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# Influence of Diluents, Media, and Membrane Filters on Detection of Injured Waterborne Coliform Bacteria

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Pure cultures of *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes*, and *Citrobacter freundii* were injured (>90%) in water from a dead-end section of the Bozeman, Montana, distribution system. The effects of the following laboratory variables on the enumeration efficiency of injured and undamaged control cells were examined: (i) diluent composition, temperature, and time of exposure; (ii) media, using various formulations employed in enumerating gram-negative bacteria; and (iii) surface pore morphology of membrane filters. The addition of peptone or milk solids to diluents and low temperature (4°C) maximized the recovery of injured cells, but had little effect on undamaged cells. Control cells were recovered with high efficiencies on most media tested, but recoveries of injured cells ranged from 0 to near 100%. Most of the media commonly used in water analysis recovered less than 30% of injured cells. This was explained in part by the sensitivity of injured bacteria to deoxycholate concentrations greater than 0.01%, whereas control cells were unaffected by 0.1%. Membrane filter surface pore morphology (at 35°C) had a negligible effect on total coliform recoveries. Recommendations are made regarding procedures to improve the recovery of injured coliforms by routine laboratory practices.

The detection of coliform bacteria in drinking water and wastewater can be inhibited by a variety of factors, including excessive numbers of heterotrophic bacteria, turbidity, and a process known as sublethal injury (17, 32). As a result, the actual number of indicator organisms present may be underestimated. Sublethal injury has been established as an important factor in determining the safety of foods exposed to physical and chemical treatments that damage indicator bacteria (7, 22). Such debilitated organisms are often unable to grow on selective media (27), but can regain that capability through a resuscitation process (1, 3, 7, 9, 22, 29) under nonrestrictive conditions. The concept of sublethal injury was first associated with the suppression of waterborne indicator bacteria when it was noted that coliform enumeration data from waters containing toxic wastes or chlorine were consistently higher by the multiple tube fermentation–most-probable-number method than by the membrane filtration procedure (6, 9, 22, 29, 35). More than 90% of the indicator bacteria present may become injured when exposed to natural waters for less than 1 week (3, 4). Injury is an important factor in underestimating numbers of waterborne indicator bacteria which may lead to inaccurate public health assessments.

Injury in the aquatic environment may be related to a number of factors, including time and temperature of exposure, disinfection levels, strain of organism, concentration of nutrients, presence of heavy metal ions, antagonistic standard plate count bacteria, and possibly other, undefined chemical and physical parameters (3, 9, 17, 28, 42–44). In addition, laboratory manipulations involving exposure to diluents, selective media, and membrane filtration may cause further underestimations of bacterial densities in aquatic environments. The importance of diluent composition in the accurate bacteriological analysis of water samples was pointed out in earlier studies (8, 42). More recent reports (3, 6, 23, 24, 32, 37, 41, 43) have supported the previous conclusions and suggested the value of low concentrations of organic additives such as peptone, tryptone, or milk solids. In most studies, however, little attention has been given to injured bacteria. Selective media have likewise been reported as a factor that causes variable recovery efficiencies of indicator bacteria (4, 11, 12, 18, 21, 32, 34). Bacteria injured in water have been shown to be more sensitive to selective ingredients in various enumeration media (4, 11) because of damage to the bacterial envelope (44). This realization stimulated the successful development of resuscitation procedures and media for the improved enumeration of injured fecal coliforms, particularly in chlorinated wastewater effluents (3, 4, 19, 25, 38). A new section describing these methods, entitled "Stressed Organism Recovery" (section no.

921), has been added to the 15th edition of *Standard Methods for the Examination of Water and Wastewater* (2). Recently, the choice of membrane filter type has also been reported to affect the recovery of indicator organisms (16, 31, 36, 39). Sladek et al. (36) reported that optimal recovery of fecal coliform bacteria on membrane filters with the high-temperature test (2) was a function of the surface pore morphology of the filters, but this concept was not applied to injured total coliforms grown at 35°C. However, coliform bacteria exposed to chlorine levels found in drinking water for 10 min or more may be recovered less efficiently by membrane filtration than on spread plates of the same medium (20).

There has been general agreement that characteristics of diluents, selective media, and membrane filters influence the recovery efficiency of waterborne indicator bacteria, but little attention has been devoted to understanding the involvement of prior cellular injury in these laboratory manipulations. The research reported here examined the recovery of coliforms both before and after substantial cellular injury in drinking water with respect to: (i) the composition, exposure time, and temperature of diluents; (ii) the composition of various media commonly used in water quality testing; and (iii) the surface pore morphology of membrane filters at 35°C.

## MATERIALS AND METHODS

**Cultures.** Cultures were obtained from surface and distribution water by the membrane filter technique (2). The isolates were identified with the API 20E system (Analytab Products, Plainview, N.Y.) as *Escherichia coli* (two strains), *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Citrobacter freundii*. The cultures were streaked periodically on *m*-Endo agar (Difco Laboratories, Detroit, Mich.), and a colony with a metallic sheen was selected. Cultures were stored on slants of tryptic soy broth (Difco) plus 0.3% yeast extract (Difco), 1.0% lactose (Difco), and 1.5% agar (Difco) (TLY agar) at 4°C.

**Organism preparation and laboratory injury.** Eighteen-hour, stationary-phase organisms grown in TLY broth (tryptic soy broth without glucose [Difco] supplemented with 1% lactose and 0.3% yeast extract) were harvested by centrifugation at 3,000 × *g* in a model RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), washed twice in sterile refrigerated reagent-grade water (obtained with a Milli-Q reagent-grade water system [Millipore Corp., Bedford, Mass.] supplied with single-distilled water), and suspended to concentrations of approximately $10^9$ colony-forming units (CFU) per ml. After being washed, at the beginning of each experiment, cultures were plated out to assess whether injury had occurred due to preparatory procedures. Because of the short washing time and use of cold reagent-grade water, no injury occurred in any of the preparatory procedures. Injured populations of coliforms were obtained by placing washed cells into a membrane dialysis chamber (28) immersed in water drawn from a dead-end main of the Bozeman, Montana, drinking-water distribution system. Various water characteristics were monitored, including conductivity, pH, and free residual chlorine. None of these characteristics correlated uniquely to the rate or occurrence of injury in coliforms, indicating the complex nature of aquatic stress. Samples were periodically withdrawn and tested for injury by comparing viable counts obtained by the spread-plate technique (2) on TLY agar (TLY broth plus 1.5% agar) and TLY agar with 0.1% deoxycholate (TLY-D). The percent injury was calculated by the following equation: (percent injury) = {[(TLY count − TLY-D count)/(TLY count)] × 100}. A population was considered "injured" when at least 90% of the cells failed to grow on the selective medium (TLY-D).

**Effect of diluent composition on coliform recovery.** Five diluents were tested with injured and noninjured *Escherichia coli* suspensions at 4 and 23 to 24°C (room temperature) to determine the effect of diluent composition, temperature, and time of exposure on the recovery of injured coliforms from water. Diluents used were: reagent-grade water, phosphate buffer (2), 0.1% peptone water (2), phosphate buffer amended with 0.1% peptone, and 1.0% milk (Difco). Diluents were sampled in triplicate at 30-min intervals by the membrane filter technique (2), using HC filters (Millipore Corp.). Filters were placed on *m*-Endo agar (Difco) and incubated for 22 to 24 h at 35°C. The percent recovery was determined by dividing the average count obtained throughout the experiment by the zero time count.

**Effect of media composition on coliform recovery.** Injured and noninjured coliforms were plated on various media to determine which formulation permitted the best recovery from water. The media tested included: triple sugar iron agar, tergitol 7 broth without indicator, lactose broth, EE broth, brilliant green broth, Levine eosin methylene blue agar, lauryl tryptose broth, *m*-Endo broth, deoxycholate lactose agar, brilliant green bile 2% broth, violet red bile, *m*-FC broth, eosin methylene blue agar, MacConkey agar, GN broth, and XLD agar (all from Difco). Also tested were nutrient alginate (13), boric acid broth (10), purple serum agar (30), minerals modified glutamate (3), TLY agar, TLY-D, TLY agar plus 0.1% Tween 80 (Difco), and 3V agar (Millipore Corp.). To all broths 1.5% agar (Difco) was added to make a solid medium. Plates were prepared at least 4 h before inoculation to allow for adequate drying. Appropriate dilutions of the organisms were made in sterile reagent-grade water at 4°C. Quintuplicate plates of each medium were inoculated by the spread-plate technique (2) and incubated for 18 to 24 h at 35°C, except boric acid agar, which was held at 35°C for 48 h. After incubation, an average count from each medium was compared with the average count obtained on TLY agar to determine the percent recovery.

**Effect of membrane filter type on the recovery of coliforms.** Experiments were conducted to compare the recovery of injured and noninjured *Escherichia coli* on HC and HA membrane filters (Millipore Corp.). Appropriate dilutions of the organism were made in sterile reagent-grade water at 4°C and processed within 30 min. Five 1-ml samples were filtered through the membrane filters and placed on TLY agar

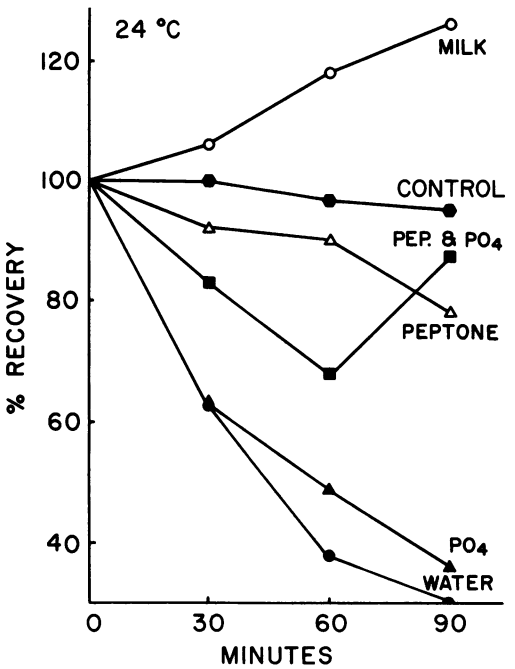


FIG. 1. Effect of diluent composition and exposure time on the recovery of injured (90%) and uninjured control *Escherichia coli* suspensions at 24°C. The control line is an average of all data obtained when uninjured cells were exposed in each diluent. Diluents are 1.0% milk, 0.1% peptone, reagent-grade water, phosphate buffer, and phosphate buffer amended with 0.1% peptone (PEP). Values are a mean of three experiment replications.

and TLY-D. Dilutions were also plated on TLY agar and TLY-D by the spread-plate technique, and all the plates were incubated at 35°C for 18 h.

Throughout the course of this study, a quality assurance program was followed as outlined in *Standard Methods for the Examination of Water and Wastewater* (2) and *Microbiological Methods for Monitoring the Environment* (5).

## RESULTS

**Effects of diluents on the recovery of injured coliforms.** The enumeration efficiency of injured and healthy suspensions of *Escherichia coli* was examined after exposure to five diluents. Sample dilutions are generally required in the analysis of polluted waters, and phosphate dilution water or 0.1% peptone water are the recommended diluents (2). What effect these diluents and others might have on the recovery of injured and healthy suspensions of *Escherichia coli* was investigated in relation to time and temperature of diluent exposure. Injured organisms diluted in various diluents at 24°C varied widely in the efficiency of recovery during the 90-min span of the experiments (Fig. 1). Injured bacteria held for 90 min in phosphate buffer or reagent-grade water were recovered at only 30 to 35% of the initial levels. Dilutions made in peptone water or phosphate buffer with 0.1% peptone were the most stable, yielding recoveries of 80 to 90% after 90 min of exposure. Repair of the injured coliforms occurred in milk at 24°C, increasing the bacterial counts on *m*-Endo agar by greater than 20%. Densities of uninjured control cells remained virtually unchanged throughout the experiment regardless of the diluent used.

When the diluents were used at 4°C, the effect of the different formulations (Fig. 2) was much less than at 24°C. The recovery response of the injured cells was very similar to the response of uninjured cells when exposed in each diluent. This was particularly true in the first 30 min.

**Effect of various media on recovery.** The recovery efficiency of injured and control coliforms was tested on a variety of selective media. Media were chosen from among those used in various applications where enteric bacteria are enumerated. Uninjured control cells were recovered at nearly 100% efficiency on most of the media tested, whereas injured cells were recovered at lower rates (Table 1). The media are arranged in three groups, based on the response of the injured cells.

Some of the media most commonly used in water testing were evaluated on a year-round basis. The ranges and average recovery rates for these media were calculated from seven repetitions, using five coliform species. Four genera of coliforms, *Escherichia coli*, (two strains), *K. pneumoniae*, *C. freundii*, and *Enterobacter aerogenes*, were used to determine whether varia-

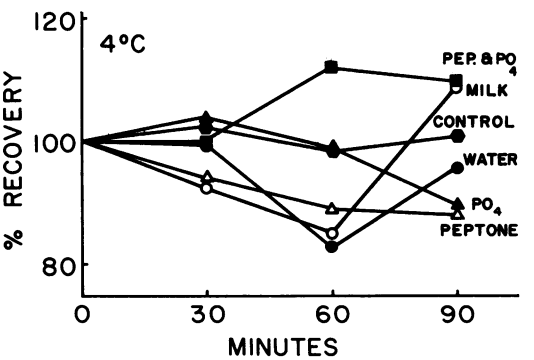


FIG. 2. Effect of diluent composition and exposure time on the recovery of injured (90%) and uninjured control *Escherichia coli* suspensions at 4°C. The control line is an average of all data obtained when uninjured cells were exposed in each diluent. Diluents are 1.0% milk, 0.1% peptone, reagent-grade water, phosphate buffer, and phosphate buffer amended with 0.1% peptone (PEP). Values are a mean of three experiment replications.

TABLE 1. Media and the recovery of injured and healthy coliforms from water[a]

| Medium | % Recovery (range)[b] | | % Deoxycholate or related compounds |
|---|---|---|---|
| | Injured | Healthy | |
| Group I | | | |
| Triple sugar iron | 181 | 106 | 0 |
| Nutrient alginate | 125 | 88 | 0 |
| Minerals modified glutamate | 99 | 106 | 0 |
| Tergitol 7 | 86 (71–101) | 99 | 0 |
| Boric acid | 84 | 92 | 0 |
| TLY + 0.1% Tween 80 | 72 | ND[c] | 0 |
| Group II | | | |
| Lactose broth | 72 (47–98) | 102 | 0 |
| *m*-Endo | 66 (30–102) | 93 | 0.1; 0.005[f] |
| Lauryl tryptose | 56 (34–79) | 98 | 0.01[f] |
| Levines EMB | 42 (37–47) | 119 | 0 |
| 3V | 39 | 95 | NA[g] |
| Purple serum | 38 | 56 | 0 |
| EE | 38 | 106 | 2.0[d] |
| Brilliant green bile 2% | 34 (18–51) | 106 | 2.0[d] |
| Deoxycholate lactose | 26 | 94 | 0.05 |
| Group III | | | |
| Eosin methylene blue | 24 (7–42) | 102 | NA |
| Violet red bile | 12 | 99 | 1.5[e] |
| *m*-FC at 44.5°C | 7 (4–10) | 105 | 1.5[e] |
| MacConkey | 5 | 97 | 0.1[e] |
| GN | 4 | 71 | 0.05 |
| TLY-D | 2 | 82 | 0.10 |
| XLD | 0 | 40 | 0.25 |

[a] Coliforms tested include: *Escherichia coli* (two strains), *K. pneumoniae*, *C. freundii*, and *Enterobacter aerogenes*.

[b] (Percent recovery) = {[(CFU selective medium)/(CFU TLY)] × 100}. Injury was between 90 and 99%. The range for injured coliforms is calculated from seven repetitions, using five coliforms over a 1-year period.

[c] ND, Not done.

[d] Oxgall.

[e] Bile salts.

[f] Lauryl sulfate.

[g] NA, Not available.

tions occurred in recoveries of different coliforms. No substantial variation occurred between the different genera in their response to different media compositions. Of the 16 most suppressive media (groups II and III, Table 1), 11 contain deoxycholate or bile salts. Because of this observation, an experiment was done to examine the relationship between deoxycholate concentration and recovery efficiency on a complete medium. Whereas uninjured cells are virtually unaffected by deoxycholate concentrations of less than 0.1%, injured cells are severely inhibited at all concentrations greater than 0.01% (Fig. 3).

**Membrane filter surface pore morphology and recovery.** Populations of injured *Escherichia coli* were enumerated on complete selective (TLY-D) and complete nonselective (TLY) media, using two filters. The filters were selected to examine the effect of the mean surface pore diameter on the recovery efficiency of injured *Escherichia coli* at 35°C. The membranes with a 2.4-μm surface pore opening (HC) and those with openings of less than 1.0 μm (HA) yielded nearly identical results (Table 2). Similar results were seen when uninjured control cells were examined under the same conditions.

## DISCUSSION

The dilution step, as shown by the data in Fig. 1 and 2, can greatly influence the enumeration efficiency of injured coliforms. Early studies done in 1927 (42) and 1932 (8) suggest a critical role of diluents in some bacteriological examinations. More recent reports have further emphasized this conclusion with nonstressed bacterial suspensions (23, 24, 32) and cells that were injured by freezing (41) and exposure to water (3, 37, 43). Diluents containing 0.1 or 0.05% peptone have been useful in the recovery of attenuated organisms from food, industrial wastes, and waters containing heavy metals

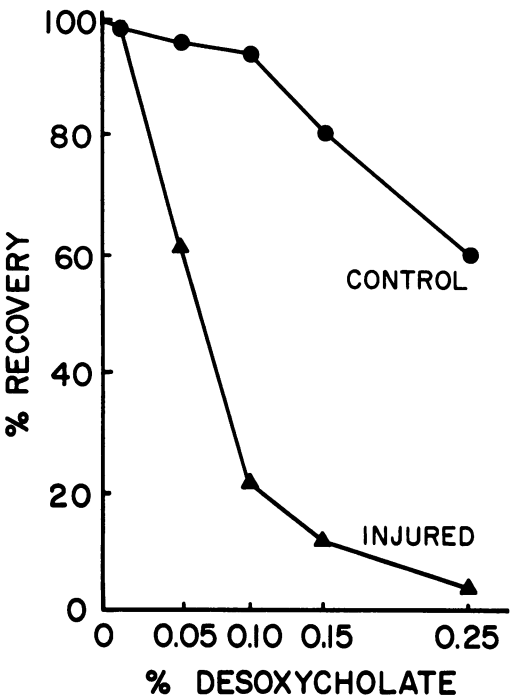


FIG. 3. Effect of various concentrations of deoxycholate on the recovery efficiency of injured and control *Escherichia coli* suspensions. Deoxycholate was added to TLY agar, and the spread-plate method was used.

(37), but bacterial multiplication can occur if the time between sample dilution and plating exceeds 40 min (15). Phosphate buffer containing magnesium phosphate (2) has been used to dilute samples containing metabolically injured cells. Samples held for more than 30 min have been reported to result in loss of viable cells (15). Because of these considerations, *Standard Methods* (2) imposes a 30-min time limit on the processing of diluted samples at room temperature. Our findings indicate the importance of the diluent composition, temperature, and the time of exposure. If diluents are maintained at approximately refrigerator temperatures (ca. 4°C), their compositions and exposure times are likely to have a minimal impact on enumeration efficiency. However, substantially lower enumeration recoveries are associated with room temperatures and extended exposure times. The enrichment of diluents with low concentrations of organic materials such as peptone, gelatin, tryptone, or milk has been demonstrated to be of value in the enumeration of aquatic bacteria (3, 23, 24, 32, 37, 41–43). The results reported here indicate that these factors are even more important in situations where injured coliforms are enumerated. This consideration is of particular significance in applications such as the enumeration of coliforms in chlorinated drinking water and wastewater effluents, where the disinfectant is known to cause substantial injury (6, 9, 27, 34). Additional factors, such as the presence of metals, probably also act as stressors to waterborne indicator bacteria.

The selection of media to be used in determining coliform numbers in water and wastewater can also significantly affect results. Several recent studies (11, 12, 21, 27) have shown that various selective media yield markedly different coliform enumeration results. Substantially reduced plating efficiencies on selective media have been associated with coliforms damaged from exposure to injurious chemicals in the environment (3, 4, 6, 34). Therefore, it is not surprising that coliforms injured in aquatic environments have been recovered with efficiencies of 10% or less on commonly used media (3, 4, 6, 18, 27). Our results (Table 1) demonstrate that, in comparison to uninjured cells, injured coliforms yielded reduced plating efficiencies on a variety of selective media used for the recovery of gram-negative enteric bacteria. The ranking of the various media (Table 1) agrees with results reported by others (4, 6, 18, 21) where fewer media were compared. Of practical significance is the finding that media widely used for water quality estimations (*m*-Endo, *m*-FC, eosin methylene blue, and MacConkey agars) generally ranked in the lower half of all media tested, with recoveries ranging from 5 to 66%. Lauryl tryptose broth had low recoveries of injured

TABLE 2. Effect of membrane filter composition on recovery of coliforms

| Enumeration technique | % Recovery of:[a] | | | | | |
|---|---|---|---|---|---|---|
| | Healthy cells | | | Injured cells | | |
| | TLY | TLY-D | % Injury | TLY | TLY-D | % Injury |
| Spread plate | 62 | 49 | 21 | 321 | 8.4 | 97.4 |
| Membrane filter HA[b] | 68 | 44 | 35 | 309 | 11.8 | 96.2 |
| Membrane filter HC[b] | 92 | 60 | 35 | 327 | 13.0 | 96.0 |

[a] Counts are an average of two experiments of five replicates each ($\times 10^7$ CFU/ml) for healthy cells and three experiments of five replicates each ($\times 10^6$ CFU/ml) for injured cells.
[b] Millipore Corp.

coliforms (average, 56%; range, 34 to 79%), possibly accounting for reports of failing to find injured coliforms in water by the recommended resuscitation technique (14).

Little variation occurred between different genera of injured coliforms tested (*Escherichia coli* [two strains], *K. pneumoniae, C. freundii*, and *Enterobacter aerogenes*) in their response to different media compositions. However, seasonal variations in the physical and chemical properties of the distribution water used to injure the coliform organisms may be responsible for the large variations in recoveries of certain media (*m*-Endo, 30 to 102%).

In an attempt to explain the relatively poor performance of the media tested, the selective agents in the different formulations were noted. Seven of the eight media in group III (Table 1) having recovery efficiencies between 0 and 24%, as well as four of the media in group II, contained either bile salts or deoxycholate. For that reason, we tested the recovery of both injured and healthy *Escherichia coli* in various concentrations of deoxycholate added to a complete medium (Fig. 3). Control cells were virtually unaffected by concentrations of 0.1% or less, whereas injured cells were severely inhibited by greater than 0.05% deoxycholate. Not surprisingly, concentrations of bile salts or deoxycholate in media that were most inhibitory to injured coliforms (group III, Table 1) were between 0.05 and 2.5%.

A recent report (44) concludes that a major location of the injury in water-injured coliforms is in the cell envelope. Such cells become more sensitive to surfactants, including bile salts and deoxycholate (44). The formulation of an improved medium, based on an understanding of the physiology of injury, is now possible. This medium must, however, be highly selective for use in the analysis of water and still recover the injured coliforms efficiently. The feasibility of this suggestion is supported by previous work (1, 4) indicating that injured fecal coliform bacteria can recover the ability to grow on selective media (19, 25, 33, 38).

Dissatisfaction with the performance of membrane filters was summarized by reports (H 16, 31) that some types of membrane filters were more effective in the recovery of waterborne fecal coliform bacteria from a variety of sources. A subsequent study by Sladek et al. (36) identified surface pore morphology as the critical physical characteristic of membrane filters that influenced fecal coliform recovery on selective media in the high-temperature (44.5°C) test. This conclusion was not, however, extended to the enumeration of total coliforms at 35°C or to injured waterborne indicator bacteria, although it has been reported that chlorine-injured coliforms may be recovered less efficiently with membrane filters than by spread plating (20). For this reason, experiments were carried out to compare the recovery of a highly injured *Escherichia coli* suspension on membranes of large (2.4 μm) and small (less than 1.0 μm) surface pore openings with spread plates, using the same media. Membrane filter surface pore morphology is not important in the recovery of injured total coliform bacteria since the counts with both filters and spread plates were comparable (Table 2). This conclusion is consistent with the results of other reports (26, 40) where the same filters were used, but the level of injury was not controlled or reported.

Reversible bacterial injury caused by stresses in aquatic environments (3, 4, 6, 27) is an important factor contributing to the suppression of indicator bacterial enumeration (9, 19, 25, 33, 38, 43). The results presented in this report demonstrated that diluent composition, exposure time, and temperature reduce the recovery efficiency of injured coliform bacteria. In addition, media can play a critical role, since many of the currently accepted media formulations used are detrimental to recovery of injured coliforms from water. These findings represent a supportive rationale for the development of new methodologies allowing a more complete enumeration of injured coliforms in potable water and providing more accurate and dependable water quality information.

## ACKNOWLEDGMENTS

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