

# 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 h 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 h, E. coli was more active than S. faecalis, as measured by nucleic acid composition. ~~Both~~ E. coli and S. faecalis survived and remained active in this tropical rain forest watershed for more than 5 days, in this natural environment, both would seem to be unsuitable as indicators of recent fecal contamination in tropical waters.

Consequently

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## 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<sup>(USGS)</sup> reported that 54 out of 67 water sampling stations on rivers in Puerto Rico exceeded the recommended maximum contaminant levels (MCL) for recreational waters (i.e. <1,000 fecal coliforms per 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 per 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 that fecal coliform densities that were just as high as most downstream sites (7).

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 claimed by the Canadian investigators. Thus all the methods gave significantly higher false positive and false negative 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 the isolates 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. Escherichia coli is usually found in higher densities than other enteric pathogens. However, as noted in Botswana (30) and Sierra Leone (31) Salmonella spp., a pathogen, may be found in the complete absence of E. coli.

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 which receive little or no treatment prior to use. In addition, contaminated tropical waters may harbour<sup>?</sup> 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 (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 taken with a dissolved oxygen meter (Yellow Springs Instrument, Co., Yellow Springs, Ohio). Three liters of sample water were 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.** Pure cultures of Streptococcus faecalis (CBSC 15-5600A) and Escherichia coli (ATCC 11775) were grown in nutrient broth at 35°C for 24 h. Cell densities were adjusted to  $10^8$  cells  $ml^{-1}$  and the suspension placed into sterile diffusion chambers just prior to immersion at the study site. The chambers and their use are as previously described (6, 14, 18, 19). Four replicate 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 for 105 h. Half of each sample was incubated with INT as described by Zimmermann et al. (33). The other half was incubated with [ $^3H$ ]thymidine ( $75 \mu Ci/m mol^{-1}$ ) for 2.5 h as described by Tabor and Neihof (28). Samples were fixed in 10% phosphate-buffered Formalin



(pH 7), after the appropriate incubation (28, 33). Bacterial activity was expressed as respiration (INT reduction (33)), [ $^3\text{H}$ ]thymidine uptake positive (microautoradiography, (31)), and activity by AODC (6, 18, 19). Direct count densities were determined by acridine orange direct counts (19) and Coulter Counter (14).

**Data analysis.** Programs developed for a Macintosh computer were used for all statistical analyses. Data were subjected to the appropriate transformation before statistical analysis as described by Zar (32). Any statistical probability less<sup>than</sup> or equal to 0.05 was considered significant.

## RESULTS AND DISCUSSION

Densities of S. faecalis were not significantly different than E. coli by either direct count method over time (Fig. 2 and 3). Both bacteria decreased by less than one order of magnitude over the course of the study and the overall trend for the two bacteria is similar. Thus the calculated time for S. faecalis to decrease by 90%, or one order of magnitude, under in-situ conditions was > 300 h, while E. coli would decrease by 90% after 276 h. McFeters et al. (20) reported that densities of S. faecalis decreased by 90% within 29 h when exposed to temperate water in-situ conditions using diffusion chambers. Bissonnette (Ph. D. thesis, Department of Microbiology, Montana State University, Bozeman, 1974) reported that even in the most sewage contaminated temperate river that S. faecalis survival never exceeded 130 h. They also reported that E. coli showed a 90% reduction in cell density after 30 h of exposure. Other studies have reported that Streptococcus spp. survive slightly longer than coliforms in temperate waters (10). However other studies by 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 (32), or Sierra Leone (33). In addition, while 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 (Wright, 1982; Feachem, 1974; Curtis et al., 1984; Fujioka and Shizumura, 1985; Evison and James, 1973).

No significant difference in the percentage of respiring cells was observed (Fig. 4). The percentages of respiring cells fluctuated between 3 to 21% for both bacteria, within the same range reported for naturally occurring bacteria from diverse environmental samples (30, 35). This was not surprising considering the oligotrophic nature of the waters in this study, as indicated by the low phosphate concentrations (0.07 mg/ml, Table 1), confirming earlier studies of the same sites (6). However, López-Torres et al. (20) observed a decrease in the percentage of respiring cells for E. coli at this site from 100% at time 0 to 10% after 96 h. 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 was even lower than in the present study. The level of respiration observed for both bacteria was high enough to suggest that neither bacteria was under severe stress (30, 35).

The proportion of bacterial cells that incorporated [ $^3\text{H}$ ]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 [ $^3\text{H}$ ]thymidine uptake were also typical for naturally-occurring temperate bacterial populations (30). Again suggesting that both bacteria were moderately active.

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  $32$ ,  $P < 0.0001$ ). But these percentages were lower than those reported for E. coli at the same site (6, 20), 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 (21), for E. coli ranged from 55 to more than 90%, while 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, 20, 21).

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, since these were direct count measurements, the physiological activity of the bacteria must be demonstrated. AODC activity indicates, like 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 (35). Since both of these bacteria are facultative anaerobes, low levels of respiration did not indicate that these bacteria were not physiologically active. Low levels of [<sup>3</sup>H]thymidine uptake would not indicate that these bacteria were physiologically inactive, only that they were not growing rapidly (30). Since the high levels of AODC activity indicated that protein synthesis was occurring (20, 21), and direct counts indicated increases in density after the initial acclimation period, than 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 has been well demonstrated (14). It has also been suggested that E. coli is a better indicator for water quality than fecal coliforms (5, 9), and that fecal streptococci may be better indicators than fecal coliforms. The present study suggests that these assumptions are incorrect for tropical freshwater. Indeed, E. coli, fecal coliforms, S. faecalis, 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, 17, 25). Thus, the best indicator for tropical freshwaters is no indicator at all, i.e. enumerate pathogens like Salmonella spp. directly. The technology is available to directly and very specifically monitor or detect pathogens using DNA probes (16) and immunofluorescence techniques (24). Maximum contaminant levels for tropical source waters, based upon certain resistant pathogens, will be more realistic and attainable regulations that represent a more realistic public health risk. Current tropical source water MCL's based upon 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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## Figure Legends

- Figure 1. Location of study site in Mameyes River watershed, Puerto Rico.
- Figure 2. Changes in total density as measured by AODC for S. faecalis and E. coli (mean  $\pm$  one standard error, n=4).
- Figure 3. Changes in total density as measured by Coulter Counter for S. faecalis and E. coli (mean  $\pm$  one standard error, n=4).
- Figure 4. Changes in percent INT-positive cells as measured by INT-reduction for S. faecalis and E. coli (mean  $\pm$  one standard error, n=4).
- Figure 5. Changes in percent microautoradiograph positive as measured by [<sup>3</sup>H]thymidine uptake for S. faecalis and E. coli (mean percent of MA  $\pm$  one standard error, n=4).
- Figure 6. Changes in percent activity as measured by AODC for S. faecalis and E. coli (mean percent of activity  $\pm$  one standard error, n=4).

Table 1. Water quality parameters in the Mameyes River watershed.

TIME	WTEMP	ATEMP	NO <sub>2+3</sub>	SO <sub>4</sub>	TP	PO <sub>4</sub>
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

ATEMP = air temperature (°C), WTEMP = water temperature (°C), SO<sub>4</sub> = sulfates (mg/L) NO<sub>2+3</sub> = nitrites plus nitrates (mg/L), TP = total phosphorus (mg/L), PO<sub>4</sub> = orthophosphates (mg/L), Mean = mean ± one standard error, TIME = hours.

# Mameyes River Watershed

*Atlantic Ocean*

Luquillo Beach













