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Survival of Coliform Bacteria in Natural Waters: Field and Laboratory Studies with Membrane-Filter Chambers

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Chambers with membrane-filter side walls were designed for studies of the survival of coliform bacteria in natural and artificial waters. Experiments were carried out in the field and in the laboratory. The initial uptake rate of inorganic ions, total carbon, and glucose into the chamber was greater than twice as fast as the accumulation of each into dialysis tubing. When the survival of a water-isolated fecal coliform bacterium was examined in two adjacent mountain streams, it was found that the organism persisted longer in Bozeman Creek than in Middle Creek. These data may be a reflection of the water chemistry because the concentration of inorganic constituents of the former was greater. Laboratory studies of the survival of a fecal coliform bacterium in artificial and natural water with continuous flow were used to determine the effect of chemical composition, temperature, and *p*H. The relation of this type of data to the use of fecal coliform bacteria as indicators of health hazard in water is discussed.

The occurrence of fecal coliform bacteria in water is regarded as the single most important indicator of public health hazard from infectious agents. However, to isolate the microorganism from water, it must survive in that environment for an indefinite time. In this connection, the fecal coliform bacteria have been found to be among the first of the microorganisms to die in the aquatic environment (6, 8, 14). Under some circumstances, therefore, the coliform bacteria in a polluted body of water could fail to survive while other bacteria or viruses persist to maintain the health hazard in the absence of the indicator organism.

The physical and chemical factors responsible for variations in the survival of fecal coliforms are extremely complex in water from different localities (4, 8, 9, 14). Recently, Geldreich (6) suggested that an examination of factors responsible for differences in the persistence of fecal coliforms is needed.

Results of studies carried out in our laboratories (12, 15) prompted the examination of coliform survival in the natural waters. Consistently higher coliform counts had been detected in the Mystic watershed (Bozeman Creek) than in the adjacent Hyalite watershed (Middle Creek). Also, analyses showed significantly higher concentrations of most inorganic ions in the water from Bozeman Creek. These observations suggested that differential survival rates, possibly brought about by the dissimilar chemical compositions, might be partially responsible for an exaggeration of the bacteriological inequality of the two streams.

This report describes the development of a technique in which a dialysis chamber with membrane side walls is used for the study of bacterial survival, both in situ and in the laboratory under controlled conditions. The findings are used to help explain microbiological data from the field and some environmental parameters relative to the survival of a coliform bacterium.

MATERIALS AND METHODS

Bacteria and media. The bacterium used in these studies was isolated from a stream within the Bozeman Municipal Watershed. It was identified as *Escherichia coli* by the following cultural characteristics: growth on Brilliant Green lactose bile broth, characteristic colonies on eosin methylene blue agar, growth and gas production in 24 hr in EC broth at 44.5 C, and indole, methyl red, Voges-Proskauer, citrate reactions of ++--. This bacterium was designated as *E. coli* MH 3427.

All cultures used in these experiments were grown in Trypticase soy broth supplemented with 0.3% yeast extract and 0.5% glucose (TSY broth) for 24 hr

at 37 C. Cells were harvested by centrifugation (3,020 × *g*) for 10 min and washed twice with sterile phosphate buffer solution (1). After the final wash, the cells were suspended in the sterile buffer and diluted to the desired population density. The bacteria were enumerated by standard methods (1).

Chambers. The dialysis culture chambers were constructed of 6.5-mm (0.25-inch) Plexiglas (Fig. 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. Membrane sheets (HAWP 304FO, Millipore Corp.) were cut into circular pieces with a diameter of 7.5 cm. The tear-resistant microweb membranes were useful in the field experiments. When the chamber was assembled, these were inserted on either side of the central spacer and held in place by 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. These chambers are similar in general design to devices that are commercially available (Chemical Rubber Co. and Bel Art Products) but differ in chamber geometry and in the surface to volume ratio.

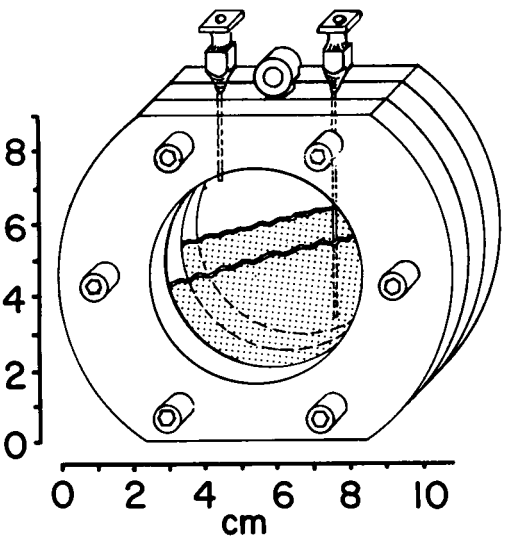

FIG. 1. *Schematic drawing of the membrane dialysis chamber.*

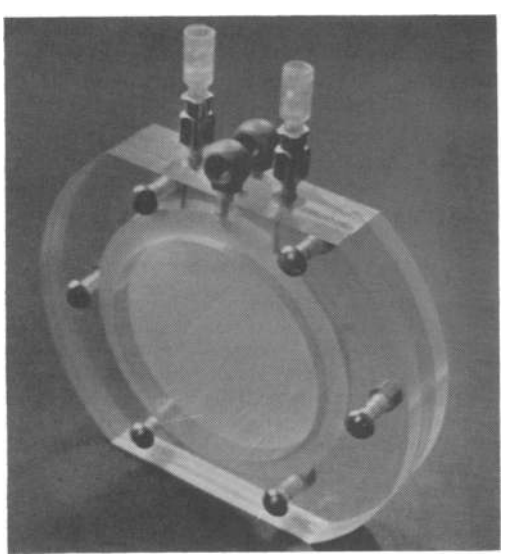

FIG. 2. *Photograph of a membrane dialysis chamber that is assembled and ready for use.*

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 chambers (Fig. 1). Dust caps were made by filling the hubs of plastic syringes with plastic cement. The Plexiglas parts of the chambers were sterilized by autoclaving, and the membranes were irradiated with ultraviolet light. Prior to assembly of the chambers, the membranes were soaked in sterile dilution buffer for 15 min. The chambers were assembled aseptically by placing the membranes between the central spacer and the retainers and securing them with stainless-steel bolts and nuts. (The chambers may be purchased through the authors.)

Field experiments. Chambers loaded with suspensions of washed cells were placed in streams within the Bozeman Municipal Watersheds. These sites were in the vicinity of the diversion dams, 6 to 7 miles (9.7 to 11.3 km) downstream in Bozeman Creek and Middle Creek, as described by Stuart et al. (12). The chambers were immersed in quiet areas where the flow was less than 15 cm per sec. Heavy string was used to suspend the chambers from an overhead support. This allowed some rotational movement of the chambers within the stream. Samples were removed daily with sterile syringes for bacterial enumeration. At the same time, the conductivity of the stream was measured, and a recording immersible thermograph was used in the vicinity of each chamber to monitor the water temperature.

Laboratory experiments. Bacterial survival experiments were also performed in the laboratory. The chambers were immersed in water that filled Plexiglas overflow vessels which were suspended in a refrigerated water bath to control the temperature. Constant mixing in the overflow vessels was accomplished by magnetic stirrers that were driven by submersible pneumatic turbines (Van Waters and Rogers, Brisbane, Calif.). The flow of water into the overflow vessel was regulated by a Harvard peristaltic pump at 2 ml/min. Stream water used in these experiments was stored at 4 C to retard physical and biological alteration.

Other experiments were conducted in the laboratory to determine the uptake of various solutes into the chambers as compared with uptake through standard viscose dialysis tubing sacs. The surface to volume ratio of the dialysis sac was equal to that of the chamber. This dialysis tubing sac was fitted with a rubber stopper which contained a hypodermic needle for sample withdrawal. A chamber and a dialysis tubing sac were each filled with 20 ml of distilled water and immersed in 2 liters of specially prepared

water with continuous mixing. This water was obtained near the outflow of the Bozeman Wastewater Treatment Plant; it was filtered through Whatman no. 2 filter paper and then supplemented with 50 μg of Dextran 500/ml (molecular weight, 500,000). The conductivity of the water was 220 μmhos, and the total carbon content was 95 μg/ml. In separate experiments in which the uptake of glucose was followed, the glucose (final concentration, 1.7 mM) was added to distilled water that surrounded the chamber and the sac.

Analytical procedures. Carbon was analyzed as total carbon by use of a Beckman laboratory carbonaceous analyzer calibrated with a standard containing 100 μg of carbon/ml as glucose.

A Lab Line Lectro MHO meter measured conductivity, which was used as an indicator of inorganic ions in the water. Standard methods were employed for the analysis of anions and cations (1).

The glucose concentration was determined by the Glucostat, glucose-oxidase peroxidase system made by the Worthington Biochemical Corp.

RESULTS

Permeability to various solutes. The permeability of the chambers to various chemicals was examined to evaluate the responsiveness of this system to changes in the environment. This was done by examining the uptake of solutes of different size into chambers as compared with their uptake into viscose dialysis tubing sacs. The accumulation of total carbon differed markedly between the two devices when high-molecular-weight dextran was the major solute (Fig. 3). The final concentration of carbon was one-third greater in the chamber than in the sac. In addition, the initial uptake rate of both carbon and inorganic components, which are responsible for conductivity, into the chamber was more than twice as fast as into the dialysis sac. As expected, the final conductivity of the contents of the sac and the chamber were not significantly different.

A separate experiment was performed to determine the comparative permeability of the chamber for glucose. As before, the initial uptake rate into the chamber was greater than twice as fast as into the dialysis sac, and the final concentration was greater in the chamber (Fig. 4).

Control experiments. Experiments were performed to establish various operational parameters of the dialysis chambers. To determine that the sterilization and assembly had been done properly, a chamber was prepared as described above, filled with sterile phosphate buffer, and placed in distilled water. Samples (1.0 ml) were withdrawn twice daily for 5 days and plated on TSY agar. None of the plates revealed any contamination when incubated for 24 hr at 37 C.

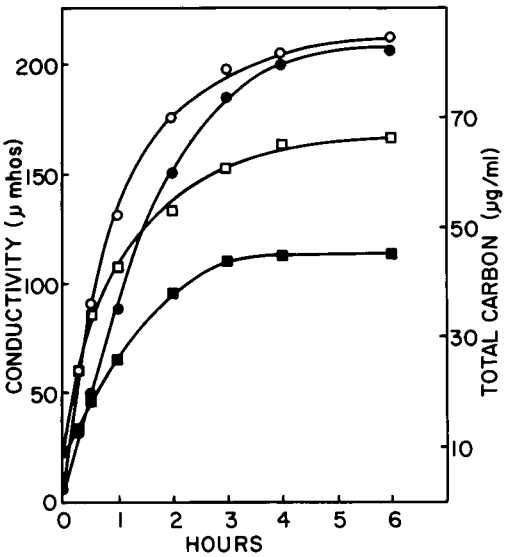

FIG. 3. *Comparative uptake of carbon-containing compounds (squares) and ions responsible for conductivity (circles) into a membrane dialysis chamber (open symbols) and a dialysis tubing sac (closed symbols). The exclosures were filled with distilled water and submerged in natural water enriched with Dextran 500 and mixed continuously. At the indicated times, samples were withdrawn from inside each exclosure and analyzed for total carbon and conductivity. The water temperature was 23 C.*

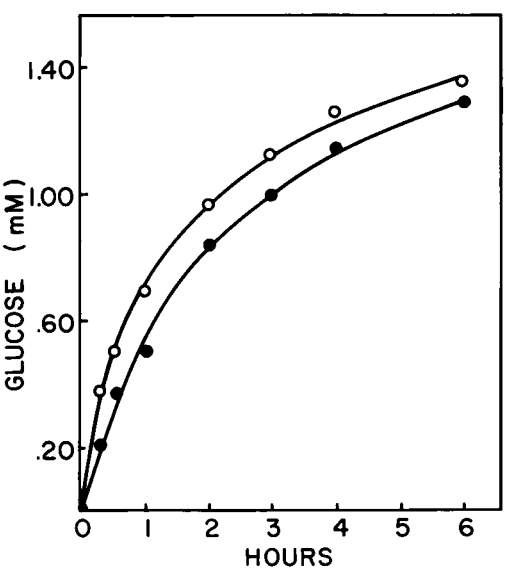

FIG. 4. *Comparative uptake of glucose into a membrane dialysis chamber (O) and a dialysis tubing sac (●). The exclosures were filled with distilled water and submerged in distilled water containing glucose with continuous mixing. Samples were removed from the exclosure and analyzed for glucose concentration at timed intervals. The water temperature was 22.5 C.*

The chambers were also tested for leakage of coliform bacteria by comparing the coliform population of the water going in to the overflow vessel in a laboratory experiment with that of the effluent. This was done daily for 5 days with Van Donsel's agar (D. J. Van Donsel, R. W. Twedt, and E. E. Geldreich, Bacteriol. Proc., p. 25, 1969) as the plating medium. No coliform bacteria were detected in either location.

Next, the effect of membrane filters on coliform survival was tested. A washed suspension of *E. coli* MH 3427 in diluted phosphate buffer or river water (2×10^4 cells/ml) was added to two sterile tubes. One contained strips of ultraviolet-sterilized membrane and the other did not. These tubes were incubated at 10 C, and the standard plate count was determined daily for 3 days. These experiments revealed no significant difference between the tubes containing the membrane and those that did not.

Bacterial survival in situ. Experiments were done to compare the survival of *E. coli* MH 3427 in natural river water from two sources. A washed suspension of cells in dilution buffer (10^8/ml) was added to chambers that were placed in Bozeman Creek and Middle Creek. Standard plate counts done at daily intervals from the two chambers revealed greater survival in Bozeman Creek (Fig. 5). This experiment, repeated with the location of the chambers, was changed to be sure this was not a localized phenomenon and that it was reproducible. The results, however, were very similar.

Through the course of the experiment illustrated in Fig. 5, the mean daily water temperature changed from 6 to 4 C with less than 1 degree of difference between the two sites. There was a diurnal variation of 2 to 3 degrees through the duration of the experiment. The conductivity of the Bozeman Creek site was 150 μmhos and the Middle Creek site was 95 μmhos. This difference remained constant through the 5 days of the experiment and is also reflected in the chemical composition of the water from the two streams (Table 1).

Bacterial survival in the laboratory. Experiments carried out in a controlled physical and chemical environment were impractical in the field. Therefore, a series of experiments was initiated in the laboratory. Because of the logistical limitations, the flow rate was greatly diminished as compared with the field studies. This altered the magnitude of the bacterial survival such that, whereas only 0.2% of the original population remained viable after dialysis culture in the field, in a laboratory experiment in which the same water was used at the same temperature 60% remained viable. The effect of temperature on the survival of *E. coli* MH 3427 was examined in the laboratory with the water from Middle Creek. In these studies, temperature of the bath was adjusted so that the contents of the overflow vessel were at 5.0 ± 0.1 C. Samples were removed daily and the bacteria were enumerated. At 3-day intervals, the temperature was increased 5 degrees. The resulting data were replotted as the time for a 50% decrease of the viable population at each temperature (Fig. 6). The response of survival to temperature was inversely proportional between 5 and 15 C. Above 15 C, temperature became less important in coliform survival. In different experiments, various temperature shifts were made to confirm these data, with very reproducible results.

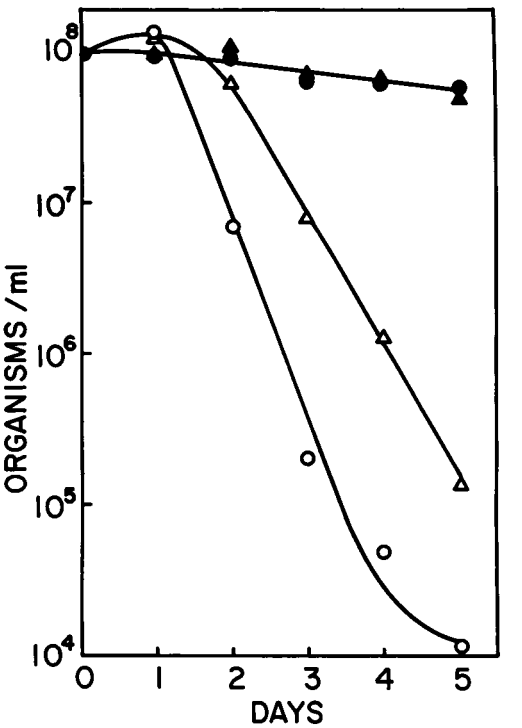

FIG. 5. *Survival of E. coli MH 3427 in situ and in the laboratory. Washed cells that were placed in membrane dialysis chambers were immersed in Bozeman Creek (Δ) and Middle Creek (O) and in the laboratory simulator in the water from Bozeman Creek (▲) and Middle Creek (●). Water samples were brought to the laboratory daily to be used in the simulator. Samples were removed from the chambers daily for bacterial enumeration.*

Experiments were also done in the laboratory to evaluate the effect of *p*H on the survival of *E. coli* MH 3427. A washed-cell suspension (10^4/ml) was prepared as before and loaded into

TABLE 1. *Comparison of the chemical characteristics of the water from Bozeman Creek and Middle Creek*[a]

Location	pH[b]	Hardness as $CaCO_3$	Ca^{2+}	Mg^{2+}	SO_4^{2-}	Na^+	K^+	Conductivity (μmhos)
Bozeman Creek	8.37	95.8	26.0	7.48	6.5	4.60	1.68	150
Middle Creek	8.10	55.8	16.5	3.50	2.25	2.07	1.29	95

[a] The amounts of each component are given in micrograms per milliliter.
[b] Determined in the field.

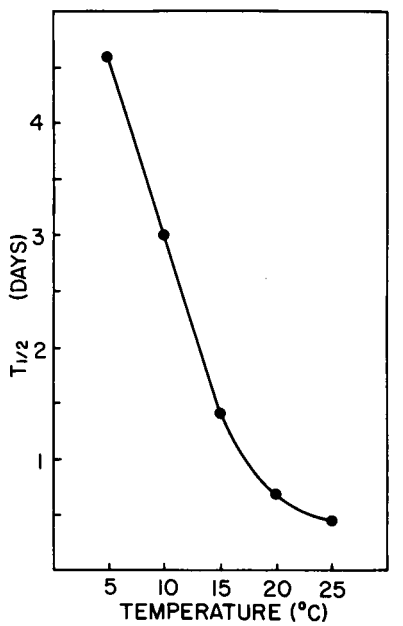

FIG. 6. *Effect of temperature on the survival of E. coli MH 3427. Washed cells (10⁴/ml) in chambers were placed in the laboratory simulator with water from Middle Creek at various temperatures. Samples were withdrawn daily for bacterial enumeration. The flow rate of the water into the simulator was 2 ml/min. The time for a 50% reduction in the viable population (T½) at each temperature was plotted against that temperature.*

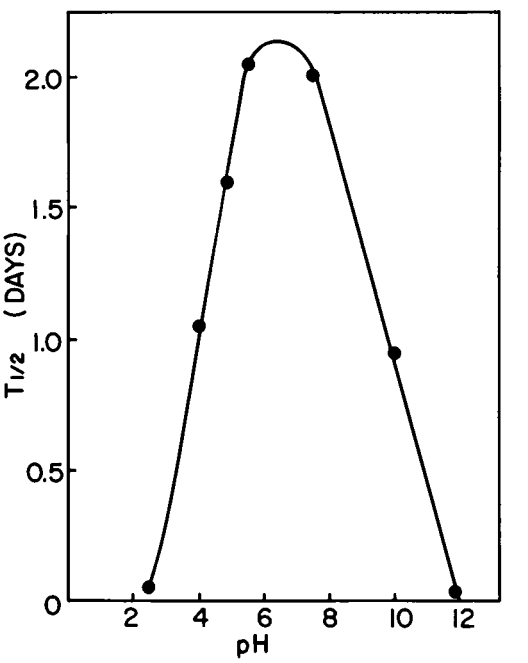

FIG. 7. *Effect of pH on the survival of E. coli MH 3427. Washed cells (10⁴/ml) were placed in the laboratory simulator with deionized distilled water at different pH values. Samples were withdrawn daily for bacterial enumeration. The flow rate of the water into the simulator was 2 ml/min. Time for a 50% reduction in viable population (T½) at each pH was plotted against that pH.*

chambers which were immersed in deionized water of various pH values. These experiments were carried out at 10 C, and the pH of each solution was checked at that temperature. Either KOH or HCl was used to adjust the pH to the desired range. The data from these experiments (Fig. 7) indicate an optimum for survival between pH 5.5 and 7.5, with rapid decline both above and below these values.

When the survival of *E. coli* MH 3427 in the water from Middle Creek and Bozeman Creek was compared in the laboratory, no significant difference was seen (Fig. 5). This is in contrast to the results of experiments performed in situ (Fig. 5). In this connection, 23% of the original population survived after 3 days in a laboratory dialysis experiment with distilled-deionized water at 10 C, whereas 50% survived in river water or a synthetic river water at the same temperature. This synthetic river water was prepared with distilled-deionized water and contained the approximate concentration of the major cations and anions found in the natural creek waters (Table 1). When the effect of distilled water on coliform survival was examined, only 0.1% of the original population remained viable after 1 day. Furthermore, when the synthetic river water was prepared with distilled water, survival was only slightly greater than when distilled water alone was used.

DISCUSSION

A review by Schultz and Gerhardt (10) dealing with the theory and design of dialysis culture methods was used in the development of the dialysis survival technique described in this communication. In this procedure, porous membranes retain a viable suspension of bacteria in a natural or artificial aqueous environment for study or enumeration. The membrane allows the water and solutes in that environment to diffuse readily through the chamber and to interact with the bacterial suspension. A significant advantage of this system over procedures in which bacteria are studied in a limited and unchanging water sample is that a continuous exchange of water and solutes come in contact with the bacteria under investigation. This characteristic allows the system to be responsive to physical and chemical changes that may occur in the surrounding water. This can be seen in the uptake of organic and inorganic solutes of differing sizes into the chamber (Fig. 3 and 4). The difference in the initial uptake rate between the dialysis tubing and the chamber was most marked when high-molecular-weight dextran was added. However, there was greater than a two-fold difference in the initial uptake rate of each of the solutes tested. The difference in final concentration between the chamber and the sac was dependent on the molecular weight of the particular solute under investigation. In the case of total carbon, when the predominant solute was dextran, the final concentration was significantly greater in the chamber. In contrast, when the solutes taken up were inorganic ions, the difference in final concentration was slight. These differences can be explained by the dissimilarity in the porosity of the material involved. The average pore diameter in the dialysis sac is on the order of 3 nm, whereas that of the membrane used in the chambers is 450 nm, or 150 times larger.

The experiments done in situ illustrate one application of the chamber dialysis technique: to aid in the interpretation of the results from water microbiology studies. Previous work compared the water microbiology of two wooded mountain watersheds that serve as the municipal water supply for Bozeman, Mont. (12, 15). A significant difference was found in the microflora as well as the water chemistry between the two streams, with Bozeman Creek being higher in both categories. Although the origin of this disparity in bacterial population was believed to be the presence of wild animals in the Bozeman Creek Watershed (12), the question was raised as to whether the water chemistry had caused an exaggerating effect on the difference of the numbers of organisms found in the two streams. From the survival experiments carried out in the lower reaches of these two watersheds (Fig. 5), it was concluded that coliform bacteria did persist longer in Bozeman Creek. Hence, differential survival had a tendency to accentuate the difference found in the microbial population of the two streams.

In experiments carried out in the laboratory by use of a membrane-filter chamber, bacterial survival was studied with the physical and chemical make-up of the water controlled. However, the reduced flow that was necessary in the laboratory was probably responsible for the greater survival seen in those experiments. As a result, the data obtained in this manner probably do not reflect the actual condition in the stream but are related to it. Studies of the effect of temperature on coliform survival is only practical in the laboratory. The importance of temperature in the survival of enteric bacteria was reported in 1927 (9). More recently, others (3, 14) have observed the role of seasonal temperature variation in the persistence of the *Enterobacteriaceae* in aqueous environments. The results of our laboratory experiments (Fig. 6) demonstrate the relationship between temperature and the survival of fecal coliforms. These data indicate that below 15 C survival was inversely related to temperature but above 15 C this relationship became less critical. One situation pointing to the importance of this observation was reported (11) in which enteric pathogens and coliform bacteria were recovered 73 miles (4 days of flow time) below their source. This was noted when a river which was enriched with sugar beet wastes was ice-covered and the water temperature was described as being very cold.

In comparing the survival studies done in the laboratory and in the field, it should be noted that the laboratory experiments were less sensitive to differences in the chemical composition of the water. This was seen in the similarity of the bacterial survival results obtained in the laboratory in water from Bozeman and Middle Creeks, whereas at the same time the field data shown in Fig. 5 revealed marked differences between the two streams. In this connection, laboratory experiments showed only slight differences of coliform survival in river water and the synthetic river water that was prepared in deionized distilled water. Similar experiments in which distilled water was used both alone and in the form of synthetic river water revealed that the survival of coliform bacteria was

less than one-hundredth of that when deionized distilled water was used. These data point to some toxic material in the distilled water that was removed when the distilled water was deionized.

Another important implication of these studies relates to the use of survival techniques in gaining a more complete understanding of the significance of either the presence or absence of viable coliform bacteria in natural waters of various physical and chemical compositions. Numerous workers have justified the use of fecal coliform bacteria as an indicator of fecal pollution and hence a possible public health hazard (1, 4-6). However, depending on the physical and chemical make up of the water, the coliform indicators may or may not survive to be identified. If they do not survive, the question may be asked, does this make the water safe to drink if pathogens are present? In the case of *Salmonella* species, the answer to the question may be yes, as the fecal coliforms and the salmonellae have very similar survival properties in natural water (1, 6, 9, 13). On the other hand, this says nothing about other pathogenic bacteria and viruses, because it has been shown that the fecal coliforms and *Salmonella* species have the most rapid decline in natural water of all of the microorganisms of public health significance tested thus far (6, 8, 13).

Elevated temperature resulting from clear-cut logging, practiced in many municipal watersheds, may raise the water temperature of a stream significantly. Studies done in the coastal area of Oregon (2) indicated that the daytime maximal water temperature increased about 8 C (from 13.5 to 22 C). From Fig. 5, it can be seen that this temperature increase represents a significant decrease in the survival potential of fecal coliforms. Likewise, thermal pollution found in various areas is important in this regard.

In addition to temperature, many other physical and chemical parameters probably modulate the survival rate of fecal coliform bacteria, such as toxic and nontoxic chemicals, aeration, sediment, and *p*H. The need therefore exists to examine these factors under different circumstances with different bacteria (5-7). The dialysis survival technique described in this communication is suitable for use in these studies in the field as well as in the laboratory.

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