatin phosphate buffer treatment. Non-lethal injury to surviving cells of the well water treatment was generally greater than that with gelatin phosphate buffer, yet less than that with the standard phosphate buffer.

Treatment of separate cell suspensions of *E. coli* EC2075 with regular phosphate buffer and gelatin phosphate buffer was studied. Enumeration over a 48 h period with non-selective TSY agar and selective DLA agar, using pour plate procedures, revealed the results found in Table 4. 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 using the regular phosphate buffer was substantially greater than that for treatment with the gelatin phosphate buffer.

Influence of Differences in Water Quality on Injury

A number of early experiments were conducted to determine whether aquatic environments of differing chemical and physical

Table 3. Effect of regular phosphate, gelatin phosphate, and well water diluents upon death and injury to suspension of *S. faecalis* MS304 in membrane filter chambers suspended in well water over a 24 h exposure period

Exposure time (h)	% Death ^a			% Injury ^b		
	Regular phosphate	Gelatin phosphate	Well water	Regular phosphate	Gelatin phosphate	Well water
0	0.0	0.0	0.0	18.2	26.1	19.0
6	40.9	30.4	19.0	80.8	18.8	23.5
12	66.8	39.1	33.3	87.8	7.1	31.4
24	80.5	52.2	52.4	86.0	34.5	66.0

a = Determined from counts obtained with TSY pour plates

b = Determined by differences between counts obtained on TSY and KF pour plates

Table 4. Effect of regular phosphate and gelatin phosphate diluents upon death and injury to suspensions of *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
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 DLA pour plates

characteristics would induce varying degrees of stress upon coliforms and fecal streptococci. In preliminary experiments, the effects of 3 aquatic environments with varying water quality characteristics were examined: these sites were M3A, EG6, and the well water (Figure 3). These particular sites were chosen because they represented waters of widely different physical and chemical characteristics. The water environment of site M3A consisted of high mountain snowmelt run-off of high quality with a minimal amount of contamination from agricultural practices. In contrast, the aquatic environment of site EG6 consisted of highly contaminated water derived from agricultural and domestic sewage effluent. Parallel enumeration on non-selective and selective media allowed for calculation of survival and injury characteristics at these sites.

In one of the early experiments, membrane filter chambers containing a suspension of *E. coli* WC959 were immersed in water at sites M3A, EG6, and the well water. Simultaneously, membrane filter chambers containing a suspension of *K. pneumoniae* 1881 were located at the same sites. Pour plate enumeration with non-selective TSY agar and selective DLA agar allowed for compilation of survival and injury characteristics over the 3 day exposure period as found in Table 5. The amount of survival, as determined by counts on the TSY medium, was observed to vary according to the site location for both organisms.

Table 5. Percentages of survival and injury for suspensions of *E. coli* WC959 and *K. pneumoniae* 1881 in membrane filter chambers located at 3 different sites (Well, M3A, and EG6) over a 3 day exposure period

	Exposure time (days)	<i>E. coli</i>			<i>K. pneumoniae</i>		
		Well	M3A	EG6	Well	M3A	EG6
% Survival ^a	0	100.0	100.0	100.0	100.0	100.0	100.0
	1	72.3	79.7	68.3	35.0	79.1	29.2
	2	69.2	71.2	4.6	58.3	16.4	3.9
	3	60.0	54.2	0.5	44.2	0.8	1.6
% Injury ^b	0	7.7	3.4	9.0	8.3	9.1	8.3
	1	2.1	25.5	23.3	0.0	16.1	22.9
	2	22.2	19.0	79.3	15.7	33.3	34.0
	3	0.0	18.8	87.1	22.6	44.3	21.1

a = Determined from counts obtained with TSY pour plates

b = Determined by differences between counts obtained on TSY and DLA pour plates

The persistence of *E. coli* WC959 at the well water site and site M3A appeared to be relatively similar with between 50 to 60% of the cells remaining viable after 3 days exposure. In contrast, the ability of *E. coli* WC959 to persist at site EG6 was considerably less relative to the other two sites as evidenced by only 4.6% of the cells being recovered after 2 days and less than 1% being recovered after 3 days exposure. Considerable variation in survivability of *K. pneumoniae* 1881 was similarly observed as evidenced by a greater degree of persistence in the well water as opposed to the environments of sites M3A and EG6. Over 44% of *K. pneumoniae* 1881 cells were found to be viable after 3 days exposure at the well water site, whereas less than 2% of the cells could be detected on TSY medium at sites M3A and EG6.

Considerable variation among the 3 site locations in terms of non-lethal injury was also observed (Table 5). The greatest degree of injury occurred to *E. coli* WC959 cells exposed to the aquatic environment of EG6 as evidenced by approximately 79% and 87% of the cells being injured after 2 and 3 days exposure, respectively. The populations of *E. coli* cells exposed to the environments at the well water site and site M3A were less susceptible to non-lethal injury, with less than 26% of the cells found to be injured for any exposure period. In general, the greatest degree of injury for the population of

K. pneumoniae 1881 occurred to those cells exposed to the environmental stresses at site M3A. Whereas *E. coli* had been found to be very susceptible to non-lethal injury at site EG6, *K. pneumoniae* was observed to be resistant to injury since only 21% of the surviving population had lost its ability to produce colonies on selective DLA agar after 3 days exposure to the stream environment. Minimal injury was also observed for *K. pneumoniae* 1881 cells immersed in a chamber at the well water site.

A similar experiment was conducted at the same sites (M3A, EG6, and well water) using two different species of fecal streptococci: *S. faecalis* var. *liquefaciens* ES1730 and *S. durans* GS255. Variation in survival capability according to site locations, as determined by pour plate counts on TSY agar, was observed for *S. faecalis* var. *liquefaciens* (Table 6). Only 4.1% of the original population of *S. faecalis* var. *liquefaciens* could be detected after 3 days exposure at site EG6. More than 61% of the cells were found to be viable on TSY medium after 3 days exposure in well water while about 14% of the cells located at site M3A could be detected after the same exposure period. The persistence of *S. durans* GS255 at the well water site and site M3A was found to be quite similar. As observed with *S. faecalis* var. *liquefaciens*, the greatest amount of fatality for

Table 6. Percentages of survival and injury for suspensions of *S. faecalis* var. *liquefaciens* ES1730 and *S. durans* GS255 in membrane filter chambers located at 3 different sites (Well, M3A, and EG6) over a 3 day exposure period

	Exposure time (days)	<i>S. faecalis</i> var. <i>liquefaciens</i>			<i>S. durans</i>		
		Well	M3A	EG6	Well	M3A	EG6
% Survival ^a	0	100.0	100.0	100.0	100.0	100.0	100.0
	1	67.9	66.3	62.5	73.3	74.4	70.5
	2	60.3	17.5	11.0	57.8	48.8	38.6
	3	61.5	13.8	4.1	51.1	44.2	8.6
% Injury ^b	0	5.1	2.5	8.8	4.4	9.3	4.5
	1	11.3	35.8	56.0	0.0	9.4	19.4
	2	14.9	28.6	98.8	3.8	4.8	23.5
	3	16.7	64.5	99.8	8.7	10.5	63.2

a = Determined from counts obtained with TSY pour plates

b = Determined by differences between counts obtained on TSY and KF pour plates

S. durans occurred with those cells exposed to the environment at EG6, resulting in approximately 9% of the original population surviving 3 days of exposure.

Differences in simultaneous counts obtained on selective KF medium and non-selective TSY medium indicated variation in the degree of injury inflicted upon cells located at the 3 sites. After 3 days exposure, more than 99% of the surviving population of *S. faecalis* var. *liquefaciens* ES1730 located at site EG6 was found to be non-lethally injured, whereas about 65% of the survivors were observed to be injured upon the same exposure period to the environment of M3A. In contrast, approximately 17% of the population of *S. faecalis* var. *liquefaciens* located at the well water site exhibited injury. Similarly, *S. durans* GS255 illustrated variability in susceptibility to injury after 3 days exposure to the water environments, ranging from a low of approximately 10% at both the well water site and site M3A to a high of over 63% at site EG6.

Data from these as well as other early experiments led to an experiment whereby eight chambers, each containing a suspension of *E. coli* C320MP25, were placed at eight different locations (Figure 3). Enumeration of cells over an exposure period of 4 days was done using TSY and DLA agar with surface-overlay techniques. Resulting counts on both media for two of the sites are shown in Figure 5. Comparison

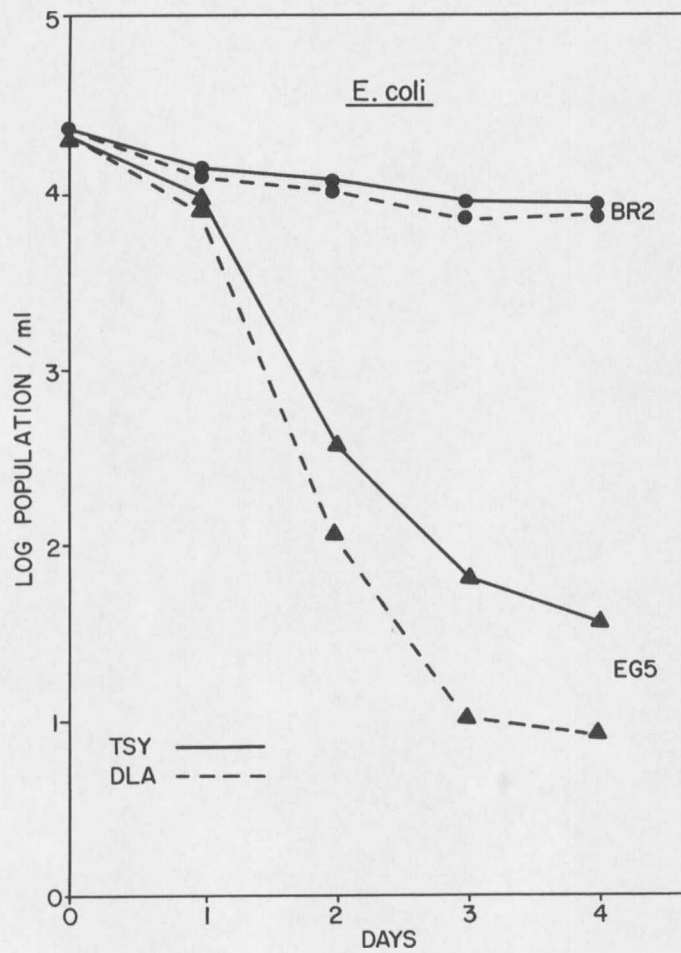


Figure 5. Comparative recovery of *E. coli* C320MP25 in membrane filter chambers located at sites BR2 (●) and EG5 (▲) over a 4 day exposure period. Samples were surface-overlay plated using TSY (——) and DLA (----) agar.

of the survival curves on TSY agar revealed a very rapid die-away pattern for those cells exposed at site EG5, while only a slight decline in survivability was observed at site BR2. Additionally, non-lethal injury at site BR2 appeared to be minimal when compared with site EG5 as evidenced by differences in counts obtained with the selective and non-selective media. Table 7 summarizes both the survival and injury characteristics at all eight sites (Figure 3). The amount of fatality varies considerably among the sites, being the greatest at sites EG4, EG5, and R1 and being the least at site BR2. The data calculated for non-lethal injury are presented in Figure 6 and also indicate substantial variability among the sites. The extreme of injury after 4 days exposure were from a low of less than 10% at site BR2 to a high of greater than 96% at site EG4.

A comparable experiment for a strain of fecal streptococci was done with eight chambers being placed at eight different sites (Figure 3). The test organism was *S. faecalis* RS1009. Enumeration of cells over an exposure period of 4 days was obtained using TSY and KF agar with pour plate techniques. Resulting counts on both media for both sites BR2 and EG5 are shown in Figure 7. Profiles of survival on the rich TSY agar reveal a considerably faster die-away rate for those cells exposed at site EG5 than those exposed at site BR2. Furthermore, the losses in recovery of the viable population with the

Table 7. Percentages of survival and injury for suspensions of *E. coli* C320MP25 in membrane filter chambers located at 8 different sites over a 4 day exposure period

	Exposure time (days)	Sites							
		M3	M3A	H3	BR2	RI	EG4	EG5	EG6
% Survival ^a	0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	1	45.8	59.1	21.3	60.9	42.4	35.4	40.0	43.5
	2	40.0	50.0	10.0	52.2	8.2	12.5	1.6	36.1
	3	27.1	22.7	6.1	38.3	1.2	4.1	0.3	26.5
	4	16.3	14.1	5.2	37.0	1.6	0.9	0.2	14.8
% Injury ^b	0	8.3	4.5	4.3	0.0	15.2	8.3	8.7	8.7
	1	0.0	15.4	24.5	7.1	54.3	3.5	16.3	9.0
	2	22.9	9.1	14.4	8.3	91.5	78.3	70.3	74.7
	3	3.1	18.0	63.6	18.2	70.7	93.9	84.6	86.7
	4	30.8	29.0	90.0	9.4	79.2	96.7	77.1	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

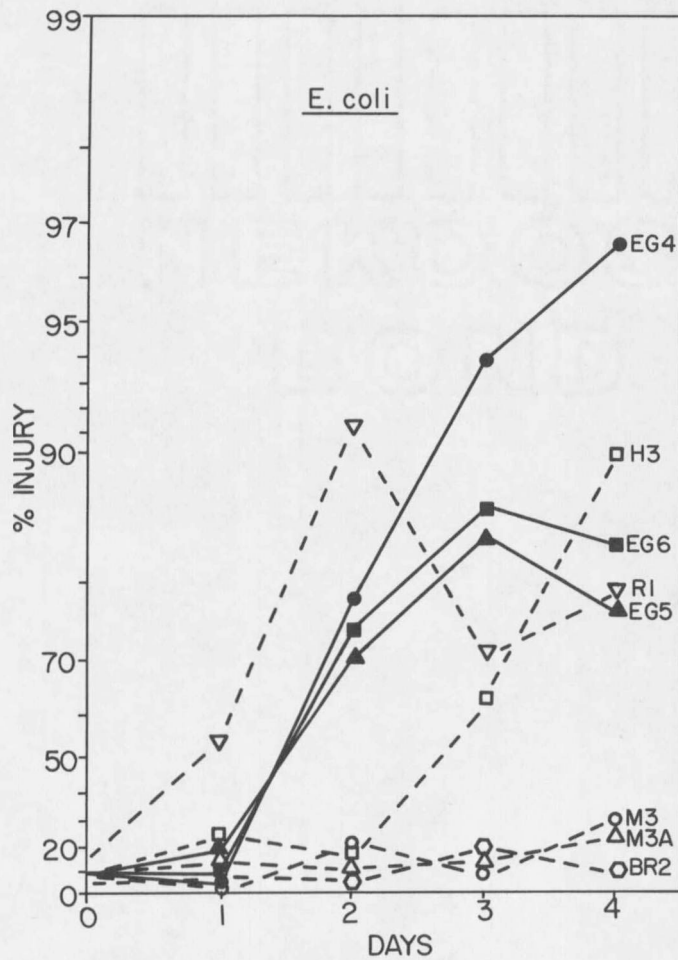


Figure 6. Comparative injury of *E. coli* C320MP25 in membrane filter chambers located at sites M3 (○), M3A (△), H3 (□), BR2 (◇), RI (▽), EG4 (●), EG5 (▲), and EG6 (■) during a 4 day exposure period. Calculations of injury to survivors were determined by differences between counts obtained on TSY and DLA surface-overlay plates.

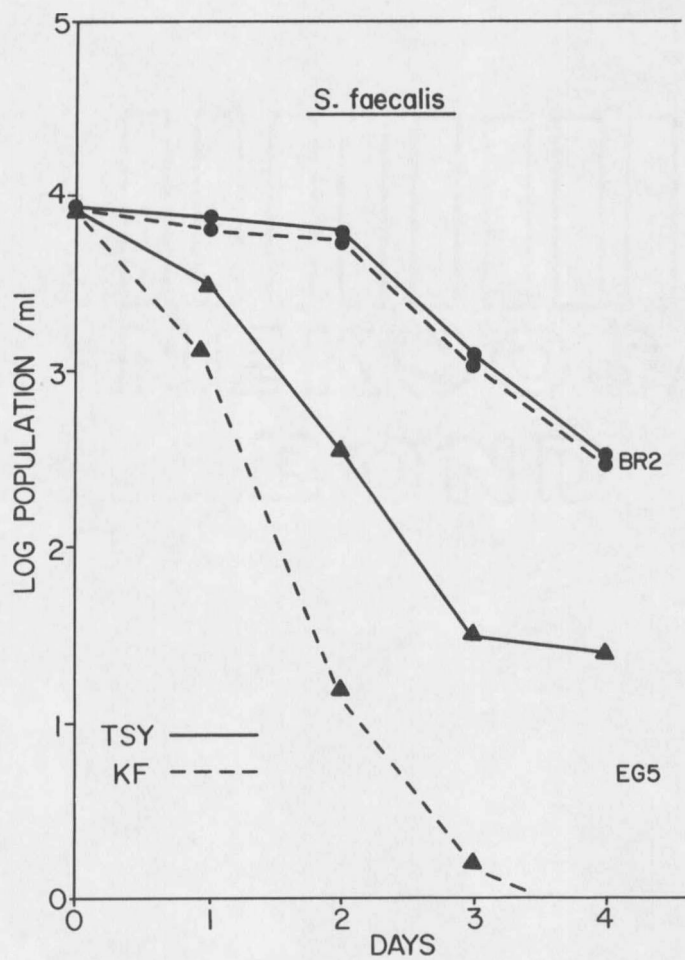


Figure 7. Comparative recovery of *S. faecalis* RS1009 in membrane filter chambers located at sites BR2 (●) and EG5 (▲) during a 4 day exposure period. Samples were pour plated using TSY (—) and KF (---) agar.

selective medium at site BR2 is minimal in contrast to very substantial losses in recover at site EG5. Table 8 summarizes both the survival and injury characteristics at all eight sites. As observed with the strain of *E. coli*, there was considerable variation of survivability as a function of the different environmental sites. The greatest degree of lethality occurred at sites R1, EG4, EG5, and EG6 with less than 1% of the population surviving after 4 days of exposure. In contrast, almost 15% of the population survived the same exposure period at site H3. The data for non-lethal injury are presented in Figure 8. The variability in injury to survivors extends from a low of approximately 3% at site BR2 to a high of 100% at sites R1, EG4, EG5, and EG6 after 4 days exposure.

Comparison of Survival and Injury Characteristics of Indicator Organisms

An experiment was designed so as to allow comparison of survival and recovery characteristics of various species of bacteria. Six chambers, each containing a different species, were immersed in stream water at site EG6. The test organisms were *E. coli* C320MP25, *E. aerogenes* EC2072, *K. pneumoniae* 1881, *S. faecalis* RS1009, *S. faecium* BS895, and *S. bovis* C3SE17. The chambers were sampled at 24 h intervals over an exposure period of 4 days. *E. coli*, *E. aerogenes*, and *K. pneumoniae* were enumerated simultaneously on TSY

Table 8. Percentages of survival and injury for suspensions of *S. faecalis* RS1009 in membrane filter chambers located at 8 different sites over a 4 day exposure period

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

a = Determined from counts obtained with TSY pour plates

b = Determined by differences between counts obtained on TSY and KF pour plates

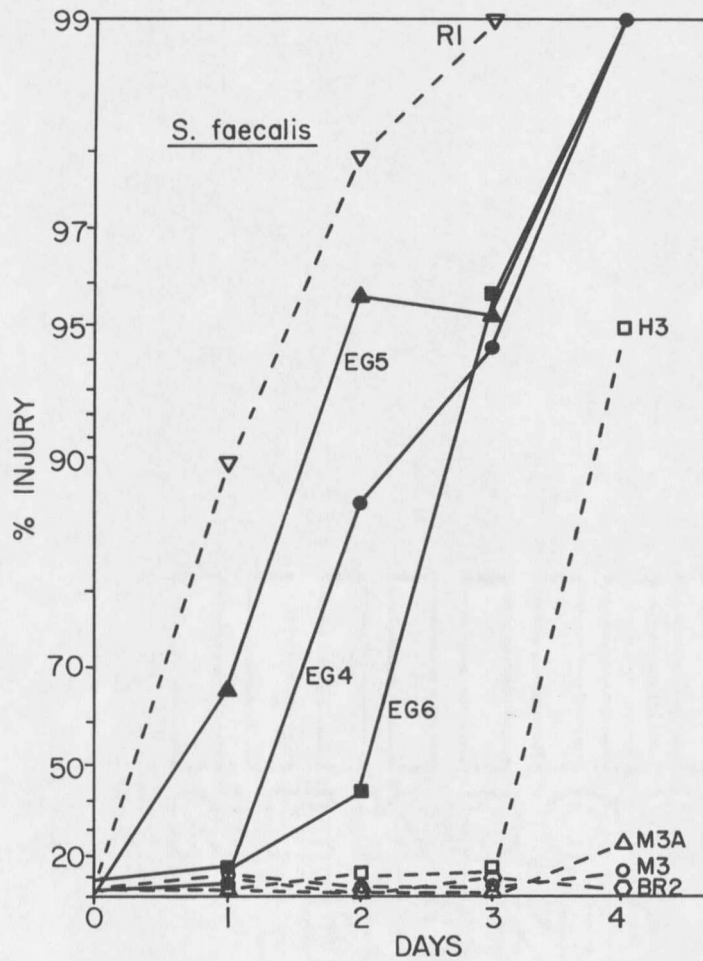


Figure 8. Comparative injury of *S. faecalis* RS1009 in membrane filter chambers located at sites M3 (○), M3A (△), H3 (□), BR2 (○), RI (▽), EG4 (●), EG5 (▲), and EG6 (■) during a 4 day exposure period. Calculations of injury to survivors were determined by differences between counts on TSY and KF pour plates.

and DLA agar using pour plate procedures. Pour plate techniques were also used to enumerate fecal streptococcal species employing TSY and KF agar.

The data obtained on the non-selective and selective media were used to plot graphs of survival (Figure 9) and injury (Figure 10). The profiles in Figure 9 show the variability of survival of the six organisms in terms of their recoverability on the rich TSY agar. At all sampling times over the entire exposure period, *K. pneumoniae* was found to be the most resistant to death with approximately 20% of the original population remaining viable after 4 days exposure. *E. aerogenes* was observed to be slightly more resistant to death than *E. coli*. Only 1% of the original population of *S. faecium* survived after 4 days, while *S. faecalis* could not be detected on the fourth day. Of all the organisms tested, *S. bovis* was found to be the most sensitive to the environment. Only 7% of the population of *S. bovis* could be detected after 1 day, while the population was found to be completely non-viable after 3 days exposure.

S. bovis and *S. faecalis* were found to be the most susceptible to non-lethal injury (Figure 10). Approximately 90% of the surviving population of these two species could not be recovered on selective KF agar after 2 days exposure, *E. coli* was also susceptible to injury as evidenced by approximately 96% of the cells being injured after

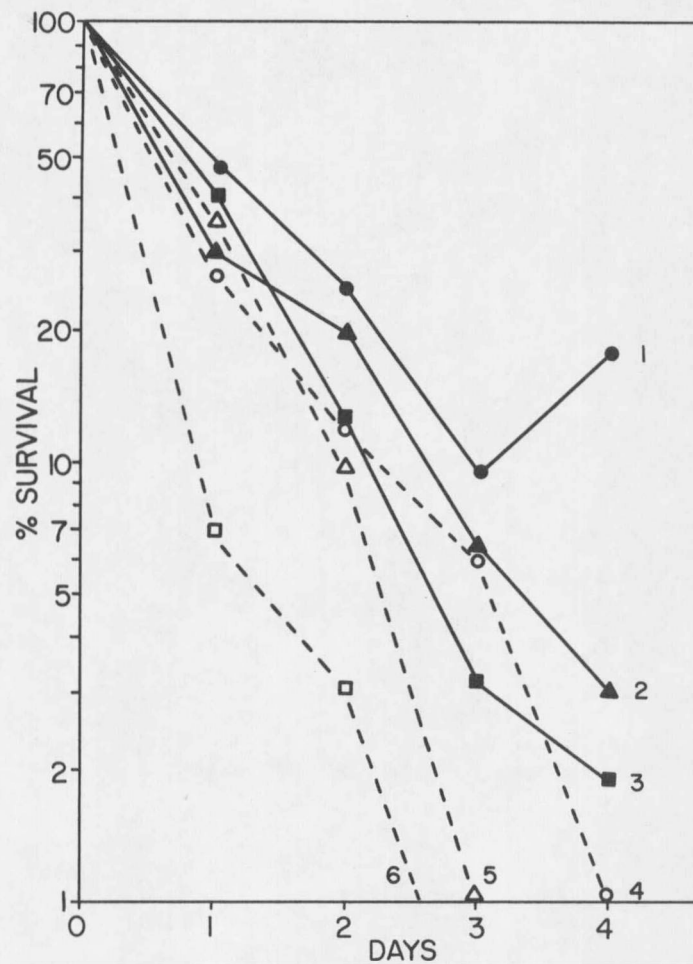


Figure 9. Comparative survival of *K. pneumoniae* 1881 (1, ●), *E. aerogenes* EC2072 (2, ▲), *E. coli* C320MP25 (3, ■), *S. faecium* BS895 (4, ○), *S. faecalis* RS1009 (5, △), and *S. bovis* C3SE17 (6, □) in membrane filter chambers during a 4 day exposure period located at site EG6. Calculations of survival were determined by counts obtained on TSY pour plates.

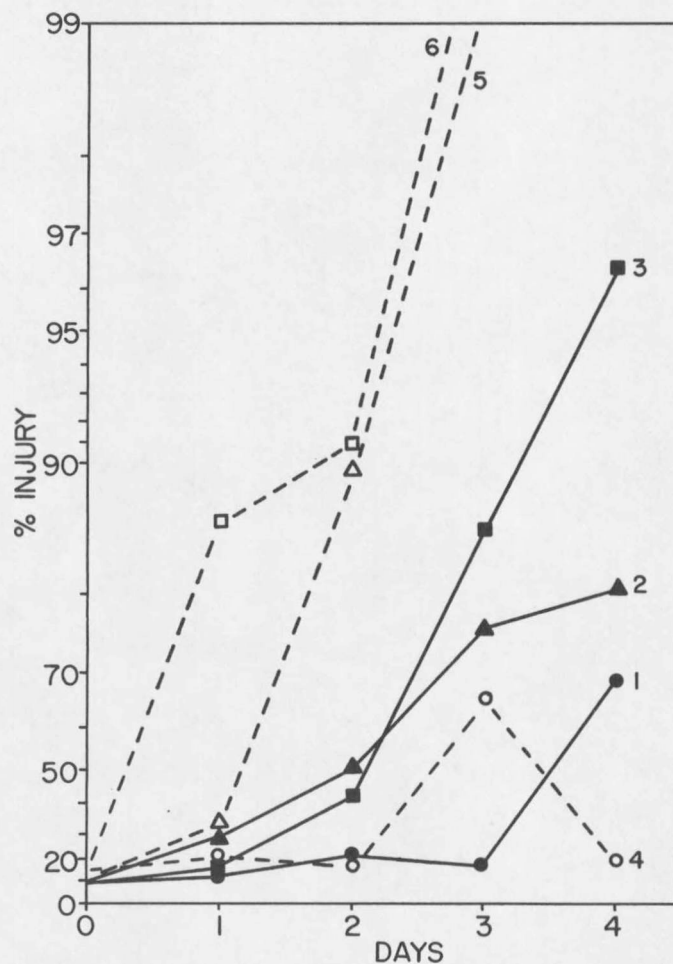


Figure 10. Comparative injury of *K. pneumoniae* 1881 (1, ●), *E. aerogenes* EC2072 (2, ▲), *E. coli* C320MP25 (3, ■), *S. faecium* BS895 (4, ○), *S. faecalis* RS1009 (5, △), and *S. bovis* C3SE17 (6, □) in membrane filter chambers over a 4 day exposure period located at site EG6. Calculations of injury to survivors were determined by differences between counts obtained on TSY and DLA pour plates for the coliforms and TSY and KF pour plates for the fecal streptococci.

4 days exposure. About 78% of the surviving population of *E. aerogenes* was not detectable on the selective medium after 4 days. *K. pneumoniae* remained extremely resistant to damage over the first three days whereafter an increase in injury to approximately 65% resulted on the fourth day. *S. faecium* was also found to be extremely resistant to injury as evidenced by about 15% loss in recovery on selective KF agar after 4 days of exposure to the environment of site EG6.

Comparison of Recovery Efficiency of Various Enumeration Methods

Several experiments were so designed as to allow comparison of recovery efficiency of various methods used to enumerate bacteria from water. A chamber, containing a suspension of *E. coli* C320MP25 at an original concentration of approximately 10^6 cells/ml, was immersed in the stream water at site EG6 (Figure 3). Periodically, samples were withdrawn and analyzed simultaneously utilizing MPN, plating, and membrane filtration methods. TSY, lactose, and BGB broths were used for MPN determinations. Both surface-overlay and pour plate techniques were conducted using TSY, DLA, and VRB agars. Enumeration by membrane filtration methods was done using TSY, M-Endo MF, and M-FC media.

The counts obtained on the rich TSY medium by the respective procedures are shown in Figure 11. The graph shows that counts

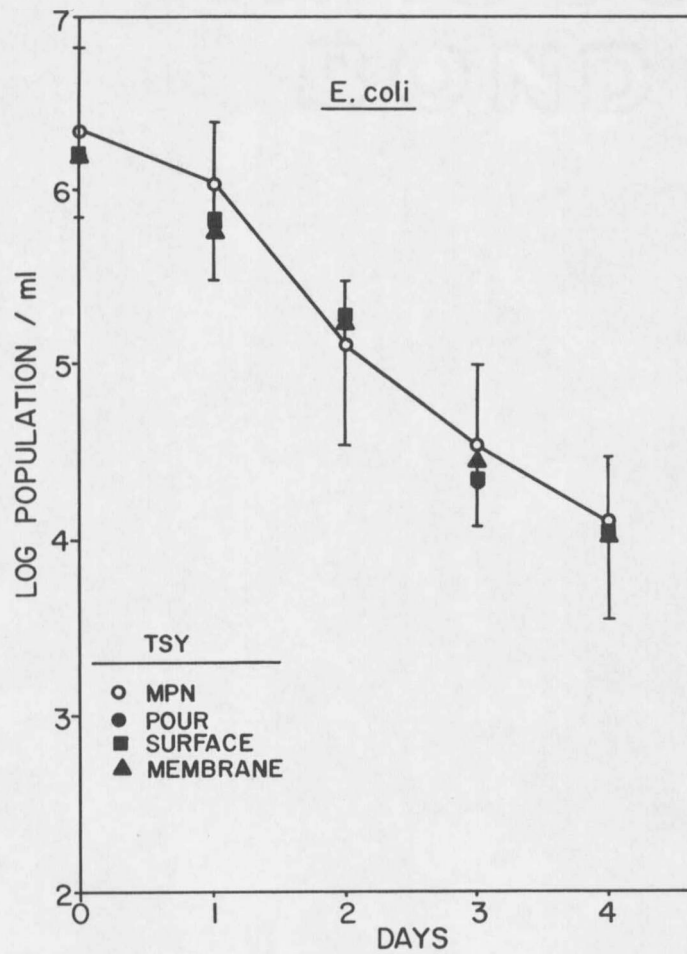


Figure 11. Comparative recovery of *E. coli* C320MP25 in a membrane filter chamber located at site EG6 on TSY medium with MPN, pour plate, surface-overlay plate, and membrane filtration procedures over a 4 day exposure period. Brackets indicate 95% confidence limits for the MPN estimate.

obtained by the different methods on TSY medium fall within the 95% confidence intervals of the MPN estimate; therefore, arithmetic averages of the counts on TSY medium by the different methods were calculated and are plotted in Figure 12 over the 4 day exposure period. Figure 12 also includes profiles of the counts obtained with the selective media by the various procedures (VRB counts not depicted in Figure 12). The graph illustrates the superior recovery efficiency of the selective liquid media over both plating and membrane filtration methods. The least efficient method of enumeration was by membrane filtration procedures.

A summarization of the recovery data is presented in Table 9. Lactose broth MPN estimates were found to give the best recovery efficiency in comparison to counts from rich TSY medium. Membrane filtration using M-FC medium in conjunction with elevated temperature incubation was found to be the least effective in terms of recovering the viable population as evidenced by only 0.3% of the cells being detected after 2 days exposure by this method. Use of M-Endo MF medium was found to be slightly more efficient than M-FC medium. Overall, plating procedures gave better recovery than membrane filtration; however, surface-overlay techniques did not appear to be superior to conventional pour plating. Differences between recovery using DLA or VRB agar were minimal. Table 9 also indicates that the greatest

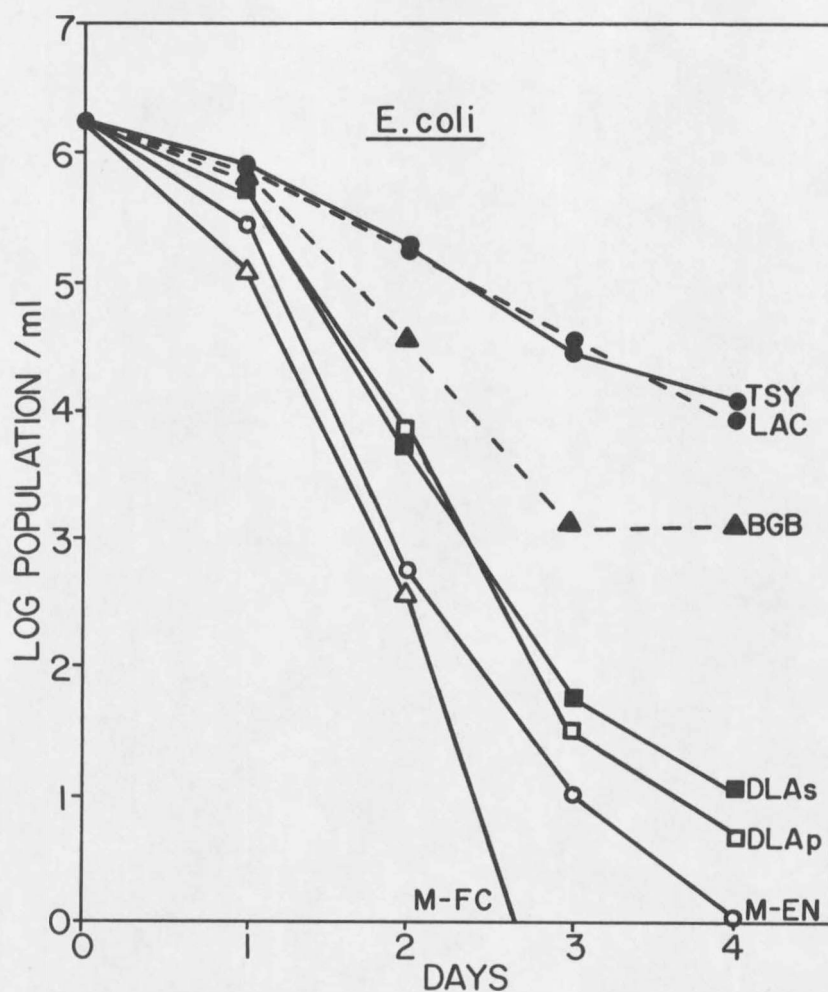


Figure 12. Comparative recovery of *E. coli* C320MP25 in a membrane filter chamber located at site EG6 with LAC MPN (●), BGB MPN (▲), DLA surface-overlay plate (■), DLA pour plate (□), M-Endo MF membrane filtration (○), and M-FC membrane filtration (Δ) procedures during a 4 day exposure period. TSY counts (●) represent average counts on TSY medium when using MPN, pour plate, surface-overlay plate, and membrane filtration procedures.

Table 9. Recovery of *E. coli* C320MP25 suspended in a membrane filter chamber at site EG6 with selective and non-selective media by different enumeration methods over a 4 day exposure period

Exposure time (days)	Percent ^a cells detected by								
	Ave. ^b	MPN		Pour plate		Surface overlay		Membrane filtration	
	TSY	LAC	BGB	DLA	VRB	DLA	VRB	M-Endo	M-FC
0	100.0	122.2	94.4	83.3	83.3	88.9	88.9	94.4	77.8
1	100.0	105.3	105.3	81.3	73.3	78.7	73.3	36.0	18.7
2	100.0	94.4	18.3	3.8	1.6	2.5	0.6	0.4	0.3
3	100.0	129.6	4.8	0.1	<0.1	0.2	0.1	<0.1	0.0
4	100.0	65.8	10.8	<0.1	0.0	0.1	0.1	0.0	0.0

a = As a percentage of the average of counts obtained by MPN, pour plate, surface-overlay plate, and membrane filtration using TSY medium

b = Average counts on TSY medium when using MPN, pour plate, surface-overlay plate, and membrane filtration

increase in non-lethal injury on most selective media occurred between the first and second day of exposure to the aquatic environment as evidenced by a large loss in recovery efficiency upon enumeration after two days of exposure.

A similar experiment to examine recovery efficiencies of various enumeration methods was conducted using *S. faecalis* RS1009. A chamber, containing a suspension of cells at approximately 10^6 cells/ml, was immersed in the stream water at site EG6 (Figure 3) and examined daily over an exposure period of 4 days. Liquid media used in the MPN method were TSY, AD, EVA, and PSE broths. Enumeration by pour plate methods was done using TSY, KF, M-Enterococcus, and PSE agars. Determinations based on membrane filtration methods were performed using TSY, KF, M-Enterococcus, and PSE agars.

The counts observed on the rich TSY medium by the respective procedures are shown in Figure 13. Counts obtained with TSY medium by pour plate and membrane filtration methods were found to be included within the 95% confidence intervals for the MPN estimates. Therefore, as with the case of *E. coli*, arithmetic averages of the counts on TSY medium by the different methods were utilized for comparison to recovery efficiencies on selective media (Table 10). Examination of Table 10 reveals that the greatest amount of non-lethal injury was observed with PSE medium. Substantial loss in recovery

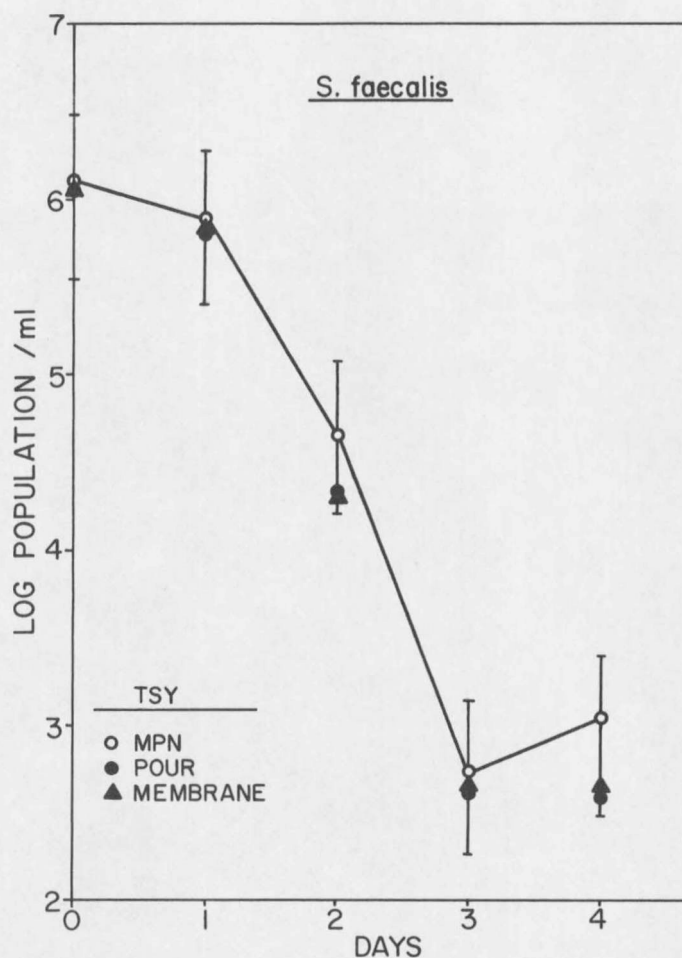


Figure 13. Comparative recovery of *S. faecalis* RS1009 in a membrane filter chamber located at site EG6 on TSY medium with MPN, pour plate, and membrane filtration procedures during a 4 day exposure period. Brackets indicate 95% confidence limits for the MPN estimate.

Table 10. Recovery of *S. faecalis* RS1009 suspended in a membrane filter chamber at site EG6 with selective and non-selective media by different enumeration methods over a 4 day exposure period

Exposure time (days)	Percent ^a cells detected by									
	Ave. ^b		MPN		Pour plate			Membrane filtration		
	TSY	AD	EVA	PSE	KF	ME	PSE	KF	ME	PSE
0	100.0	78.3	78.3	108.3	91.7	91.7	91.7	91.7	83.3	83.3
1	100.0	64.8	98.6	46.5	71.8	88.7	19.7	66.2	95.8	38.0
2	100.0	7.6	11.4	1.7	3.8	51.7	0.3	1.7	55.2	1.0
3	100.0	14.9	13.4	5.5	12.8	44.7	2.1	14.9	31.9	8.5
4	100.0	33.8	41.5	2.6	7.7	40.0	0.8	11.5	29.2	0.8

a = As a percentage of the average of counts obtained by MPN, pour plate, and membrane filtration using TSY medium

b = Average counts on TSY medium when using MPN, pour plate, and membrane filtration

efficiency was observed when pour plate, membrane filtration, as well as MPN methods were used in conjunction with PSE medium. The maximal amount of recovery was found with M-Enterococcus medium in the pour plate and membrane filtration techniques. The recovery of *S. faecalis* cells on KF medium was not as efficient as on M-Enterococcus medium, yet substantially superior to PSE medium. The recovery efficiencies of AD and EVA broths using MPN methods were found to be essentially equal; however, both of these were substantially more efficient than PSE broth. Comparison of MPN, pour plate, and membrane filtration methods as a whole (excluding M-Enterococcus medium from consideration), reveals that MPN methods gave slightly better recovery efficiency than either pour plate or membrane filtration procedures during the later 3 days of exposure. Recovery efficiencies with pour plate and membrane filtration techniques were observed to be essentially similar.

Comparison of recovery efficiencies of various enumeration methods were also conducted with natural suspensions of raw sewage. In one such experiment, a membrane filter chamber containing a suspension of raw sewage was immersed in stream water at site EG6 (Figure 3). Samples were withdrawn from the chamber on a daily basis over a 4 day exposure period. Coliforms were enumerated using DLA and VRB agar in both the pour plate and surface-overlay techniques,

as well as by membrane filtration procedures using M-Endo MF and M-FC media. Confirmation of probable coliform organisms was accomplished by picking colonies into BGB broth. At the same time samples were analyzed for coliforms, enumeration of fecal streptococci was performed using both plating and membrane filtration techniques. KF and M-Enterococcus agars were used with pour plate techniques. These same two media were also used with the membrane filtration procedure. Probable fecal streptococcal colonies were picked into EVA broth for confirmation.

Data obtained for raw sewage over the 4 day exposure period with membrane filtration methods are shown in Figure 14. The rate of die-away for the total coliform population was found to be fairly constant, beginning with an original concentration of approximately 10^5 coliforms/ml and ending with about 3.0×10^3 coliforms/ml after 4 days. The fecal coliform portion of the total coliform population was observed to die-away at a very rapid rate, beginning with over 10^4 fecal coliforms/ml and ending with only 10^1 fecal coliforms/ml after 4 days. As a group, the fecal streptococci were more resistant to die-off than the fecal coliforms. Evidence that the fecal streptococci were more persistent than fecal coliforms can be seen from the profile of the FC/FS ratio. The original FC/FS ratio was 3.9 and rapidly dropped to 1.2 after 1 day and eventually to approximately 0.1 after 4 days. The decreasing ratio over the 4 day exposure period

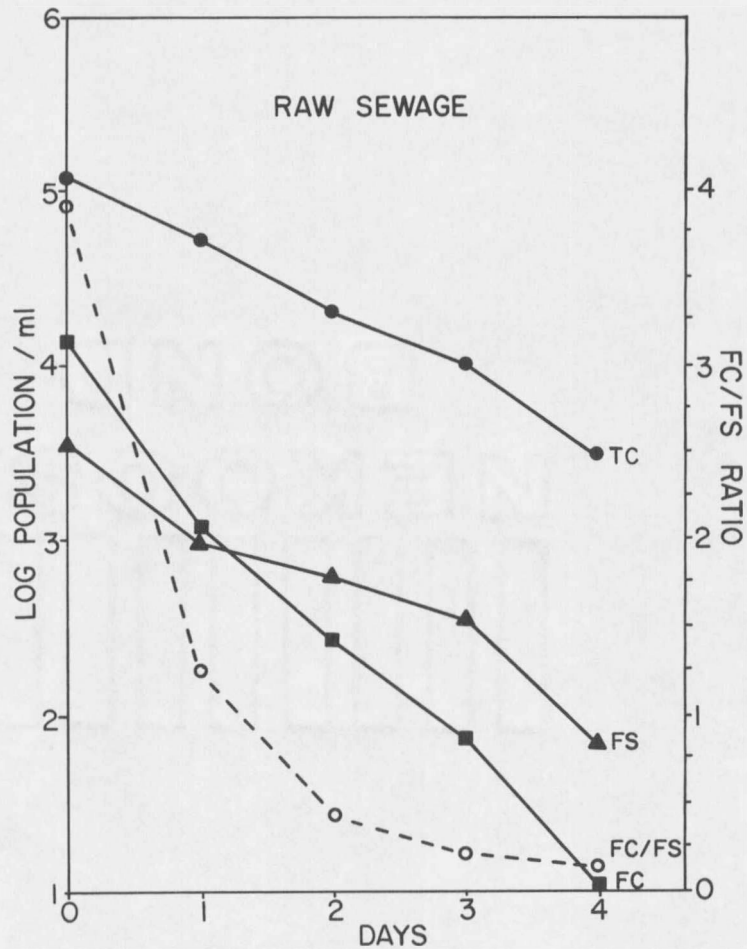


Figure 14. Recovery of total coliforms (TC, ●), fecal coliforms (FC, ■), and fecal streptococci (FS, ▲) by membrane filtration methods from a suspension of raw sewage in a membrane filter chamber located at site EG6 during a 4 day exposure period. The FC/FS profile (O - - O) represents the ratio of fecal coliforms to fecal streptococci in the raw sewage at each specific sampling time.

indicates that the fecal coliform group was more susceptible to death in the water environment than that of the fecal streptococcal group, at least within the limitations of the recovery efficiencies of the procedures used.

A comparison of the recovery efficiencies of the various methods of enumerating coliforms and fecal streptococci from raw sewage is presented in Table 11. The efficiency of coliform recovery by the various methods is compared to recovery on DLA surface-overlay plates. Pour plate procedures were not as efficient as surface-overlay techniques, DLA agar gave slightly superior recovery than did VRB agar with either pour plate or surface-overlay methods. M-Endo MF medium with membrane filtration was found to be the least efficient as a recovery method, especially during the last 2 days of exposure to the aquatic environment. Comparison of recovery efficiency of fecal streptococci reveals somewhat higher recovery using pour plate methods as opposed to membrane filtration techniques. Negligible differences in recovery efficiency between KF and M-Enterococcus media are also evident in Table 11.

A similar experiment was conducted to compare the recovery efficiency of coliforms by various methods from a natural suspension of raw sewage in a membrane filter chamber immersed in stream water at site EG6 (Figure 3). Samples were withdrawn from the chamber on a

Table 11. Recovery of coliforms and fecal streptococci from a suspension of raw sewage suspended in a membrane filter chamber at site EG6 with selective media by different enumeration methods over a 4 day exposure period

Exposure time (days)	Coliforms ^a					Fecal Streptococci ^b				
	Percent ^c cells detected by					Percent ^d cells detected by				
	Surface- overlay		Pour		Memb. filt.	Pour		Memb. filt.		
	DLA	VRB	DLA	VRB	M-Endo	KF	M-E	KF	M-E	
0	100.0	100.0	92.9	92.9	85.7	100.0	102.0	72.0	70.0	
1	100.0	96.0	78.7	72.0	72.0	100.0	109.0	89.1	65.5	
2	100.0	88.0	96.0	76.0	84.0	100.0	100.0	90.1	71.8	
3	100.0	77.8	94.4	72.2	55.6	100.0	100.0	92.3	82.1	
4	100.0	68.8	68.8	43.1	19.4	100.0	88.9	82.4	82.4	

a = Counts based on confirmation in BGB broth

b = Counts based on confirmation in EVA broth

c = As a percentage of counts on DLA surface-overlay plates

d = As a percentage of counts on KF pour plates

daily basis over a 2 day exposure period. Enumeration of total coliforms was accomplished using lactose MPN, surface-overlay plating with DLA agar, and membrane filtration with M-Endo MF medium. BGB broth was used to confirm probable coliform organisms. Also, transfers from positive BGB broth tubes were made into EC medium in order to develop probable fecal coliform densities. The direct isolation of fecal coliforms was done by using M-FC membrane filtration procedures at the elevated temperature incubation. Probable fecal coliform colonies on M-FC media were also confirmed in BGB and EC media.

A comparison of the recovery efficiencies of the various methods of enumerating coliforms and fecal coliforms from raw sewage is presented in Table 12. The recovery efficiency of coliforms by surface-overlay plating and MPN techniques were found to be quite similar. However, membrane filtration was found to be the least efficient in detecting coliforms as evidenced by the detection of only 61% of the coliforms by membrane filtration in comparison to detection by surface-overlay procedures at 0-h. After 2 days exposure, only 34% of the coliforms were recovered by M-Endo MF membrane filtration as compared to DLA agar surface-overlay plating.

Detection of fecal coliforms from raw sewage was also found to be least efficient when using membrane filtration methods (Table 12). The use of direct isolation of fecal coliforms by elevated

Table 12. Recovery of coliforms and fecal coliforms from a suspension of raw sewage suspended in a membrane filter chamber at site EG6 with selective media by different enumeration methods over a 2 day exposure period.

Exposure time (days)	Coliforms ^a			Fecal coliforms ^b			
	Surface overlay		Membrane filtration		Surface overlay		Membrane filtration
	MPN		Percent ^c cells detected by		MPN		MPN
	DLA	LAC	M-Endo	DLA	LAC	M-Endo	M-FC
0	100.0	92.9	60.7	100.0	108.3	65.8	50.0
1	100.0	75.7	64.9	100.0	73.7	52.6	28.9
2	100.0	106.3	34.4	100.0	113.8	21.0	10.3

a = Counts based on confirmation in BGB broth

b = Counts based on confirmation in BGB broth and EC medium

c = As a percentage of counts on DLA surface-overlay plates

temperature methods was found to be the least efficient recovery method, ranging from 50% recovery at 0-h to 10% recovery after 2 days exposure when compared to surface-overlay techniques. Membrane filtration methods using M-Endo MF medium followed by identification of fecal coliforms with EC medium was observed to improve recoverability of fecal coliforms to a slight extent. Both surface-overlay plating and MPN procedures were found to give superior recovery of fecal coliforms than by membrane filtration methods.

Repair Experiments

Experiments were conducted to determine whether cells injured by environmental stress in natural waters had the capability to repair themselves when exposed to a suitable environment. A total of three repair experiments were conducted: *E. coli* C320MP25 was utilized for a 2 day exposure period, *E. coli* WC959 was utilized for a 7 day exposure period, and *S. faecalis* RS1009 was utilized for a 2 and 4 day exposure period. The location for all these experiments was at site EG6 (Figure 3). The methods used in these experiments have been previously described in the Materials and Methods section.

The data observed with *E. coli* C320MP25 using non-selective TSY agar and selective DLA agar are plotted in Figure 15 as a function of time of exposure in rich TSY broth. The control or 0-h curves, using both media, represent growth curves for a population of cells which

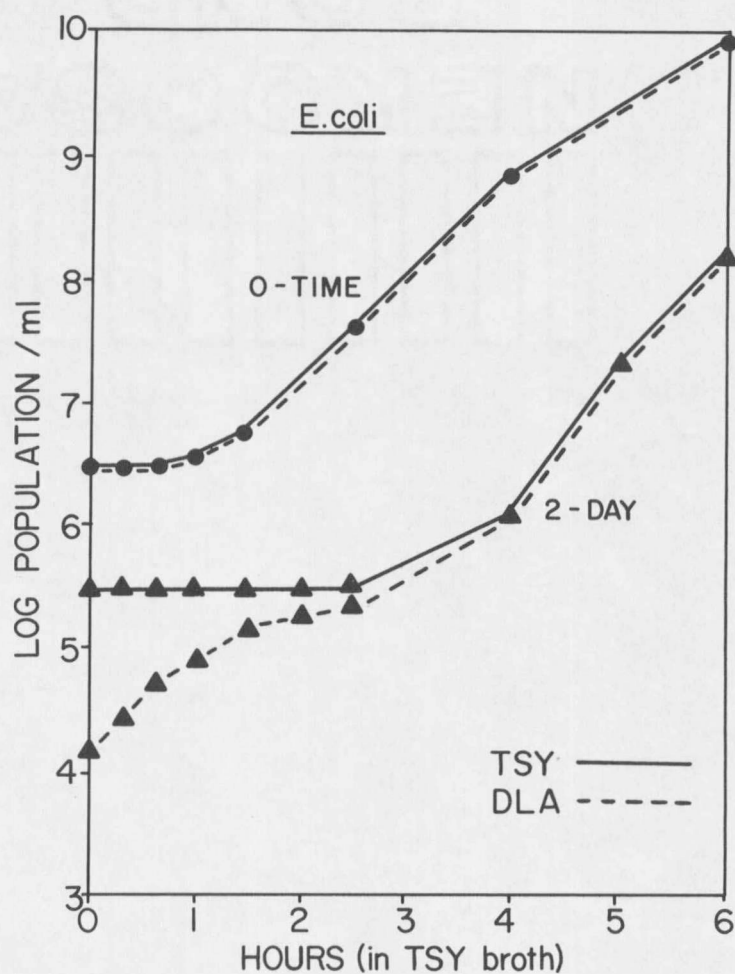


Figure 15. Repair of injury in TSY broth for *E. coli* C320MP25 cells having been exposed to the stream environment of site EG6 for 2 days. Control or 0-h cells (●) and 2 day exposed cells (▲) were enumerated over a 6 h growth period in TSY broth using TSY (——) and DLA (- - - -) surface-overlay plates.

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 using either the non-selective 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 recovery 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 produce colonies on the selective DLA agar. After $2\frac{1}{2}$ - 3 h of exposure to the TSY broth, counts obtained with both the non-selective and selective media were approximately equal, indicating that the injured proportion of the population had rapidly repaired and recovered from injury in the rich TSY medium. During the $2\frac{1}{2}$ - 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. Subsequent to this $2\frac{1}{2}$ - 3 h repair period, multiplication was initiated as evidenced by concomitant increases in counts on both the TSY and DLA agar.

Membrane filtration methods were used for enumeration of *E. coli* WC959 in a second repair experiment at site EG6 (Figure 3). As in the previous experiment, it was observed that relatively equal counts for the control population were obtained with the non-selective medium and selective media over the 8 h growth period in TSY broth (Figure 16). Also, a similar 1 h lag period prior to beginning the logarithmic phase of multiplication was observed for the control population in this experiment. A large proportion of cells were found to be injured as a result of 7 days exposure to the aquatic environment as evidenced by the loss in recovery on the selective medium as compared to TSY medium. It should be noted that during the third to fifth day of exposure to the water, the entire cell suspension became frozen with the membrane filter chamber due to extremely cold weather conditions. Water temperatures rose above freezing during the sixth and seventh days of exposure such that a sample was obtained on the seventh day. As the injured population of cells was exposed to the rich TSY broth over the 8 h time period, it was found that increasing proportions of the injured cells were able to rapidly repair the non-lethal injury such that they could produce colonies on the selective M-Endo MF and M-FC media. After approximately 4 h of exposure to the TSY broth, counts obtained with the non-selective medium and two selective media were essentially equal, indicating complete repair

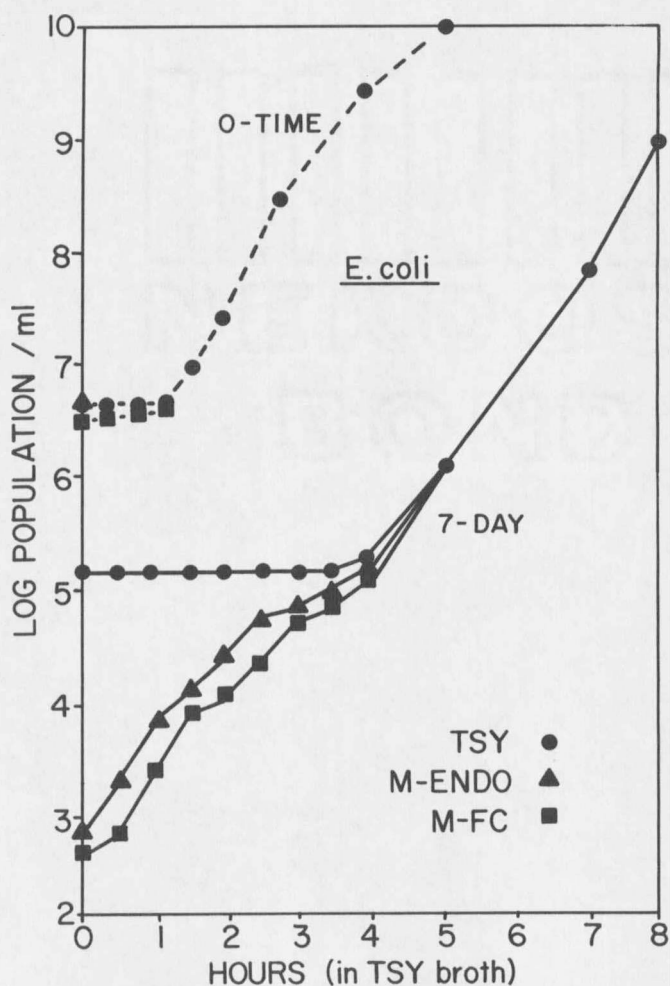


Figure 16. Repair of injury in TSY broth for *E. coli* WC959 cells having been exposed to the stream environment of site EG6 for 7 days. Control or 0-h cells (---) and 7 day exposed cells (—) were enumerated over an 8 h growth period in TSY broth using TSY (●), M-Endo MF (▲), and M-FC (■) media with membrane filtration procedures.

and recovery of the injured population. An extended lag period of 4 h, as evidenced by uniformity of counts on non-selective TSY medium, was observed for the population of stressed cells as opposed to the 1 h lag period for the control population. A simultaneous increase in counts on the non-selective medium and the two selective media after approximately 4 h in the TSY broth indicated that cell multiplication had begun.

A third repair experiment was conducted using a fecal streptococcal species, *S. faecalis* RS1009. The membrane filter chamber was again located at site EG6. Enumeration was by membrane filtration methods after 2 days and 4 days exposure to the natural water as well as an enumeration of the control population, which was not exposed to the aquatic environment. As previously observed with the two strains of *E. coli*, relatively equal counts for the control population of *S. faecalis* were obtained with the non-selective TSY medium and the selective KF medium over the growth period in TSY broth (Figure 17). Similarly, a 1 h lag phase prior to logarithmic growth was observed in this experiment for the control population. A small portion of the population of cells was found to be injured after 2 days exposure to the stream environment. This injured population was observed to be capable of rapidly repairing the non-lethal injury in TSY broth such that the population was completely repaired and could produce colonies

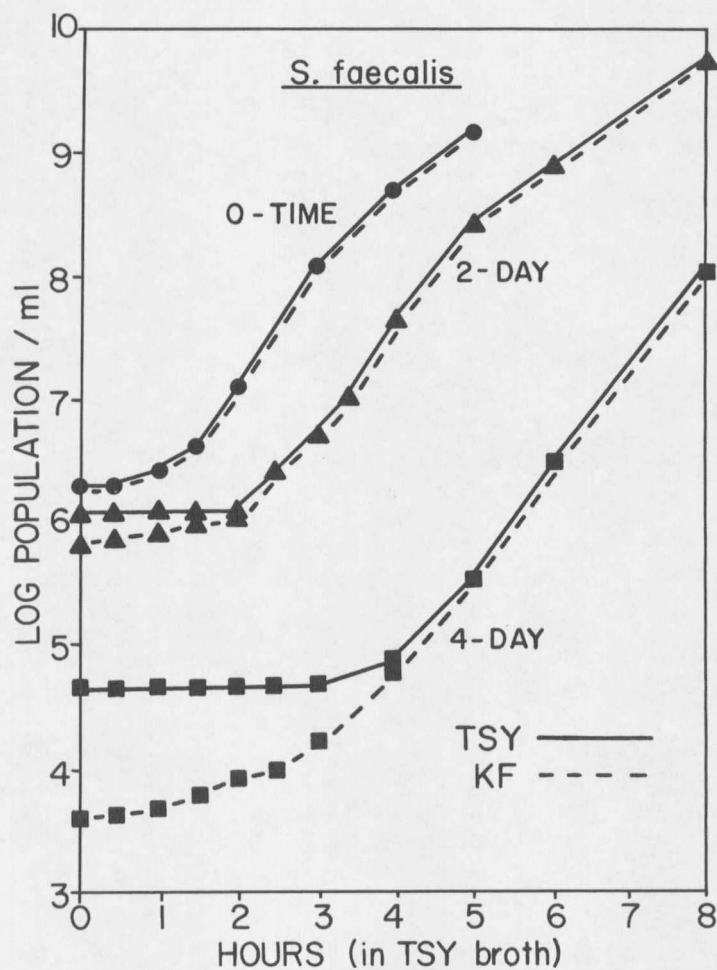


Figure 17. Repair of injury in TSY broth for *S. faecalis* RS1009 cells having been exposed to the stream environment of site EG6 for 2 and 4 days. Control or 0-h cells (●), 2 day exposed cells (▲), and 4 day exposed cells (■) were enumerated over an 8 h growth period in TSY broth using TSY (—) and KF (- - -) media with membrane filtration procedures.

on selective KF medium after 2 h in TSY broth. A larger proportion of the cells were found to be injured after 4 days exposure in the stream environment, resulting in a longer time commitment of 3½ to 4 h in order to complete the repair of the injured population in the TSY broth. Additionally, a longer lag phase of 3½ to 4 h prior to multiplication was observed for those cells exposed for 4 days as compared to a 2 h lag phase for those cells exposed for 2 days to the stresses of the aquatic environment.

Enrichment Experiments

An enrichment procedure was compared to the conventional membrane filtration direct procedure for the recovery of a suspension of *E. coli* C320MP25 exposed to the stream environment. The membrane filter chamber was located at site EG4 (Figure 3) and examined daily over an exposure period of 3 days. The specific procedures have been previously described. The data (Figure 18) indicated that a short enrichment period of 2 h on rich, non-selective TSY medium prior to exposure to specific selective media substantially enhanced the recovery of the total viable population relative to recovery by direct primary exposure to selective media.

Table 13 presents a comparison of the recovery efficiencies of direct and enrichment procedures as a function of the enumeration with TSY medium. The application of direct primary exposure to the

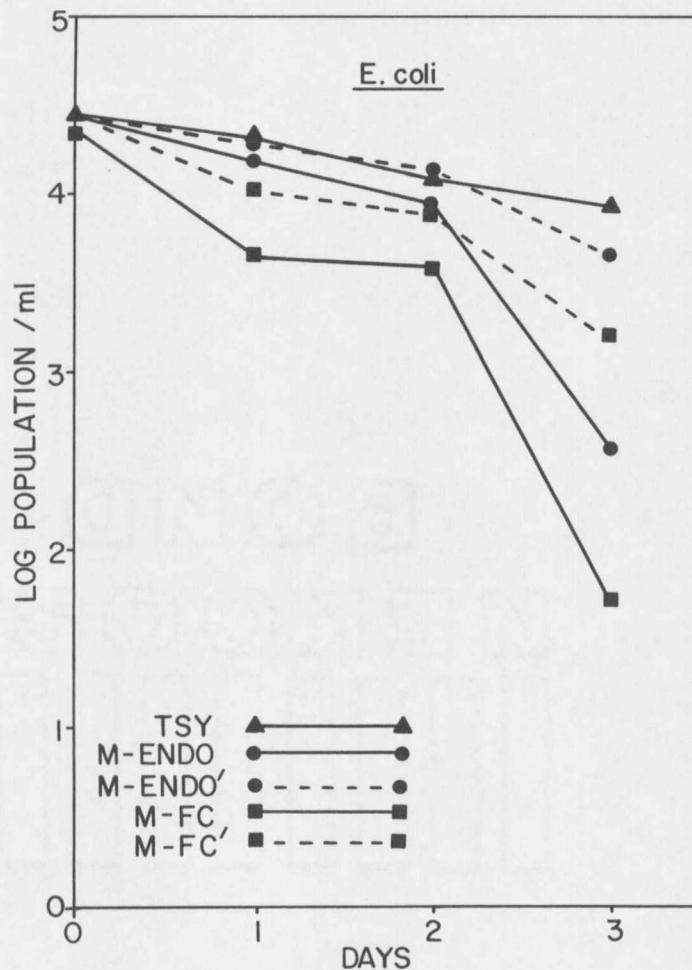


Figure 18. Comparison of direct and enrichment membrane filtration techniques for the recovery of a suspension of *E. coli* C320MP25 located at site EG6 over a 3 day exposure period. Direct techniques (—) consisted of the direct transfer of membrane filters to TSY (▲), M-Endo MF (●), and M-FC (■) media. Enrichment techniques (---) consisted of 2 h incubation on TSY medium prior to transfer of filters to M-Endo MF (●) and M-FC (■) media.

Table 13. Comparison of direct and enrichment membrane filtration techniques on the recovery of *E. coli* C320MP25 suspended in a membrane filter chamber at site EG4 over a 3 day exposure period

Exposure time (days)	Percent ^a cells detected by				
	M-Endo MF			M-FC	
	TSY	Direct ^b	Enrichment ^c	Direct ^b	Enrichment ^c
0	100.0	103.8	115.4	88.5	115.4
1	100.0	75.0	105.0	22.5	50.0
2	100.0	71.5	100.0	31.5	68.5
3	100.0	4.6	50.6	0.6	16.9

a = As a percentage of counts on TSY membrane filter plates

b = Direct transfer of membrane filter to respective selective media

c = A 2 h enrichment on TSY medium prior to transfer to respective selective media

elevated temperature of 44.5 C with M-FC medium was found to severely limit the recoverability of *E. coli* as evidenced by the fact that the recovery of the total viable population was approximately 23% after 1 day, 32% after 2 days, and less than 1% after 3 days exposure to the stream environment. Enrichment of membrane filters on TSY medium for 2 h prior to transfer to M-FC medium incubated at the elevated temperature was found to more than double the recovery of *E. coli* for all exposure periods, indicating that a proportion of the injured cells were capable of repair during the 2 h enrichment period.

The direct use of M-Endo MF medium was also found to inhibit the recovery of *E. coli* as compared to TSY medium, yet primary exposure to M-Endo MF medium was found to be less harmful than direct use of M-FC medium (Table 13). An enrichment period of 2 h prior to transfer to M-Endo MF medium was observed to substantially improve recovery of stressed cells of *E. coli*. Sufficient repair of the injured cells occurred during the enrichment period such that essentially equal recoverability was observed on M-Endo MF medium as compared to TSY medium during the first 2 days of exposure to the aquatic environment. Approximately 51% of the population was recovered by enrichment - M-Endo MF procedures after 3 days of exposure. For all exposure periods, enrichment on TSY medium followed by transfer to M-Endo MF medium was found to be the most efficient selective method

of recovering the viable population of *E. coli*, when compared to counts obtained on the rich, non-selective TSY medium.

Recovery efficiencies of total coliforms and fecal coliforms were also examined using a natural suspension of raw sewage in a membrane filter chamber immersed at site EG4. Comparison of the recovery of total coliforms using direct and enrichment techniques with membrane filtration procedures is shown in Figure 19. Over the 3 day exposure period it was found that improved recovery efficiency could be attained by using a 2 h enrichment on TSY medium prior to exposure to the selective M-Endo MF medium as opposed to direct application of selective M-Endo MF medium. The effectiveness of the enrichment step became more evident during the latter 2 days of the experiment.

Recovery efficiency of direct and enrichment methods for fecal coliforms from raw sewage is depicted in Figure 20. A 2 h enrichment on TSY medium prior to application of selective M-Endo MF medium was found to substantially enhance the recovery of fecal coliforms over that recovered by direct enumeration on M-Endo MF medium. Similarly, enrichment before transfer of filters to M-FC medium at the elevated incubation temperature resulted in improved recoverability of fecal coliforms in comparison to recovery efficiency using direct exposure to selective M-FC medium at 44.5 C. Throughout the entire exposure period of the raw sewage to the stressful environment of the stream

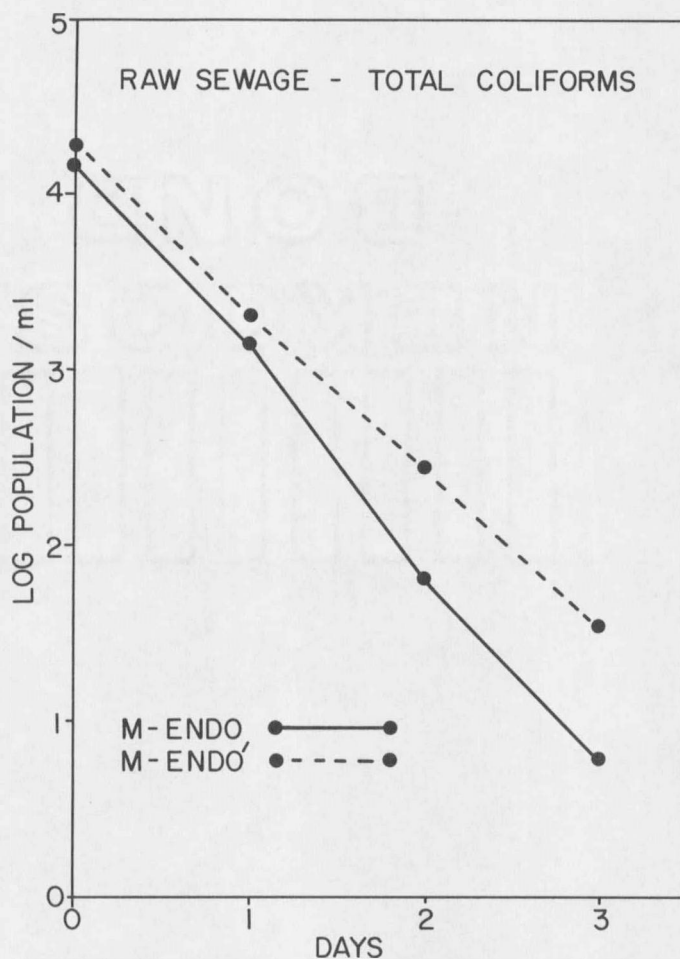


Figure 19. Comparison of direct and enrichment membrane filtration techniques for the recovery of total coliforms from a suspension of raw sewage located at site EG4 over a 3 day exposure period. Direct techniques consisted of the direct transfer of membrane filters to M-Endo MF (●—●) medium. Enrichment techniques consisted of 2 h incubation on TSY medium prior to transfer of filters to M-Endo MF (● - - ●) medium.

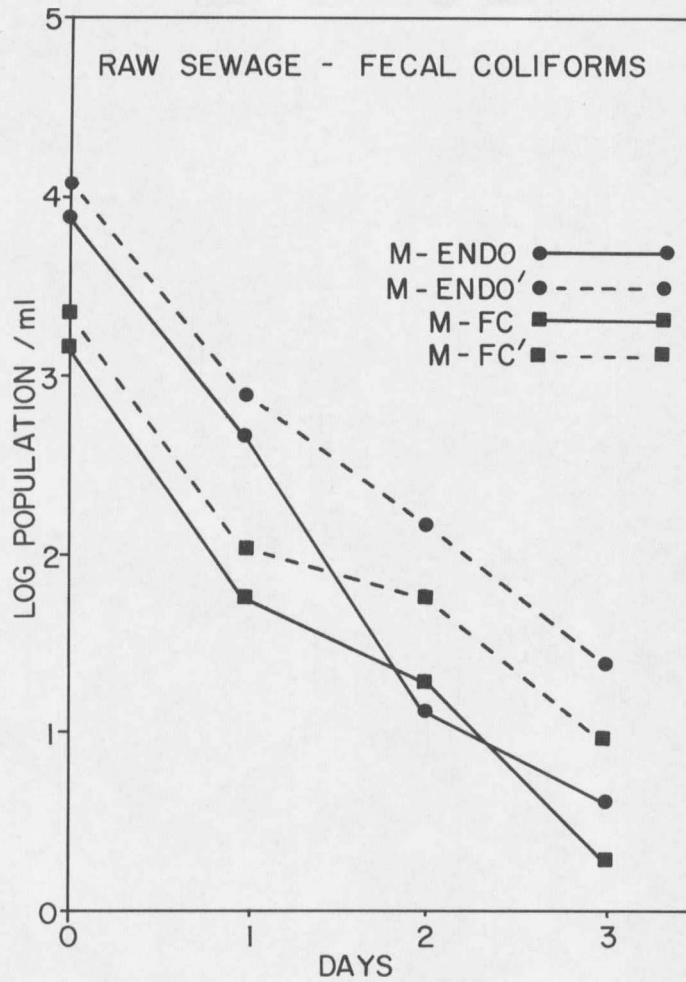


Figure 20. Comparison of direct and enrichment membrane filtration techniques for the recovery of fecal coliforms from a suspension of raw sewage located at site EG4 over a 3 day exposure period. Direct techniques (—) consisted of the direct transfer of membrane filters to M-Endo MF (●) and M-FC (■) media. Enrichment techniques (---) consisted of 2 h incubation on TSY medium prior to transfer of filters to M-Endo MF (●) and M-FC (■) media.

water it was observed that the most efficient method for the recovery of fecal coliforms was by a short 2 h enrichment on rich TSY medium followed by transfer to selective M-Endo MF medium. Direct enumeration by primary application of either M-Endo MF or M-FC media were found to be the most inhibitory toward recovery of coliforms of fecal origin, especially during the last 2 days of exposure to the water environment. The least efficient method of recovery of fecal coliforms from raw sewage was observed when using direct application of M-FC medium in conjunction with the 44.5 C elevated incubation temperature.

Chemical and Physical Results

Chemical and physical analyses of water samples collected from locations for membrane filter chambers were done periodically throughout the course of the study. Concentrations of various constituents for the ten site locations during a single sampling period is shown in Table 14. Variation in water quality characteristics in terms of anion, cation, and nutrient concentrations among the various sites is readily evident. Sites located below the outfall of the Bozeman Wastewater Treatment Plant (EG5 and EG6) reflect the contribution of nutrients (phosphate, nitrate, ammonia, and nitrite) from the domestic sewage as well as the contribution from agricultural practices in the proximity of the river. Lower concentrations of anions, cations, and

Table 14. Chemical and physical parameters for the 10 site locations (all parameters were measured from samples taken on 9/11/73 unless otherwise specified)

Determination	Site Location								
	M3	M3A	H3	BR2	R1	EG4	EG5	EG6	Well ^a
Water temperature (C)	5.5	7.5	6.5	9.5	8.5	9.5	10.0	10.5	9.5
pH	7.98	7.87	7.70	7.99	8.22	8.01	7.92	7.95	7.55
Specific Conductance (micromhos @ 25C)	219	328	122	398	425	378	405	391	-- ^b
Total hardness (mg/l CaCO ₃)	103.6	163.6	53.6	192.2	211.0	189.8	195.8	190.8	201.0
Calcium (mg/l)	29.8	46.5	16.0	55.8	57.7	53.0	55.3	58.1	55.8
Magnesium (mg/l)	7.1	11.5	3.3	12.8	16.2	13.9	14.0	11.1	14.9
Sodium (mg/l)	3.0	4.1	2.4	10.8	7.6	7.8	9.4	10.6	9.9
Potassium (mg/l)	1.3	2.0	1.1	0.8	1.9	1.8	2.1	2.4	3.6
Total alkalinity (mg/l CaCO ₃)	101.5	164.5	55.5	192.0	191.0	185.0	189.5	181.5	207.5
Sulfate (mg/l)	10.8	8.0	5.8	19.2	36.3	20.6	21.0	21.8	13.2
Chloride (mg/l)	2.0	0.5	0.4	1.6	1.9	2.1	4.2	4.1	4.0

a = Determined on 4/28/73

b = Not determined

Table 14 (continued).

Determination	Site Location								Well ^a
	M3	M3A	H3	BR2	R1	EG4	EG5	EG6	
Total phosphate (mg/l PO ₄ -P)	0.047	0.043	0.058	0.018	0.028	0.034	0.180	0.400	--- ^b
Orthophosphate (mg/l PO ₄ -P)	0.039	0.033	0.051	0.010	0.017	0.024	0.150	0.250	0.070
Nitrate (mg/l NO ₃ -N)	0.019	0.000	0.037	0.077	0.057	0.200	0.330	0.470	0.400
Nitrite (mg/l NO ₂ -N)	0.000	0.000	0.001	0.002	0.003	0.006	0.003	0.008	0.000
Ammonia (mg/l NH ₃ -N)	0.006	0.006	0.005	0.021	0.017	0.010	0.130	0.220	0.160
Total carbon (mg/l)	23	37	16	46	46	45	47	45	---
Organic carbon (mg/l)	4	4	5	6	7	5	6	8	---
Inorganic carbon (mg/l)	19	33	11	40	39	40	41	37	---
Residual chlorine (mg/l)	--	--	--	--	--	0.00	0.06	0.00	---

a = Determined on 4/28/73

b = Not determined

nutrients at sites M3, M3A, and H3 reflect a high quality water originating from snowmelt in the adjacent mountains.

Chapter 5

DISCUSSION

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. This new aquatic environment is foreign to enteric bacteria, being much different from the surroundings of the intestine. In the intestinal tract, the bacteria are exposed to a constant temperature of 37 C in contrast to the relatively much colder and fluctuating temperatures of aquatic environments. Additionally, the available nutrients for growth and survival are relatively very minimal in the aquatic system as compared to the very nutrient rich confines of the intestine. In a sense, the microorganisms are presented with starvation conditions in the aquatic environment due to a lesser availability of growth nutrients. In short, the microorganisms leave the favorable environment of the intestine and enter a very new and foreign aquatic habitat.

Extensive documentation of survival characteristics of enteric bacteria has been made by a number of investigators. In general, it has been observed that natural waters are unfavorable as a growth environment, as evidenced by bacterial survival data exhibiting die-away characteristics. Thus, essentially two sub-populations of cells have

been considered in most survival studies. One fraction is those cells which can withstand the foreign aquatic environment as reflected by their detection and enumeration using standard laboratory procedures. The other sub-population is those cells which cannot persist in the unfavorable environment, resulting in death and non-detection.

The present study indicates that careful consideration should be given to the possible existence of a third sub-population of cells. This third population of cells would be that portion of the total population that has become physiologically debilitated, damaged, or injured as a result of exposure to the stresses of the aquatic environment. It should be emphasized that this environmental stress may merely injure the cell but not necessarily kill or destroy it.

With the aid of membrane filter chambers developed by McFeters and Stuart (56), it was possible to follow the survival and recovery characteristics of a population of cells as a function of time of exposure in the aquatic environment. This membrane filter chamber was a system open to the influences of the water environment, since it permitted 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 and physical constituents of natural waters.

Proper consideration must also be given to the fact that certain inherent disadvantages are associated with the use of these membrane filter chambers. For instance, the natural effects of predation by protozoa upon bacterial populations are not reflected by this technique. Also, the bacteria are confined in a localized area of water and are not subject to dispersion by dilution, adsorption, and settling. Consideration must be given to the possibility of cryptic growth within the confines of the membrane filter chamber. Cryptic growth occurs from the leakage and lysis products of dead organisms which may support the growth of survivors. Additionally, the possibility of adhesion of bacteria to the membrane filter side walls or Plexiglass must be considered, since recovery of survivors would be underestimated due to the possible adhesion of cells to the chamber components. Even if substantial numbers of bacteria do adhere to the membrane filter chamber, it was not considered to affect the objectives of this research since detection of injured microorganisms was dependent upon simultaneous enumeration on non-selective and selective media from the same sample withdrawn from the chamber. Unless a differential selectivity of adhesion to the membrane filter chamber components exists for non-injured and injured cells, this disadvantage of using the membrane filter chamber is greatly diminished.

Samples, which were periodically withdrawn from the membrane filter chamber, were simultaneously enumerated with a rich, non-selective medium and with a specific selective medium containing inhibitory agents. The count obtained with the non-selective medium was considered to be the total viable population, i.e., both injured and non-injured cells. The count obtained with the selective medium was considered to be that portion of the total population which was non-injured. The difference in counts obtained on the non-selective and selective media for a given exposure period was defined as that part of the total population which was physiologically debilitated or injured.

Routinely, the detection and enumeration of microorganisms from water involves the direct application of specific selective media. These selective media, containing various inhibitory agents, are utilized to allow for the development of the specific types being sought to the exclusion of a multitude of unwanted types. Thus, the microbiologist is forced to use inhibitory media in order to suppress the growth of those organisms which may interfere with the detection and enumeration of specific desired types. However, the use of selective media often complicates the detection of injured microorganisms. The environmental conditions and/or added inhibitory agents utilized to allow for development of the specific types being sought may, in fact,

exert unsuspected inhibition, especially to those cells subjected to stress. Thus, the combination of environmental stress with subsequent utilization of specific selective media may result in significantly lowered recovery of the population of injured cells.

The data obtained from various experiments using different species of indicator organisms indicate that, in fact, a substantial proportion of a population of cells may become non-lethally injured during exposure to the stresses of the aquatic environment. A suspension of *E. coli* C320MP25 located at site EG5 was found to be very susceptible to both death and non-lethal injury (Figure 5). The rapidly decreasing counts on the non-selective TSY medium indicated that large proportions of the population were dying upon increased exposure time to the water environment. Also, it was observed that substantial amounts of non-lethal injury, as reflected by differences in counts on non-selective and selective media was sustained by *E. coli* C320MP25 cells exposed to the stream environment of site EG5. In general, the amount of injury to survivors tended to become a greater proportion of the total viable population as the exposure period increased.

Profiles of recovery of *E. coli* C320MP25 at site BR2 with both the non-selective and selective media indicate that both overall survival and injury varied according to different sites (Figure 5). At site BR2 the population of *E. coli* C320MP25 was found to be very stable

in terms of recovery on the non-selective TSY medium. Also, non-lethal injury was observed to be minimal to almost non-existent over the 4 day exposure period at site BR2.

The varying influences of aquatic environments with differing chemical and physical characteristics is evident in Table 7 and Figure 6. Among the eight site locations, considerable variation in survival and injury characteristics were observed, especially from the second to fourth days of exposure. In other words, *E. coli* C320MP25 had a wide range of ability to resist death and injury, and this variation was a function of differing chemical and physical characteristics associated with each individual site (Table 14).

This lack in uniformity of resistance to either overall death or non-lethal injury among the different aquatic environments was also evident for microorganisms of the fecal streptococcus group. Table 8 illustrates the wide range of ability for *S. faecalis* RS1009 to resist death and injury as a function of the eight different sites.

Statistical analyses verified the fact that considerable variation in non-lethal injury occurred to *E. coli* C320MP25 and *S. faecalis* RS1009 (Tables 17 and 18) among the eight sites. The statistical analyses indicated that at some sites there was not any significant difference between enumeration on non-selective and selective media. Yet, statistically significant differences between counts

on non-selective and selective media were found after exposure to several of the stream environments. The fact that varying degrees of injury were observed at the various sites indicates that it well may be necessary to initiate improved enumeration methods in order to more efficiently recover the population of injured cells.

It was not the objective of this research to attempt to identify the chemical or physical factor(s) responsible for death or injury to bacterial populations. However, measurements of chemical and physical characteristics were conducted (Table 14) and indicated that variation of death and injury observed to populations of bacteria were associated with varying chemical and physical characteristics associated with each site. The magnitude of death and injury observed at sites EG5 and EG6 may be thought to be the influence of residual chlorine in domestic sewage effluent entering the river above these two sites. However, less than 0.1 mg/l residual chlorine could be detected at site EG5 while it could not be detected at site EG6. Other unmeasured factors, such as heavy metals, may account for the accelerated death and injury to bacteria at these sites. It is interesting to note that equal, and sometimes greater, death and injury were observed for bacterial populations located remotely from the influence of domestic sewage, such as sites EG4 and R1. The greater enhanced survival and minimal injury observed at site BR2 is also difficult to

explain in terms of the chemical or physical aquatic environment. In short, the variation in survival and injury of bacterial populations at the different site locations perhaps result from a combination of several chemical and physical factors, which may be different at the various sites.

While conventional methods may be adequate to enumerate cells from those waters exerting minimal environmental stress, careful consideration should be given to developing new techniques in order to recover non-lethally injured cells from stressful water environments. Since injured cells were found to be a large proportion of the total population when exposed to certain environments, it is important that this damaged population be recovered in order to correctly evaluate bacteriological water quality.

In addition to the injury that occurred in the aquatic environment, it was found that substantial injury could be induced through certain laboratory manipulations. Some of the early experiments indicated that part of the injury attributed to the stress of the stream environment was, in fact, injury inflicted upon cells as a result of laboratory techniques. Since it was the objective of this research to determine whether bacteria could be physiologically debilitated as a result of environmental stress, it was necessary to identify and minimize any apparent laboratory injury.

Perhaps the most substantial amount of laboratory induced death or injury identified in this research was that of the deleterious effects of standard phosphate buffered diluent (2). Specifically designed experiments showed that treatment of pure culture suspensions with phosphate buffer during centrifugation-washing procedures, transport of chambers to site locations, transport of samples back to the laboratory, and general laboratory dilution procedures often resulted in substantial death and/or injury. Treatment of a suspension of *S. faecalis* ES1913 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 (Figure 4). Regular phosphate buffer was also found to contribute to non-lethal injury of survivors of a suspension of *E. coli* EC2075 to a greater degree than gelatin phosphate buffer (Table 4). In general, it appeared that *E. coli* was slightly more resistant to the toxic effects of regular phosphate buffer than was *S. faecalis*.

The statistical analyses verified the fact that significant differences existed as a result of enumeration on non-selective and selective media when using the regular phosphate buffer (Tables 19 and 20). Furthermore, the statistical analyses indicated that use of gelatin phosphate buffer resulted in statistical non-significance for

enumeration on the non-selective and selective media, indicating that the gelatin phosphate buffer afforded protection from laboratory injury.

The accelerated die-off and injury of indicator organisms in conventional phosphate dilution water may possibly be related to the level of heavy metal ion impurities present in the distilled water. The use of the gelatin phosphate buffer seemed to afford substantially greater protection to bacterial cells. Even the use of sterilized well water was found to be less harmful to cell suspensions than was the regular phosphate diluent (Table 3).

The physiological age of laboratory cultures used for survival and injury studies has been shown to have definite influence upon resulting persistence patterns (1,73,76,102). In general, young cultures in the logarithmic phase of growth have been found to be more sensitive to various environmental stresses than are older cultures in the stationary phase of growth. In the present research, only pure cultures in the stationary phase of growth were utilized for persistence studies. It was felt that any detection of death or injury to this hardier population of cells would more realistically emphasize the influence of environmental stresses upon recovery of bacterial cells from water than would the same phenomenon occurring to a younger, more fragile population.

Consideration must also be given to the fact that a certain degree of bias may have been introduced into those experiments using pure cultures. The pure cultures of coliforms and fecal streptococci were isolated by conventional detection methods whereby direct application of selective media was used. If, in fact, populations of injured cells existed in the water sample, it may well be that isolated pure cultures were the progeny of non-injured cells. Therefore, by a selection process, the pure cultures used for survival and injury studies consisted of those strains of organisms which were most resistant to stress. Assuming that the hardest strains of microorganisms were used, it may be said that any observed death or injury to this population would even add more emphasis to the importance of considering the influence of both environmental and added laboratory stresses upon recovery of bacteria from water.

Data from this research indicated that individual species of the coliform and fecal streptococcal groups exhibited distinct differences in regard to the relative extent of death and injury incurred as a result of environmental stress. In a specifically designed experiment, five species of indicator organisms, as well as a strain of *K. pneumoniae*, were simultaneously examined for recovery characteristics (Figures 9 and 10) after periods of exposure to the water environment of site EG6. In terms of resistance to death, the two species

of coliforms (*E. coli* and *E. aerogenes*) were found to be more persistent than the three species of fecal streptococci (*S. faecium*, *S. faecalis*, and *S. bovis*). *K. pneumoniae* was observed to be the hardiest of the test organisms, while *S. bovis* was found to be the most susceptible to death. Inspection of Figure 10 reveals that a wide variation in susceptibility to non-lethal injury was observed for the test organisms. The range of injury observed over the 4 day exposure period extended from the extreme susceptibility to injury for *S. bovis* and *S. faecalis* to the maximum resistances to injury for *K. pneumoniae* and *S. faecium*. Both *E. aerogenes* and *E. coli* exhibited high susceptibility to injury although not as pronounced as that of *S. bovis* and *S. faecalis*.

Other workers (7,35,93) have observed that the species *S. bovis* of the fecal streptococcus group exhibited extremely rapid die-away when exposed to the aquatic environment of either fresh or salt water. The present research has shown that selective media further complicate the recovery of this very fragile species from natural waters. However, a considerable portion of the injury observed to occur to pure cultures of *S. bovis* in the aquatic environment may be influenced by predisposition of the pure culture to laboratory growth conditions. It was found that growth in Trypticase soy broth containing 0.5% carbohydrate for preparation of test cell suspensions resulted in

substantial injury to *S. bovis* even before exposure to the aquatic environment. By reducing the carbohydrate concentration to 0.1%, a substantial decrease in non-lethal injury was observed. It appeared that the greater injury observed for those cultures grown in 0.5% carbohydrate may have been a reflection of sensitivity of *S. bovis* to acid end-products during growth in the broth.

Presently, increasing attention is being given to the possible significance of the potential pathogen *K. pneumoniae* in aquatic environments (15). In terms of resistance to both death and injury, it was observed that *K. pneumoniae* was better able to withstand the antagonism of environmental stress at site EG6 when compared to members of the coliform or fecal streptococcal group.

Although comparison has been made among the indicator organism species and *K. pneumoniae*, it should be emphasized that the recovery data obtained represents only survival and injury characteristics for these species at a single site location. Comparison of persistence and recovery characteristics at other sites, having varying degrees of stress, may very well reveal a different relative pattern. Evidence of this possibility may be observed in Table 5 whereby it was found that a strain of *E. coli* was better able to withstand the stresses of site M3A than was a strain of *K. pneumoniae* both in terms of resistance to death as well as injury. It seems likely, therefore, that a

given species may be more susceptible to death and injury than some other species in a given water environment while, conversely, this same species may be more resistant to death and injury than the other species in a different aquatic environment.

Numerous investigations have been made regarding comparison of MPN and membrane filtration recovery of indicator organisms from aquatic environments (23,44,47,54,58,74,89,90). Comparison of the recovery efficiency of various methods used to enumerate bacteria from water in the present study yielded information indicating that some techniques and media were more efficient than others when attempting to detect populations of injured cells. Specially designed experiments were conducted in which recovery was quantified using three general enumeration procedures in conjunction with a variety of media. The three methods employed were enumeration by plating methods using solid media, multiple tube dilution extinction methods using liquid media, and membrane filtration procedures.

In general, results from this research indicated that MPN methods gave superior recovery of coliforms than did plating or membrane filtration procedures. Table 9 and Figure 12 show the efficient recovery of a pure culture of *E. coli* C320MP25 when using MPN methods; in fact, lactose broth MPN procedures were almost equally efficient in recovery of the total viable population as was the rich, non-selective

TSY medium. Although the more selective BGB broth MPN determination was not as efficient as the lactose broth MPN determination, BGB broth was observed to be substantially superior to either plating or membrane filtration techniques. The deficiency in recovery of *E. coli* with membrane filtration using M-FC medium indicated that the selectivity of M-FC medium in conjunction with a selective elevated incubation temperature of 44.5 C contributed to substantial injury of cells even after 1 and 2 days of exposure to the stream environment of site EG6. Interestingly, surface plating techniques were not observed to improve recovery of this organism in comparison to pour plating procedures. Apparently, the added stress of subjecting cells to 46 C agar did not contribute any appreciable injury to the survivors.

It should be emphasized that a pure culture of *E. coli* C320MP25 was used in the previous experiment to examine recovery efficiency of various methods and media. In the routine analysis of a water sample for coliform organisms the presence of many other types of organisms complicate the detection and enumeration of the coliform group. In order to suppress the growth of the unwanted organisms and to minimize the probability of false-positives, it is necessary to use selective media. Yet, as shown in Table 9, some media and methods are highly inhibitory toward injured cells of the coliform group. It appears that the environment of the selective broths allow the "weakened" cells

to recover more readily than does the conditions of growth dictated by the selective media on solid agar or membrane filters.

Comparison of recovery efficiencies of enumeration methods and various selective media for a pure culture of *S. faecalis* RS1009 (Table 10) reveals that the greatest amount of non-lethal injury occurred when using PSE medium. This inhibited recovery of injured cells was observed regardless of whether the PSE medium was used in MPN, pour plating, or membrane filtration techniques. The maximal amount of recovery of *S. faecalis* was not obtained when using MPN methods. Rather, the most efficient recovery was observed when using M-Enterococcus medium with either the pour plate or membrane filtration technique. However, excluding M-Enterococcus medium from consideration, MPN methods using AD or EVA broths were found to be superior to pour plating and membrane filtration when using KF or PSE media.

The inhibitory effects of PSE medium on injured cells of this particular strain of *S. faecalis* are quite evident. The observation that recovery of *S. faecalis* with KF agar was not as efficient as with M-Enterococcus agar may be a reflection of the fact that KF agar is believed to more specific for species of animal origin and less specific for those of human fecal origin (62). The particular *S. faecalis* strain used for this experiment was isolated from domestic

raw sewage, making identification of the original source improbable. Again, as in the case of the similar experiment with *E. coli*, it should be emphasized that a pure culture of *S. faecalis* was used. Furthermore, since the fecal streptococci consist of several other species, it seems probable that these other species may show a different response than this strain of *S. faecalis* to the recovery methods and media employed.

Comparison of recovery efficiencies of various methods and media for coliforms from raw sewage also exhibited considerable variation (Table 11). As observed with the pure culture of *E. coli* C320MP25, recovery of injured populations of coliforms from raw sewage was inhibited by membrane filtration procedures using M-Endo MF medium. The lack of recovery by M-Endo MF medium became most evident during the last two days of exposure to the stream environment when only 56% and 19%, respectively, of the population was detected in comparison to populations enumerated with DLA surface-overlay techniques. Apparently, stressed coliform organisms were not able to recover in the growth environment of M-Endo MF medium as well as in the growth environment of DLA and VRB surface-overlay or pour plates. Similar lack of recovery of coliforms from raw sewage with membrane filtration methods was observed in another experiment (Table 12).

The adverse effects of membrane filtration procedures upon recovery of stressed cells were even more evident when attempting to

enumerate the fecal coliform population (Table 12). The poor recovery observed with the direct use of M-FC medium in conjunction with elevated temperature incubation of 44.5 C indicates that perhaps additional stress is imposed on cells as a result of the elevated incubation temperature. Somewhat improved recovery was attained by enumeration with M-Endo MF medium incubated at the conventional temperature of 35 C, followed by confirmation in EC broth. Even greater recovery of fecal coliforms was observed when using primary isolation at 35 C with lactose broth MPN or DLA surface-overlay methods, followed by confirmation in EC broth. Although this lower incubation temperature does require additional labor, time, and materials in order to verify the presence of fecal coliforms, it does allow for a considerable improvement in extent of recovery of fecal coliforms from raw sewage.

This conclusion regarding the effects of incubation temperature on recovery is in contrast to those of Mossel and Vega (65) who observed that the direct enumeration of fecal coliforms at 44 C was equal to that of enumeration at 37 C, followed by elevated temperature confirmation, when using MacConkey agar. Also, Geldreich et al. (34) reported an average 88% recovery of fecal coliform bacteria on the membrane filter with M-FC medium at 44.5 C based on counts of pour plates incubated at 35 C with tryptone-glucose-extract agar.

Reduced recoveries by the membrane filtration procedure were also observed when attempting to enumerate fecal streptococci from a suspension of raw sewage (Table 11). Pour plate enumeration, using either KF or M-Enterococcus agar, was found to more efficiently recover fecal streptococci from raw sewage over the 4 day exposure period. However, the results indicated that inhibition of recovery of fecal streptococci when using membrane filtration procedures was not as great as the inhibition of recovery of coliforms or fecal coliforms when using the same technique.

The overall lack of recovery of total coliforms and fecal coliforms when examining either pure cultures or raw sewage may, in part, be due to inhibition arising from characteristics of the membrane filters. Comparison of Gelman and Millipore membrane filters, by Presswood and Brown (75), showed that Gelman GN-6 filters recovered statistically more coliform and fecal coliform colonies than did Millipore HAWG 047 SO filters incubated at 35 C or 44.5 C. It was suggested that differences in methods of sterilization might possibly account for these observations. Since Millipore membrane filters were used in the present study, perhaps the observed deficiency in recovery of coliforms and fecal coliforms may be partially due to this same phenomenon.

Fecal coliform to fecal streptococci ratios (FC/FS) are believed to offer a valuable investigative tool when attempting to

determine sources of pollution (27,33,35). When the FC/FS ratio is greater than or equal to 4.0, it may be taken as strong evidence that pollution derives from human wastes. Ratios less than 0.7 suggest that the pollution is derived from fecal discharges of warm-blooded animals other than humans. The observation of FC/FS ratios between 0.7 and 4.0 make interpretation of specific sources more difficult and indicate mixed pollution from both human and animal wastes. The present research indicated that the FC/FS ratio of a suspension of raw sewage will rapidly decrease upon exposure to the aquatic environment. Beginning with a FC/FS ratio of approximately 4.0 in raw sewage, it was observed that the ratio rapidly dropped to approximately 1.2 after 24 h exposure to the stream environment and to less than 0.4 after 48 h exposure (Figure 14). The rapidly decreasing ratio was a result of differences in relative persistence of fecal coliforms and fecal streptococci. The accelerated die-off of the fecal coliform population relative to a more persistent fecal streptococcal population was reflected by the decreasing FC/FS ratio over the 4 day exposure period (Figure 14). This observation indicates that FC/FS ratios lose significance when examining water containing fecal wastes of more than 24 h. Evans et al. (27) stated that FC/FS ratios are more meaningful if applied to effluent discharges and receiving waters not more than 24 h flow time downstream of the point of pollutional discharge. The observation that the FC/FS ratio decreased from approximately 4 to 1 within

24 h questions the 24 h limitation. It appears that a much shorter time limitation should be attached to interpretation of FC/FS ratios since problems with bacterial die-off were found to obscure meaningful ratios even within 24 h.

In order to recover those cells which have undergone stress and resulting injury, it is necessary that such cells have the opportunity to repair themselves before they can multiply and divide. In general, appreciable repair from injury is obtained by first exposing the injured cells to a rich, non-selective medium for a short time to permit repair of the damaged cells prior to use of selective media. Exposure of injured cells to a nutritionally rich liquid medium allows for repair of injury such that the recovered cells will have the ability to grow on selective media. This capability of repair of non-lethal injury in rich, non-selective media has been observed for bacterial populations damaged by heating (19,61,68,70,98,104), freezing (43,63,78,79,81,110), and freeze-drying (76,77).

In the present studies, similar repair of injury was observed for populations of cells exposed to the stresses of aquatic environments. In one experiment, it was observed that the injury acquired by a population of *E. coli* C320MP25, during 2 days of exposure to the aquatic environment, could be rapidly repaired within 2½-3 h in nutritionally rich non-selective TSY broth (Figure 15). 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 non-selective and selective media within $2\frac{1}{2}$ -3 h. During the $2\frac{1}{2}$ -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 $2\frac{1}{2}$ -3 h in TSY broth indicate that these injured cells are capable of repair if exposed to a suitable nutritionally rich growth medium.

Similar repair of injury of a stressed population of *E. coli* WC959 was observed (Figure 16). A repair period of 4 h in TSY broth was required before similar counts were obtained on the non-selective and selective media. An extended lag period of 4 h was required prior to initiation of multiplication. The 4 h repair and lag periods for this experiment indicate that the longer exposure period of 7 days in the aquatic environment, as compared to 2 days in the previously discussed experiment, may result in longer time requirements in rich media in order to complete the repair processes. However, several other variables must be considered which may account for this difference.

First, the enumeration methods and selective media used were different for the 2 experiments (surface-overlay plate and membrane filtration). Also, two different strains of *E. coli* were used. Perhaps the 2 strains had relatively different degrees of sensitivity to the stresses of the water environment. The fact that the experiments were done approximately 2 weeks apart must be considered since the overall chemical and physical environment of the water can change. In addition, during the 7 day exposure period (Figure 16), cold weather conditions resulted in a frozen cell suspension within the stream environment. Conceivably the freezing process could have resulted in additional stress on the population of surviving cells.

In addition to the repair of sub-lethally injured cells of *E. coli*, it was found that injured populations of *S. faecalis* RS1009 were capable of the same general repair phenomenon in a nutritionally rich, non-selective medium (Figure 17). After 2 h in rich TSY medium, the injured population of *S. faecalis*, which had undergone 2 days of stress in the stream environment, had completely repaired the non-lethal injury such that essentially the entire population could grow on selective KF medium. An even longer repair period of 3½-4 h was required for those cells exposed to the aquatic environment for 4 days. As observed with the strains of *E. coli*, an extended lag phase prior to multiplication relative to a control population, was exhibited by those cells exposed to the water environment. The data in Figure 17 do

indicate that the longer exposure of cells to environmental stress in water results in a longer time requirement in a rich, non-selective medium in order to manifest complete repair and recovery of the damaged population.

The observed rapid repair of injured cells in nutritionally rich, non-selective media lends support to the use of an enrichment technique to improve the recovery of stressed cells. The use 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. The enrichment technique appears to be especially applicable to the membrane filtration procedure, which has been previously shown in this study to have low efficiency in recovering injured cells. Various enrichment techniques have been advocated as an efficient method of improving recovery of total coliform populations from water when using the membrane filtration procedure (20,21,47,51,53,55,62,90). Similarly enrichment procedures for the detection of fecal streptococci from water have been emphasized (62,82).

In the present research, it was observed that a short 2 h enrichment on rich TSY medium prior to exposure to selective media substantially improved recovery of *E. coli* C320MP25 relative to recovery by direct primary exposure to selective media (Figure 18 and Table 13). The enrichment step was found to be extremely efficient in recovering

the total viable population when using M-Endo MF medium as the selective medium. In fact, the enrichment-M-Endo MF procedure was found to be equally efficient as rich TSY medium in recovering the *E. coli* cells during the first 2 days of exposure to the aquatic environment. A preliminary enrichment prior to application of M-FC medium incubated at 44.5 C was also found to improve recovery over that of the direct use of M-FC medium at the elevated temperature. The fact that the enrichment-M-FC technique was not as efficient as the enrichment - M-Endo MF technique perhaps indicates that a proportion of the stressed cells remained sensitive to the elevated incubation temperature even after the 2 h enrichment step. Possibly, an extension of the enrichment step may result in a more complete repair of damage such that cells would become less sensitive to the elevated incubation temperature.

In addition to improved recovery of a pure culture suspension of *E. coli* during exposure to environmental stress, enrichment techniques were found to substantially improve recovery of total (Figure 19) and fecal coliform (Figure 20) populations from a natural suspension of raw sewage. The direct application of selective M-Endo MF or M-FC media did not allow for efficient recovery of injured cells relative to enrichment techniques. Throughout the entire exposure period of the raw sewage to the aquatic environment it was observed that the most efficient method for the recovery of fecal coliforms was by a short 2 h enrichment on rich TSY medium followed by transfer to selective

M-Endo MF medium. This enrichment - M-Endo MF technique substantially improved recovery of fecal coliforms over that of recovery by the conventional direct elevated temperature technique even before the raw sewage was exposed to the environment of site EG4 (Figure 20). Thus, it should be realized that the so-called "0-h sample" represented a sewage sample that, in fact, had undergone exposure to environmental stress within the sewage itself prior to additional stress during the 4 day exposure period at site EG4. As the suspension of raw sewage was exposed to the aquatic environment, the lack of efficiency of direct detection methods became more evident relative to recovery observed with enrichment techniques.

The inhibiting and/or bactericidal influence shown by M-Endo MF and M-FC media against "weak" coliforms appears to be extremely damaging during the first few hours of incubation on these selective media. Therefore, this inhibition of recovery of damaged cells by direct application of selective media strongly suggests the need for an enrichment or resuscitation period. Such a period appears to provide a non-toxic environment for the gradual adjustment and repair of the injured microorganisms.

The fact that certain aquatic environments have been shown to have the ability to induce stress upon a substantial proportion of cells such that these cells become physiologically injured and cannot be detected by direct, conventional selective procedures lends support

to the use of enrichment techniques. The adoption of enrichment techniques perhaps would help overcome some of the inadequacies and limitations arising from the direct application of selective media for the detection and enumeration of bacteria in water and wastewater. In a sense, an enrichment step for improved recovery of injured organisms possibly would provide the necessary bridge for bacterial cell adjustment between the environment of the natural water and that of selective media.

Chapter 6

SUMMARY

The recovery characteristics of indicator organisms exposed to the natural surroundings of the aquatic environment were examined using membrane filter chambers (56). The membrane filter chambers, containing pure culture suspensions of indicator organisms or natural suspensions of raw sewage, were immersed in the stream environment at a number of different sites having water of varying physical and chemical composition. Periodically, samples were withdrawn from the chamber and enumerated using both non-selective and selective media. The differences between counts obtained on the non-selective and selective media indicated that substantial proportions of cells may become physiologically debilitated, damaged, or injured as a result of exposure to the stresses of some aquatic environments. It should be emphasized that this environmental stress may merely injure the cell but not necessarily kill or destroy it. Apparently, the foreign aquatic environment has the capability to induce stress on large proportions of the population such that they may become sensitive to inhibitory agents contained within selective media. Thus, the direct application of specific selective media merely complicates the recovery of the population of non-lethally injured cells.

Using pure culture suspensions of coliforms and fecal streptococci, it was found that considerable variation in terms of both

death and non-lethal injury existed among the various stream environments utilized for exposure of bacterial suspensions within membrane filter chambers. Thus, it appears that the varying degrees of observed death and injury to bacterial populations exposed to the influences of various aquatic environments may be partly a function of the differing chemical and physical characteristics associated with each individual stream environment.

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 dilution water during preparation and dilution of pure culture suspensions. The use of gelatin phosphate buffer afforded greater protection from such laboratory induced death and/or injury.

Comparison of the recovery efficiency of various methods used to enumerate bacteria from water yielded information indicating that some techniques and media were more efficient than others when attempting to detect populations of injured cells. In general, results from this research indicated that multiple tube fermentation techniques gave superior recovery of coliforms than did plating or membrane filtration procedures whether pure cultures or natural suspensions were used as the test system. The least efficient method of recovery of coliforms or fecal streptococci was by membrane filtration procedures. Especially poor recovery of fecal coliforms was noted

with the direct application of M-FC medium at the elevated incubation temperature of 44.5 C.

The injury inflicted upon pure cultures of *E. coli* and *S. faecalis* by the aquatic environment was found to be reversible and could be repaired in a medium such as Trypticase soy yeast extract broth. As the injured populations of cells were exposed to the rich, non-selective broth, increasing proportions of cells were able to repair themselves such that they became insensitive to inhibitory agents in selective media.

The observed rapid repair of injured cells in a nutritionally rich, non-selective broth lends support to the use of an enrichment technique to improve the recovery of stressed cells. It was observed that a short 2 h enrichment on a rich, non-selective medium prior to exposure to selective media substantially enhanced recovery of coliforms and fecal coliforms from raw sewage relative to recovery by direct primary exposure to selective media. Such an enrichment period appears to provide a non-toxic environment for the gradual adjustment and repair of the injured microorganisms. The enrichment technique appeared to be especially applicable to the membrane filtration procedure. The adoption of enrichment techniques perhaps would help overcome some of the inadequacies and limitations arising from the direct application of selective media for the detection and enumeration of bacteria in water and wastewater.

APPENDIX

Table 15. Biochemical reactions of strains of *E. coli*, *E. aerogenes*, and *K. pneumoniae*.

Organism and Strain No.	Indole	Methyl Red	Voges-Proskauer	Simon's Citrate	H ₂ S (TSI)	Urea	Motility	Lysine Decarboxylase	Ornithine Decarboxylase	Glucose	Lactose	Mannitol	Dulcitol	Sorbitol	Arabinose	EC @ 44.5 C
<i>E. coli</i> C320MP25	+	+	-	-	-	-	-	+	-	AG	AG	AG	AG	AG	AG	+
<i>E. coli</i> WC959	+	+	-	-	-	-	+	+	+	AG	AG	AG	-	AG	AG	+
<i>E. coli</i> RC1435	+	+	-	-	-	-	+	+	+	AG	AG	AG	AG	AG	AG	+
<i>E. coli</i> EC2075	+	+	-	-	-	-	-	+	-	AG	AG	AG	-	AG	AG	+
<i>E. aerogenes</i> RC1282	-	-	+	+	-	-	+	+	+	AG	AG	AG	-	AG	AG	-
<i>E. aerogenes</i> EC2027	-	-	+	+	-	-	+	+	+	AG	AG	AG	AG	AG	AG	-
<i>K. pneumoniae</i> 1881	-	-	-	+	-	+	-	+	-	AG	AG	AG	AG	AG	AG	+

A = Acid reaction in carbohydrate

G = Gas production in carbohydrate

+ = Compound is utilized by, or characteristic is positive for, the strain shown

- = Compound is not utilized by, or characteristic is negative for, the strain shown

Table 16. Biochemical reactions of strains of fecal streptococci.

Organism and Strain No.	EVA	6.5% NaCl	BHI - 10 C	BHI - 45 C	Catalase	NH ₃ from Peptone	Gelatin	Starch	Growth on 0.04% tellurite	Glucose	Lactose	Mannitol	Dulcitol	Sorbitol	Arabinose	Melezitose	Melibiose
<i>S. faecalis</i> ES1913	+	+	+	+	+	+	-	-	+	A	A	A	-	A	-	A	A
<i>S. faecalis</i> BS905	+	+	+	+	+	+	-	-	+	A	A	A	-	A	-	-	A
<i>S. faecalis</i> MS304	+	+	+	+	+	+	-	-	+	A	A	A	-	A	A	A	-
<i>S. faecalis</i> RS1009	+	+	+	+	+	+	-	-	+	A	A	A	-	A	-	A	-
<i>S. faecium</i> BS895	+	+	+	+	+	+	-	-	-	A	A	A	-	A	A	-	A
<i>S. faecium</i> ES2017	+	+	+	+	+	+	-	-	-	A	A	A	A	-	A	-	A
<i>S. faecalis</i> var. <i>liquefaciens</i> ES1730	+	+	+	+	+	+	+	-	+	A	A	A	-	A	-	-	-
<i>S. durans</i> GS255	+	+	+	+	+	+	-	-	-	A	A	-	-	-	-	-	-
<i>S. bovis</i> C3SE17	+	-	-	+	+	-	-	+	-	A	A	-	-	-	A	-	-

A = Acid reaction in carbohydrate

+ = Compound is utilized by, or characteristic is positive for, the strain shown

- = Compound is not utilized by, or characteristic is negative for, the strain shown

Table 17. 95% confidence limits for percentages of injury for suspensions of *E. coli* C320MP25 located at 8 different sites over a 4 day exposure period.

Exposure time (days)	M3	M3A	H3	BR2
0	- 5.7 to 18.5	- 7.6 to 17.6	- 9.3 to 16.0	-12.8 to 12.9
1	-19.4 to 17.7	* 2.8 to 32.0	-27.1 to 33.1	- 7.0 to 24.0
2	* 4.7 to 38.0	-15.9 to 20.7	* 3.6 to 26.5	-12.4 to 21.7
3	-24.0 to 24.4	- 8.4 to 39.7	*53.8 to 70.7	- 1.8 to 34.6
4	* 9.7 to 44.3	* 6.2 to 45.4	*85.4 to 93.9	-14.0 to 26.4

Exposure time (days)	R1	EG4	EG5	EG6
0	* 6.3 to 25.4	- 2.5 to 20.9	- 1.6 to 22.1	- 5.1 to 19.5
1	*44.3 to 63.4	-18.9 to 22.7	- 4.2 to 32.2	- 8.3 to 27.4
2	*88.5 to 93.8	*74.3 to 82.4	*56.2 to 84.0	*70.1 to 79.2
3	*48.5 to 80.2	*90.3 to 97.4	*77.1 to 91.4	*83.1 to 89.4
4	*76.2 to 82.2	*94.8 to 98.4	*63.9 to 88.6	*80.6 to 87.1

* Significant at alpha = 0.05

Table 18. 95% confidence limits for percentages of injury for suspensions of *S. faecalis* RS1009 located at 8 different sites over a 4 day exposure period.

Exposure time (days)	M3	M3A	H3	BR2
0	-16.7 to 24.0	-18.3 to 23.4	-14.1 to 20.1	-19.7 to 22.3
1	- 0.2 to 27.6	-20.2 to 30.0	-17.1 to 27.7	-11.1 to 30.3
2	-19.8 to 27.5	-27.2 to 24.1	-16.8 to 31.5	-23.3 to 25.3
3	-28.3 to 28.8	-35.8 to 23.2	-27.1 to 47.9	- 7.2 to 25.5
4	* 1.0 to 26.5	*11.2 to 42.7	*92.1 to 97.8	-34.6 to 32.3

Exposure time (days)	R1	EG4	EG5	EG6
0	-19.7 to 22.3	-22.8 to 20.5	-15.3 to 25.0	-11.2 to 28.1
1	*86.5 to 92.3	-21.9 to 30.5	*51.3 to 82.4	-23.0 to 39.9
2	*95.8 to 99.9	*86.4 to 92.0	*93.6 to 97.7	*32.4 to 54.8
3	*96.5 to 98.9	*80.7 to 104.8	*89.4 to 100.5	*90.7 to 99.8
4	*44.9 to 115.1	*98.4 to 100.5	*94.0 to 101.8	*96.2 to 101.2

* Significant at alpha = 0.05

Table 19. 95% confidence limits for percentages of injury for separate suspensions of *S. faecalis* ES1913 treated with regular phosphate and gelatin phosphate diluents. Membrane filter chambers were suspended at the well water site for a 48 h exposure period

Exposure time (h)	Regular phosphate	Gelatin phosphate
0	*17.7 to 50.1	-19.0 to 21.5
6	*97.9 to 98.5	- 9.0 to 8.3
10	*97.6 to 98.6	- 9.9 to 7.3
24	*97.4 to 98.7	- 4.9 to 11.6
48	*98.0 to 99.9	* 1.0 to 18.2

* Significant at $\alpha = 0.05$

Table 20. 95% confidence limits for percentages of injury for separate suspensions of *E. coli* EC2075 treated with regular phosphate and gelatin phosphate diluents. Membrane filter chambers were suspended at the well water site for a 48 h exposure period

Exposure time (h)	Regular phosphate	Gelatin phosphate
0	* 9.4 to 33.4	-13.4 to 17.4
6	*28.1 to 52.2	-18.3 to 15.5
10	*52.7 to 70.7	- 3.7 to 28.8
24	*36.2 to 60.8	- 5.9 to 27.9
48	*43.5 to 67.7	-21.4 to 19.4

* Significant at $\alpha = 0.05$

LITERATURE CITED

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1. Allen, L.A., S.M. Pasley, and M.A.F. Pierce. 1952. Some factors affecting the viability of faecal bacteria in water. J. Genl. Microbiol., 7: 36-43.
2. American Public Health Association. 1971. Standard methods for the examination of water and wastewater. 13th Ed., A.P.H.A., New York.
3. Andre, D.A., H.H. Weiser, and G.W. Malaney. 1967. Survival of bacterial enteric pathogens in farm pond water. J. Amer. Water Works Assoc., 59: 503-508.
4. Ayres, J.C., A.A. Kraft, H.E. Snyder, and H.W. Walker, editors. 1962. Chemical and biological hazards in food. Iowa State University Press, Ames, Iowa.
5. Ballentine, R.K., and F.W. Kittrell. 1968. Observation of fecal coliforms in several recent stream pollution studies. U.S.D.I., F.W.P.C.A., Cincinnati.
6. Barnes, H. 1959. Apparatus and methods of oceanography. Interscience Publishers.
7. Bartley, C.H., and L.W. Slanetz. 1960. Types and sanitary significance of fecal streptococci isolated from feces, sewage, and water. Amer. J. Public Health, 50: 1455.
8. Beard, P.J., and N.F. Meadowcroft. 1935. Survival and rate of death of intestinal bacteria in sea water. Amer. J. Public Health, 25: 1023-1026.
9. Bonde, G.J. 1962. Bacterial indicators of water pollution. Teknisk Forlag, Copenhagen.
10. Boyd, W.L., B.P. Klubek, and J.W. Boyd. 1972. Sewage disposal at Point Barrow, Alaska. Office of Naval Research, Technical Rpt. #1.
11. _____, _____, and _____. 1972. Clean water ecology in the Polar Region. Naval Research, 17-24.
12. Brasfeild, H. 1972. Environmental factors correlated with size of bacterial populations in a polluted stream. Appl. Microbiol., 24: 349-352.

13. Brezenski, F.T., and R. Russomanno. 1969. The detection and use of salmonellae in studying polluted tidal estuaries. J. Water Pollut. Contr. Fed., 41: part 1, 725-737.
14. _____, _____, and P. DeFalco, Jr. 1965. The occurrence of *Salmonella* and *Shigella* in post-chlorinated sewage effluents and receiving waters. Health Lab. Sci., 2: 40-47.
15. Brown, C., and R.J. Seidler. 1973. Potential pathogens in the environment: *Klebsiella pneumoniae*, a taxonomic and ecological enigma. Appl. Microbiol., 25: 900-904.
16. Buck, J.D., and R.C. Cleverdon. 1960. The spread plate as a method for the enumeration of marine bacteria. Limnology and Oceanography, 5: 78-80.
17. Burman, N.P. 1961. Some observations on coli-aerogenes bacteria and streptococci in water. J. Appl. Bacteriol., 24: 368-376.
18. Butler, C.E., and P.P. Ludovici. 1969. Survival and recovery of *Salmonella* in Tuscon's wastewater reclamation program. J. Water Pollut. Contr. Fed., 41: part 1, 738-744.
19. Clark, C.W., and Z.J. Ordal. 1969. Thermal injury and recovery of *Salmonella typhimurium* and its effect on enumeration procedures. Appl. Microbiol., 18: 332-336.
20. Clark, H.F., E.E. Geldreich, H.L. Jeter, and P.W. Kabler. 1951. The membrane filter in sanitary bacteriology. Public Health Reports, 66: 951-977.
21. _____, H.L. Jeter, E.E. Geldreich, and _____. 1952. Domestic and European molecular filter membranes. J. Amer. Water Works Assoc., 44: 1052-1056.
22. Cohen, J., and H.I. Shuval. 1973. Coliforms, fecal coliforms, and fecal streptococci as indicators of water pollution. Water, Air, and Soil Pollution, 2: 85-95.
23. Collet, E., R. Johnston, L.F. Ey, and C.C. Croft. 1953. Isolation of *Salmonella typhosa* from well water by the membrane filter technic. Amer. J. Public Health, 43: 1438-1440.
24. Deaner, D.G., and K.D. Kerri. 1969. Regrowth of fecal coliforms. J. Amer. Water Works Assoc., 61: 465-468.

25. Edwards, P.R., and W.H. Ewing. 1972. Identification of Enterobacteriaceae. Third edition, Burgess Publishing Co., Minneapolis.
26. Environmental Protection Agency. 1971. Methods for chemical analysis of water and wastes. Cincinnati.
27. Evans, F.L., E.E. Geldreich, S.R. Weibel, and G.G. Robeck. 1968. Treatment of urban stormwater runoff. J. Water Pollut. Contr. Fed., 40: part 2, R162-R170.
28. Fair, J.F., and S.M. Morrison. 1967. Recovery of bacterial pathogens from high quality surface water. Water Resour. Res., 3: 799-803.
29. Gallagher, T.P., and D.F. Spino. 1968. The significance of numbers of coliform bacteria as an indicator of enteric pathogens. Water Res., 2: 169-175.
30. Geldreich, E.E. 1967. Fecal coliform concepts in stream pollution. Water and Sewage Works, Ref. Number, R-98.
31. _____. 1970. Applying bacteriological parameters to recreational water quality. J. Amer. Water Works Assoc., 62: 113-120.
32. _____, L.C. Best, B.A. Kenner, and D.J. Van Donsel. 1968. The bacteriological aspects of stormwater pollution. J. Water Pollut. Contr. Fed., 40: part 1, 1861-1872.
33. _____, H.F. Clark, and C.B. Huff. 1964. A study of pollution indicators in a water stabilization pond. J. Water Pollut. Contr. Fed., 36: 1372-1379.
34. _____, _____, _____, and L.C. Best. 1965. Fecal coliform organism medium for the membrane filter technique. J. Amer. Water Works Assoc., 57: 208-214.
35. _____, and B.A. Kenner. 1969. Concepts of fecal streptococci in stream pollution. J. Water Pollut. Contr. Fed., 41: part 2, R336-352.
36. Goodrich, T.D., D.G. Stuart, and W.G. Walter. 1973. Enteric bacteria found in elk droppings in a municipal watershed in Montana. J. of Environ. Health, 35: 374-377.

37. Gordon, R.C. 1972. Winter survival of fecal indicator bacteria in a Subarctic Alaskan River. EPA Technology Series, EPA - R2 - 72 - 013.
38. Greenberg, A.E. 1956. Survival of enteric organisms in sea water. Public Health Reports, 71: 77-86.
39. Gyllenberg, H., S. Niemelä, and T. Sormunen. 1960. Survival of bifid bacteria in water as compared with that of coliform bacteria and enterococci. Appl. Microbiol., 8: 20-22.
40. Hall, H.E. 1970. Effects of diluents on the recovery of microorganisms from foods. J. Milk Food Technol., 33: 311-313.
41. Halton, J.E., and W.R. Nehlsen. 1968. Survival of *Escherichia coli* in zero-degree centigrade sea water. J. Water Pollut. Contr. Fed., 40: 865-868.
42. Hendricks, C.W., and S.M. Morrison. 1967. Multiplication and growth of selected enteric bacteria in clear mountain stream water. Water Res., 1: 567-576.
43. Janseen, D.W., and F.F. Busta. 1973. Influence of milk components on the injury, repair, and death of *Salmonella anatum* cells subjected to freezing and thawing. Appl. Microbiol., 26: 725-732.
44. Jeter, H.L., E.E. Geldreich, and H.F. Clark. 1954. Type differentiation of coliform organisms with membrane filter technique. J. Amer. Water Works Assoc., 46: 386-394.
45. Jordan, E.O., H.L. Russel, and H.R. Zeil. 1904. The longevity of the typhoid bacillus in water. J. Infect. Dis., 1: 641-689.
46. Joyce, G., and H.H. Weiser. 1967. Survival of enteroviruses and bacteriophage in farm pond water. J. Amer. Water Works Assoc., 59: 491-501.
47. Kabler, P.W. 1954. Water examination by membrane filter and most probable number procedures. Am. J. Public Health, 44: 379-386.
48. Kittrell, F.W., and S.A. Furfari. 1963. Observations of coliform bacteria in streams. J. Water Pollut. Contr. Fed., 35: 1361-1385.

49. Klein, D.A., and S. Wu. 1974. Stress: a factor to be considered in heterotrophic microorganism enumeration from aquatic environments. *Appl. Microbiol.*, 27: 429-431.
50. Klock, J.W. 1971. Survival of coliform bacteria in wastewater treatment lagoons. *J. Water Pollut. Contr. Fed.*, 43: 2071-2083.
51. Laubausch, E.J., E.E. Geldreich, and H.L. Jeter. 1953. Membrane filter procedure applied in the field. *Public Health Reports*, 68: 1118-1122.
52. Lehmann, E.L. 1959. Testing statistical hypotheses. Wiley Publishing Co., New York.
53. Levin, G.V., and E.J. Laubausch. 1954. Membrane filter and emergency water supply control. *Am. J. Public Health*, 44: 55-63.
54. Lin, S. 1973. Evaluation of coliform tests for chlorinated secondary effluent. *J. Water Pollut. Contr. Fed.*, 45: 498-506.
55. McCarthy, J.A., J.E. Delaney, and R.J. Grasso. 1961. Measuring coliforms in water. *Water and Sewage Works*. 108: 238-243.
56. McFeters, G.A., and D.G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane filter chambers. *Appl. Microbiol.*, 24: 805-811.
57. Mallman, W.L., and W. Litsky. 1951. Survival of selected enteric organisms in various types of soil. *Amer. J. Public Health*, 41: 38-44.
58. Mara, D.D. 1973. Coliform counts of polluted waters: a comparison of media and methods. *Water Res.*, 7: 1889-1903.
59. Masharipov, N.P. 1970. Survival of dysentery pathogens in well water. *Hygiene and Sanitation*, 35: 146-147.
60. Maxcy, R.B. 1970. Non-lethal injury and limitations of recovery of coliform organisms on selective media. *J. Milk Food Technol.*, 33: 445-448.

61. Maxcy, R.B. 1973. Condition of coliform organisms influencing recovery of subcultures on selective media. J. Milk Food Technol., 36: 414-416.
62. Millipore Corporation. 1973. Biological analysis of water and wastewater - application manual AM302.
63. Moss, C.W., and M.L. Speck. 1966. Identification of nutritional components in trypticase responsible for recovery of *Escherichia coli* injured by freezing. J. Bacteriol., 91: 1098-1104.
64. Morris, W., and R.H. Weaver. 1954. Streptococci as indices of pollution in well waters. Appl. Microbiol., 2: 282-284.
65. Mossel, D.A.A., and C.L. Vega. 1973. The direct enumeration of *Escherichia coli* in water using MacConkey's agar at 44 C in plastic pouches. Health Lab. Sci., 10: 303-307.
66. Mundt, J.O. 1973. Litmus milk reaction as a distinguishing feature between *Streptococcus faecalis* of human and non-human origins. J. Milk Food Technol., 36: 364-367.
67. O'Connor, P.B. 1972. Survival characteristics of *Salmonella* in the Fort Loudon Reservoir. MS Thesis, Univ. of Tennessee, Knoxville.
68. Ordal, Z.J. 1970. Current developments in detection of micro-organisms in foods: influence of environmental factors on detection methods. J. Milk Food Technol., 33: 1-5.
69. Orlob, G.T. 1956. Viability of sewage bacteria in sea water. Sewage and Industrial Wastes, 28: 1147-1167.
70. Pierson, M.D., R.I. Tomlins, Z.J. Ordal. 1971. Biosynthesis during recovery of heat-injured *Salmonella typhimurium*. Appl. Microbiol., 105: 1234-1236.
71. Platt, A.E. 1935. The viability of *Bact. coli* and *Bact. aerogenes* in water. A method for the rapid enumeration of these organisms. Amer. J. Hygiene, 35: 437-448.
72. Postgate, J.R. 1969. Viability measurements and the survival of microbes under minimum stress. Advances in Microbial Physiology, 1: 1-23.

73. Postgate, J.R., and J.R. Hunter. 1962. The survival of starved bacteria. *J. Genl. Microbiol.*, 29: 233-263.
74. Presnell, M.W., W. Arcisz, and C.B. Kelley. 1954. Comparison of the MF and MPN techniques in examining sea water. *Public Health Reports*, 69: 300-304.
75. Presswood, W.G., and L.R. Brown. 1973. Comparison of Gelman and Millipore membrane filters for enumeration of fecal coliform bacteria. *Appl. Microbiol.*, 26: 332-336.
76. Ray, B., J.J. Jezeski, and F.F. Busta. 1971. Effect of rehydration on recovery, repair, and growth of injured freeze-dried *Salmonella anatum*. *Appl. Microbiol.*, 22: 184-189.
77. _____, _____, and _____. 1971. Repair of injury in freeze-dried *Salmonella anatum*. *Appl. Microbiol.*, 22: 401-407.
78. _____, and M.L. Speck. 1972. Repair of injury induced by freezing *Escherichia coli* as influenced by recovery medium. *Appl. Microbiol.*, 24: 258-263.
79. _____, and _____. 1972. Metabolic process during the repair of freeze-injury in *Escherichia coli*. *Appl. Microbiol.*, 24: 585-590.
80. _____, and _____. 1973. Discrepancies in the enumeration of *Escherichia coli*. *Appl. Microbiol.*, 25: 494-498.
81. _____, and _____. 1973. Enumeration of *Escherichia coli* in frozen samples after recovery from injury. *Appl. Microbiol.*, 25: 499-503.
82. Rose, R.E., and W. Litsky. 1965. Enrichment procedure for use with the membrane filter for the isolation and enumeration of fecal streptococci in water. *Appl. Microbiol.*, 13: 106-108.
83. Roth, L.A., M.E. Stiles, and L.F.L. Clegg. 1972. Communal growth response of a heat-injured strain of *Escherichia coli*. *Canad. J. Microbiol.*, 18: 1776-1777.
84. Rudolfs, W., L.L. Falk, and R.A. Ragotzkie. 1950. Literature review on the occurrence and survival of enteric, pathogenic, and relative organisms in soil, water, sewage, and sludges, and on vegetation. *Sewage and Industrial Wastes*, 22: 1261-1281.

85. Savage, H.P., and N.B. Hanes. 1971. Toxicity of sea water to coliform bacteria. *J. Water Pollut. Contr. Fed.*, 43: 854-861.
86. Scarpino, P.V. 1971. Bacterial and viral analysis of water and wastewater. In *Water and water pollution handbook* (L.L. Ciaccio, Ed.), 2: 639-761.
87. Scheusner, D.L., F.F. Busta, and M.L. Speck. 1971. Injury of bacteria by sanitizers. *Appl. Microbiol.*, 21: 41-45.
88. _____, _____, and _____. 1971. Inhibition of injured *Escherichia coli* by several selective agents. *Appl. Microbiol.*, 21: 46-49.
89. Schuettepelz, D.H. 1969. Fecal and total coliform tests in water quality evaluation. Research Report 42, Dept. of Natural Resources, Madison, Wisconsin.
90. Shipe, E.L., and G.M. Cameron. 1954. A comparison of the membrane filter with the most probable number method for coliform determinations from several waters. *Appl. Microbiol.*, 2: 85-88.
91. Shuval, H.I., J. Cohen, and R. Kolodney. 1973. Regrowth of coliforms and fecal coliforms in chlorinated wastewater effluent. *Water Res.*, 7: 537-546.
92. Skaliy, P., and R.G. Eagon. 1972. Effect of physiological age and state on survival of dessicated *Pseudomonas aeruginosa*. *Appl. Microbiol.*, 24: 763-767.
93. Slanetz, L.W., and C.H. Bartley. 1965. Survival of fecal streptococci in sea water. *Health Lab. Sci.*, 2: 142-148.
94. Smith, R.J., R.M. Twedt, and L.K. Flanigan. 1973. Relationships of indicator and pathogenic bacteria in stream waters. *J. Water Pollut. Contr. Fed.*, 45: 1736-1745.
95. Snedecor, G.W., and W.G. Cochran. 1967. Statistical methods, 4th ed., Iowa State University Press, Ames, Iowa.
96. Solorzano, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnology and Oceanography*, 14: 799-801.

97. Sorrells, K.M., M.L. Speck, and J.A. Warren. 1970. Pathogenicity of *Salmonella gallinarum* after metabolic injury by freezing. Appl. Microbiol., 19: 39-43.
98. Speck, M.L. 1970. Selective culture of spoilage and indicator organisms. J. Milk Food Technol., 33: 163-167.
99. Stersky, A.K., and T.I. Hedrick. 1972. Inhibition of growth of airborne coliforms and other bacteria on selective media. J. Milk Food Technol., 35: 156-162.
100. Straka, R.P., and J.L. Stokes. 1957. Rapid destruction of bacteria in commonly used diluents and its elimination. Appl. Microbiol., 5: 21-25.
101. _____, and _____. 1959. Metabolic injury to bacteria at low temperatures. J. Bacteriol., 78: 181-185.
102. Strange, R.E., and F.A. Dark. 1962. Effect of chilling on *Aerobacter aerogenes* in aqueous suspension. J. Genl. Microbiol., 29: 719-730.
103. Thomas, D.G. 1970. Exact confidence limits for the odds ratio in a 2 x 2 table. Appl. Statist., 20: 105-110.
104. Tomlins, R.I., and Z.J. Ordal. 1971. Requirements of *Salmonella typhimurium* for recovery from thermal injury. J. Bacteriol., 105: 512-518.
105. Van Donsel, D.J., E.E. Geldreich, and N.A. Clarke. 1967. Seasonal variations in survival of indicator bacteria in soil and their contribution to stormwater pollution. Appl. Microbiol., 15: 1362-1370.
106. van Soestbergen, A.A., and C.H. Lee. 1969. Pour or streak plates? Appl. Microbiol., 18: 1092-1093.
107. Wagenaar, R.O., and J.J. Jezeski. 1952. The influence of the type of diluent on the growth and survival of *Pseudomonas putrefaciens*. J. Dairy Science, 35: 738-754.
108. Wang, W.L.L., S.G. Dunlop, and R.G. DeBoer. 1956. The survival of *Shigella* in sewage. I. An effect of sewage and fecal suspensions on *Shigella flexneri*. Appl. Microbiol., 4: 34-38.

109. Wang, W.L.L., S.G. Dunlop, and P.S. Munson. 1966. Factors influencing the survival of *Shigella* in wastewater and irrigation water. J. Water Pollut. Contr. Fed., 38: 1775-1781.
110. Warseck, M., B. Ray, and M.L. Speck. 1973. Repair and enumeration of injured coliforms in frozen foods. Appl. Microbiol., 26: 919-924.
111. Weiler, W.A., and S.E. Hartsell. 1969. Diluent composition and the recovery of *Escherichia coli*. Appl. Microbiol., 18: 956-957.
112. Wheeler, J.M. 1906. The viability of *B. typhosus* under various conditions. J. Med. Res., 15: 269.
113. Wiley, B.N., and S.C. Westerberg. 1969. Survival of human pathogens in composted sewage. Appl. Microbiol., 18: 994-1001.
114. Will, L.A., S.L. Diesch, and B.S. Pomeroy. 1973. Survival of *Salmonella typhimurium* in animal manure disposal in a model oxidation ditch. Am. J. Public Health, 63: 322-326.
115. Won, W.D., and H. Ross. 1973. Persistence of virus and bacteria in sea water. J. Environmental Engineering Division, ASCE, 99: 205-211.

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