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A study of the survival of Escherichia coli and Streptococcus faecalis in water

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A STUDY OF THE SURVIVAL OF *ESCHERICHIA COLI* AND *STREPTOCOCCUS FAECALIS* IN WATER

By

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CHAPTER I

INTRODUCTION

Ideally, microorganisms used as indicators of fecal pollution of natural waters should be present whenever the presence of pathogens is an imminent danger, should not be able to proliferate to any greater degree than the pathogens in the aqueous environment, should be easily recoverable using relatively simple techniques, and should be able to grow independently of other microorganisms present (Bonde, 1966).

Traditionally the coliform group of bacteria has been accepted as the most reliable indicator of fecal pollution. According to Standard Methods for the Examination of Water and Wastewater (1960) the coliform group includes all of the aerobic and facultative anaerobic, Gram-negative, nonsporeforming, rod-shaped bacilli which ferment lactose with gas formation within 48 hours at 35 C.

With the advent, however, of relatively simple and reliable media and techniques for the recovery of the fecal streptococci (Litsky et al., 1953; Slanetz and Bartley, 1957) the use of coliforms as indicator organisms has been re-evaluated. Primary studies have indicated that the fecal streptococci may be better indicators of recent and dangerous pollution.

The ubiquitous sources of coliforms in nature suggest they are able to multiply for unlimited time periods under a wide variety of environmental conditions independent of an animal host.

Studies on the survival of the coliform group in surface waters

have indicated that Escherichia coli is able to survive for long periods of time in an aqueous environment, and may not be indicative of recent pollution. Results of Hendricks and Morrison (1967) suggest that E. coli and Aerobacter aerogenes are able to grow and multiply in dilute nutrient (river bottom sediment), and at low temperatures (environments representative of a cold mountain stream). Niemela and Tirronen (1967) attributed the occurrence of high coliform/enterococci (C/F) ratios during periods of high temperatures and high water levels to the multiplication of coliforms in the river.

Comparisons of survival of enteric bacteria in soil also indicate that coliforms may withstand environmental conditions for long periods of time. Soil runoff from such areas may contribute to bacterial counts and cause errors in sanitational surveys of neighboring water sources (Van Donsel et al., 1967). Bergner-Rabinowitz (1955) found, in soil irrigated once with sewage during the winter, the coliform count maintained a stable low-level population during a period of 70 days. Litsky and Mallmann (1951) reported that coliforms were able to survive up to 3 years and 7 months in various types of soils, whereas fecal streptococci died out within a period of 11 weeks, after one initial seeding. In a similar experiment, Salmonella typhosa survived for only 19 days.

Coli-aerogenes bacteria have been isolated from soils free of fecal contamination by Geldreich et al. (1962) and Randall (1956). In a study of 369 undisturbed soil samples adjacent to water supply reservoirs, Medrek and Litsky (1960) isolated coliform bacteria from 73.4% of the samples, and isolated enterococci from only 2.2% of the

same samples. Van Donsel et al. (1967) reported evidence of "after-growth" of fecal and nonfecal coliforms in the soil as a result of temperature and rainfall variations.

Another objection to the use of total coliform counts as pollution indicators has been that not all of the species originate from fecal sources (Geldreich et al., 1962; Slanetz and Bartley, 1964; Bonde, 1966; Gallagher and Spino, 1968). Kabler and Clark (1961) have divided the coliforms into three groups on the basis of their most probable origin in nature: the Escherichia coli group, indicative of recent fecal contamination; the intermediate-aerogenes-cloacae (I.A.C.) group which originates from nonfecal sources, and has a longer survival time than the fecal coliforms; the Aerobacter aerogenes and cloacae group, which has been isolated from various types of vegetation. All of the above may be present in surface waters and may yield similar laboratory results.

Studies by Glantz and Krantz (1965) and Geldreich and Clark (1966) indicate E. coli is able to survive and multiply in the fish intestine when water temperatures are 20 C or higher. They have suggested that fish may influence the coliform index of their habitat by acting as conveyors of microorganisms.

Hajna and Perry (1942) found that the absence of enterococci appeared to offer a wider margin of safety in swimming waters because they were more resistant to chlorination than coliform bacteria.

Gallagher and Spino (1968) found little correlation between levels of total or fecal coliforms and the isolation of an enteric pathogen, Salmonella typhimurium. Data indicated that rates of growth and

"die-off" of both species are different with regard to changes in temperature and nutritional conditions in the environment. During winter and early spring large reductions in fecal coliforms were accompanied by very little reduction in the potential pathogen (in this case, Salmonella).

The enterococci include Streptococcus faecalis (var. zymogenes and liquefaciens) and S. durans (Sherman, 1938). S. bovis and S. equinuus, the predominate species in fecal material of cattle and horses, and S. faecium are also classified as enterococci in the literature (Shattock, 1949; Deibel et al., 1963).

Litsky et al. (1953), Clarke et al. (1960), Raibaud et al. (1961), Mundt (1962), Rogers and Sarles (1964), and Maki and Picard (1965) have reported the presence of the fecal streptococci (enterococci) in large numbers in the gastrointestinal tract and feces of a wide variety of wild and domestic mammals and fowl, as well as in human feces.

In studies by Winter and Sandholzer (1946), Kjellander (1960), Slanetz and Bartley (1964) and Bonde (1966) enterococci were always present in water contaminated by sewage and almost virtually absent from water free from fecal contamination.

Studies of C/F ratios in fresh and sea waters, and sewage show that under most environmental conditions, coliforms are present in much greater numbers and may persist for greater distances from a source of pollution than enterococci (Winter and Sandholzer, 1946; Walter and Bottman, 1967). Investigators such as Litsky et al. (1953), Kjellander (1960), and Slanetz and Bartley (1964) have concluded that high C/F ratios in water and sewage suggest that enterococci are better indicators of recent pollution because they are not able to survive in nature once

released from the mammalian digestive tract.

Others have reported data which contradict this conclusion. Bergner-Rabinowitz (1955) found that enterococci and coliforms persisted in soil samples for similar periods of time. Both enterococci and coliforms survived at similar population levels up to 38 days.

Geldreich et al. (1964) found that 646 streptococcus cultures isolated from plants and 226 streptococcus cultures isolated from insects were predominantly S. faecalis.

It has also been reported that enterococci are able to establish an epiphytic relationship with various kinds of vegetation, (Mundt et al., 1958; Eaves and Mundt, 1960; Mundt et al., 1962; Mundt, 1964). In studies of the growth of enterococci on plants, Mundt and associates (Mundt et al., 1958; Mundt, 1961; Mundt, 1962; Mundt et al., 1962) showed that the fecal streptococci may be regarded as temporary residents on plants. They are capable of limited reproduction on plant parts and are disseminated among plants and spread to soil by the action of insects, wind, gravity, and rain.

High C/F ratios in waters contaminated with feces and the absence of enterococci in water free from fecal contamination suggest that the fecal streptococci may be a more significant index of recent pollution, because they are unable to survive in natural waters for long periods of time. Yet, the literature indicates the enterococci occur independent of the mammalian digestive tract in soil and as epiphytic and transient residents on plants and insects.

Niemela and Tirronen (1967) have suggested several reasons for high C/F ratios in water, among which is the net effect of death/multiplica-

tion of the two groups varying with environmental factors such as water temperature.

In nature microorganisms normally occur in mixed populations. The population equilibrium which results may be influenced by two sets of interacting factors. Physio-chemical limitations such as temperature, pH, and nutrient supply may alter population levels.

Nutrient availability in a limited environment has been studied by Klein and Casida (1967). They found that protection against die-out of E. coli in soil, acheived by the addition of a small portion of autoclaved soil to normal soil was associated with organic carbon (glucose) availability. These authors concluded that the inability of E. coli to step down its metabolic rate to meet the low availability of usuable nutrients is a major factor in low population levels of the organism in natural soils.

Bacterial competition for available nutrients has been shown to affect the population equilibrium of mixed cultures. Barnes (1931), Lockhart and Powelson (1953), and Mossel and Ingram (1955) described significant inhibition of test cultures caused by exhaustion of nutrients by an associated bacterial species. Dagley et al. (1952) and Charlton (1955) reported inhibition of Aerobacter strains in mixed bacterial populations caused by competition for gaseous nutrients.

Inhibition of Shigella flexneri by E. coli, Aerobacter aerogenes, and Proteus vulgaris due to competition for fermentable carbon sources under reduced conditions has been described by Freter (1962) and Hentges and Freter (1962).

Repression of Staphylococcus aureus by other food bacteria was

attributed to competition for available nutrients by Troller and Frazier (1963) and Iandolo et al. (1965).

Gibson (1957) reported pronounced microbial population changes when the pH of the rumen of sheep was lowered by indigestion due to a change in diet. Numbers of protozoa were greatly reduced while S. bovis numbers increased. Cuthbert et al. (1955) cited pH as a factor governing the survival of Bacterium coli and S. faecalis in soil. Both organisms were able to persist for several weeks in limestone soils, but died out rapidly within a few days in peat soils. Goodman (1965) reported pH as a factor governing the population equilibrium between two bacterial cultures isolated from apple buds.

Peterson et al. (1962) found that the predominant bacterial population in thawing food can be varied by changing the temperature of defrost.

In a study of the influence of systematic alterations in the environment, Hentges and Fulton (1964) found that the population equilibrium of Klebsiella and Shigella could be altered by changes in temperature, pH, aeration, and concentration of nutrient.

The associative effects of microbial interactions, both competitive, and antagonistic, among all individuals or individual species may also alter or control the population equilibrium in a specific biological niche.

Interactions among soil microorganisms have been studied by Mitchell and Alexander (1963). They reported that a strain of Bacillus cereus isolated from soil was capable of lysing Fusarium oxysporium cells. Mukerji (1968), in a study of the mutual relationships among

soil microorganisms, reported that species of Chaetomium are antagonistic towards other fungi. Hattingh and Louw (1969) found that, out of 1091 bacteria isolated from the rhizoplane of inoculated pasture clovers, 83 isolates (most of which belonged to the genus, Pseudomonas) inhibited the growth of Rhizobium trifolii. Chatterjee and Gibbons (1969) cited antagonism of Erwinia amylovora by Erwinia herbicola, isolated from apple buds.

Changes in the population equilibrium by microbial interaction in vivo have been reported of S. flexneri by other intestinal bacteria (Freter, 1962; Hentges and Freter, 1962), of bacteria by Herpes simplex virus (Schneierson, 1963), and of Salmonella typhimurium by Proteus mirabilis (Greenberg, 1969).

Mechanisms of bacterial antagonism have been studied in vitro. Von Wikullil (1932) using Bail's (1929) concept of "M-concentration" developed the theory of space antagonism to explain changes of population dynamics due to inhibition of one bacterial species by another. He found that when two organisms that possessed different M-concentrations (maximum number of cells which may be produced by a bacterial species in a particular biological space), were grown together in a broth medium, the final population was made up almost entirely of the species with the largest M-concentration, because it grew more rapidly, and consequently utilized a greater proportion of the available space.

In 1934 Nuefeld and Kuhn originated the concept of "direct antagonism" in which close contact was essential between living cells of the antagonist and those of the inhibited species. Subsequent studies of bacterial antagonism by Aerobacter strains (Wynne, 1947; Bowling and

Wynne, 1951) and by Gaffkya tetragena of test strains including Escherichia and Streptococcus species (Frankel and Wynne, 1951) have substantiated this theory.

Gratia's discovery (1925) of an antibiotically active E. coli strain has stimulated interest in the role of antibiotic producing bacteria in microbial antagonism. Gratia and Fredericq (1947) and Fredericq and Levine (1947) cited representative test species from almost all members of the Enterobacteriaciae which were sensitive to antibiotic substances (colicins) produced by Escherichia strains. Wynne and Norman (1953) reported that certain strains of Aerobacter and Gaffkya were capable of producing antibiotic substances. In 1954 Levine and Tanimoto isolated Escherichia strains from feces, streams, and sea water which possessed a wide antibiotic spectrum against other Gram-negative bacteria.

In a study of mixed cultures of Shigella paradysenteriae and Escherichia coli that produced peritoneal infections in adult mice, Friedman and Halbert (1960) reported that an antibiotic producing E. coli caused a reduction in shigellae. Concomitantly there was in vivo secretion of the antibiotic.

Antibiotic production by anaerobic soil bacteria against common aerobic soil organisms was reported by Sturgen and Casida (1962).

Bacterial strains capable of producing staphylolytic enzymes have been reported by Zysking et al. (1965), Coles and Gilbo (1967) and Burke and Pattee (1967).

Metabolic by-products released into the growth media have been listed as the cause of inhibition of Bacillus polymyxa by Proteus vulgaris

(Yeoh et al., 1968), of Clostridium, Bacillus, and Lactobacillus species by enterococci (Kafel and Ayres, 1969), and of Shigella flexneri by fecal coliforms (Hentges, 1969).

Managan (1969) attributed the inhibition of germination of Aspergillus terreus spores to pigment production by Pseudomonas aeruginosa.

In a study of interactive phenomena among microorganisms indigenous to man, Rosebury et al. (1954) found that mutual inhibition between Streptococcus mitis and either Streptococcus pyogenes or S. faecalis was dependent on the relative concentration of each species in the paired association.

In a study of the suppression of Salmonella typhimurium in various areas of the digestive tract of the blow fly, Calliphora vicinia by known populations of bacteria, Greenberg (1969) found that the equilibrium among the bacterial populations depended on the number of bacterial species involved in the interaction. In dibiotic interactions (two different species) Salmonella dominated S. faecalis and was dominated by Proteus mirabilis. When a third organism was added to the interaction (tribiotic infection) a synergistic suppression of S. typhimurium occurred.

A variety of these same factors have been studied in relationship to the survival of E. coli in sea water. Rosenfeld and Zobell (1947), Vaccaro et al. (1950), and Mitchell (1968) isolated marine microorganisms antibiotically active against E. coli.

Mitchell et al. (1967) suggested that lysis of E. coli in water may be due in part to indigenous microflora in the sea, specifically

Bdellovibrio.

Carlucci and Pramer (1959, 1960) and Mitchell (1968) reported that die-off of E. coli in a marine environment may be due to physiochemical factors such as salinity, pH, and nutrient availability. Seasonal fluxuation of E. coli populations due to the lethal effect of sunlight was reported by Gameson and Saxon (1967). Halton and Nehlsen (1968) have found that low seawater temperatures favor the survival of large numbers of E. coli.

Little is known, however, about the effect of environmental and bacterial interactive phenomena on the survival of E. coli and the enterococci in fresh water. Niemela and Tirronen (1967) and Gallagher and Spino (1968) have shown that seasonal variations of enterococcus and coliform numbers do occur in a fresh water environment. The exact factors causing these variations in population levels have yet to be elucidated.

STATEMENT OF THE PROBLEM

Although the fecal streptococci and Escherichia coli are commonly used as indicator organisms of water pollution, very little is known about the influence of environmental factors on the survival of these organisms in fresh waters once they have been released from the mammalian intestinal tract.

This investigation consisted of a study of the effects of selected environmental conditions on the survival of E. coli and Streptococcus faecalis in pure and mixed culture in fresh water. The effects of variations in the environment on the population equilibrium of E. coli and S. faecalis in mixed culture were also studied.

CHAPTER II

METHODS AND MATERIALS

I. General Methods and Materials

(1) Bacterial Strains Used

Escherichia coli B (stock culture, University of Montana) and Streptococcus faecalis, obtained from Bayliss, Montana State University, Bozeman, were used throughout this study. Stock cultures were maintained on Stock Culture Agar (Difco).

Stock cultures were transfered approximately once every two months by inoculating a 125 ml flask containing 50 ml Brain Heart Infusion Broth (Difco) with the organism being transfered. After incubating each flask at 37 C for 24 hours Gram stained preparations were made of each broth culture to check for purity. A loopful of each flask culture was then streaked onto a fresh Stock Culture Agar slant, incubated for 24 hours at 37 C, and stored at room temperature.

(2) Routine Media

Stock cultures were maintained on Stock Culture Agar (Difco) slants. Brain Heart Infusion Broth (Difco) was used for growth of inoculum cells, and for growth of large numbers of cells during transfer of stock cultures to fresh stock culture agar slants.

Water obtained from the Rattlesnake Creek, in a closed watershed approximately one mile above a city reservoir, Missoula, Montana, was used as the menstruum for the survival studies. The water was collected in a sterile, five liter, screw top, glass jar at various times during

a two year period, by immersion, mouth upstream, in an area of rapidly running water. The water was stored at 10 C. Each five liter volume was stored for approximately three days before use in order to allow the debris to settle. All water used as culture media was sterilized in a Seitz Bacteriological Filter (air pressure filter), model F 1540, before use.

For each survival study culture samples were plated on Plate Count Agar (Difco) to obtain viable cell counts. Samples were also plated on M-Enterococcus Agar (Difco), a selective medium for the enumeration of S. faecalis in mixed culture, and on Endo Agar (Difco), a differential medium for the enumeration of E. coli in mixed culture. All plates were dried in a 37 C incubator for 24 hours before use, and stored at 10 C.

(3) Inoculum

To insure an inoculum that would give reproducible results, separate screw cap tubes containing 7 ml of Brain Heart Infusion Broth (Difco) were inoculated with a loopful of E. coli and S. faecalis, respectively, and incubated for 12 hours at 37 C. A one ml aliquot of each culture was then transfered to a second tube of Brain Heart Infusion Broth and reincubated for 12 hours at 37 C. One ml aliquots of each culture were then serially diluted in 9 ml sterile water blanks to a dilution of 10^{-8}. One ml aliquots of the 10^{-7} and 10^{-8} dilutions were plated on Plate Count Agar, M-Enterococcus Agar and Endo Agar to determine the number of cells of each organism per ml of BHI broth after 12 hours incubation at 37 C.

This procedure was repeated three times. Counts obtained for

each organism were averaged to find the approximate number of cells per ml (see Table 1). These averages were used as standard values when preparing inocula for each survival study.

The inoculum for each survival test was prepared in the following manner. A screw cap tube containing 7 ml of BHI broth was inoculated with a loopful of the stock culture and incubated for 12 hours at 37 C. One ml of each culture was then transfered to a fresh tube of BHI broth and reincubated for 12 hours at 37 C.

Inoculum cells were harvested by transfering one ml aliquots of each BHI culture to sterile centrifuge tubes, covered with aluminum foil. The samples were centrifuged in a Servall Angle Centrifuge at 6000 R.P.M. for 20 minutes. The supernatant fluid was removed aseptically with sterile capillary pipettes. Cells were resuspended in 2 ml of sterile saline and recentrifuged for 20 minutes at 6000 R.P.M. The supernatant was again removed with sterile capillary pipettes, and cells were resuspended in 1 ml aliquots of the survival test culture medium.

For each survival study three 250 ml Erlenmyer flasks containing 200 ml sterile survival test medium were inoculated so that the final concentration of cells in each flask was similar to that listed in Table 2.

(4) Plating Methods

Viable cell counts were made by plating dilutions of the flask cultures at 0, 12, 24, 36, 48, and 72 hours, and subsequent varied intervals (determined by length of survival time under varied growth conditions) up to 480 hours (20 days).

Samples were plated on Plate Count Agar, M-Enterococcus Agar and

TABLE 1. Number of viable cells per ml of a 12 hour culture in BHI broth

Trial	*S. faecalis**	*E. coli**
1	2.50×10^9	4.70×10^8
2	2.20×10^9	4.90×10^8
3	2.69×10^9	5.20×10^8
Average No. viable cells/ml	2.46×10^9	4.96×10^8

*Represents only counts of 30-300 colonies per plate.

TABLE 2. Final concentration of cells in flask cultures after addition of inoculum

Flask	Organism	Final Concentration (No. cells/ml)
A	*S. faecalis*	2×10^6
	E. coli	none
B	*S. faecalis*	none
	E. coli	2×10^6
C	*S. faecalis*	2×10^6
	E. coli	2×10^6

Endo Agar by spreading 0.1 ml aliquots of the desired dilution on the agar surface with a sterile bent glass rod. All plates were incubated at 37 C for 24 to 36 hours. Plate counts were made with the aid of a Quebec colony counter.

All dilutions were made by adding one ml aliquots of the flask culture to a tube containing 9 ml of sterile distilled water and diluting to the desired dilution by making successive decimal dilutions in sterile 9 ml water blanks.

The number of viable cells/ml of the original culture (undiluted) was recorded for each time period for plate counts of values of 30-300. Survival curves were made by plotting the age of the sample against the number of viable cells/ml of each organism.

All colonies growing on the M-Enterococcus Agar were assumed to be S. faecalis, and all metallic, red colonies growing on the Endo Agar were assumed to be E. coli. Since both bacteria grew on the Plate Count Agar, colonies plated from the mixed cultures were easily distinguished on the basis of colonial characteristics, such as color, size marginal chape, and opacity. Counts obtained on Plate Count Agar were compared to counts obtained on the selective media to test for accuracy of counting methods.

The pH of the survival test culture medium of each flask was measured at the onset and the termination of each survival study in which a buffering system was used to insure a constant pH throughout the period of incubation. The pH of each flask remained constant $\pm$ 0.1 during the experimental procedures.

II. Environmental Conditions

(1) Standard Conditions

Under standard control conditions, the organisms were maintained in sterile, nutrient deficient, potassium phosphate buffer, pH 7.0, in a 16 C General Electric, Precision-Scientific air incubator, model 805.

(2) Temperature Alterations

Cultures maintained at 37 C were incubated in a Precision-Thelco air incubator, model 6; 10 C cultures were incubated in a General Electric Precision-Scientific air incubator, model 805; 4 C cultures were incubated in a General Motors Frigidaire air incubator; and 27 C cultures were incubated at room temperature in a closed drawer. Cultures were removed from incubators periodically for 5-10 minute intervals to remove samples to obtain viable cell counts.

(3) pH Alterations

Potassium phosphate buffer was used as a basal medium. The pH was altered by preparing the buffer according to Table 3.

All pH measurements were made with a Coleman Metrion IV pH meter. All buffer solutions were filter-sterilized in a Seitz Bacteriological Filter, model F 1540.

To insure that the buffer solution remained at a constant pH under different temperatures of incubation when bacterial cells were added, test flasks containing 200 ml buffer, pH 6.0, were inoculated with 4×10^8 S. faecalis cells and 4×10^8 E. coli cells so that the final concentration of each flask was 4×10^6 cells/ml. Flasks were incubated at 37 C, 27 C, and 10 C. The pH of the buffer remained relatively constant irrespective of temperature and inoculum during the 72 hour

TABLE 3. Composition of Potassium Phosphate Buffer

pH	Solution A (ml)*	+	Solution B (ml)**	+	H_2O (ml)***
6.0	87.7		12.3		100
7.0	39.0		61.0		100
8.0	5.3		94.7		100

*0.2 M solution of monobasic potassium phosphate (27.2 g KH_2PO_4 in 1000 ml water from the Rattlesnake Creek).

**0.2 M solution of dibasic potassium phosphate (45.75 g $K_2HPO_4 \cdot 3H_2O$ in 1000 ml water from the Rattlesnake Creek).

***Water from the Rattlesnake Creek.

period (see Table 4).

(4) Aeration Alterations

(a) Increased Aeration

Air from an air outlet in the laboratory was bubbled into each culture flask continuously for 480 hours. The air was sterilized by passing it through a fiberglas filter (see Fig. I).

10 C was used as the standard temperature of incubation instead of 16 C because of difficulties encountered when trying to aerate three flasks in a closed incubator. The temperature was maintained at approximately 10 C by incubating the culture flasks in an open water bath. Tap water was continuously run ito the water bath and allowed to overflow for the duration of the incubation period. The temperature was measured every 24 hours.

Depth of the water bath was approximately half the height of the culture flasks to prevent contamination of the cultures due to overflow of the water bath. A loose cover of aluminum foil was placed over the apparatus to minimize any effects on the survival of test bacteria by direct sunlight.

The air filter was constructed by stuffing a one liter, side arm filtering flask with pyrex wool filtering fibre. A rubber stopper fitted with a glass tube which protruded down into the fiberglas was used to stopper the flask. A piece of rubber tubing was connected to the outside end of the glass tube, and sealed at the open end with sluminum foil. The flask side arm was also stuffed with pyrex wool fibre and sealed with aluminum foil. A strip of aluminum foil was also wrapped around the top of the flask and the stopper.

TABLE 4. Ability of Potassium Phosphate Buffer* to maintain a constant pH

Hour	pH 37 C	25 C	10 C
0	6.0	6.0	6.0
12	6.1	6.0	6.1
24	6.1	6.0	6.0
36	6.1	6.0	6.0
48	6.0	5.9	6.0
60	6.0	5.9	5.9
72	6.0	5.9	6.0

*Containing 2 x 10^6 S. faecalis cells/ml and 2 x 10^6 E. coli cells/ml.

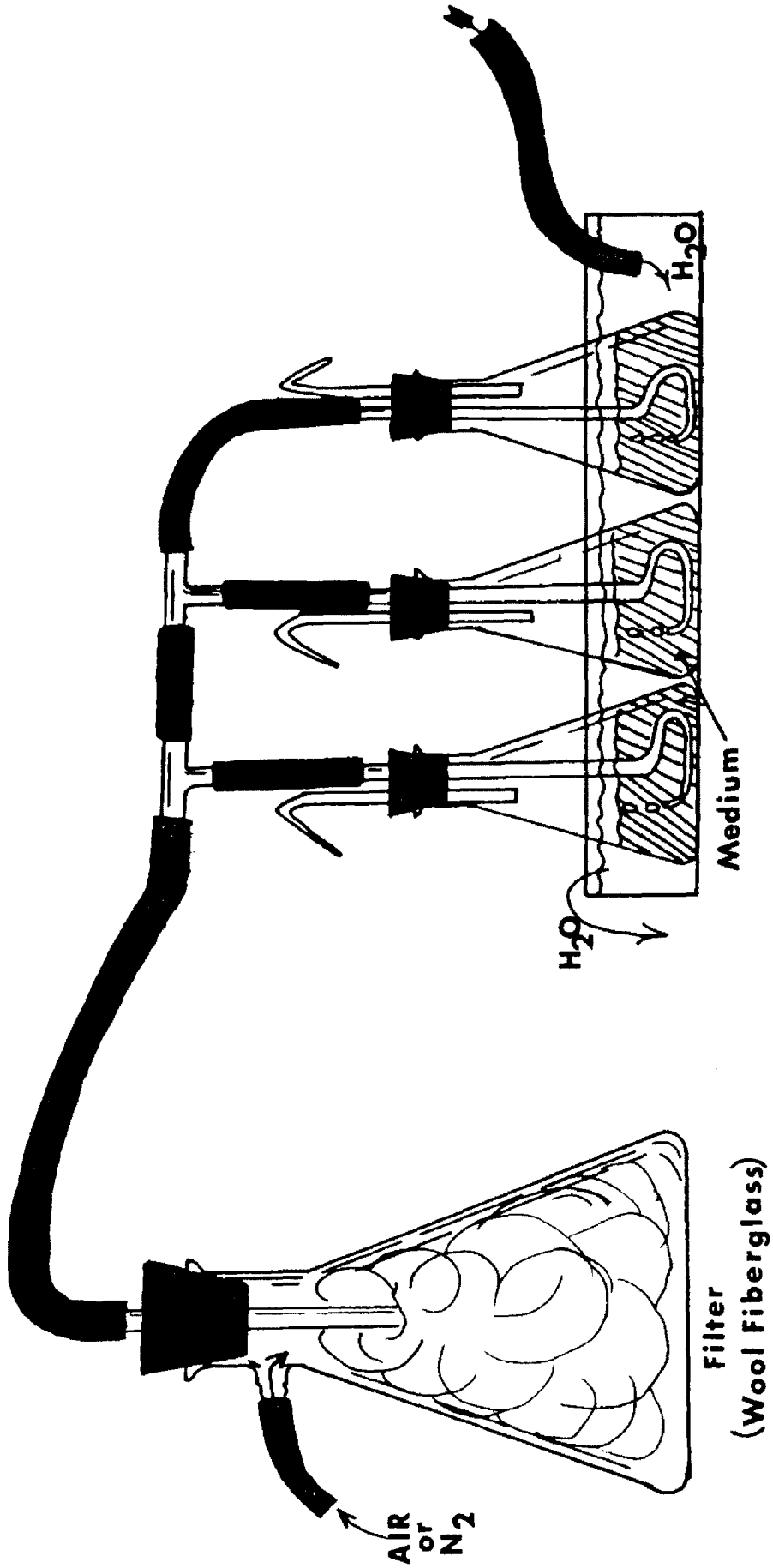

FIGURE I. Aeration Apparatus

Each of the culture flasks was stoppered with a rubber stopper containing an air inlet and an air outlet fashioned from capillary pipettes (see Fig. I). The end of each air outlet was sealed with a small piece of masking tape, and the top of each air inlet was sealed with aluminum foil. A strip of aluminum foil was also wrapped around the top of each flask and stopper, so that the air inlet was covered.

To connect the filtering apparatus to the flasks, sections of rubbing tubing were attatched to two Kimax T-joints so that air could pass from one air outlet into each of three flasks (see Fig. I). To sterilize, each open end of tubing was sealed with aluminum foil, and the entire tubing-T-joint aparatus was wrapped in brown paper.

The filtering flask with rubber tubing, the culture flasks, and the tubing-T-joint apparatus were autoclaved separetly at 121 C, 15 lb. pressure, for 20 minutes.

The entire apparatus was assembled and connected to the air outlet, and 200 ml of sterile survival test culture medium was added aseptically to each flask just prior to the onset of the experiment.

(b) Decreased Aeration

To achieve an atmosphere of decreased aeration, water-pumped nitrogen was bubbled into each culture flask for ten minute periods approximately once every 24 hours for 480 hours. On days when samples were removed from each flask to determine viable cell counts, nitrogen was added immediately after the sampling period. On days when samples were not removed, nitrogen was added approxi-

mately 24 hours after the last sampling period. It was impossible to add nitrogen continuously over a period of 480 hours because of a limited nitrogen volume.

The same apparatus and system of incubation used in the increased aeration experiment was used in this experiment (see Fig. I).

Air outlet tubing in each flask stopper was opened during the addition of nitrogen to prevent build up of pressure inside the flasks. To prevent bacterial contamination and exchange of the flask and room atmospheres, each air outlet was taped shut with a small piece of masking tape during periods of static incubation.

(5) Nutrient Alterations

Culture medium of standard strength was designated as 1.0 X. The concentration of the standard strength medium was arbitrarily set at 0.00125 g vegetative debris per ml. In dilute medium, designated as 0.1 X, the concentration of the vegetative material was reduced to 0.1 times the standard concentration. In concentrated medium, designated as 10 X, the concentration of vegetative material was increased to 10 times the standard concentration.

Organic material consisted of dead hedge grasses, and fallen alder and vine maple leaves collected in the late fall (November), from the ground, on the banks and neighboring area of the water collection point. After collection, the vegetative material was stored in plastic bags at 10 C.

The standard strength culture medium was prepared by first emulsifying 1.0 g of dry vegetative debris in 200 ml potassium phosphate

buffer, pH 7.0, in a Waring Blender, model 702 B. The emulsion was distributed into four 250 ml screw cap flasks so that each flask contained 50 ml of the emulsion. The flasks were then autoclaved at 121 C, 15 lb. pressure, for 20 minutes. Sterile potassium phosphate buffer, pH 7.0, was then added aseptically to the flasks so that each contained a final volume of 200 ml.

The dilute medium was prepared by emulsifying 0.1 g of vegetative debris in 200 ml of potassium phosphate buffer, pH 7.0, and proceeding as described above.

The concentrated medium was prepared by emulsifying 10 g of vegetative debris in 200 ml potassium phosphate buffer, pH 7.0, and proceeding as described above.

CHAPTER III

RESULTS

(1) General Procedure

The results are reported as viable counts of pure and mixed cultures of Escherichia coli and Streptococcus faecalis. The mixed culture counts were done in triplicate, once on Plate Count Agar, once on Endo Agar, for the enumeration of E. coli in mixed culture, and once on M-Enterococcus Agar for enumeration of S. faecalis in mixed culture.

Four environmental factors were tested, each at three levels, except temperature, which was tested at five levels. Each experiment was carried out only once due to the length of time involved.

Each experiment was terminated after a maximum incubation period of 480 hours (20 days). If die-out (failure to recover viable cells at a 10^0 dilution) of either S. faecalis or E. coli occurred before the 480 limit, the experiment was stopped.

To obtain a visual representation of the population differences, graphs were made for each level of each environmental factor studied by plotting viable cell numbers versus time of incubation (days). Since colonies of each genus were easily distinguishable on Plate Count Agar in mixed culture, and because counts obtained on M-Enterococcus Agar and Endo Agar matched those obtained on Plate Count Agar (see Table 5) only viable cell counts obtained on P.C.A. were used to formulate survival curves.

Since the initial inoculum of each organism for each experiment

TABLE 5. Viable Streptococcus faecalis and Escherichia coli populations in mixed culture*

Day	S. faecalis		E. coli	
	Plate Count Agar	M-Enterococcus Agar	Plate Count Agar	Endo Agar
0	3.00×10^6	3.27×10^6	2.70×10^6	2.46×10^6
1	2.02×10^6	2.23×10^6	2.76×10^6	2.50×10^6
2	2.32×10^6	2.55×10^6	2.55×10^6	2.59×10^6
3	2.01×10^6	1.79×10^6	1.99×10^6	2.02×10^6
6	1.80×10^6	1.59×10^6	1.58×10^6	1.52×10^6
10	1.19×10^6	1.23×10^6	6.50×10^5	5.10×10^5
15	1.00×10^6	9.50×10^5	5.00×10^5	4.90×10^5
20	2.01×10^5	2.67×10^5	1.05×10^5	1.48×10^5

*pH 6.0, standard conditions of temperature, aeration, and nutrient supply.

varied between 1.5×10^6 and 3×10^6 the ratio of the initial concentration to the final concentration was calculated to obtain a more absolute comparison of population changes. Ratios >1 represent a decrease in viable cell numbers below the initial concentration, ratios <1 represent an increase in numbers above the initial concentration, ratios = 1 represent no change, and ratios = NS indicate a failure to recover viable cells at a 10^0 dilution at the hour of termination.

(2) Standard Control Conditions

When no buffer was used to maintain the pH the experiment was terminated at 240 hours due to complete die-out of S. faecalis in pure culture. When incubated in river water, initial pH 7.0, without the buffering system, at 16 C, both organisms were able to survive better in mixed culture even though the initial inoculum of each organism in mixed culture was slightly smaller than in pure culture (see Fig. X).

Fig. X also shows that after a period of 240 hours (10 days) E. coli cells greatly out numbered S. faecalis cells in both pure and mixed culture. Although population levels of both organisms were reduced from the initial concentration, greater reduction of the Streptococcus population occurred under these conditions, even to the point of complete die-out in pure culture. In comparison, after 10 days E. coli population levels dropped only to 10^6 cells/ml in mixed culture, and 10^5 cells/ml in pure culture.

When a potassium phosphate buffer was used as the menstruum (see Fig. III) S. faecalis survived slightly better than E. coli in both pure and mixed culture, and both organisms were able to maintain high level populations up to 20 days.

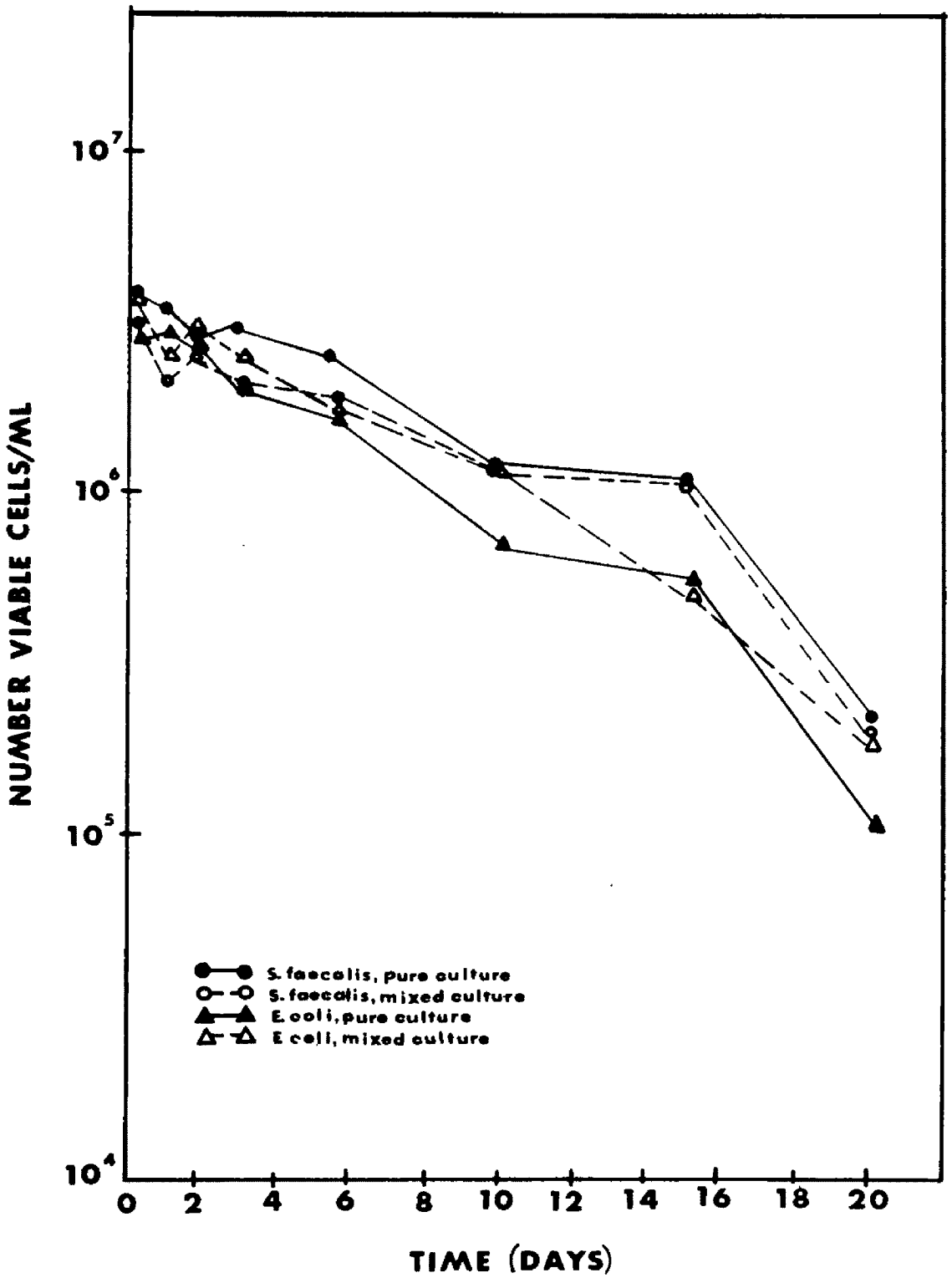

FIGURE II. Survival curve of S. faecalis and E. coli in pure and mixed culture, pH 6.0 (16 C, static, 0.0X nutrient concentration).

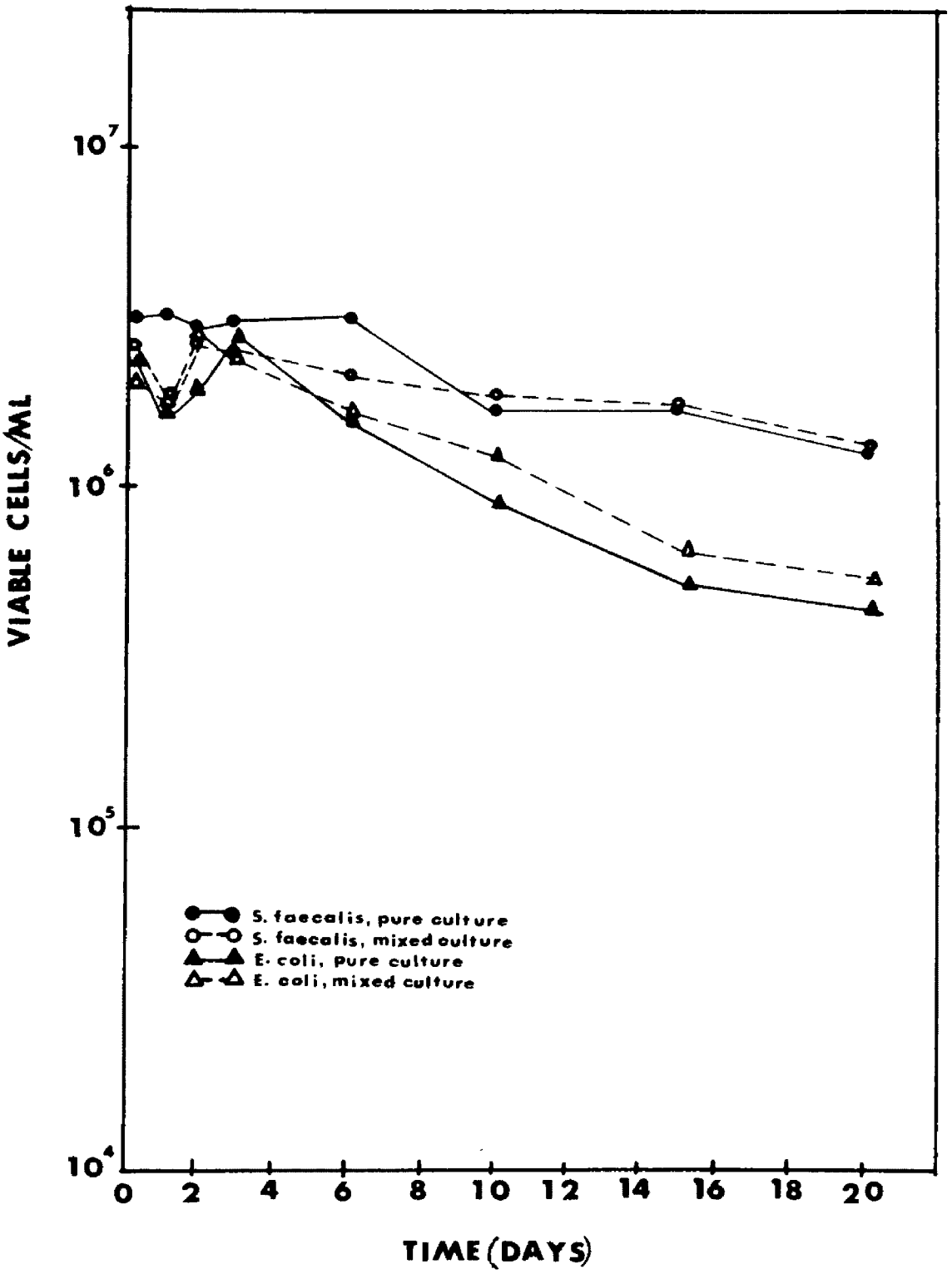

FIGURE III. Survival curve of S. faecalis and E. coli in pure and mixed culture, pH 7.0 (16 C, static, 0.0X nutrient concentration).

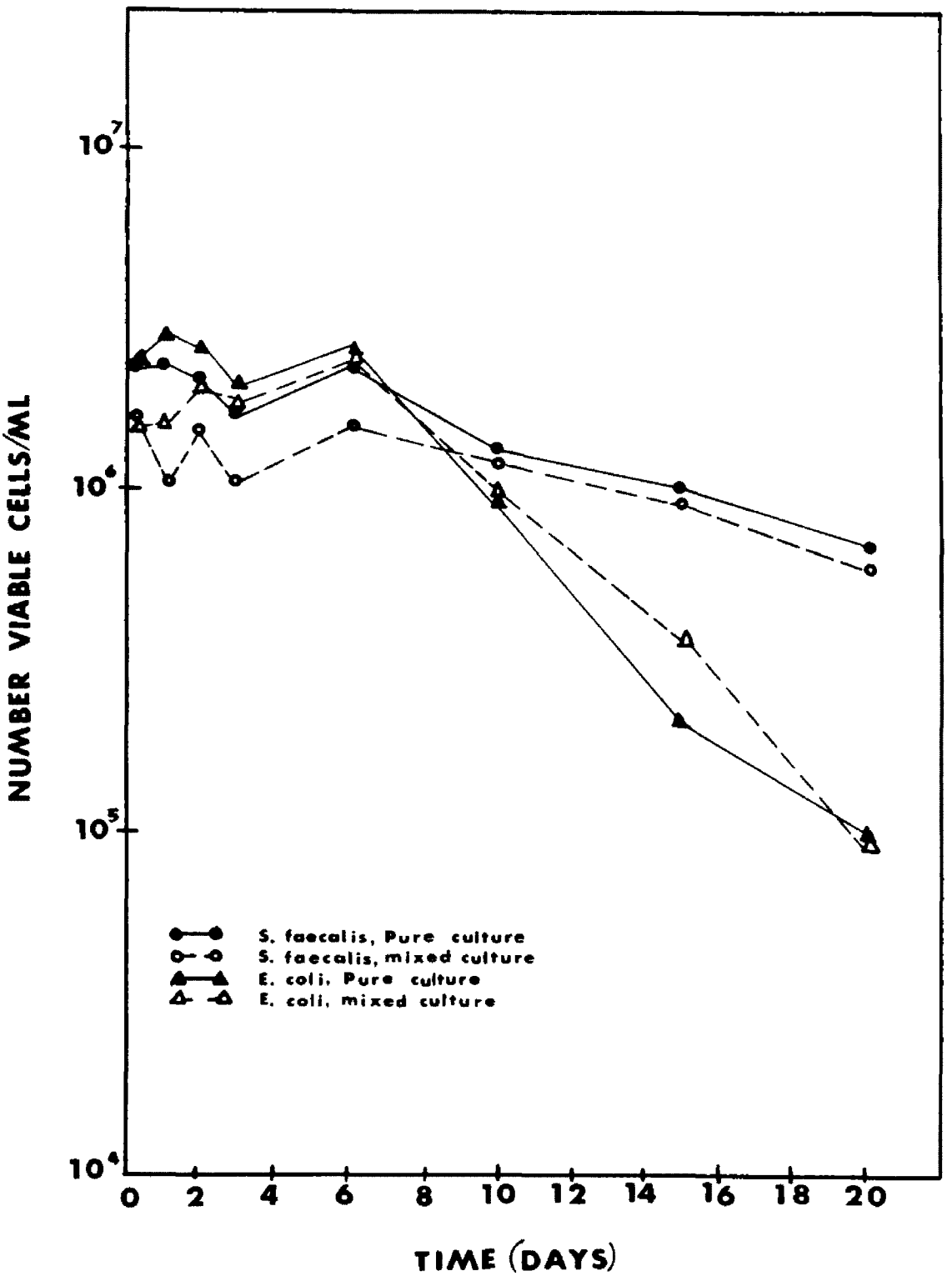

FIGURE IV. Survival curve of S. faecalis and E. coli in pure and mixed culture, pH 8.0 (16 C, static, 0.0X nutrient concentration).

TABLE 6. Ratio of initial:final concentration*

pH of the Medium	Streptococcus		Escherichia	
	pure	mixed	pure	mixed
6.0	16.36	15.00	19.44	24.54
7.0 (buffer)	2.40	1.92	4.75	4.60
7.0 (no buffer)†	NS	202.89	20.73	1.81
8.0	3.54	3.80	24.50	21.10

*After 480 hours incubation in potassium phosphate buffer, 16 C.

†Standard control conditions, after 240 hours incubation in river water, initial pH 7.0.

(3) pH Alterations

The ratios in Table 6 show that at each pH there was a greater reduction of E. coli cell numbers than of Streptococcus cell numbers in both pure and mixed culture after 20 days. The greatest difference in cell number reduction occurred at pH 8.0.

No appreciable differences in population levels of mixed versus pure cultures can be noted from the ratios in Table 6 for either organism except at pH 6.0. At this pH E. coli survived slightly better in mixed culture.

Both E. coli and S. faecalis survived best at pH 7.0 when a buffering system was used. The table indicates that at pH values above 7.0 S. faecalis is able to survive better than at pH values below 7.0. E. coli, on the other hand, is able to survive equally well at pH values above or below 7.0.

(4) Nutrient Alterations

Fig. V through VII show the population differences which occurred when different amounts of nutrient material were added to the river water. Addition of the lowest amount of nutrient material (0.1 X) resulted in population levels far greater for both organisms after 240 hours than in river water containing no nutrient material and no buffering system. When organisms were incubated in a buffered medium containing no nutrient, population changes were similar to those observed in the dilute nutrient (0.1 X) medium.

The graphs suggest that the ability of E. coli and S. faecalis to undergo active multiplication is directly proportional to the amount of nutrient material added. Active multiplication of E. coli occurred

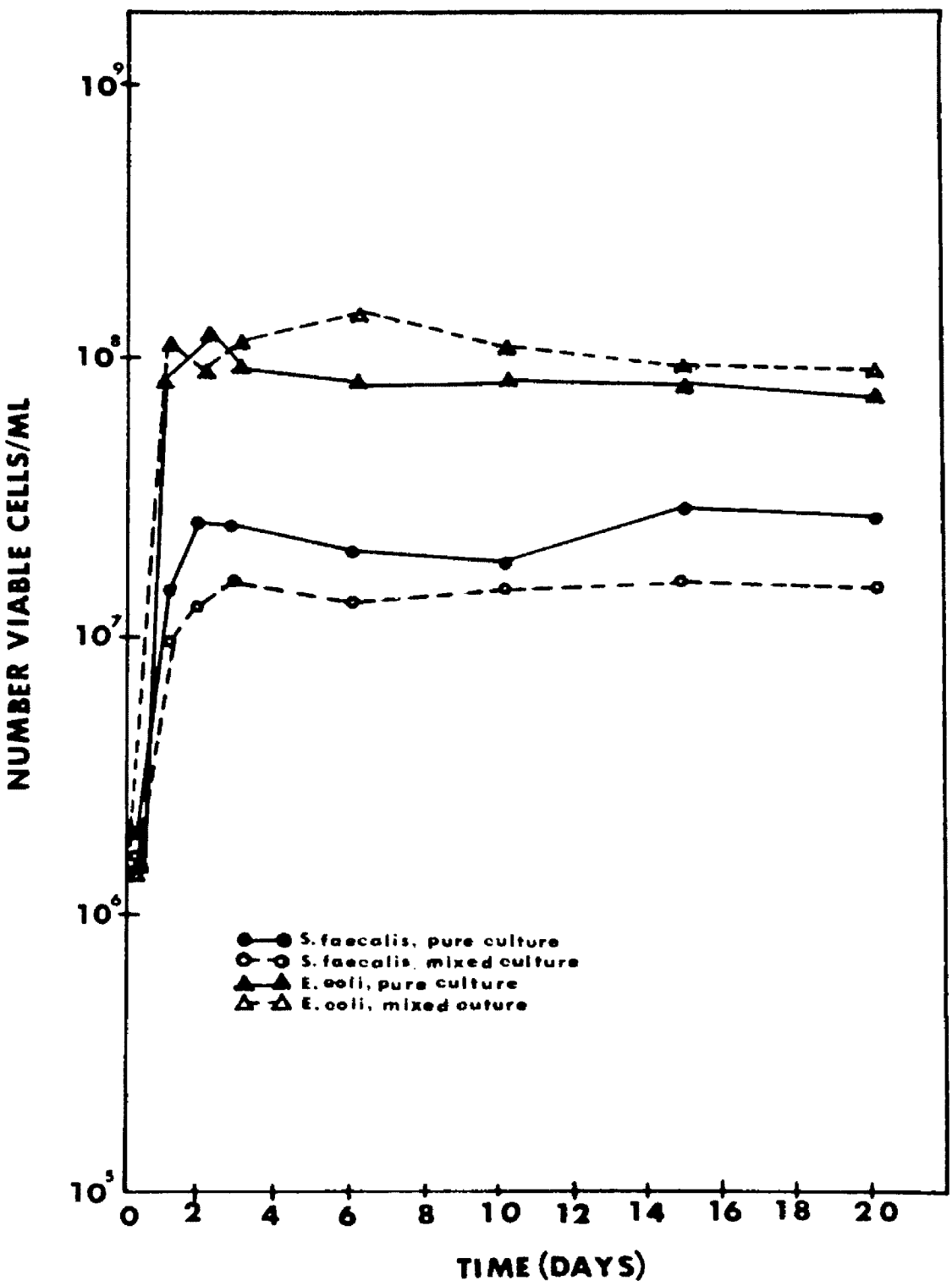

FIGURE V. Survival curve of S. faecalis and E. coli in pure and mixed culture, 10X nutrient concentration (16 C, static, pH 7.0).

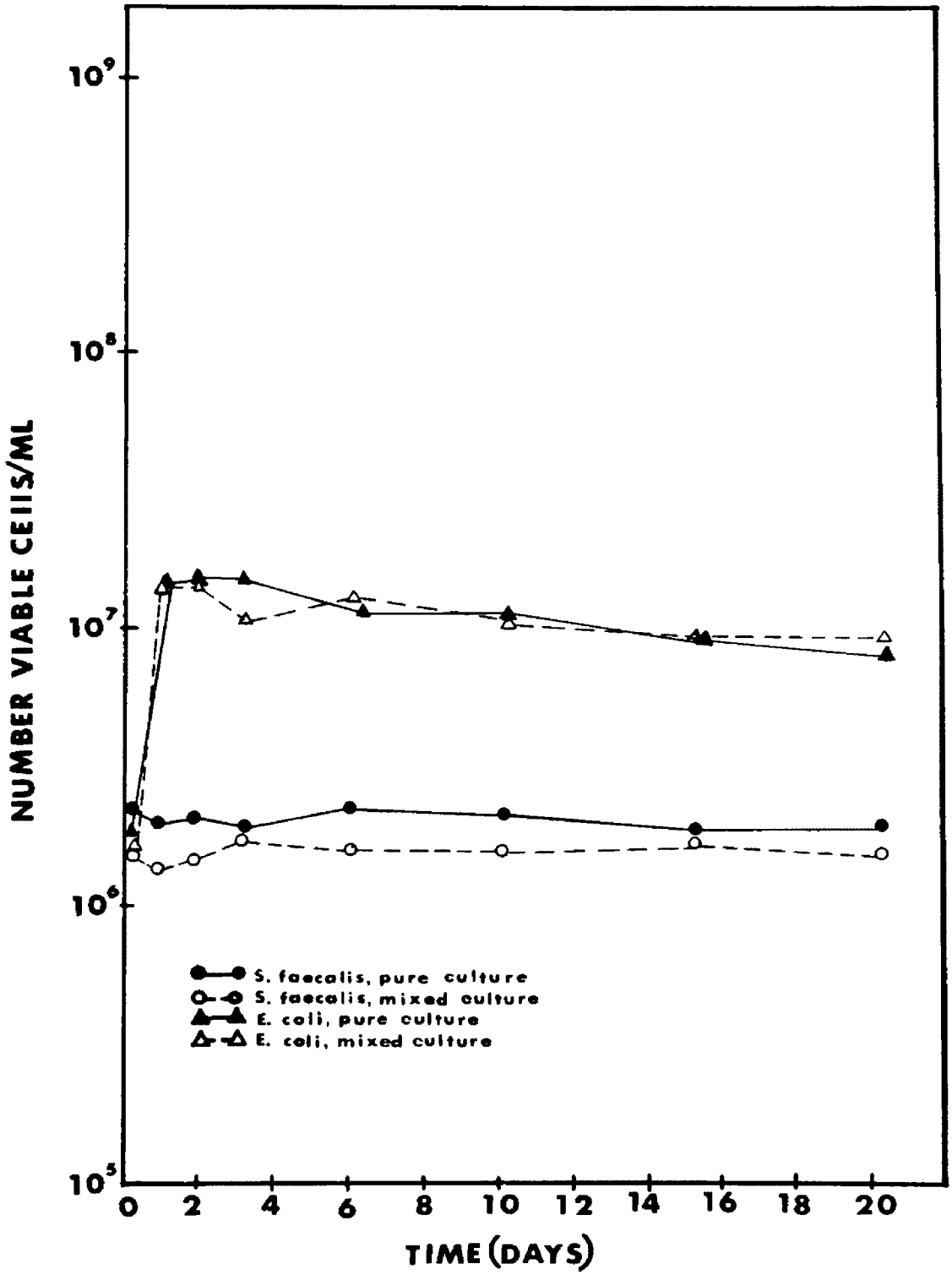

FIGURE VI. Survival curve of S. faecalis and E. coli in pure and mixed culture, 1.0X nutrient concentration (16 C, static, pH 7.0).

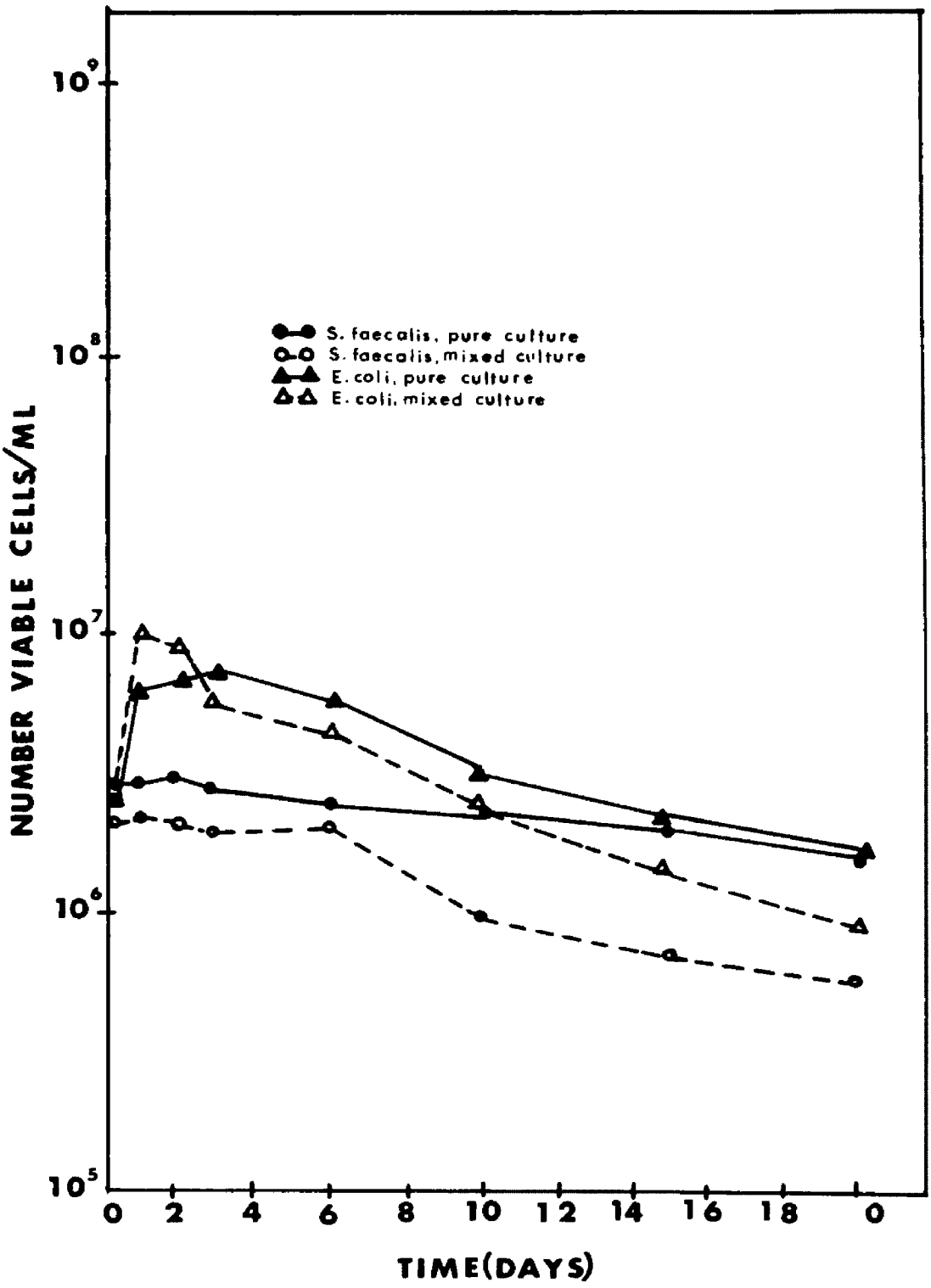

FIGURE VII. Survival curve of S. faecalis and E. coli in pure and mixed culture, 0.1X nutrient concentration (16 C, static, pH 7.0).

TABLE 7. Ratio of initial:final concentration*

Nutrient Concentration	Streptococcus		Escherichia	
	pure	mixed	pure	mixed
10.0 X	0.07	0.10	0.02	0.02
1.0 X	1.09	0.95	0.22	0.18
0.1 X	1.85	3.37	1.81	3.37
0.0 X+	NS	202.89	20.73	1.81
0.0 X++	2.40	1.92	4.75	4.60

*After 480 hours incubation, 16 C.

+Standard control conditions, after 240 hours incubation in river water, 16 C.

++Standard control conditions, after 480 hours incubation in potassium phosphate buffer, 16 C.

in both pure and mixed culture in all of the nutrient containing media. The greatest amount of multiplication occurred in the concentrated (10 X) medium. The population level was maintained to the 20th day in the concentrated (10 X) and standard (1.0 X) strength media. In the dilute (0.1 X) media, after an initial increase, population levels had fallen to values below the initial concentration after 480 hours.

Active multiplication of S. faecalis occurred only in the concentrated medium. In the standard strength medium the streptococci were only able to maintain population levels equal to the initial concentration. In dilute media cell numbers were reduced to values below the initial concentration.

No significant differences in population levels of each organism in pure versus mixed culture were observed accept in the dilute medium, in which both organisms survived slightly better in pure than in mixed culture. It is interesting to note that under standard control conditions when no nutrient material was added, the opposite occurred.

Fig. V through VII show E. coli population levels in mixed culture were dominant in the concentrated and standard strength media. In dilute media, although E. coli numbers increased initially, and the Streptococcus numbers did not, mixed culture population levels of each organism were similar after 20 days. Under standard control conditions, when no nutrient was added, the streptococci maintained higher population levels than did E. coli during the 20 day period.

When comparing the ratios in Table 7 of each organism in the standard strength and dilute media, it is important to keep in mind that E. coli populations increased initially, whereas the S. faecalis popula-

tions did not. Therefore, although the values may be the same for E. coli and S. faecalis, as is the case in dilute media indicating comparable reductions in viable cell numbers, the decrease is actually greater for E. coli populations than for S. faecalis cell numbers, because of their initial increase.

(5) Temperature Alterations

Table 8 shows that S. faecalis was able to survive a great deal better in mixed than in pure culture especially at 27 C (see Fig. IX). In mixed culture S. faecalis was able to maintain higher population levels for longer periods of time, whereas in pure culture S. faecalis die-out occurred within time periods proportional to the temperature changes at every temperature except 4 C.

E. coli survived better in mixed culture in a cold environment (16 C and 10 C), and better in pure culture in a warm environment (27 C and 37 C). At 4 C only a negligable difference could be noted.

Fig. VIII through XII indicate that at each of the temperatures, when no buffering system is used in the medium, the survival time of S. faecalis and E. coli is inversely proportional to the temperature of incubation. As the temperature was decreased both organisms were able to maintain higher population levels for longer periods of time. At warm temperatures (37 C and 27 C) a rapid die-out of pure culture S. faecalis populations was observed within 48 hours, accompanied by a significant decrease of E. coli population levels and S. faecalis mixed culture population levels within 72 hours after initial inoculation. As the temperature dropped, organisms were able to survive for longer time periods. At the coldest temperature, 4 C, both organisms were able

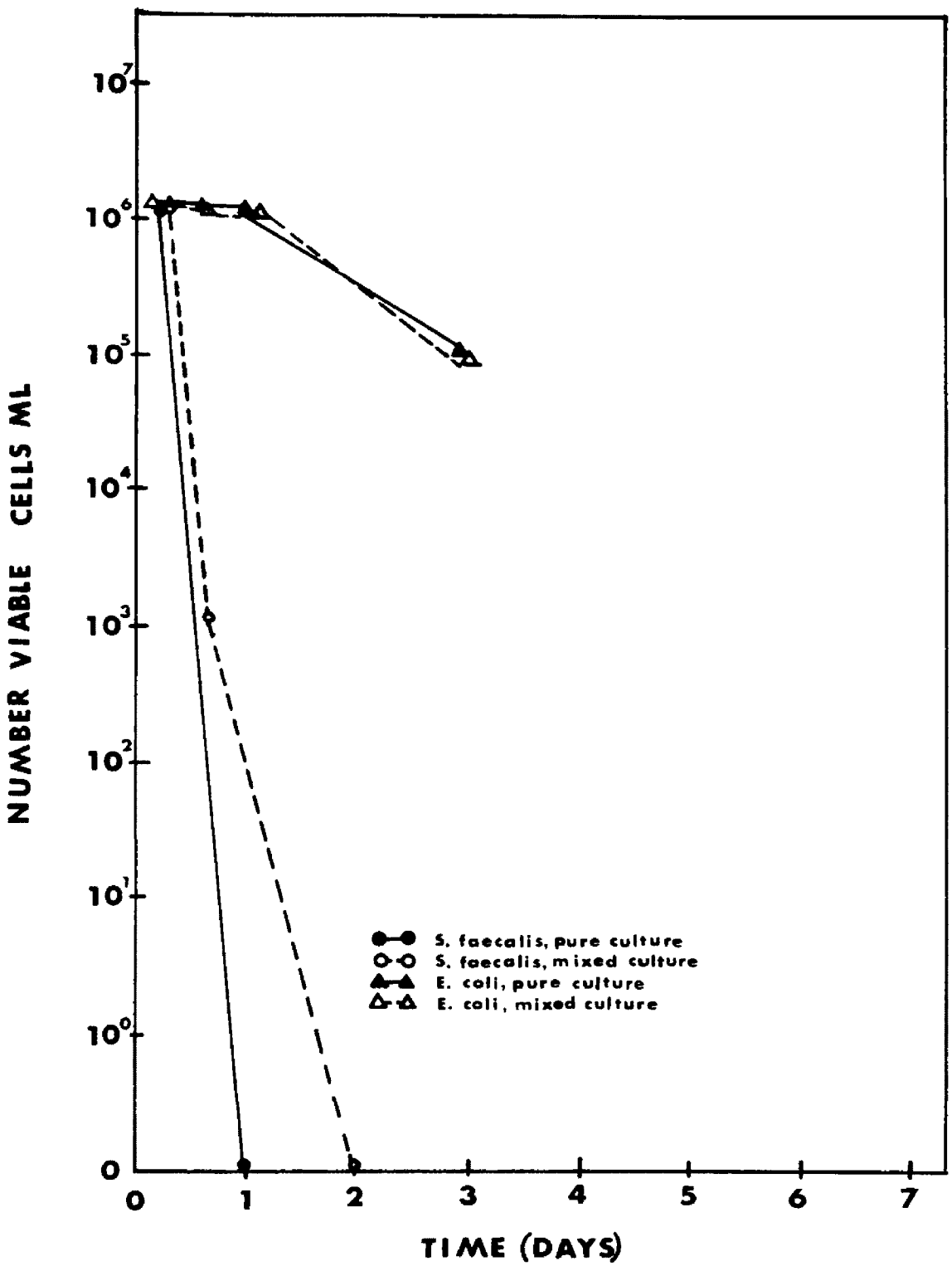

FIGURE VIII. Survival of S. faecalis and E. coli in pure and mixed culture, 37 C (static, 0.0X nutrient concentration, initial pH 7.0).

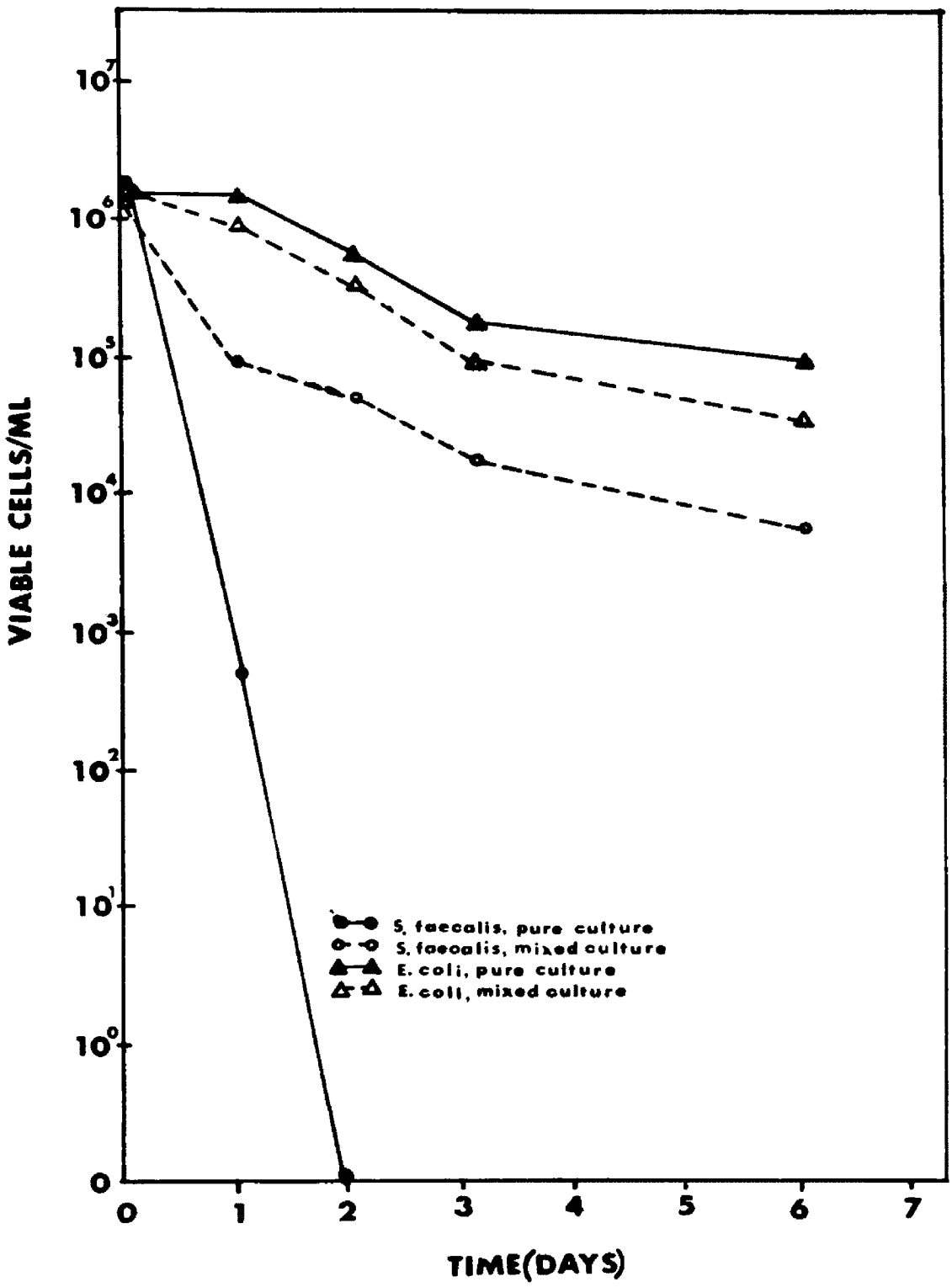

FIGURE IX. Survival curve of S. faecalis and E. coli in pure and mixed culture, 27 C (static, 0.0X nutrient concentration, initial pH 7.0).

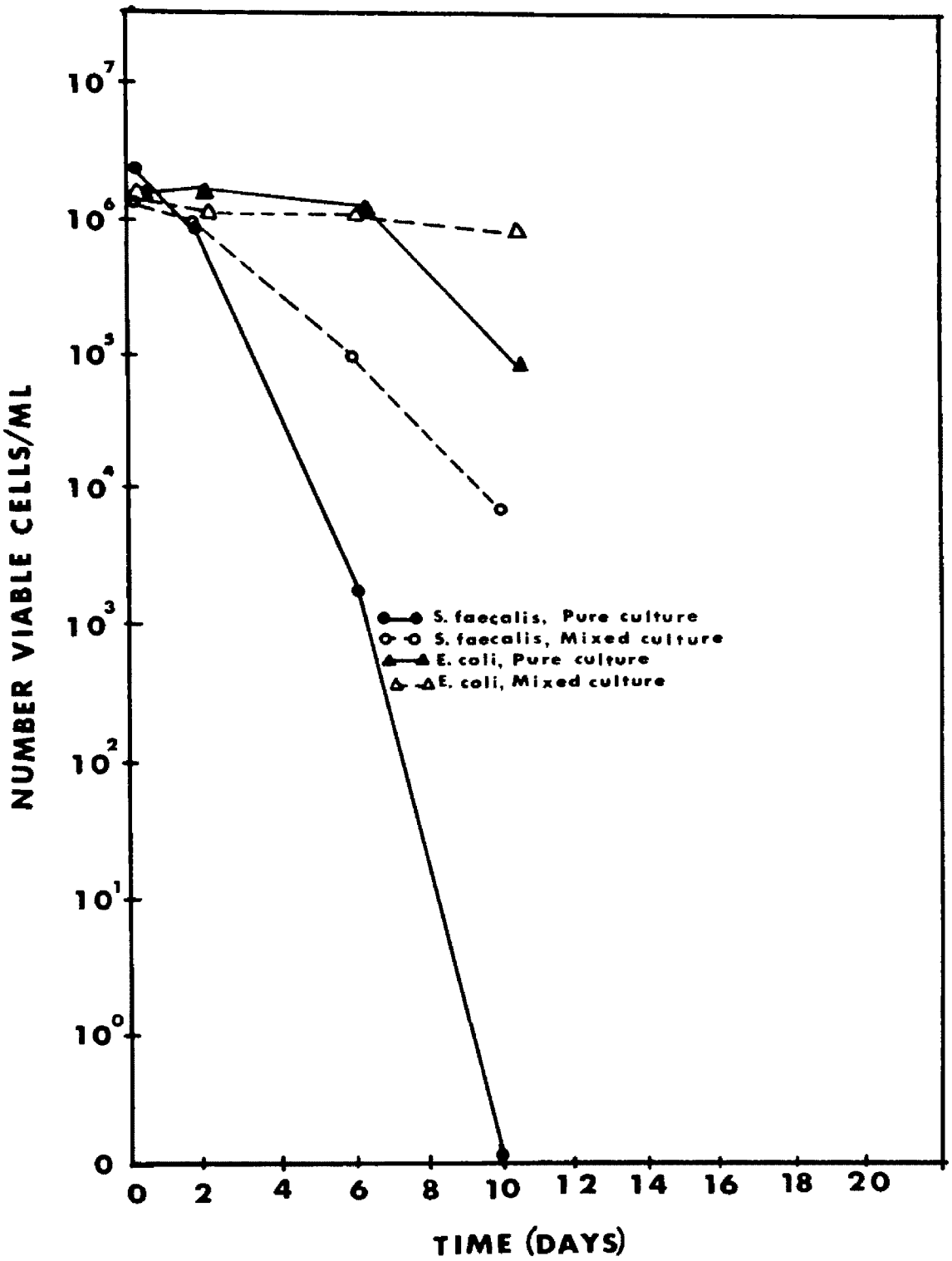

FIGURE X. Survival curve of S. faecalis and E. coli in pure and mixed culture, 16 C (static, 0.0X nutrient concentration, initial pH 7.0).

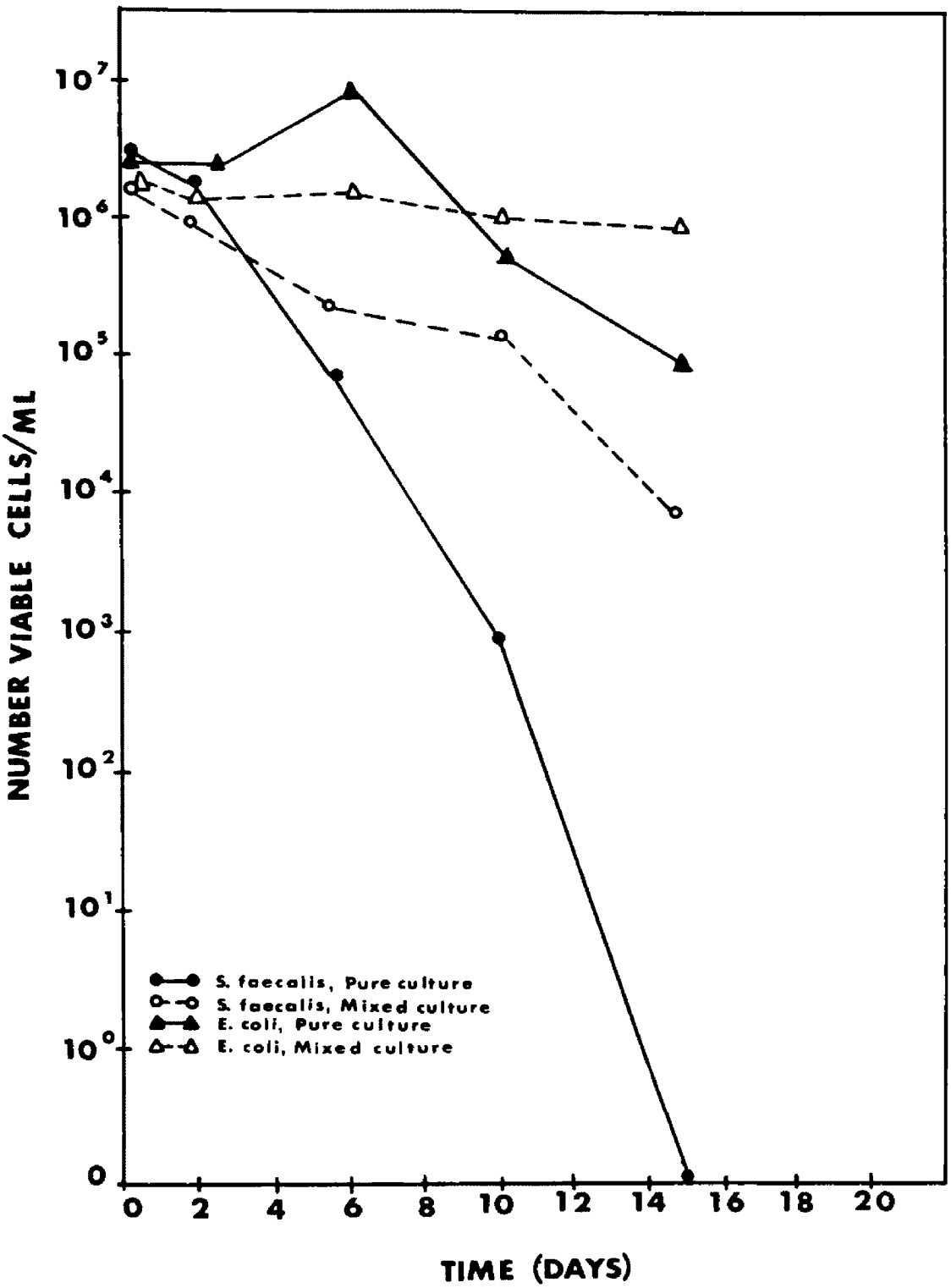

FIGURE XI. Survival curve of S. faecalis and E. coli in pure and mixed culture, 10 C (static, 0.0X nutrient concentration, initial pH 7.0).

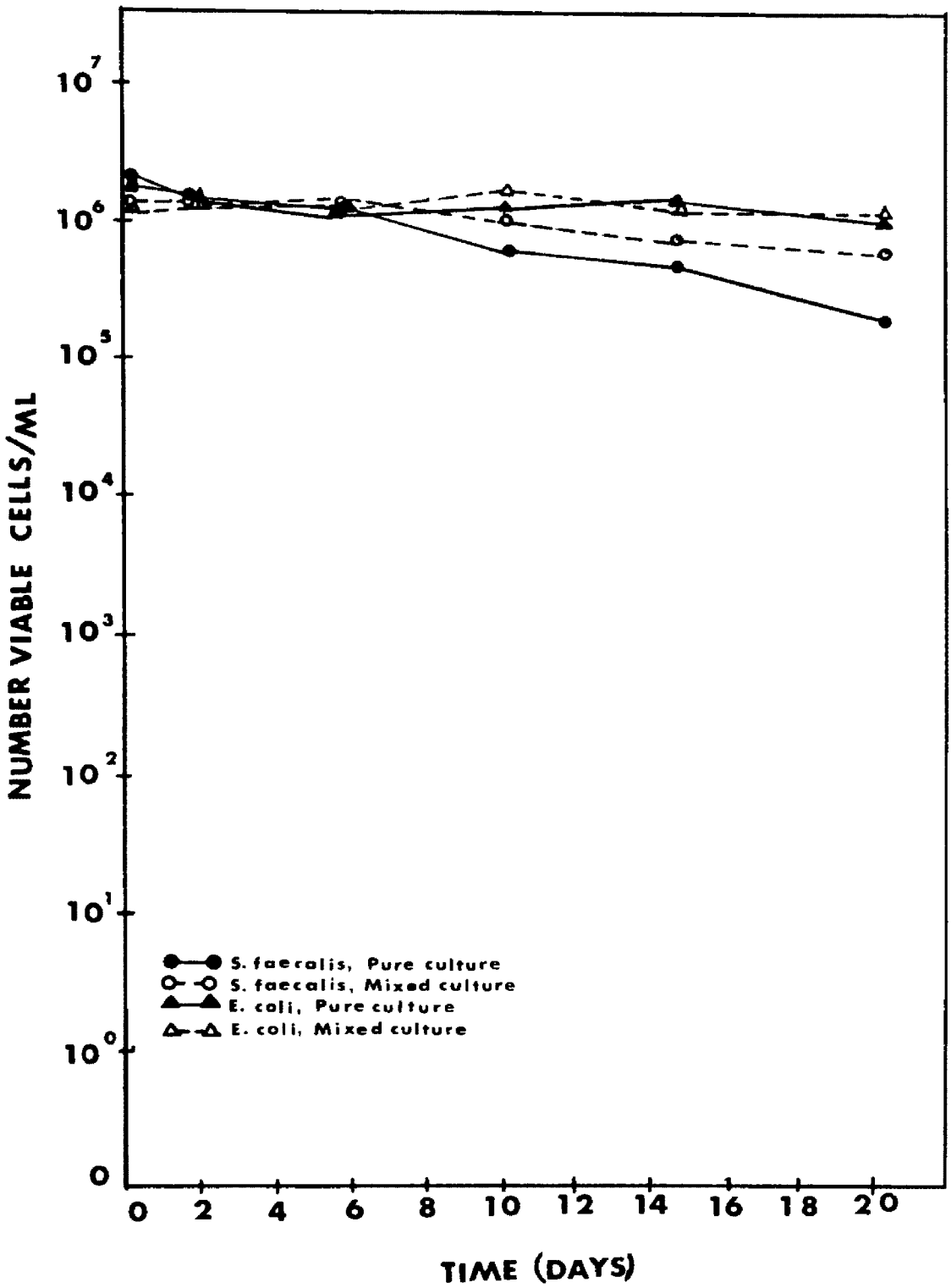

FIGURE XII. Survival curve of S. faecalis and E. coli in pure and mixed culture, 4°C (static, 0.0X nutrient concentration, initial pH 7.0).

TABLE 8. Ratio of initial:final concentration

Incubation Temperature	Streptococcus		Escherichia	
	pure	mixed	pure	mixed
4 C*	11.37	2.27	1.70	1.12
10 C**	NS	245.90	31.25	2.01
16 C+	NS	202.89	20.73	1.81
27 C***	NS	232.73	17.91	46.29
37 C****	NS	NS	15.50	25.36

*After 480 hours incubation in river water.

**After 360 hours incubation in river water.

***After 144 hours incubation in river water.

****After 72 hours incubation in river water.

+Standard Control Conditions, after 240 hours incubation in river water.

to maintain population levels in both pure and mixed culture equal to or only slightly less than the initial concentration during the 20 day incubation period.

Again it must be noted that the decrease in cell numbers of E. coli at 37 C is actually larger than it appears in Table 8 due to an initial increase in viable cell numbers within the first 12 hours of incubation.

(6) Aeration Alteration

Fig. XIII and XIV show the effect of increased and decreased aeration on the survival of E. coli and S. faecalis, and also show the temperature fluxuations of the tap water. The water temperature was measured once every 24 hours in the morning. Mean temperatures were similar for both experiments. There were no apparent fluxuations of population levels which would correspond to temperature changes during the incubation period.

Ratios in Table 9 show that in both the aerated and nitrogen environments E. coli survived better in pure than in mixed culture. In the nitrogen environment S. faecalis was unable to survive in either the pure or mixed culture. In the aerated atmosphere the S. faecalis pure culture population level was greatly reduced, whereas in mixed culture S. faecalis was able to maintain a population level approximately equal to the E. coli pure culture population.

Evidence suggests that both increased aeration, and decreased aeration by the addition of nitrogen, are detrimental to the survival of both E. coli and S. faecalis, especially the latter. The ratios in Table 9 show that reductions in population levels of both organisms

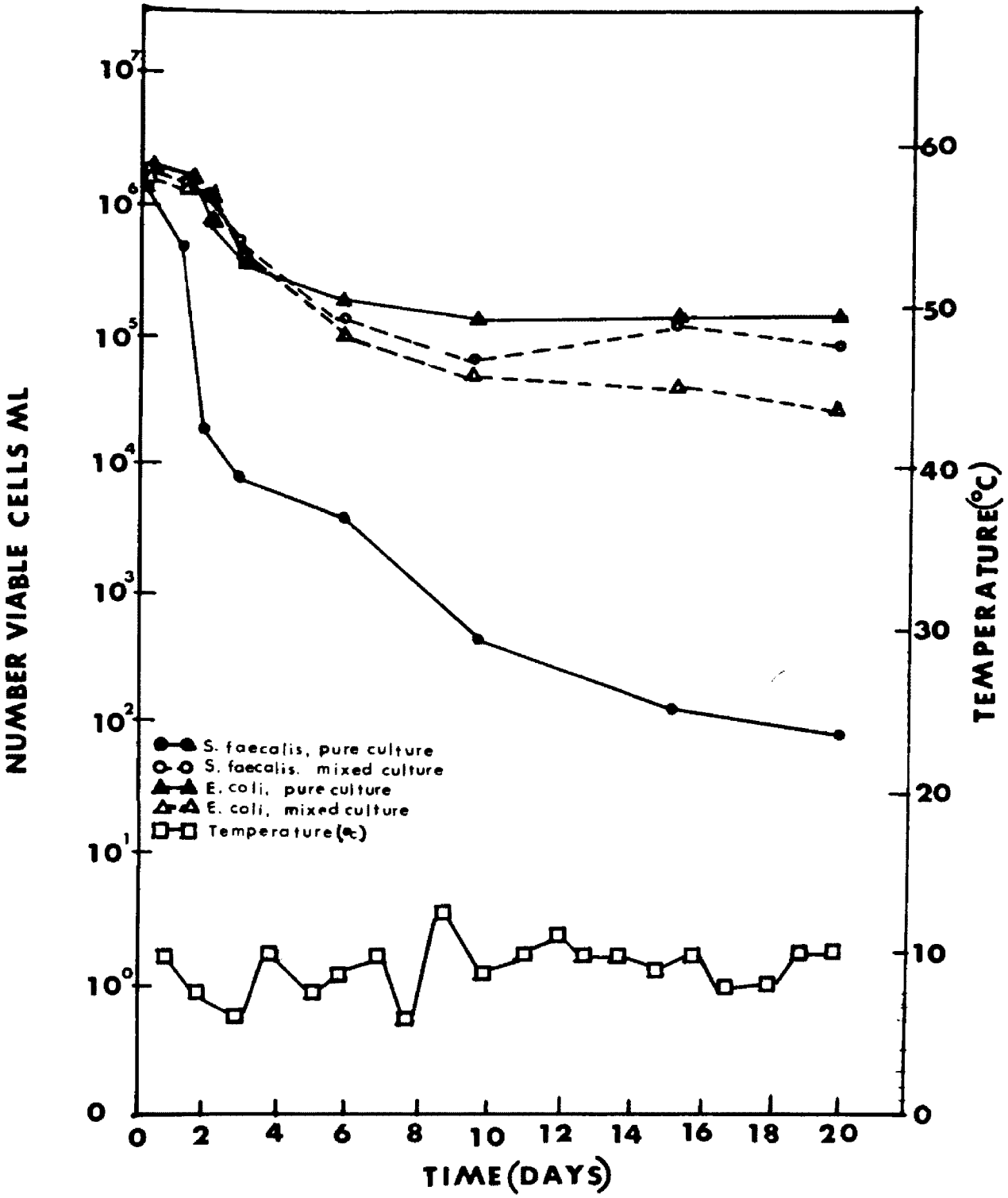

FIGURE XIII. Survival curve of S. faecalis and E. coli in pure and mixed culture, increased aeration (16 C, 0.0X nutrient concentration, pH 7.0).

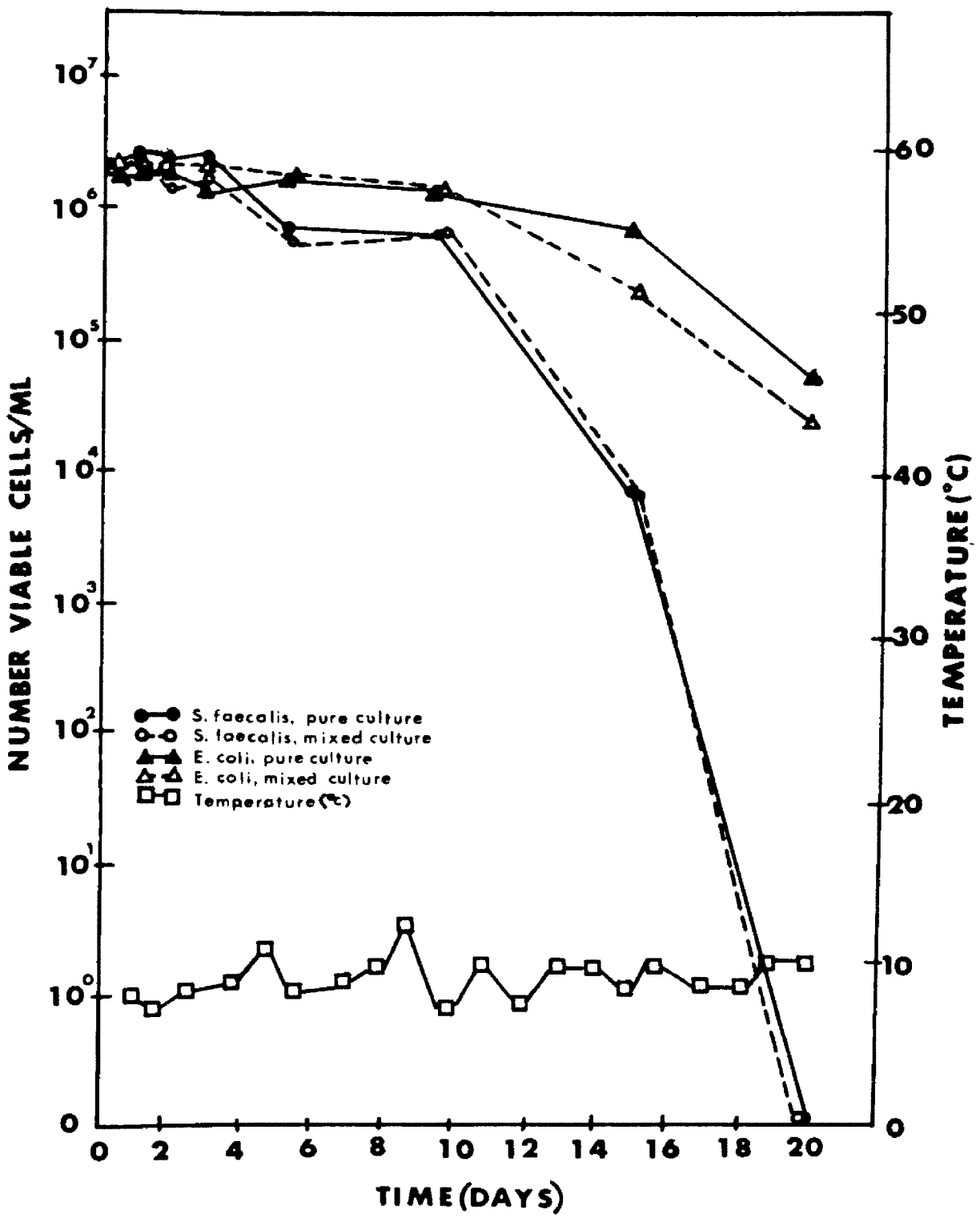

FIGURE XIV. Survival curve of S. faecalis and E. coli in pure and mixed culture, decreased aeration (16 C, 0.0X, nutrient concentration, pH 7.0).

TABLE 9. Ratio of initial:final concentration*

Oxygen Supply	Streptococcus		Escherichia	
	pure	mixed	pure	mixed
Increased	1,875.00	18.30	12.36	62.80
Static+	2.40	1.92	4.75	4.60
Static++	NS	202.89	20.73	1.81
Decreased	NS	NS	3.62	99.59

*After 480 hours incubation in potassium phosphate buffer, 16 C.

+Standard Contro Conditions, potassium phosphate buffer, 16 C.

++Standard Control Conditions, river water, initial pH 7.0, 16 C.

which occurred in each altered environment were greater than reductions which occurred in the control atmosphere when a buffering system was used.

CHAPTER IV

DISCUSSION

The experimental evidence indicates that the environment greatly influences the survival of S. faecalis and E. coli in both pure and mixed culture. With the exception of a few alterations, each change in the environment resulted in either, an altered ability to maintain constant high level populations for 480 hours, or in active multiplication to population levels much greater than the initial concentration. Changes in population size occured in both pure and mixed cultures.

In general, both organisms survived as well or slightly better in mixed culture than in pure culture. When nutrient material was added and when the oxygen supply was increased the opposite effect occurred. This suggests that in a completely nutrient deficient environment the organisms exerted a protective effect on one another in mixed culture.

Nioh and Furusaka (1968) have shown that bacteria are able to reproduce in nutrient deficient, heat-killed cell suspensions of the same organism or other organisms. They found that the amount of growth was dependent on the concentration and kind of heat-killed cells. This same phenomenon may be partially responsible for the protective effect of mixed cultures observed in this study, particularly when the temperature of incubation was varied. Each organism may be able to utilize dead cells or cellular debri of the associated species as a nutrient substrate better than cells of its own kind.

When nutrient was added in the form of vegetative debris (see Fig.

V through VII) both E. coli and S. faecalis survived slightly better in pure culture, and when the amount of aeration was increased (Fig. XIII) E. coli survived slightly better in pure culture. These results substantiate the findings of Barnes (1931), Lockhart and Powelson (1953), Mossel and Ingram (1955), Dagley et al. (1952), Charlton (1955), Freter (1963), and Iandolo et al. (1965). When a small amount of nutrient is present bacterial competition for the nutrient material may regulate population levels of E. coli and S. faecalis in mixed culture.

In this study temperature alterations were tested first. To insure the most natural conditions, unbuffered river water at an initial pH 7.0, which had been filter sterilized was used, except at 16 C. At this temperature survival of E. coli and S. faecalis was also studied in river water containing a buffering system.

Just prior to filtration, the pH of each river water volume was measured and each time the pH was within the range 7.0-7.2. Water was collected approximately once every two months during a one year period. Therefore it would be subject to a variety of seasonal changes, such as changes in the surrounding soil and vegetative microflora, amount of soil run-off, types and amount of fauna and flora frequenting the adjacent land area, amount of snow-melt, and the microflora of the water itself. This suggests that there may exist some type of natural buffering system in fresh surface waters which may be independent of the physical and mechanical influences of the environment. Therefore, those results obtained using river water containing a buffering system may be more representative of what actually occurs in nature rather than those results obtained using only river water at an initial pH 7.0.

Hence, in all subsequent studies a buffering system was used.

To insure a standard temperature which was representative of the mean temperature of natural waters during the warmer months of the year (June - September) when the sanitary quality of water sources is most important, two procedures were employed. The temperatures of three water sources, the Rattlesnake River, the Clark Fork River, and The Bitterroot River, which drain land in and immediately adjacent to Missoula, Montana, were measured. The measurements were made during the third week in August, 1969, following a two-week period of 90-100 F daily temperatures. All three water sources were 16 C at the respective test points.

Temperature measurements reported in other studies substantiated these findings. Niemela and Tirronen (1967) and Gallagher and Spino (1968) reported that temperatures of cold, fresh water sources measured over a one year period ranged from 5.9 - 20 C. Therefore, 16 C was chosen as the standard temperature of incubation.

Niemela and Tirronen (1967) reported that in a cold mountain stream high coliform/fecal streptococci (C/F) values were associated with high temperatures. They concluded that coliforms were able to multiply in the river at high temperatures, whereas streptococci maintained a stable population regardless of the temperature.

In this study, although C/F values were not calculated, coliforms survived better than S. faecalis at elevated temperatures. Fig. VIII through XII show that the ratio of E. coli to S. faecalis decreased as the temperature decreased during incubation periods of increasing length, after a single initial inoculum was introduced into the water. This, however, did not appear to be caused by multiplication of E. coli,

but rather to die-out of streptococci at higher temperatures. Niemela and Tirronen (1967) based their findings on viable counts from samples obtained directly from a natural water source in which the probability of direct nutrient availability is high. In this study no nutrient material was added to the temperature test media.

Indirectly, however, the results of this study tend to substantiate multiplication of E. coli at temperatures at or above 16 C. When nutrient was added in the form of emulsified vegetative debris at 16 C, E. coli was able to attain populations greater than the initial concentration even in the dilute medium, whereas S. faecalis multiplied actively only in the concentrated media.

The nutrient material which consisted of dried vegetative debris collected along the banks of a fresh water stream may not be representative of the nutritional content of the river water, and the method of emulsion and suspension of the vegetation also may not be representative of the nutrient availability in the river water.

The fact that enteric bacteria are capable of reproduction in nutritional environments similar to cold mountain streams and lakes, however, has been reported by McGrew and Mallette (1962). They showed that enteric bacteria, including E. coli, can survive and multiply in media that contains less than 5 ug/ml of glucose. Hendricks and Morrison (1967) reported measurable multiplication and protein synthesis of E. coli and Shigella flexneri in river water containing an extract of river bottom sediment.

It is conceivable that decomposed vegetative debris may enter natural water sources via torential rainfall and subsequent soil run-off,

may be carried by wind and gravity, or may fall from overhanging vegetation due to seasonal changes or weathering factors, into the river and possibly influence the nutrient content or nutrient availability of a water source. This type of occurrence is more likely to result when water movement is relatively slow or static, or where there is a great deal of dense vegetation lining the banks of the water source.

In an actively free-flowing river environment, the probablility of active multiplication of bacteria in the free-flowing water due to any factor is probably greatly reduced as a result of dilution and sedimentation of the nutrient confluent. Here multiplication would be more likely to occur in the river bottom sediment which may be influenced by external vegetative material.

Changes of population levels of bacteria due to changes in pH have been reported by Gibson (1957), Cuthbert et al. (1955), and Goodman (1965). In this study, reduced populations of S. faecalis and E. coli were obtained by altering the pH with a potassium phosphate buffering system (see Fig. II and IV).

The results indicate that when the pH of the surrounding medium was alkaline (pH 8.0) S. faecalis was able to maintain a stable high level population equal to the initial concentration for up to 20 days, whereas when the pH was acidic the streptococci were unable to adjust to the altered environment. E. coli, on the other hand, was sensitive to both the acidic and alkaline environments, undergoing similar reductions of population levels, in media at pH 6.0 and pH 8.0 after a single initial inoculum was added.

In a buffered medium at pH 7.0 (see Fig. III) the final population

levels after 480 hours were only slightly reduced or equal to the initial population levels. When organisms were maintained in media at an initial pH 7.0, with no buffering system, the results were radically different, (see Fig. X). Population reduction after 240 hours with no buffering system was much greater than or equal to population reductions after 480 hours with a buffering system. When no buffering system was used the greatest reductions occured in the S. faecalis populations.

These results suggest that neither organism was able to rapidly reduce metabolic activity, and the initial burst of metabolic activity which resulted caused the pH of the medium to decrease, possibly due to the release of acidic substances by one or both bacteria. The pH of the medium, however, was not measured at the termination of the experiment, so it is impossible to ascertain the exact cause of population reductions when the buffering system was absent.

Cuthbert et al. (1955) and Hentges and Fulton (1964) have also reported that the population equilibrium between associated pairs of bacteria can be altered by alterations in the pH. In this study, when a buffering system was used survival in mixed culture equalled survival in pure culture at each pH.

Hentges and Fulton (1964) found that the population equilibrium between Klebsiella and Shigella could be altered by increasing or decreasing the amount of aeration of the environment. The results of this study show that the population equilibrium between E. coli and S. faecalis can be altered by altering the degree of aeration. Both the aerated and nitrogen atmospheres were detrimental to the streptococci in pure culture, whereas in mixed culture increased aeration favored a high population

level and decreased aeration resulted in complete die-out (see Fig. XIII and XIV). E. coli survived at similar population levels in both the aerated and nitrogen environments, but at lower levels than those obtained in the static environment.

The fact that S. faecalis was radically reduced in pure culture but maintained a stable, high level population in mixed culture when the medium was aerated suggests that S. faecalis is sensitive to an increased oxygen tension above some optimum value. In mixed culture, utilization of oxygen by E. coli may have caused a reduced oxygen tension, resulting in higher numbers of streptococci.

E. coli, on the other hand, survived better in pure culture when the medium was aerated, suggesting that utilization of oxygen by S. faecalis prevented E. coli from attaining population levels in mixed culture equal to pure culture populations. Hence, utilization of oxygen by S. faecalis and E. coli in mixed culture may result in a bacterial interaction involving both commensalism and inhibition.

The results obtained when the nitrogen atmosphere was used support this hypothesis in part. If during metabolism only oxygen is used as the final electron acceptor by both organisms, competition for oxygen when the oxygen supply is limited should result in lower population levels of both organisms in mixed culture than in pure culture. In a nitrogen atmosphere E. coli survived better in pure than in mixed culture, whereas complete die-out of S. faecalis populations occurred in both pure and mixed cultures. These results suggest that either nitrogen may in some way be toxic for S. faecalis, or lack of an oxygen source in a nutrient deficient environment may not be compatable with survival of

S. faecalis.

Some evaporation occurred in the flasks that were aerated. This would tend to cause concentration of any toxic or stimulatory substances present in the mecium as a result of metabolism. Whether or not this was a factor effecting the survival of either organism cannot be determined from these results.

As a result of the method that was used the mean temperature of incubation when the aerated and nitrogen atmospheres were studied was 9.4 C and 9.15 C respectively. Although the temperature of incubation fluxuated as the temperature of the tap water changed from day to day, it is doubtful that this effected the experiment significantly.

Probably in nature, the amount of dissolved oxygen of a water source would depend on a vast number of factors, such as the type of water source (lake, river, bay, etc.), climatic and seasonal variations in temperature, the associated microflora, amount of turbulence, and the amount of internal vegetation. These factors may be interdependent. For example, river turbulence, which tends to cause increased aeration, is dependent on the physical features of the river bed, and the water level, which in turn is dependent on the amount of snow-melt or rain fall.

In this study only isolated environmental factors were studied. In nature, however, the effects of environmental changes are complex and dependent on many variables. Hence, the methods used in this study, although expedient in application, are not the most ideal. In a static flask culture, factors such as nutrient availability in the bottom sediment, particle adsorption and sedimentation, diluative effects of river volume and rate of flow, and associative effects of the resident

microflora have been removed. Therefore, population reductions which were observed in this study may not be entirely characteristic of what occurs in nature.

Niemela and Tirronen (1967), in an attempt to minimize the effects of artificiality which are inherent in studies of environments limited to small, static volumes in closed containers, obtained viable coliform and fecal streptococci counts by sampling a river at several points once a month for two years. In a study such as this it is difficult to ascertain what factor or what combination of factors is directly responsible for population variance.

Ultimately, when the purpose of using specific organisms as indicators of fecal pollution is being evaluated, it is not as important to know exactly what factors directly effect the survival of a particular organism, as it is to know that environmental factors do display a marked effect on the survival of microorganisms in a natural environment. This study illustrates the importance of the analysis of environmental factors when the outcome of population growth of pollution indicator organisms is being evaluated.

It is also important to remember that pathogenic as well as indicator organisms are subject to the same environmental factors. Perhaps a more effective future approach to the study of the efficiency of E. coli and the enterococci as indicators of recent and dangerous pollution should involve comparative studies of the pathogenic as well as the indicator organisms.

As a result of early studies by Streeter (1927-1929) the U.S. Public Health Service (1962) standard for acceptable water quality of

drinking water sources requiring treatment has historically been a total coliform density of 5000/100 ml and 1000/100 ml in recreational waters. Yet Gallagher (1966) reported presence of salmonellae in water containing mean total coliform concentrations of 862/100 ml and fecal coliform concentrations of 35/100 ml in Las Vegas Bay, an area used extensively for boating and swimming. At a sampling station just below an area of chlorination, no salmonellae were isolated at mean total and fecal coliform densities of 6860/100 ml and 1280/100 ml.

Gallagher and Spino (1968) showed that rates of growth and die-out of fecal coliforms and Salmonellae typhimurium are different with regard to environmental conditons. They reported that S. typhimurium was much more persistant than fecal coliforms at low temperatures. Thus, large reductions in the indicator organisms may not necessarily insure reductions in the pathogen.

To date, the fecal coliforms and the enterococci remain the most efficient indicators of water pollution. Both organisms are found in feces of a variety of animals and fowl, both are found in water known to be fecally contaminated, and both are easily recoverable using simple techniques.

The results presented in this study indicate that S. faecalis is as good, but not necessarily a better indicator of recent and dangerous pollution in a cold, fresh water environment. S. faecalis was able to maintain comparable populations in both pure and mixed culture to those of E. coli under all conditions except at elevated temperatures with no buffering system, and when the oxygen tension was reduced.

The results also illustrate the importance of the analysis of

environmental effects on enteric bacteria when the ability to survive in nature outside the mammalian digestive tract is being studied. An extention of this type of application of ecological principles to the survival of enteric pathogens may result in a reevaluation of pollution standards.

CHAPTER V

SUMMARY

1. Viable cell counts of Streptococcus faecalis and Escherichia coli in pure and mixed culture were made during maintainence in river water under predetermined conditions of temperature, nutrient, pH, and oxygen supply.
2. S. faecalis populations were reduced in both pure and mixed culture when the pH was acidic (6.0), when the oxygen supply was increased and decreased, and when cells were maintained in unbuffered media at temperatures of 10 C or higher.
3. E. coli populations were reduced in both pure and mixed culture at both acidic (pH 6.0) and alkaline (pH 8.0) conditions and in unbuffered media at temperatures of 27 C and 37 C.
4. S. faecalis populations increased above the initial concentration only in the "concentrated" nutrient medium.
5. E. coli populations increased above the initial concentration in all of the nutrient media.
6. S. faecalis survived as well or better in mixed culture than in pure culture, depending on the environment.
7. E. coli survived as well or better in mixed culture compared to pure culture, except at temperatures of 27 C and 37 C in unbuffered media, and under altered oxygen tensions in buffered media.

8. *E. coli* survived better than *S. faecalis* in pure and mixed cultures in unbuffered media at temperatures of 10 C and above, and in a buffered medium under reduced oxygen concentrations.
9. *S. faecalis* survived better than *E. coli* only when the pH of the medium was alkaline (8.0).
10. The significance of the results is discussed.

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