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 Periodically, samples were withdrawn from the chamber and enumerated using both non-selective and selective media.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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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	xi
ABSTRACT	xiv
Chapter	
1. INTRODUCTION	1
Statement of Purpose	2
2. LITERATURE REVIEW	4
Survival of Organisms in Water	4
Application of Dialysis Sacs and Membrane Filter Chambers to Survival Studies	14
Recovery of Injured Cells	19
Repair of Injured Cells	23
Injury as a Result of Laboratory Manipulation	23
Injury of Cells in the Aquatic Environment	26
Enrichment Methods to Improve Recovery of Bacteria from Aquatic Environments	28

Chapter		Page
3.	MATERIALS AND METHODS	32
	Source of Test Microorganisms and Physiological Identification	32
	Membrane Filter Chambers	35
	Preparation of Cell Suspensions	38
	Locations for Membrane Filter Chambers and Sampling Procedure	40
	Enumeration Procedures for Pure Cultures	41
	Coliforms	44
	Fecal streptococci	47
	Repair Experiments	48
	Enumeration Procedures for Raw Sewage	50
	Enrichment Procedures	52
	Effects of Wash Solutions and Diluents	53
	Calculations and Assumptions	54
	Chemical and Physical Procedures	55
	Statistical Methods	56
4.	RESULTS	57
	Physiological Identification of Pure Cultures	57
	Effects of Wash Solutions and Diluents	57
	Influence of Differences in Water Quality	61

Page

Comparison of Survival and Injury Characteristics of Indicator Organisms 75

Comparison of Recovery Efficiency of Various Enumeration Methods 81

Repair Experiments 96

Enrichment Experiments 103

Chemical and Physical Results 110

5. DISCUSSION 114

6. SUMMARY 141

APPENDIX 144

LITERATURE CITED 151

LIST OF TABLES

Table		Page
1.	Description and location of sites for membrane filter chambers	43
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	60
3.	Effect of regular phosphate, gelatin phosphate, and well water diluents upon death and injury to suspensions of *S. faecalis* MS304 in membrane filter chambers suspended in well water over a 24 h exposure period	62
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	63
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, EG6) over a 3 day exposure period	65
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	68
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	72

Table		Page
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	76
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	85
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	88
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	93
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	95
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	105
14.	Chemical and physical parameters for the 10 site locations	111
15.	Biochemical reactions of strains of *E. coli*, *E. aerogenes*, and *K. pneumoniae*	145
16.	Biochemical reactions of strains of fecal streptococci	146

Table		Page
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 . . .	147
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	148
19.	95% confidence limits for percentages of injury for separate suspensions of *S. faecalis* ES1913 treated with regular phosphate and gelatin phosphate diluents	149
20.	95% confidence limits for percentages of injury for separate suspensions of *E. coli* EC2075 treated with regular phosphate and gelatin phosphate diluents	150

LIST OF FIGURES

Figure		Page
1.	Schematic drawing of the membrane filter chamber	36
2.	Photograph of the membrane filter chamber	37
3.	Site locations for membrane filter chambers	42
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	58
5.	Comparative recovery of *E. coli* C320MP25 in membrane filter chambers located at sites BR2 and EG5 over a 4 day exposure period	70
6.	Comparative injury of *E. coli* C320MP25 in membrane filter chambers located at sites M3, M3A, H3, BR2, R1, EG4, EG5, and EG6 over a 4 day exposure period . .	73
7.	Comparative recovery of *S. faecalis* RS1009 in membrane filter chambers located at sites BR2 and EG5 over a 4 day exposure period	74
8.	Comparative injury of *S. faecalis* RS1009 in membrane filter chambers located at sites M3, M3A, H3, BR2, R1, EG4, EG5, and EG6 over a 4 day exposure period . .	77
9.	Comparative survival of *K. pneumoniae* 1881, *E. aerogenes* EC2072, *E. coli* C320MP25, *S. faecium* BS895, *S. faecalis* 1009, and *S. bovis* C3SE17 in membrane filter chambers over a 4 day exposure period located at site EG6	79
10.	Comparative injury of *K. pneumoniae* 1881, *E. aerogenes* EC2072, *E. coli* C320MP25, *S. faecium* BS895, *S. faecalis* 1009, and *S. bovis* C3SE17 in membrane filter chambers over a 4 day exposure period located at site EG6	80

Figure		Page
11.	Comparative recovery of *E. coli* C320MP25 in membrane filter chambers on TSY medium with MPN, pour plate, surface-overlay, and membrane filtration procedures over a 4 day exposure period	82
12.	Comparative recovery of *E. coli* C320MP25 in membrane filter chambers with LAC MPN, BGB MPN, DLA surface overlay plate, DLA pour plate, M-Endo MF membrane filtration, and M-FC membrane filtration procedures over a 4 day exposure period	84
13.	Comparative recovery of *S. faecalis* RS1009 in a membrane filter chamber on TSY medium with MPN, pour plate, and membrane filtration procedures over a 4 day exposure period	87
14.	Recovery of total coliforms, fecal coliforms, and fecal streptococci by membrane filtration methods from a suspension of raw sewage in a membrane filter chamber located at site EG6 over a 4 day exposure period	91
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	97
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	100
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	102
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	104

Figure | Page

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 108

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 109

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 Giaxa 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.

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*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, diffusable 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 *Paracolobactrum 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.

Andre 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 trypticase 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 1½ - 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 Fl, 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 cm^2; 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.

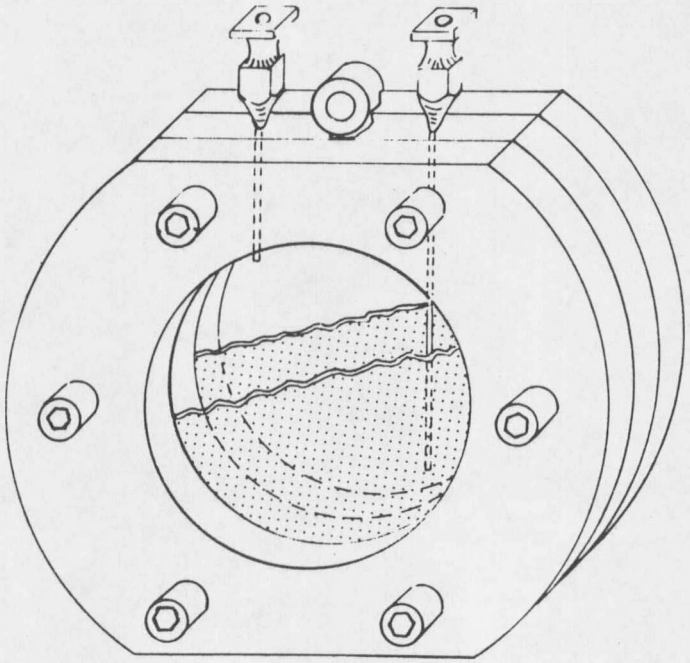

Figure 1. Schematic drawing of the membrane filter chamber.

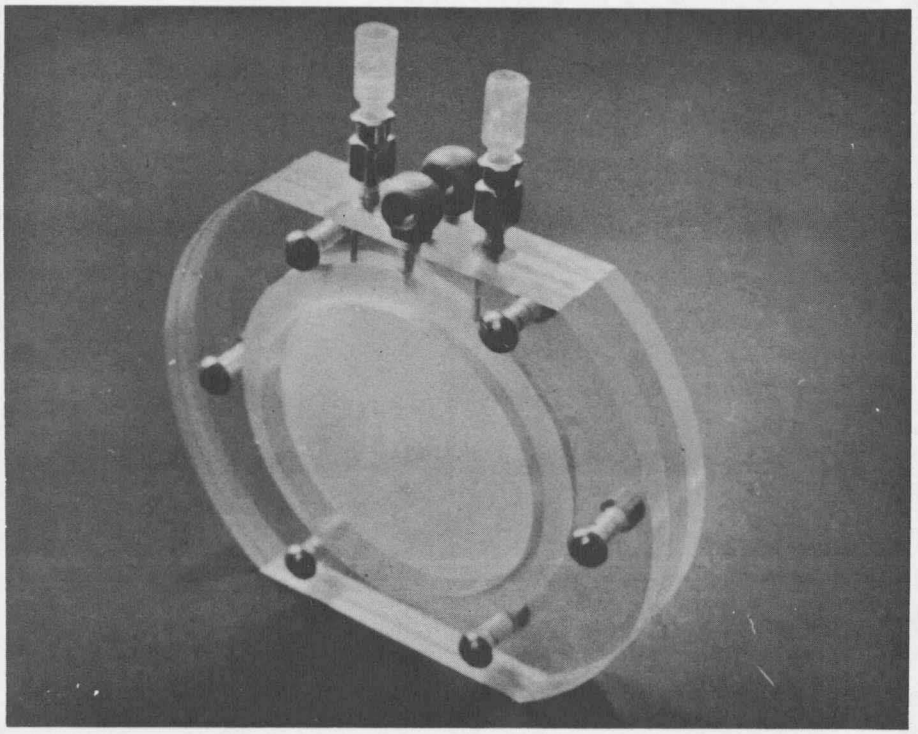

Figure 2. Photograph of a membrane filter chamber that is assembled and ready for use.

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

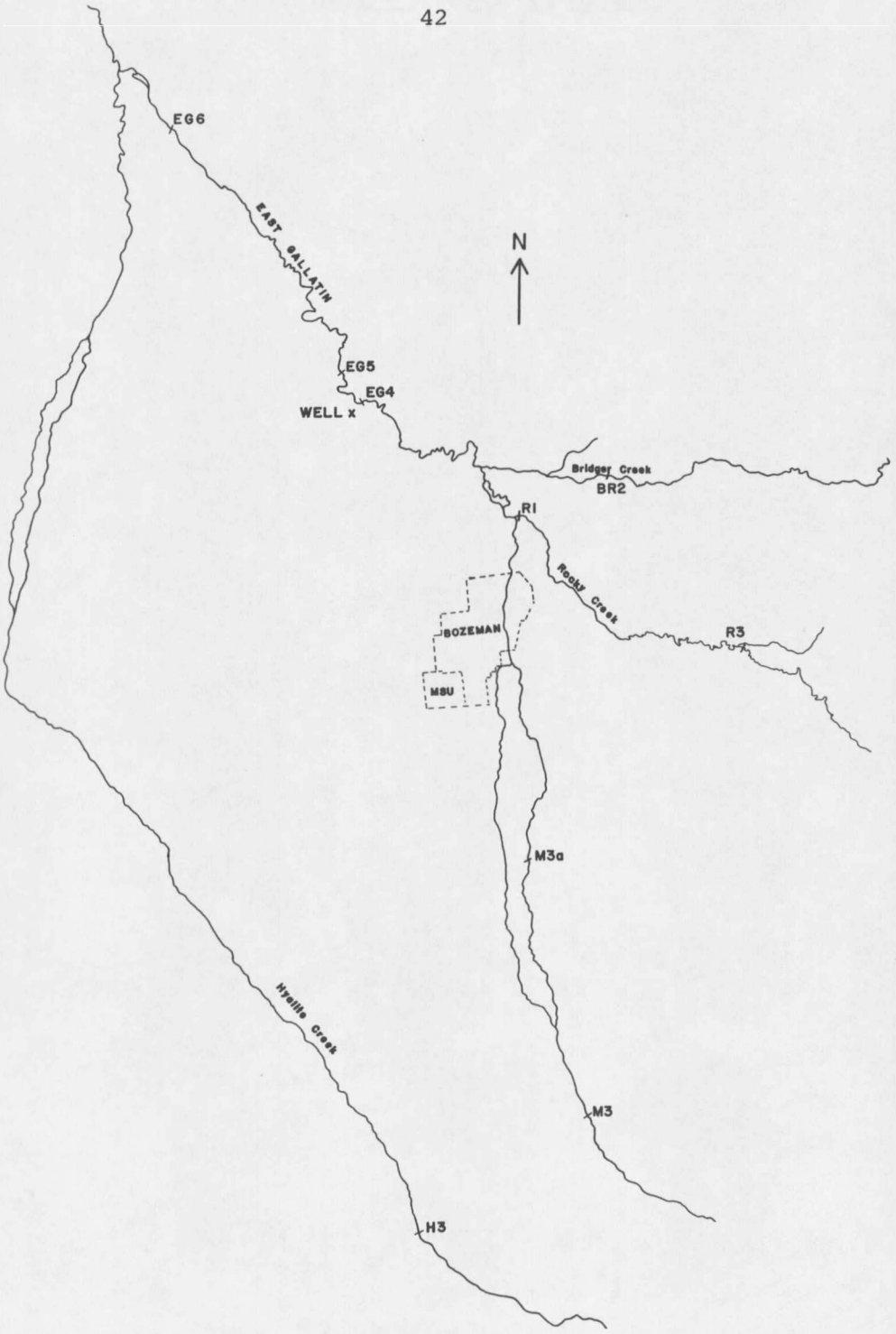

Figure 3. Site locations for membrane filter chambers.

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 prepoured 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% rosalic 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 triphenyl 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 - non-selective medium count at time t / non-selective medium count at 0-h) X 100; % survival at time t = 100% - % death at time t; and % injury at time t = (1 - selective medium count at time t / non-selective medium count at time t) X 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

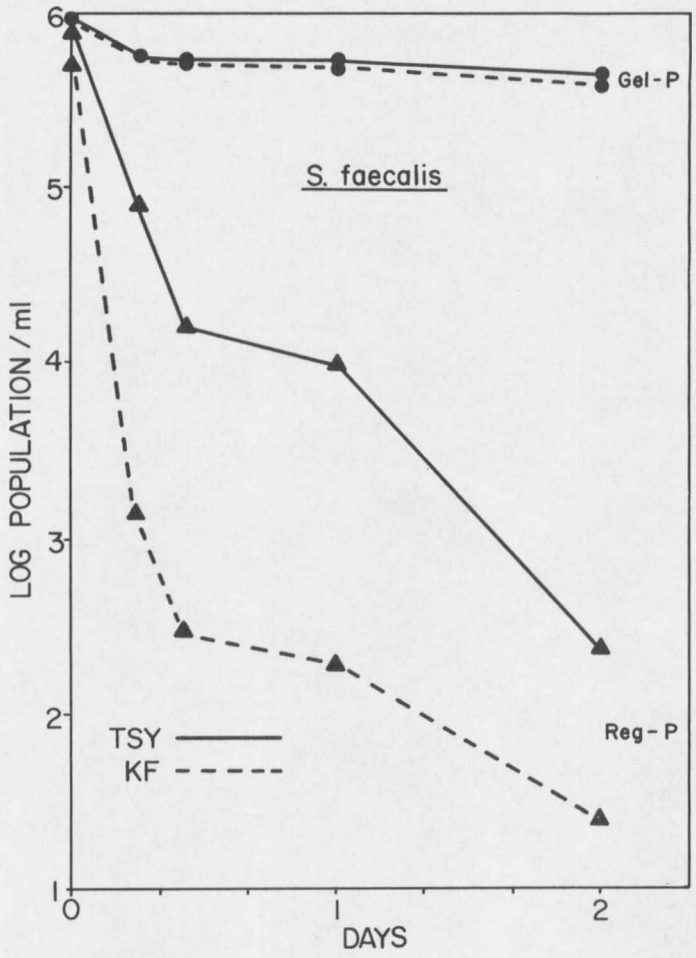

Figure 4. Effect of regular phosphate (▲) and gelatin phosphate (●) diluents upon recovery of suspensions of *S. faecalis* ES1913 in membrane filter chambers located in well water during a 48 h exposure period. Samples were pour plated using TSY (_____) and KF (- - - -) agar.

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)	*E. coli*			*K. pneumoniae*		
		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)	*S. faecalis* var. *liquefaciens*			*S. durans*		
		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 simulataneous 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

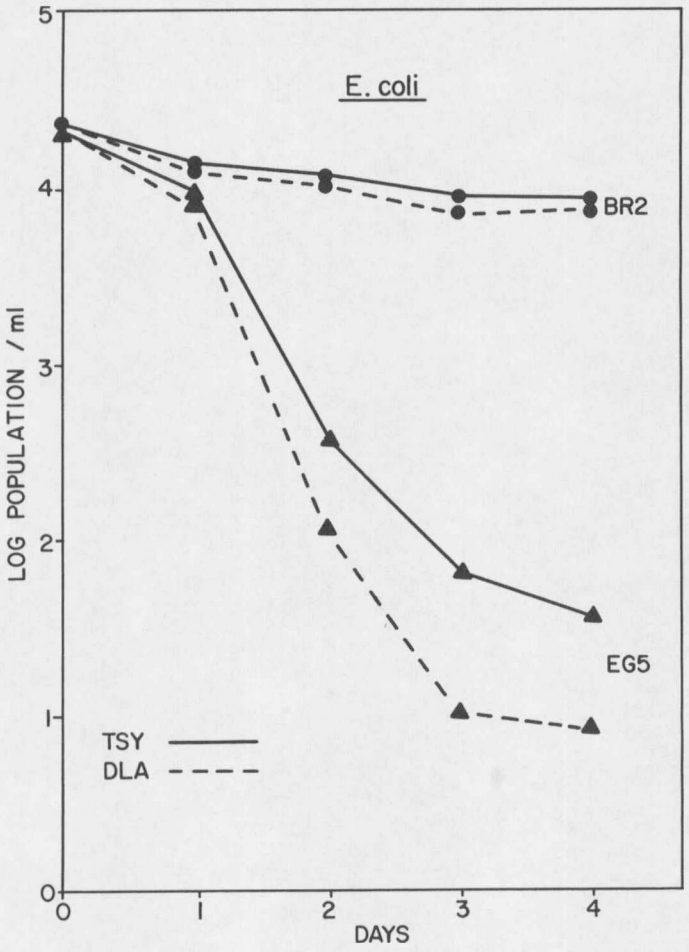

Figure 5. Comparative recovery of *E. coli* C320MP25 in membrane filter chambers located at sites BR2 (●) and EG5 (▲) over a 4 day exposure period. Samples were surface-overlay plated using TSY (_____) and DLA (- - - -) agar.

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	R1	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

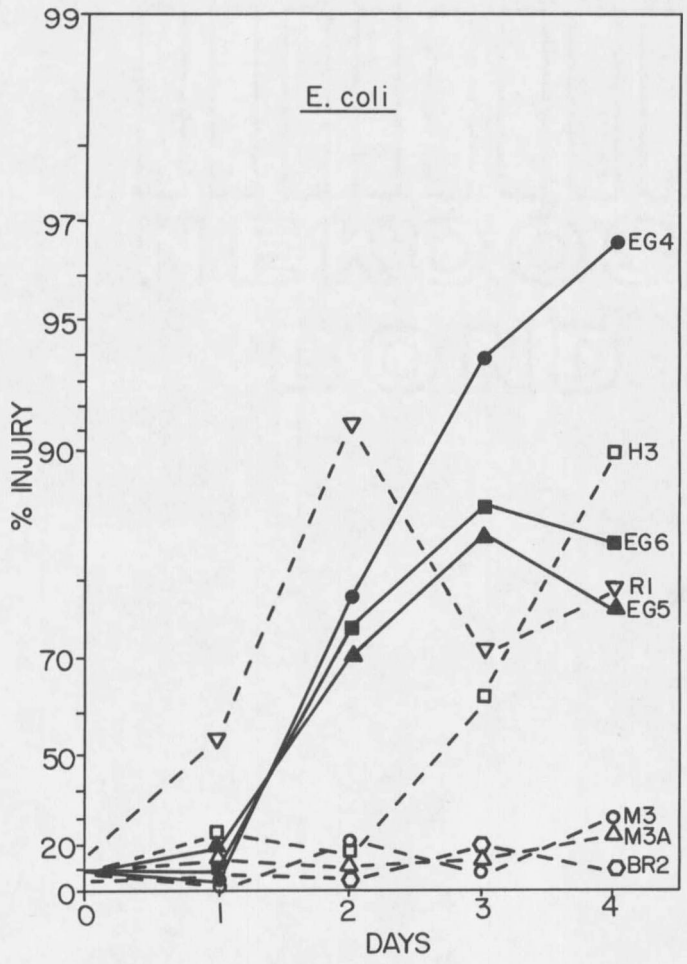

Figure 6. Comparative injury of *E. coli* C320MP25 in membrane filter chambers located at sites M3 (○), M3A (△), H3 (□), BR2 (⬡), R1 (▽), EG4 (●), EG5 (▲), and EG6 (■) during a 4 day exposure period. Calculations of injury to survivors were determined by differences between counts obtained on TSY and DLA surface-overlay plates.

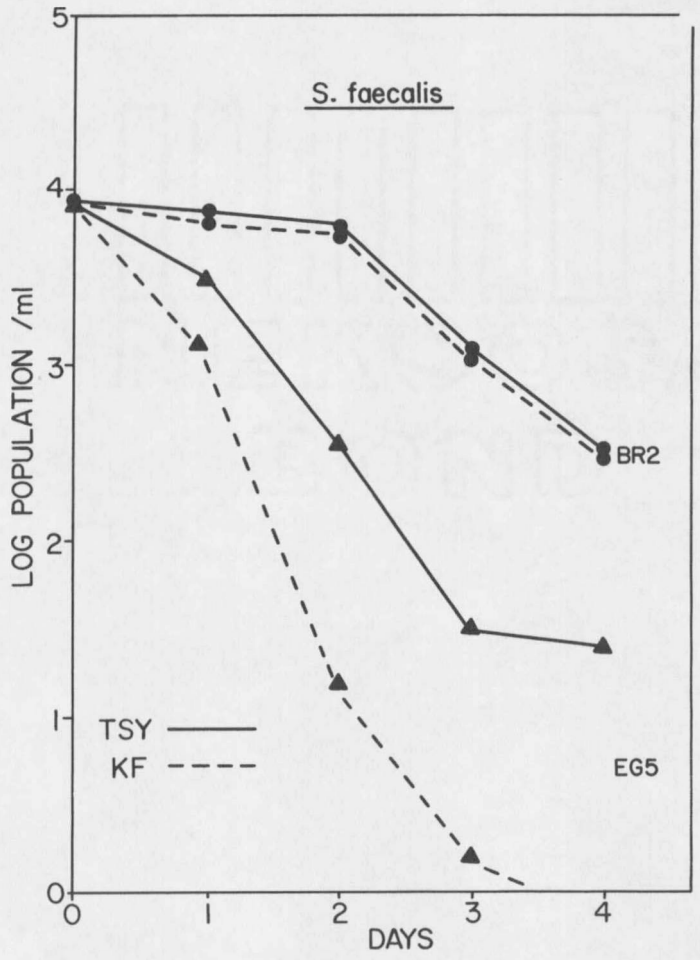

Figure 7. Comparative recovery of *S. faecalis* RS1009 in membrane filter chambers located at sites BR2 (●) and EG5 (▲) during a 4 day exposure period. Samples were pour plated using TSY (_____) and KF (- - - -) agar.

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	R1	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

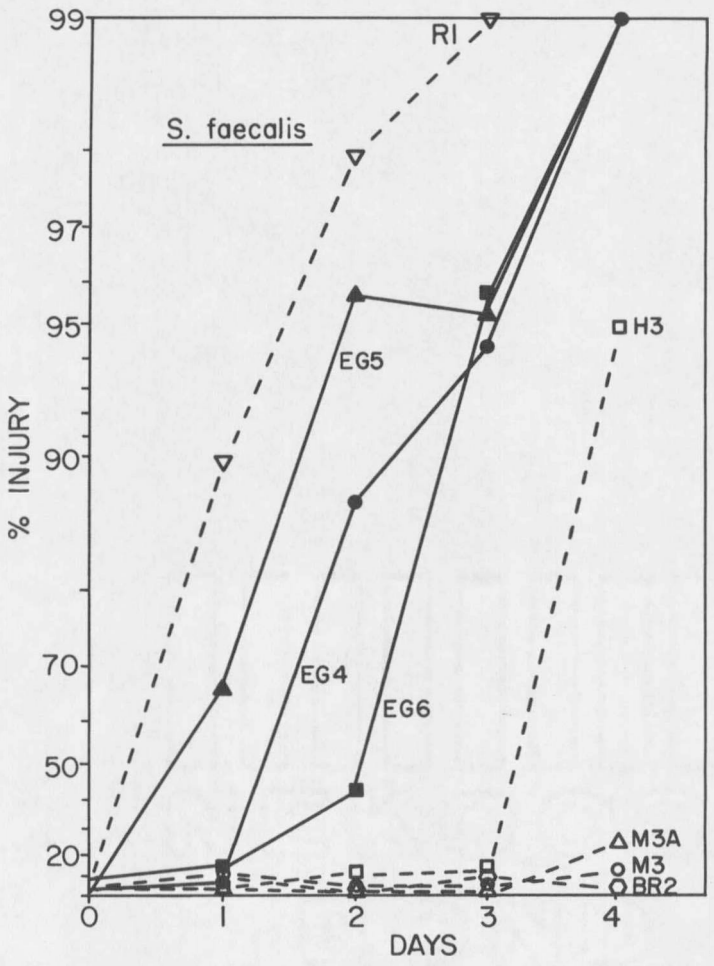

Figure 8. Comparative injury of *S. faecalis* RS1009 in membrane filter chambers located at sites M3 (O), M3A (Δ), H3 (□), BR2 (⬡), R1 (▽), EG4 (●), EG5 (▲), and EG6 (■) during a 4 day exposure period. Calculations of injury to survivors were determined by differences between counts on TSY and KF pour 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

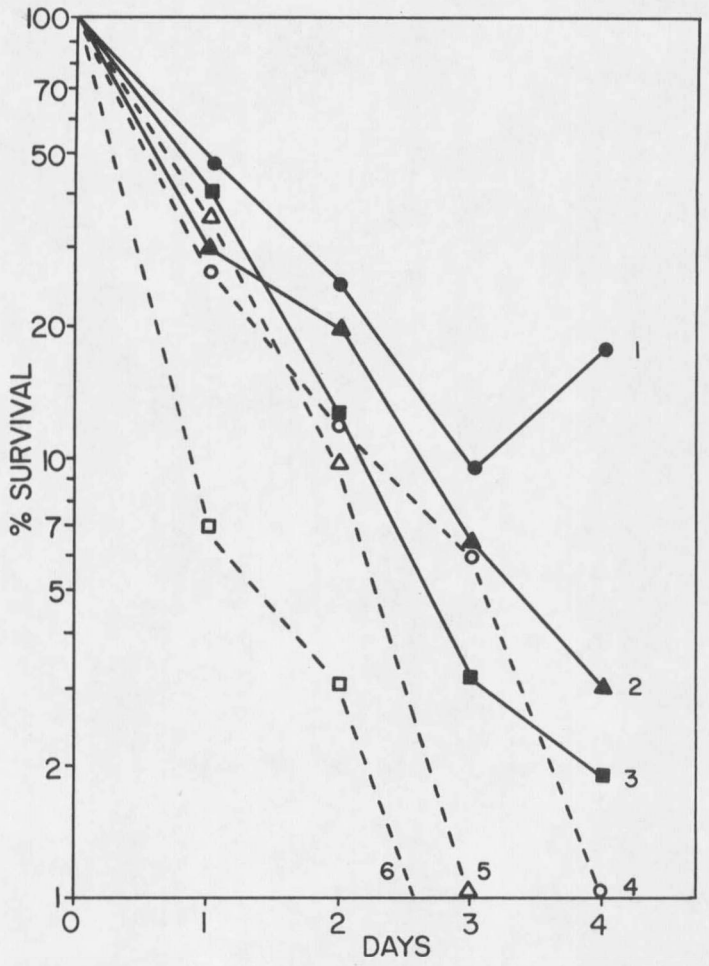

Figure 9. Comparative survival of *K. pneumoniae* 1881 (1, ●), *E. aerogenes* EC2072 (2,▲), *E. coli* C320MP25 (3,■), *S. faecium* BS895 (4, O), *S. faecalis* RS1009 (5,Δ), and *S. bovis* C3SE17 (6,□) in membrane filter chambers during a 4 day exposure period located at site EG6. Calculations of survival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

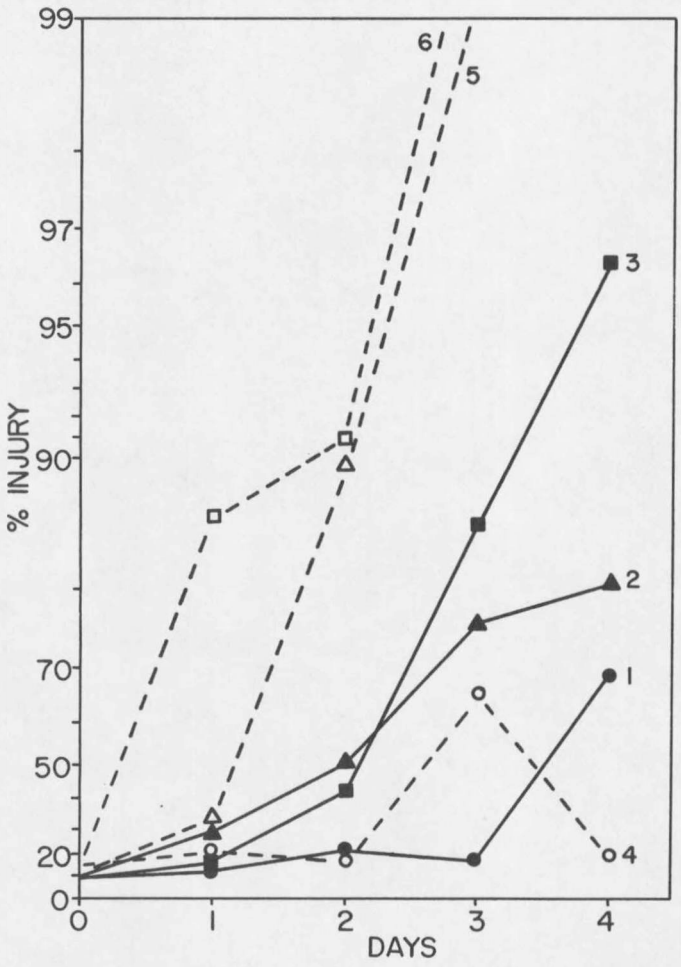

Figure 10. Comparative injury of *K. pneumoniae* 1881 (1, ●), *E. aerogenes* EC2072 (2, ▲), *E. coli* C320MP25 (3, ■), *S. faecium* BS895 (4, ○), *S. faecalis* RS1009 (5, △), and *S. bovis* C3SE17 (6, □) in membrane filter chambers over a 4 day exposure period located at site EG6. Calculations of injury 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