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Survival and activity of *Salmonella typhimurium* and *Escherichia coli* in tropical freshwater

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The survival of *Salmonella typhimurium* LT2 and *Escherichia coli* was studied *in situ* in a tropical rain forest watershed using membrane diffusion chambers. Numbers were determined by acridine orange staining and a Coulter counter. Population activity was determined by microautoradiography, cell respiration, frequency of dividing cells, and by nucleic acid composition. Numbers of *Salm. typhimurium* 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, frequency of dividing cells, and microautoradiography indicated that both bacteria remained moderately active during the entire study. After 24 h, *E. coli* was more active than *Salm. typhimurium*, as measured by nucleic acid composition, and frequency of dividing cells. Both *E. coli* and *Salm. typhimurium* survived and remained active in this tropical rain forest watershed for more than 5 d, suggesting that *Salm. typhimurium* may be of prolonged public health significance once it is introduced into tropical surface waters. As *E. coli* was active and survived for a long time in this natural environment, it would seem to be unsuitable as an indicator of recent faecal contamination in tropical waters.

Large numbers of *Escherichia coli*, an indicator of faecal contamination, have been found when a known faecal source was not detectable in tropical areas, eg. Nigeria (Oluwande *et al.* 1983), Hawaii (Fujioka & Shizumura 1983), New Guinea (Feachem 1974), Puerto Rico (Carrillo *et al.* 1985), Sierra Leone (Wright 1982), and the Ivory Coast (Lavóie 1983). Monitoring of Puerto Rican waters by the US Geological Survey showed that 54/67 water sampling stations on rivers in Puerto Rico had more than the recommended maximum contaminant levels (MCL) for recreational waters (i.e. <1000 faecal coliforms/100 ml) during 1984 (Curtis *et al.* 1984). 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 faecal coliforms/100 ml). These findings have resulted in condemnation of sewage treatment in Puerto Rico as improper and as a source of faecal pollution of natural waters (Hazen *et al.* 1987, 1988). Yet sampling sites upstream from sewage treatment plant outfalls showed that the numbers of faecal coliforms were just as high as at most downstream sites (Curtis *et al.* 1984).

The reliability of faecal coliform techniques in the tropics has also been questioned. In Puerto Rico, less than 30% of the 'faecal' coliforms isolated from natural waters are confirmed as *E. coli*, whereas more than 90% of those recovered from all temperate waters are so confirmed (Santiago-Mercado & Hazen 1987). Pagel *et al.* (1982) compared four faecal coliform counts in

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various types of freshwaters in Southern Canada. They observed that while these were somewhat variable in their abilities to detect faecal coliforms in environmental samples, they were all acceptable in terms of their specificity and selectivity. In similar studies, Santiago-Mercado & Hazen (1987) used the same technique to detect faecal coliforms in freshwaters in Puerto Rico and found that the specificity of the media (determined by its ability to restrict growth of organisms other than the target bacteria) 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 (Bermúdez & Hazen 1988; Rivera *et al.* 1988). Plasmid profiles, antibiotic sensitivity patterns, coliphage susceptibility, and physiological and biochemical characteristics confirm that *E. coli* isolated from epiphytes in trees 15 m above the ground are identical with strains of *E. coli* from clinical material (Rivera *et al.* 1988). These environmental strains have identical mole% G + C and more than 85% DNA homology with *E. coli* B (Bermúdez & Hazen 1988; Hazen *et al.* 1987). Thus, tropical source waters may contain large numbers of naturally-occurring *E. coli* in the absence of pathogens, or faecal material. *Escherichia coli* usually outnumbers pathogens in the gut and faeces, hence its choice as an indicator organism. Recently, however, it has been shown that pathogens may be in water in which *E. coli* cannot be detected, e.g. in Botswana (Thompson 1981) and Sierra Leone (Wright 1982).

Water treatment in tropical countries is often deficient (Thomson 1981; Wright 1982), resulting in a high incidence of water-borne disease. In addition, contaminated tropical waters may harbour a greater variety of human pathogens, some of which are unique to tropical climes (Hazen *et al.* 1987, 1988). Thus, the importance of understanding the survival of pathogens such as *Salmonella* spp. and its indicator, *E. coli*, is especially important in tropical aquatic environments. This point was further emphasized by a recent epidemic outbreak of salmonellosis at a Puerto Rican resort (Anon. 1986). The present study compared the *in situ* survival and activity of these two bacteria in a tropical river.

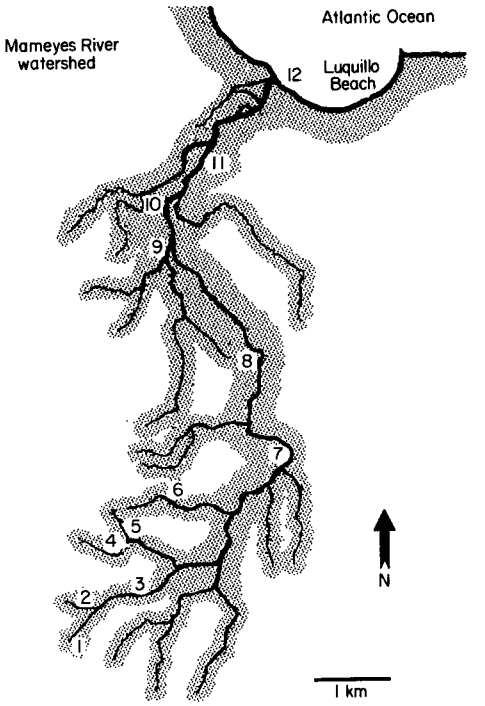

Fig. 1. Location of study site in Mameyes River watershed, Puerto Rico.

Materials and Methods

STUDY SITE

The Mameyes River is located at the north-east 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. For a more complete description of the Mameyes River watershed and site 1 see Carrillo *et al.* (1985).

WATER QUALITY

Dissolved oxygen, air temperature, and water temperature were taken with a dissolved oxygen meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). Three litres of sample water were fixed with sulphuric acid, zinc acetate, or mercuric chloride, placed on ice and transported to the laboratory for examination. Nitrates plus

Table 1. Water quality parameters in the Mameyes River watershed

Time (h)	Water temperature (°C)	Air temperature (°C)	Nitrates and nitrites (mg/l)	Sulphates (mg/l)	Total phosphorus (mg/l)	Phosphates (mg/l)
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 ± 1 SE	19.5 ± 0.5	19.6 ± 0.4	0.03 ± 0.01	0.02 ± 0.00	0.08 ± 0.01	0.01 ± 0.00

nitrites (brucine sulphate colorimetric method), sulphates (turbidimetric method), total phosphorus, and phosphates (P_i) (ascorbic acid method), were determined in the laboratory as described in *Standard Methods for the Examination of Water and Wastewater* (*Anon*, 1985).

CELL DENSITIES AND ACTIVITY

Pure cultures of *Salmonella typhimurium* LT2 (obtained from P. T. Loverde, Purdue University) and *E. coli* (ATCC 11775) were grown in nutrient broth at 35°C for 24 h. Both were isolated from clinical material. Cell densities were adjusted to 10^8 cells/ml and the suspension placed into sterile diffusion chambers just before immersion at the study site. The chambers and their use have been described by Hazen & Esch (1983), Carrillo *et al.* (1985), López-Torres *et al.* (1987, 1988). 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 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) as described by Zimmermann *et al.* (1978). The other half was incubated with [^{3}H]thymidine (75 μCi/mmol) for 2·5 h as described by Tabor & Neihof (1982). Samples were fixed in 10% phosphate-buffered formalin (pH 7), after the appropriate incubation (Zimmermann *et al.* 1978; Tabor & Neihof 1982). Bacterial activity was expressed as respiration (INT reduction; Zimmermann *et al.* 1978), [^{3}H]thymidine uptake positive (microautoradiography; Tabor & Neihof 1982), frequency of dividing cells (Hagstrom *et al.* 1979), and activity by acridine orange direct count (AODC) (Carrillo *et al.* 1985; López-Torrés *et al.* 1987, 1988). Direct count densities were

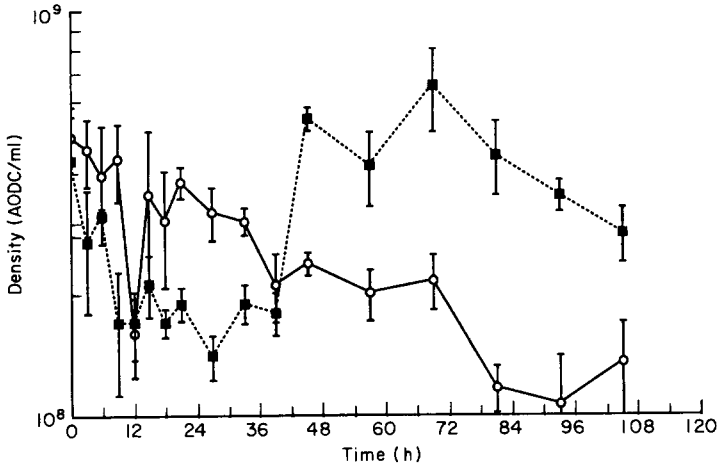

Fig. 2. Changes in total density as measured by AODC for *Salmonella typhimurium* (○) and *Escherichia coli* (■) (mean ± one standard error, $n = 4$).

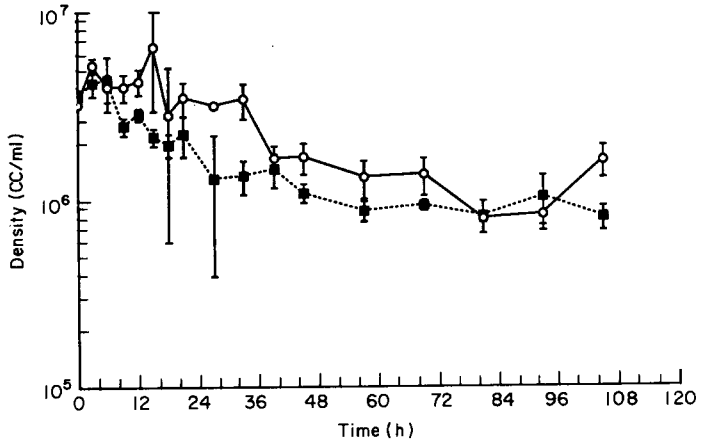

Fig. 3. Changes in total density as measured by Coulter counter for *Salmonella typhimurium* (○) and *Escherichia coli* (■) (mean ± one standard error, $n = 4$).

determined by AODCs from INT fixed samples (López-Torres *et al.* 1988) and Coulter counter (Hazen & Esch 1983).

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 (1984). Any statistical probability less or equal to 0·05 was considered significant.

Results

The water quality at the site did not change significantly for any parameter during the course of the study (Table 1). Numbers of *Salm. typhimurium* were not significantly different from those of *E. coli* by either direct count method over time (Figs 2 and 3). The numbers of both bacteria decreased by less than one order of magnitude over the course of the study and the overall trend for the two organisms is similar. No significant difference in the percentage of respiring cells was observed (Fig. 4). The respir-

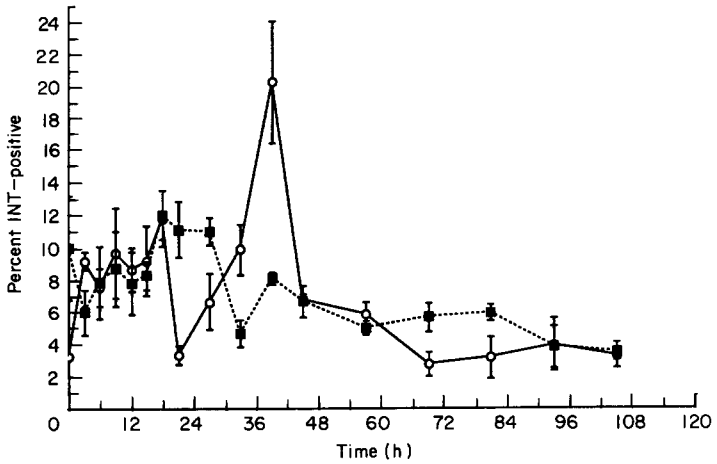

Fig. 4. Changes in percent INT-positive cells as measured by INT-reduction for *Salmonella typhimurium* (○) and *Escherichia coli* (■) (mean ± one standard error, $n = 4$).

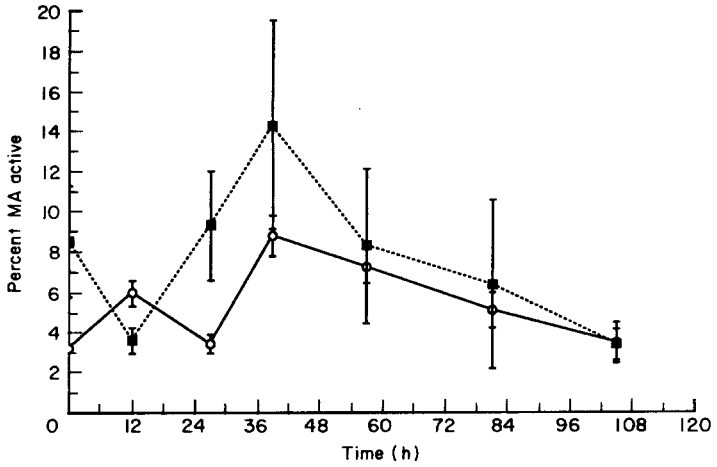

Fig. 5. Changes in percent microautoradiograph positive as measured by [^{3}H]thymidine uptake for *Salmonella typhimurium* (○) and *Escherichia coli* (■) (mean percent of MA ± one standard error, $n = 4$).

ation of both bacteria declined only slightly during the course of the study. The proportion of bacterial cells that incorporated [^{3}H]thymidine, i.e. microautoradiographic positive cells, 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). Large variability in measurements made differences insignificant for both bacteria.

The percentage of active *E. coli* cells, as measured by acridine orange staining, was significantly higher than *Salm. typhimurium* (Fig. 6; $F = 52{\cdot}9$, df = 1 and 32, $P < 0{\cdot}0001$). The red/red + green cell fluorescence indicated that the percentage of physiologically active cells for the *E. coli* population ranged from 55 to more than 90%, while *Salm. typhimurium* activity ranged from 35 to 65%. The proportion of active *E. coli* cells increased significantly during the first 24 h and then remained the same. The frequency of dividing cells (FDC) was also significantly higher for *E. coli* (Fig. 7; $F = 52{\cdot}3$, df = 1 and 10, $P < 0{\cdot}0001$), and again the proportion of the *E. coli* population that was active increased significantly during the first 24 h and then remained the same.

Discussion

The calculated time for *Salm. typhimurium* to decrease by 90%, or one order of magnitude, under *in situ* conditions was 130 h, while *E. coli* decreased by 90% after 276 h. McFeters *et al.* (1974) reported that numbers of *Salm. typhimurium* decreased by 90% within 29 h when exposed to temperate water conditions *in situ* in diffusion chambers. They also reported that *E. coli* showed a 90% reduction in numbers after exposure for 30 h. Other studies have reported that *Salmonella* spp. survive slightly longer than coliforms in temperate waters (Seligman & Reiter 1965; Andre *et al.* 1967; Gallagher & Spino 1968; McFeters *et al.* 1974). Studies by our laboratory, however, have shown that under most conditions *E. coli* will survive indefinitely in tropical freshwaters (Carrillo *et al.* 1985; López-Torres *et al.* 1987, 1988). In addition, no correlation between *E. coli* counts and presence of *Salmonella* spp. was found in tropical waters in Africa (Thompson 1981).

The numbers of both bacteria as measured by AODC were nearly 2 orders of magnitude lower by Coulter counter. This, as discussed in previous works (Carillo *et al.* 1985; López-Torres *et al.* 1987, 1988), 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 with 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

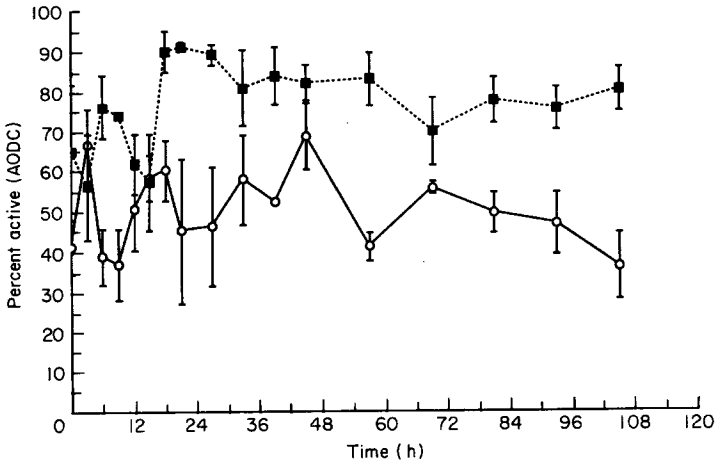

Fig. 6. Changes in percent activity as measured by AODC for *Salmonella typhimurium* (○) and *Escherichia coli* (■) (mean percent of activity ± one standard error, $n = 4$).

and controlled experimental results see López-Torres *et al.* (1988).

The percentages of respiring cells, or those with active electron transport systems, fluctuated between 3 and 21% for both bacteria, within the same range reported for naturally occurring bacteria from diverse environmental samples (Zimmermann *et al.* 1978; Tabor & Neihof 1984). Thus the proportion of bacteria that can actively respire in this environment is relatively low. This was not surprising considering the oligotrophic nature of the waters in this study, as indicated by the low phosphate concentrations (0·07 μg/ml, Table 1), confirming earlier studies of the same sites (Carrillo *et al.* 1985). López-Torres *et al.* (1987), however, 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 or that the sustaining capacity of the water was even lower. The level of respiration observed for both bacteria was high enough to suggest that neither bacteria was under severe stress (Zimmermann *et al.* 1978; Tabor & Neihof 1984). The levels of [^{3}H]thymidine uptake were also typical for naturally-occurring temperate bacterial populations (Tabor & Neihof 1984).

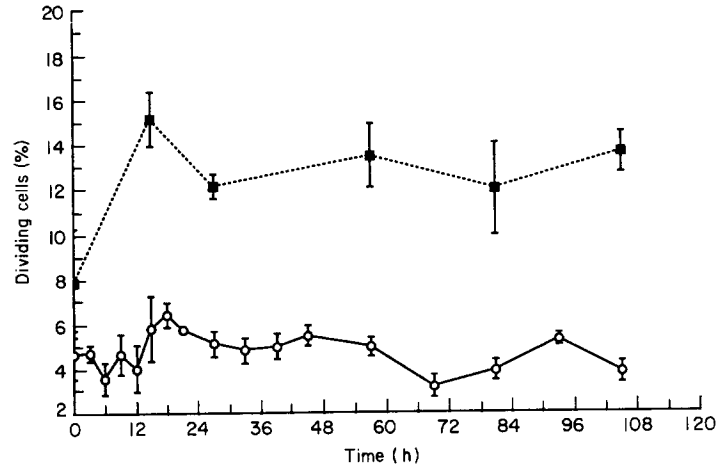

Fig. 7. Changes in the percentage of dividing cells for *Salmonella typhimurium* (○) and *Escherichia coli* (■) (mean ± one standard error, $n = 4$).

The number of MA positive bacteria seems rather low, but it must be remembered this is only a measurement of those capable of actively taking up thymidine, and assumed to be actively engaged in DNA synthesis. Again this suggests that both bacteria were moderately active.

The percentage of active *E. coli* cells, as measured by AODC, was significantly higher than *Salm. typhimurium*. But these percentages were lower than those reported for *E. coli* at the same site (Carrillo *et al.* 1985; López-Torres *et al.* 1987), 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 (López-Torres *et al.* 1988), for *E. coli* ranged from 55 to more than 90%, while *Salm. typhimurium* activity ranged from 35 to 65%. This level of AODC activity has been shown in previous studies to indicate a physiologically active population (Carrillo *et al.* 1985; López-Torres *et al.* 1987, 1988). The frequency of dividing cells (FDC) for *Salm. typhimurium* changed little over time (4–6%) and remained 10% lower than *E. coli* for all samples. Hagstrom *et al.* (1979) reported that naturally occurring bacteria in temperate environments also maintained an FDC of 3 to 15%, thus both bacteria were also moderately active by this measure.

Neither direct count technique showed dramatic changes in numbers of either bacteria so it may be assumed that they can survive for extended periods (Roszak & Colwell 1987). Since these were direct count measurements, however, the physiological activity of the bacteria must be demonstrated. AODC activity and FDC indicate, like microautoradiography (MA) and INT-reduction, that both bacteria were active *in situ* in this tropical freshwater stream. AODC activity and FDC both indicated, however, that *E. coli* remained at a higher level of activity than *Salm. typhimurium*, 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 (Zimmermann *et al.* 1978). Since both of these bacteria are facultative anaerobes, low levels of respiration did not indicate that they were not physiologically active. Low levels of [^{3}H]thymidine uptake would not indicate that they were physiologically inactive, only that they were not growing rapidly (Tabor & Neihof 1982). Since dividing cells were present, then both bacteria were growing to some extent (Hagstrom *et al.* 1979), and the high levels of AODC activity indicated that protein synthesis was also occurring (López-Torres *et al.* 1987, 1988). All of the activity measurements confirmed that both bacteria were active. Two of the four activity measurements also indicated that *E. coli* has a higher activity than *Salm. typhimurium* in tropical freshwaters. This observation is corroborated by the calculated survival times from the direct count methods, since *E. coli* survived more than twice as long.

Density of faecal coliforms and presence of *Salmonella* spp. in temperate waters has been well demonstrated (Geldreich *et al.* 1968). It has also been suggested that *E. coli* is a better indicator for water quality than faecal coliforms (Dufour 1976; Cabelli 1980). In addition, faecal coliforms and *E. coli* are considered good indicators of the presence of *Salm. newport* and *Salmonella* spp. in sediments and shellfish (Allen-Burton *et al.* 1987). The present study suggests that these assumptions are incorrect for tropical freshwater. Indeed, once introduced in tropical waters, *Salm. typhimurium* could represent a serious public health hazard due to its long-term survival. But, *E. coli* and faecal coliforms do not indicate accurately the presence of these pathogens in tropical waters because of their rate of survival, and possible indigenous nature (Hazen *et al.* 1987, 1988; Bermúdez & Hazen 1988; Rivera *et al.* 1988). Thus, the best indicator for tropical freshwaters is no indicator at all: pathogens like *Salmonella* spp. should be counted directly. Methods are now available, e.g. DNA probes (Fitts *et al.* 1983) and immunofluorescence (Roszak *et al.* 1984). 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 MCLs based upon faecal coliforms, whose target is *E. coli*, are unenforceable and may not represent a real public health risk under many circumstances.

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