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Survival and Activity of *Streptococcus faecalis* and *Escherichia coli* in Tropical Freshwater

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Abstract. The survival of *Streptococcus faecalis* and *Escherichia coli* was studied in situ in a tropical rain forest watershed using membrane diffusion chambers. Densities were determined by acridine orange direct count and Coulter Counter. Population activity was determined by microautoradiography, cell respiration, and by nucleic acid composition. Densities of *S. faecalis* and *E. coli* decreased less than 1 log unit after 105 hours as measured by direct count methods. Activity as measured by respiration, acridine orange activity, and microautoradiography indicated that both bacteria remained moderately active during the entire study. After 12 hours, *E. coli* was more active than *S. faecalis* as measured by nucleic acid composition. In this tropical rain forest watershed, *E. coli* and *S. faecalis* survived and remained active for more than 5 days; consequently, both would seem to be unsuitable as indicators of recent fecal contamination in tropical waters.

Introduction

In tropical areas of Nigeria [21], Hawaii [10], New Guinea [9], Puerto Rico [13, 15], Sierra Leone [31], and the Ivory Coast [17], high densities of *Escherichia coli,* an indicator of fecal contamination, were found in the complete absence of any known fecal source. Monitoring of Puerto Rican waters by the U.S. Geological Survey reported that 54 of 67 water sampling stations on rivers in Puerto Rico exceeded the U.S. Environmental Protection Agency recommended maximum contaminant levels (MCL) for recreational waters (i.e., <1,000 fecal coliforms/100 ml) during 1984 [7]. Thus, only 19% of all sites sampled met the recommended MCL for recreational waters, and none of these waters could meet raw source water standards (<2 fecal coliforms/100 ml). These findings have resulted in condemnation of sewage treatment in Puerto Rico as a source of fecal pollution of natural waters due to improper treatment [13, 15]. Yet, sampling sites upstream from sewage treatment plant outfalls indicated fecal coliform densities that were just as high as most downstream sites [7].

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The reliability of fecal coliform techniques in the tropics has also been questioned. In Puerto Rico, less than 30% of the fecal coliform-positive isolates from natural waters are confirmed as *E. coli*, whereas more than 90% of all temperate water isolates are confirmed as *E. coli* [25]. Pagel et al. [22] compared four fecal coliform assays in various types of freshwaters in Southern Canada. They observed that while these assays were somewhat variable in their abilities to detect fecal coliforms from environmental samples, they were all acceptable in terms of their specificity and selectivity. In similar studies, Santiago-Mercado and Hazen [25] used the same methodology to detect fecal coliforms from freshwaters in Puerto Rico and found that the specificity of the media (determined by the ability of the medium to restrict growth of organisms other than the target bacterium) was at least 20% less than the specificity reported by the Canadian investigators. Thus, all the methods gave significantly higher false-positive (mistakenly identified as *E. coli*) or false-negative (*E. coli* that were not identified as *E. coli* in the test media) errors in tropical waters. Controls using known strains of *E. coli* indicated the accuracy of the methods to be the same in both studies.

Recent studies have also shown that *E. coli* can be isolated from pristine areas of tropical rain forests in Puerto Rico [4, 23]. Plasmid profiles, antibiotic sensitivities, coliphage susceptibility, and physiological and biochemical characteristics confirm that even *E. coli* isolated from epiphytes in trees 15 m above the ground are identical to clinical isolates of *E. coli* [23]. These environmental isolates have identical mole% G + C of their DNA and more than 75% DNA homology with *E. coli* B [4]. Thus, tropical source waters may have high densities of naturally occurring *E. coli* in the absence of pathogens or fecal sources.

Water treatment in tropical countries is often deficient [30, 31]. Thus, prevalence of disease is exacerbated by the common use of traditional drinking water sources that receive little or no treatment prior to use. In addition, contaminated tropical waters may harbor a much greater variety of human pathogens, some of which are unique to tropical climes [13]. *Streptococcus faecalis* has often been suggested as a possible alternative to *E. coli* as an indicator of recent human fecal contamination [5]. The present study compares the in situ survival and activity of these two bacteria in a tropical river.

Materials and Methods

Study Site

The Mameyes River is located at the northeast corner of the island of Puerto Rico (Fig. 1). The river originates in a cloud rain forest in a pristine portion of the Luquillo Experimental Forest, U.S. Forest Service. For a more complete description of the Mameyes River watershed and site 1, see Carrillo et al. [6].

Water Quality

Dissolved oxygen, air temperature, and water temperature were measured with a dissolved oxygen meter (Yellow Springs Instrument Co., Yellow Springs, OH). Three liters of sample water were

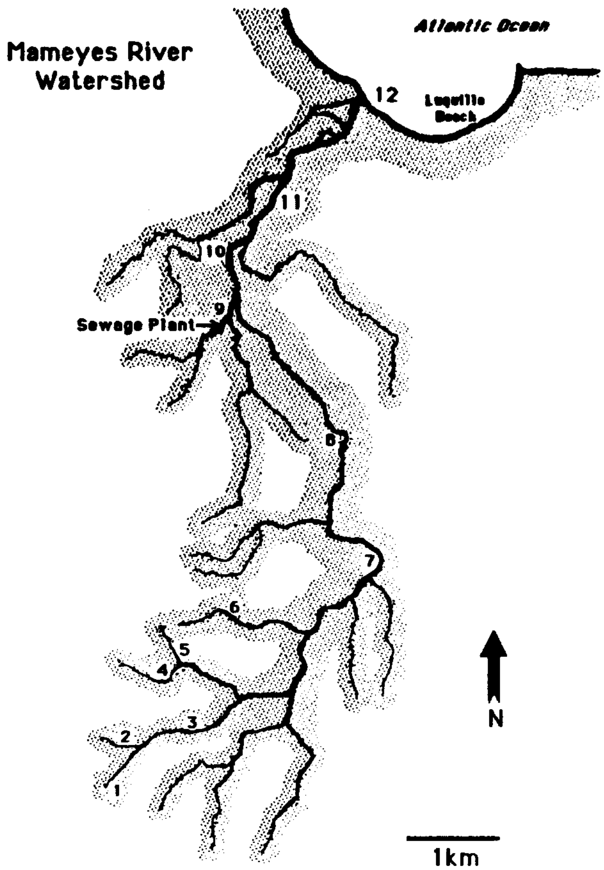

Fig. 1. Location of study site in Mameyes River watershed, Puerto Rico.

fixed with either sulfuric acid, zinc acetate, or mercuric chloride, placed on ice, and transported to the laboratory for analysis. Nitrates plus nitrites, sulfates, total phosphorus, and phosphates (P_i) were determined in the laboratory as described in Standard Methods for the Examination of Water and Wastewater [2].

Cell Densities and Activity

For survival studies, plexiglass diffusion chambers with 100-ml capacity were used with a 0.45-μm pore size, nylon reinforced Versapor membrane filters (Gelman, Instrument Co., Ann Arbor, MI) as a diffusion surface. O-rings were added to chambers to reduce leakage and contamination. Pure cultures of *S. faecalis* (CBSC 15–5600A) and *E. coli* (ATCC 11775) were grown in nutrient broth at 35°C for 24 hours. The cells were then harvested by centrifugation and resuspended in filter-sterilized phosphate-buffered saline (pH 7). Cell density was determined with a model ZF Coulter Counter (Coulter Electronics, Hialeah, FL) and adjusted to a concentration of 10^8 cells ml^{-1}. The bacterial suspensions were placed into the sterile diffusion chambers just before placing them at the study sites. Chambers were sterilized by filling with 95% ethanol 24 hours before being used. The chambers and their use are as previously described [6, 14, 18, 19]. Four chambers of each bacterial species were suspended 30 cm below the surface at site 1. Samples (2 ml) were taken with a sterile syringe at regular intervals over 105 hours beginning at 2100 hours on November 7, 1986. Half of each sample was incubated with 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltet-

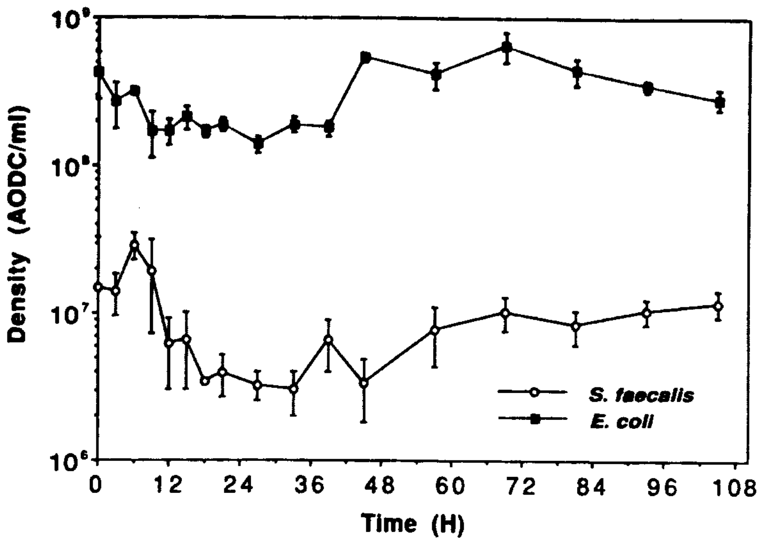

Fig. 2. Changes in total density as measured by AODC for *S. faecalis* and *E. coli* (mean ± 1 SE, n = 4).

razolium chloride (INT) as described by Zimmermann et al. [33]. The other half was incubated with [^{3}H]thymidine (75 μCi mmol^{-1}) for 2.5 hours as described by Tabor and Neihof [29]. Samples were fixed in 10% phosphate-buffered formalin (pH 7), after the appropriate incubation [29, 33]. Aliquots of the first subsample were used to determine acridine orange direct counts (AODC) [19], Coulter Counter direct counts [14], respiration (INT reduction [33]), and activity by AODC [6, 18, 19]. The second subsample was used to determine AODC [19] and [^{3}H]thymidine uptake-positive activity (microautoradiography [28]).

Data Analysis

Programs developed for a Macintosh computer were used for all statistical analyses. Density data were subjected to the log (x + 1) transformation before statistical analysis to reduce heteroscedascity as described by Zar [32]. Any statistical probability ≤0.05 was considered significant.

Results and Discussion

Densities of *S. faecalis* were not significantly different from those of *E. coli* determined by either direct count method over time (Figs. 2 and 3). The numbers of both bacteria as measured by AODC were nearly two orders of magnitude lower by Coulter Counter. This, as discussed in previous works [6, 18, 19], is because the aperture on the Coulter Counter is of sufficient size (50 μm) to allow multiple bacteria to pass at the same time, resulting in underestimation of the numbers of bacteria in the sample. This phenomenon, however, does not interfere with the ability of the instrument to detect changes over time, and when compared to the samples counted by AODC can give a good estimate of the relative changes in morphology that may be occurring in the population. For a thorough discussion and controlled experimental results, see López-Torres et al. [19].

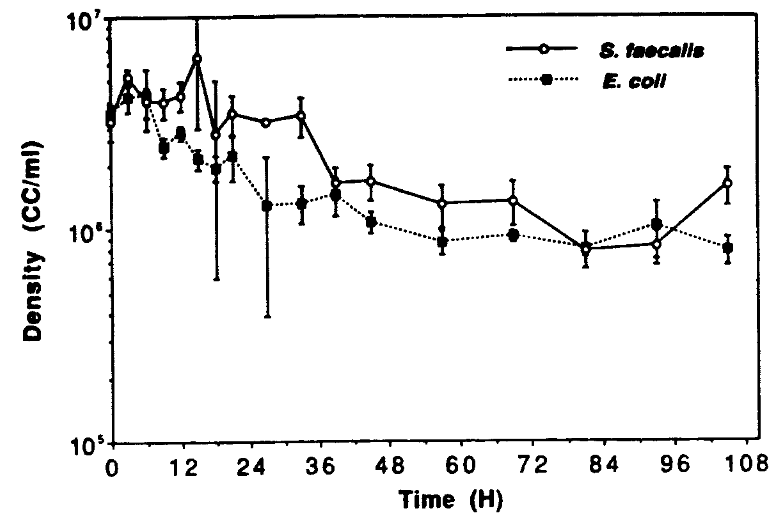

Fig. 3. Changes in total density as measured by Coulter Counter for *S. faecalis* and *E. coli* (mean ± 1 SE, n = 4).

Both bacteria decreased by less than one order of magnitude over the course of the study, and the overall trend for the two bacteria was similar. Thus, the calculated (linear regression for transformed data) time for *S. faecalis* to decrease by 90%, or one order of magnitude, under in situ conditions was >300 hours, whereas *E. coli* would decrease by 90% after 276 hours. McFeters et al. [20] reported that densities of *S. faecalis* decreased by 90% within 29 hours when exposed to temperate water in situ conditions using diffusion chambers. G. K. Bissonnette (Ph.D. thesis, Department of Microbiology, Montana State University, Bozeman, 1974) reported that even in the most sewage-contaminated temperate river, *S. faecalis* survival never exceeded 130 hours. McFeters et al. also reported that *E. coli* showed a 90% reduction in cell density after 30 hours of exposure. Other studies have reported that *Streptococcus* spp. survive slightly longer than coliforms in temperate waters [12]. However, studies in our laboratory have shown that under most conditions *E. coli* will survive indefinitely in tropical freshwaters [6, 18, 19]. In addition, no correlation between *S. faecalis* counts and presence of *Salmonella* spp., a known pathogen, was found in tropical waters in Africa [30], or Sierra Leone [31]. In addition, though densities of fecal streptococci are usually lower than densities of fecal coliforms in tropical areas, like the fecal coliforms they seem to be unrelated to known sources of fecal contamination [7, 9, 10, 31].

No significant difference in the percentage of respiring cells was observed (Fig. 4). The percentages of respiring cells fluctuated between 0 and 12% for both bacteria, within the same range reported for naturally occurring bacteria from diverse environmental samples [29, 33]. This was not surprising considering the oligotrophic nature of the waters in this study, as indicated by the low phosphate concentrations (0.03 mg/liter, Table 1), confirming earlier studies of the same sites [6]. However, López-Torres et al. [18] observed a decrease in the percentage of respiring cells for *E. coli* at this site from 100% at time 0

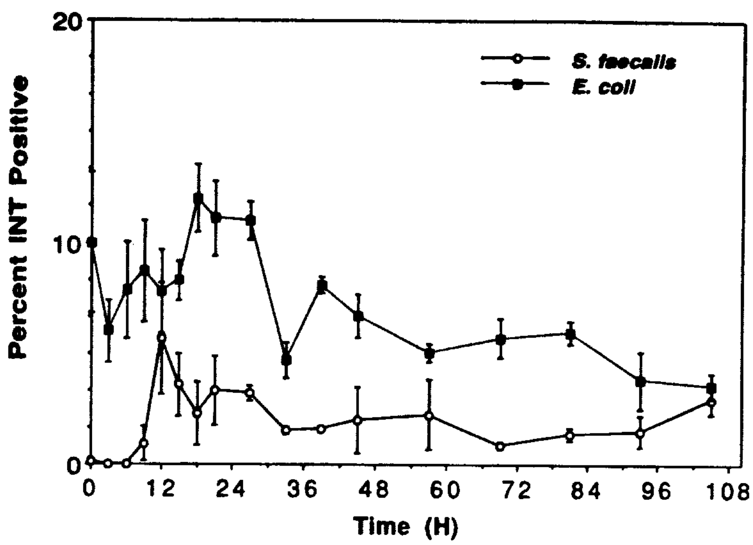

Fig. 4. Changes in percent INT-positive cells as measured by INT-reduction for *S. faecalis* and *E. coli* (mean ± 1 SE, n = 4).

to 10% after 96 hours. The lower percentages observed at the beginning and during the course of the present study suggest that the bacteria may have been stressed at the beginning of the present study or that the sustaining capacity of the water in the previous study [18] was even lower than that in the present study. The level of respiration observed for both bacteria was high enough to suggest that neither one was under severe stress [29, 33].

The proportion of bacterial cells that incorporated [^{3}H]thymidine was also not significantly different between bacteria (Fig. 5). The percentage of active cells for both bacteria fluctuated from 3 to 14%, though the average activities for *E. coli* were higher at all sampling times except one (Fig. 5). These levels of [^{3}H]thymidine uptake were also typical for naturally occurring temperate bacterial populations [29], again suggesting that both bacteria were moderately active.

Table 1. Water quality parameters in the Mameyes River watershed

Time (hours)	Wtemp (°C)	Atemp (°C)	NO_{2+3}	SO_4	TP	PO_4
0	18.0	19.0	0.04	0.02	0.08	0.01
24	20.0	20.0	0.06	0.02	0.09	0.03
48	21.0	21.0	0.03	0.03	0.05	0.01
72	19.5	19.5	0.03	0.02	0.08	0.01
96	19.0	18.5	0.02	0.02	0.08	0.01
105	ND	ND	0.02	0.01	0.07	0.01
Mean	19.5 ± 0.5	19.6 ± 0.4	0.03 ± 0.01	0.02 ± 0.00	0.08 ± 0.01	0.01 ± 0.00

Wtemp = water temperature, Atemp = air temperature, NO_{2+3} = nitrites plus nitrates (mg/liter), SO_4 = sulfates (mg/liter), TP = total phosphorus (mg/liter), PO_4 = orthophosphates (mg/liter), mean = mean ± 1 SE

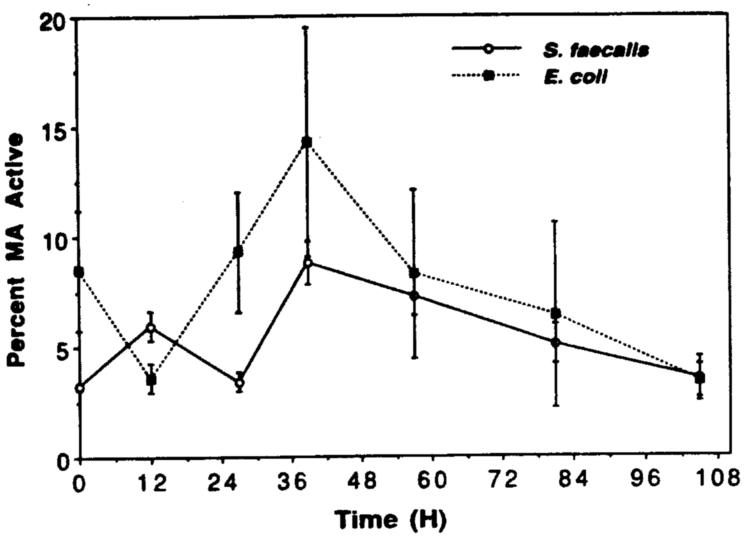

Fig. 5. Changes in percent microautoradiograph (MA)-positive bacteria as measured by [^{3}H]thymidine uptake for *S. faecalis* and *E. coli* (mean percent of MA ± 1 SE, n = 4).

The percentage of active *E. coli* cells, as measured by AODC, were significantly higher than *S. faecalis* (Fig. 6; $F = 52.9$, df = 1 and 32, $P < 0.0001$). These percentages were lower than those previously reported for *E. coli* at the same site [6, 18], further suggesting slightly more stressful conditions for the bacteria at the time of this study. AODC activities, as determined by the red/red + green cell percent [19], for *E. coli* ranged from 55 to more than 90%,

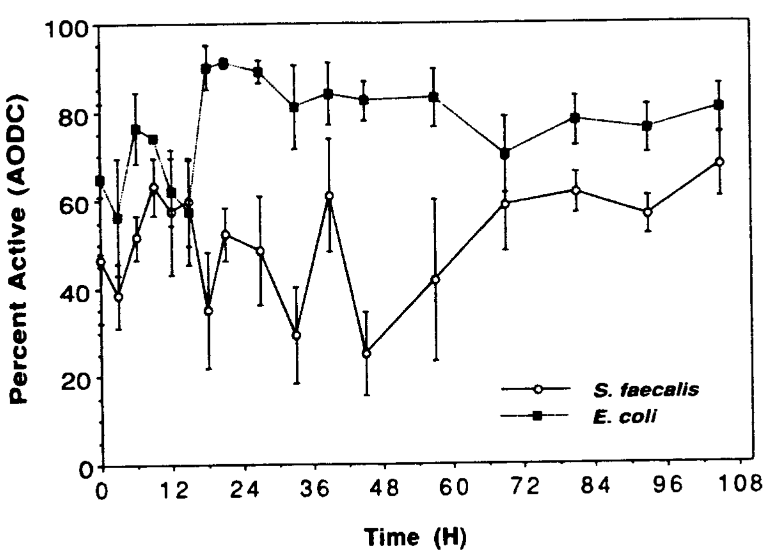

Fig. 6. Changes in percent activity as measured by AODC for *S. faecalis* and *E. coli* (mean percent of activity ± 1 SE, n = 4).

whereas *S. faecalis* activity ranged from 25 to 67%. This level of AODC activity has been shown in previous studies to indicate a physiologically active population [6, 18, 19].

Neither direct count technique showed dramatic changes in density for either bacteria, thus, it may be assumed that these bacteria can survive for extended periods [26]. However, because these were direct count measurements, the physiological activity of the bacteria must be demonstrated. AODC activity indicates, like microautoradiography (MA) and INT-reduction, that both bacteria were active in situ in this tropical freshwater stream; however, AODC activity indicated that *E. coli* remained at a higher level of activity than *S. faecalis,* unlike the assays for MA and INT-reduction. The differences in these activity measurements undoubtedly lie in the differences of each technique. The INT-reduction technique measures respiration via electron transport activity [33]. As both of these bacteria are facultative anaerobes, low levels of respiration did not indicate that these bacteria were not physiologically active. Low levels of [^{3}H]thymidine uptake would not indicate that these bacteria were physiologically inactive, only that they were not growing rapidly [29]. As the high levels of AODC activity indicated that protein synthesis was occurring [18, 19], and direct counts indicated increases in density after the initial acclimation period, then the bacteria were growing. All of the activity measurements confirmed that both bacteria were active. One of the three activity measurements also indicate that *E. coli* has a higher activity than *S. faecalis* in tropical freshwaters.

Density of fecal coliforms and presence of *Streptococcus* spp. in temperate waters have been well demonstrated [12]. It has also been suggested that *E. coli* is a better indicator for water quality than fecal coliforms [5, 8], and that fecal streptococci may be better indicators than fecal coliforms [1, 3, 11, 27]. The present study suggests that these assumptions are incorrect for tropical freshwater. Indeed, *E. coli,* fecal coliforms, *S. faecalis,* and fecal streptococci do not accurately indicate the presence of these pathogens in tropical waters due to their rate of survival, and possible indigenous nature [4, 15, 23]. Thus, the best indicator for tropical freshwaters is no indicator at all, i.e., enumerate pathogens such as *Salmonella* spp. directly. The technology is available to directly and very specifically monitor or detect pathogens using DNA probes [16] and immunofluorescence techniques [24]. Current tropical source water MCLs based on fecal coliforms, whose target is *E. coli,* are unenforceable and may not represent a real public health risk under many circumstances.

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References

1. Allen Burton Jr G, Gunnison D, Lanza GR (1987) Survival of pathogenic bacteria in various freshwater sediments. Appl Environ Microbiol 53:633–638
2. American Public Health Association (1985) Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, DC
3. Andre DA, Weiser HH, Maloney GW (1967) Survival of bacterial enteric pathogens in farm pond water. J Am Water Works Assoc 59:503–508
4. Bermúdez M, Hazen TC (1988) Phenotypic and genotypic comparison of *Escherichia coli* from pristine tropical waters. Appl Environ Microbiol 54:979–983
5. Cabelli VJ (1980) What do water quality indicators indicate? In: Colwell RR, Foster J (eds) Aquatic microbial ecology. University of Maryland, College Park, pp 305–306
6. Carrillo M, Estrada E, Hazen TC (1985) Survival and enumeration of the fecal indicators *Bifidobacterium adolescentis* and *Escherichia coli* in a tropical rain forest watershed. Appl Environ Microbiol 50:468–476
7. Curtis RE, Guzman-Ríos S, Díaz PL (1984) Water resources data: Puerto Rico and the U.S. Virgin Islands. USGS Survey Water-Data Report PR-84-1
8. Dufour AP (1976) *Escherichia coli*, the fecal coliform. In: Hoadley AW, Dutka BJ (eds) Bacterial indicators/health hazards associated with water. American Society for Testing Materials, Philadelphia, pp 48–58
9. Feachem RG (1974) Fecal coliforms and fecal streptococci in streams in the New Guinea highlands. Water Res 8:367–374
10. Fujioka R, Shizumura LK (1983) *Clostridium perfringens*: A reliable indicator of stream water quality. J Water Pollut Contr Fed 57:986–992
11. Gallagher TP, Spino DF (1968) The significance of coliform bacteria as an indicator of enteric pathogens. Water Res 2:169–175
12. Geldreich EE, Best LC, Kenner BA, Van Donsel DJ (1968) The bacteriological aspects of stormwater pollution. J Water Pollut Contr Fed 40:1861–1872
13. Hazen TC (1988) Fecal coliforms as indicators in tropical waters: A review. Int J Tox Asses 3:461–477
14. Hazen TC, Esch GW (1983) Effect of effluent from a nitrogen fertilizer factory and a pulp mill on the distribution and abundance of *Aeromonas hydrophila* in Albemarle Sound, North Carolina. Appl Environ Microbiol 45:31–42
15. Hazen TC, Fuentes FA, Santo Domingo JW (1988) *In situ* survival and activity of pathogens and their indicators. Proc IV ISME, pp 406–411
16. Hazen TC, Jiménez L (1988) Enumeration and identification of bacteria from environmental samples using nucleic acid probes. Microbiol Sci 5:340–343
17. Lavoie MC (1983) Identification of strain isolates as total and fecal coliforms and comparison of both groups as indicators of fecal pollution in tropical climates. Can J Microbiol 29: 689–693
18. López-Torres AJ, Hazen TC, Toranzos GA (1987) Distribution and *in situ* survival and activity of *Klebsiella pneumoniae* and *Escherichia coli* in a tropical rain forest watershed. Curr Microbiol 15:213–218
19. López-Torres AJ, Prieto L, Hazen TC (1988) Comparison of the *in situ* survival and activity of *Klebsiella pneumoniae* and *Escherichia coli* in tropical marine environments. Microb Ecol 15:41–57
20. McFeters GA, Bissonnette GK, Jezeski JJ, Thomson CA, Stuart DG (1974) Comparative survival of indicator bacteria and enteric pathogens in well water. Appl Environ Microbiol 27:823–829
21. Oluwande PA, Sridhar KC, Bammeke AO, Okubadejo AO (1983) Pollution level in some Nigerian rivers. Water Res 17:957–963
22. Pagel JE, Qureshi AA, Young DM, Vlassoff LT (1982) Comparison of four membrane filter methods for fecal coliform enumeration. Appl Environ Microbiol 43:787–793
23. Rivera S, Hazen TC, Toranzos GA (1988) Isolation of fecal coliforms from pristine sites in a tropical rain forest. Appl Environ Microbiol 54:513–517

24. Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379
25. Santiago-Mercado J, Hazen TC (1987) Comparison of four membrane filtration and MPN methods for fecal coliform enumeration in tropical waters. Appl Environ Microbiol 53:2922–2928
26. Santo Domingo JW, Fuentes FA, Hazen TC (1989) Survival and activity of *Streptococcus faecalis* and *Escherichia coli* in petroleum-contaminated tropical marine waters. Environ Pollut 56:263–281
27. Seligman R, Reiter R (1965) Enteropathogens in water with low *Escherichia coli* titer. J Am Water Works Assoc 57:1572–1574
28. Tabor P, Neihof J (1982) Improved microautoradiography method to determine individual microorganisms active in substrate uptake in natural waters. Appl Environ Microbiol 44:945–953
29. Tabor P, Neihof J (1984) Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl Environ Microbiol 48:1012–1019
30. Thomson JA (1981) Inadequacy of *Escherichia coli* as an indicator of water pollution in a tropical climate: A preliminary study in Botswana. South Africa J Sci 77:44–45
31. Wright RC (1982) A comparison of the levels of faecal indicator bacteria in water and human faeces in a rural area of a tropical developing country (Sierra Leone). J Hyg 89:69–78
32. Zar JH (1984) Biostatistical analysis. Prentice-Hall, Inc, Englewood Cliffs, NJ
33. Zimmermann R, Iturriaga R, Becker-Birck J (1978) Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl Environ Microbiol 36:926–934