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Survival and Distribution of *Yersinia enterocolitica* in a Tropical Rain Forest Stream

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Abstract. The survival and activity of *Yersinia enterocolitica* and *Escherichia coli* in a tropical rain forest stream were studied in situ in membrane diffusion chambers. Direct counts of *Y. enterocolitica* decreased by one order of magnitude during the first 6 h and then remained constant. Densities of *E. coli* increased over time, doubling after 2 days. Physiological activity of *E. coli* dropped initially and then stabilized at 85%. Physiological activity for *Y. enterocolitica* increased during the first 6 h, then declined to 50%. The percentage of respiring cells as measured by 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride reduction decreased for *E. coli* to 10%, whereas *Y. enterocolitica* remained near 25%; *Y. enterocolitica* is a survivor in tropical freshwater, as is *E. coli*. Indirect and direct fluorescent antibody (FA) methods were evaluated for the direct detection of *Y. enterocolitica* in natural habitats. Natural densities of FA-positive cells were always less than 10 cells ml^{-1}, and no isolates were obtained by culturing samples.

Water appears to be a significant environmental reservoir for *Yersinia enterocolitica*. It has been isolated from drinking water in Norway [16], streams and lakes of California [11], a drainage basin in Wisconsin [21], surface and well waters in Canada and New York [24, 25], distilled water [13], and potable, fresh, and marine waters in Washington [28]. Yet direct transmission of *Y. enterocolitica* in water to man has not been well established. A possible waterborne epidemic of human Yersiniosis was described from a ski resort in Montana [8], where a significant correlation was found between the amount of well water consumed and reported illness. Subsequently, *Y. enterocolitica* was isolated from unchlorinated well water, which served as the resort's water supply. However, *Y. enterocolitica* could not be implicated as the etiological agent, since stool specimens from patients with acute symptoms were not tested [11]. Langeland [15] has also suggested that drinking water may be contaminated with *Y. enterocolitica* from sources such as raw sewage and sewage sludge where it can persist for up to 1.5 years.

Recently, infections caused by *Y. enterocolitica* have been reported from South Florida [7]. This is a departure from the cold weather geographic distribution of this bacterium normally reported. Two isolates of *Y. enterocolitica* were also brought to our attention as part of routine stool analysis of two paraplegic patients at the Veterans Administration Hospital, San Juan, Puerto Rico. Chester et al. [7] suggested that infections due to *Y. enterocolitica* may be far more prevalent in tropical areas than what is presently appreciated; others have also suggested that *Y. enterocolitica* will be found wherever it is looked for [14]. The present study was undertaken to determine the distribution and survival of *Y. enterocolitica* in Puerto Rico.

Materials and Methods

Study site. The Mameyes River (18° 22' 03" E, 65° 46' 14" N) watershed begins in a natural cloud rain forest that receives approximately 762 cm of rain each year (Fig. 1). For further detailed descriptions, see Carrillo et al. [6] and López-Torres et al. [17].

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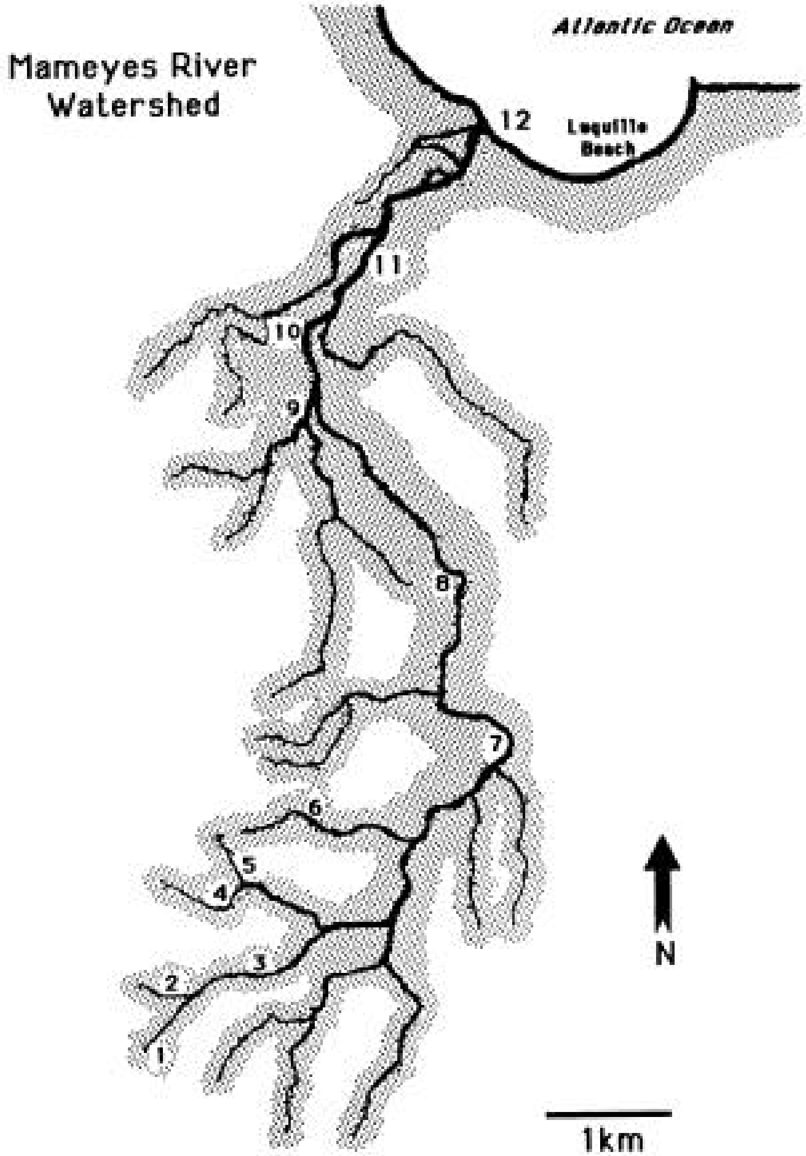

Fig. 1. Map of sampling sites in Mameyes River watershed, Puerto Rico.

Water quality analysis. Simultaneous measurements for conductivity, pH, dissolved oxygen, and water and air temperatures were taken in situ with a Hydrolab digital surveyor (model 4041, Hydrolab Co., Austin, Texas). Hardness and alkalinity were determined in the field using spectrokits (Bausch & Lomb, Rochester, New York). Water samples were preserved in situ by fixation with zinc acetate, sulfuric acid, or mercuric chloride, and transported to the laboratory for further analysis of sulfates, nitrates plus nitrites, phosphates, and total phosphorus. For chlorophyll *a* determination, water samples were placed in amber-colored plastic bottles, transported on ice, and analyzed within 12 h of collection. All analyses were done according to standard methods for water and wastewater analysis [1].

Bacteriological analysis. Freshwater samples for the isolation of *Yersinia enterocolitica* were collected from each site in sterile Whirl-Pak bags (Nasco, Ft. Wilkinson, Wisconsin), stored on ice, and transported to the laboratory. All samples were processed within 6 h of collection. Medium was prepared immediately before use, according to the mYE technique [3]. The plates were incubated for 48 h at 25°C. All bright-yellow to yellow-orange colonies ranging in size from 0.3 to 1.0 mm were counted as presumptive *Y. enterocolitica*.

After the initial incubation on the recovery broth, the membrane filters were transferred to lysine-arginine agar and incubated for 1 h at 35°C in BBL Gas-Pak anaerobic jars (Beckton-Dickinson, Cockeysville, Maryland). The plates were removed and typical colonies marked. The filter was then transferred to an absorbent pad saturated with 2 ml urease broth. After 5–10 min incubation at room temperature, colonies that changed color from yellow to a distinct green or deep bluish purple, indicating urease activity, were presumed to be *Y. enterocolitica*.

Presumptive isolates were grown on *Yersinia* Selective Agar (Difco Labs, Detroit, Michigan) at 35°C for 24 h. Small colonies with a deep-red "bull's eye" center surrounded by an outer zone that is usually translucent were stored on tryptic soy agar and further identified with API-20E strips (Analytab Products, Plainview, New York). In addition, conventional test media such as TSI, LIA, citrate, and motility were inoculated. As a control for all media and tests, *Y. enterocolitica* (ATCC 9610) was used; this strain has been characterized by Bercovier et al. [4] as *Yersinia enterocolitica* sensu stricto.

For direct bacterial counts, 1-liter samples were collected from each site in sterile Nalgene bottles. Immediately, samples were incubated with 100 ml of a 0.2% aqueous solution of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) for 40–50 min to determine respiring cells [30]. After incubation, samples were fixed in situ with 10 ml of 37% formaldehyde, kept in the dark, and transported on ice to the laboratory. Once in the laboratory, these samples were concentrated by centrifugation (5,000 *g* for 10 min) to 10 ml. Subsamples were taken from these tubes: one for the direct quantification of cells in the sample, another for the determination of the percentage of active cells and the percentage of respiring cells. Another sample was stained for the direct enumeration of *Y. enterocolitica* with fluorescent antibody.

The total direct counts of bacteria were determined by acridine orange direct count (AODC). The number of bacteria was then estimated as the total of red and green fluorescing cells. The percentage of active cells was calculated from the ratio of red cells to the total of red and green fluorescing cells. Respiring cells were enumerated by the INT reduction technique of Zimmermann et al. [30]. All techniques are as described previously [6, 17, 18].

Survival studies. Pure cultures of *Y. enterocolitica* (ATCC 9610) and *Escherichia coli* (ATCC 11775) were grown in TSB at 35°C for 24 h. Cells were concentrated by centrifugation and washed in filter-sterilized, phosphate-buffered saline (pH 7). The number of cells per milliliter was determined with a Coulter Counter (model ZM, Coulter Electronics, Inc., Hialeah, Florida) and adjusted to 10^8 cells ml^{-1}. A final bacterial suspension was placed into sterile diffusion chambers just prior to immersion at the study sites. One-ml samples were taken from each chamber at regular intervals for 70 h. The chambers and their use is as described previously [6, 17, 18].

Fluorescent antibody. Stock cultures of *Y. enterocolitica* (ATCC 9610) were used for preparation of antisera according to Fliermans and Hazen [9], except that serum was further purified through a QAE-Sephadex (Sigma Chemical, St. Louis, Missouri) A-50 column and concentrated by vacuum dialysis in PBS (pH 7) at 4°C. The antiserum was tested with the homologous antigen and a variety of heterologous organisms. The antiserum was also reacted with a local *Y. enterocolitica* isolate (courtesy of Maria Medina, Veterans Administration Hospital, San Juan).

Data analysis. The prepared programs for the Apple IIe, Macintosh, and SAS run on an IBM 4382 computer were used for the statistical analysis of the data. Analysis of variance (ANOVA) tests were performed to show significant differences between sites and time of collection. Data that were heteroscedastic were transformed with either $\log_{10}(x + 1)$ or arcsine-square-root. Probabilities equal to or less than 0.05 were considered significant [29].

Table 1. Direct and indirect fluorescent antibody (FA) reactions of *Yersinia enterocolitica* immunoglobulin with homologous and heterologous antigens[a]

Organism	Indirect	Direct
Yersinia enterocolitica (ATCC 9610)	+4	+2
Yersinia enterocolitica VA (NT)[b]	+4	+2
Yersinia enterocolitica (ATCC 23715)	+4	+2
Yersinia ruckeri (NT)	+1	+1
Aeromonas hydrophila (ATCC 7966)	+2	+2
Escherichia coli (ATCC 25922)	+0	+2
Enterobacter cloacae (ATCC E13047)	+2	+1
Pseudomonas aeruqinosa (ATCC 27853)	+1	+1
Proteus vulgaris	+1	0
Salmonella enteritidis	0	+1
Salmonella typhimurium	0	+2
Shigella sonnei	+2	+2
Serratia marcescens	0	+1
Vibrio parahaemolyticus (ATCC 17802)	0	0
Vibrio cholerae (ATCC 14035)	0	+1
Vibrio vulnificus (ATCC 27562)	0	0
Klebsiella pneumoniae (NT)	+1	+1
Klebsiella oxytoca (NT)	+1	+1

[a] FA titers were those obtained with conjugated gobulins at a protein concentration of 1 mg/ml.
[b] NT = not typed, local isolates.

Results and Discussion

Fluorescent antibody. Homologous and heterologous strains of bacteria were directly and indirectly stained with the *Yersinia enterocolitica* fluorescent antibody (Table 1). No positive fluorescence (> +2) was observed for *Salmonella* spp., *Vibrio* spp., *Escherichia coli,* or *Serratia marcescens*. Reactions with *Aeromonas hydrophila, Enterobacter cloacae,* and *Shigella sonnei* for both staining procedures gave a +2, which was considered positive although weak. In general, less cross-reactivity was observed in the indirect procedure (Table 1).

Water quality. Measurements of water quality, cell activity, cell respiration, and total direct cell counts are shown for four sites along the Mameyes River in Table 2. The highest values for water and air temperatures, conductivity, alkalinity, and hardness were measured at site 9. The lowest measurements for these same parameters were observed at site 1. Dissolved oxygen concentration varied among the different sites but was always lower at site 9. Also, nitrates plus nitrites, chlorophyll *a*, and total phosphorus concentrations were consistently higher at site 9. The low productivity and oligotrophic nature of the Mameyes River watershed, observed in this study, has been described in previous works [6].

Table 2. Water quality parameters in the Mameyes River watershed[a]

SITE	WTEMP	ATEMP	COND	DO	PH	ALKAL	HARD
1	20.0 ± 0.8	20.9 ± 0.7	73.8 ± 4.2	9.3 ± 0.2	6.2 ± 0.2	25.0 ± 5	30.0 ± 10
4	20.8 ± 0.7	23.7 ± 1.9	115.5 ± 6.9	9.1 ± 0.1	7.1 ± 0.2	40.0 ± 8.2	55.0 ± 9.6
5	21.6 ± 0.6	23.1 ± 1.4	165.8 ± 6.9	9.1 ± 0.3	7.4 ± 0.1	60.0 ± 8.2	90.0 ± 12.9
9	27.2 ± 1.2	30.2 ± 2.2	247.5 ± 11.3	7.9 ± 0.1	6.9 ± 0.2	85.0 ± 12.6	110.0 ± 23.8

SITE	CHLA	NO_{2+3}	SO_4	TP	TDC	%RC	%AC	TIFC
1	37.3 ± 0.8	0.26 ± 0.02	1.5 ± 0.2	0.02 ± 0.01	4.9 ± 1.5	36.5 ± 4.1	54.8 ± 5.8	0.31 ± 0.07
4	38.0 ± 5.3	0.27 ± 0.04	2.7 ± 0.2	0.05 ± 0.01	2.7 ± 0.7	22.9 ± 7.9	50.7 ± 10.8	0.79 ± 0.17
5	39.2 ± 1.3	0.34 ± 0.04	6.3 ± 1.3	0.06 ± 0.02	1.9 ± 1.0	24.7 ± 10.1	51.2 ± 2.9	0.49 ± 0.08
9	56.4 ± 5.7	0.46 ± 0.05	3.6 ± 0.5	0.16 ± 0.04	5.3 ± 1.6	26.6 ± 7.5	61.1 ± 3.6	0.59 ± 0.23

[a] All values are mean ± one standard error ($n = 5$). ATEMP = air temperature (°C), WTEMP = water temperature (°C), DO = dissolved oxygen (mg/liter), COND = Conductivity (umohs/cm), SO_4 = sulfates (×1000 mg/liter), ALKAL = alkalinity (mg/liter $CaCO_3$), TDC = total direct count (AODC × 10^8/ml), %RC = percentage of respiring cells, %AC = percentage of AODC active cells, NO_{2+3} = nitrites plus nitrates (mg/liter), TP = total phosphorus (mg/liter), CHLA = chlorophyll A (mg/liter), TIFC = *Y. enterocolitica* immunofluorescence (cells/ml).

The high nutrient (total phosphorus and nitrates) concentrations observed at site 9 were expected, since this site received sewage effluents. Densities of *Y. enterocolitica*-like cells as measured by immunofluorescence were always quite low at all sites (<10 cells ml^{-1}), even at site 9 where total bacteria densities (AODC) were always greater than 10^8 cells ml^{-1} (Table 2). Total bacterial activity was moderately high at all sites, as measured by INT reduction (22.9%–36.5%) and AODC activity (50.7%–61.1%). Several other studies have shown that many enteric bacteria can maintain high densities and high levels of activity in tropical freshwater [6, 17, 23].

Isolation from water. Presumptive *Y. enterocolitica* isolates were not obtained from any of the four sites. As has been seen for other bacteria in natural environments, when the immunofluorescent densities are below 10^3 cells ml^{-1}, viable count methods are unable to recover these organisms [6, 23]. However, atypical colonies on CIN (almost all presumptive colonies grew as medium-sized pink colonies with no translucent halo or the typical deep red "bull's eye" center), were also isolated. Believing that tropical environmental isolates may not behave the same as clinical isolates, we confirmed the identification. The API-20E identification profile system failed to identify any *Yersinia* among the 57 random isolates. Instead, 8% of these were identified as *Serratia marcescens*, 25% as *Enterobacter agglomerans*, and 67% as *Aeromonas hydrophila*, all with a probability of 0.94 or higher. Our results coincided with those of Highsmith et al. [13], who found that the majority of the Gram-negative isolates on CIN medium from well waters were *Aeromonas* spp., and a few were *Enterobacter* spp. Since the density of these bacteria on the plating medium was high (>100 CFU ml^{-1}), it is possible that they masked the presence of *Y. enterocolitica* colonies. The presumptive identification of waterborne *Yersinia* colonies by this procedure seems to be compromised by some distinctive characteristics that are shared by other microorganisms, thus underestimating the presence of *Y. enterocolitica* in tropical waters.

Water temperature and life competition, as suggested by Marinelli et al. [19], may adversely influence the survival of *Y. enterocolitica* in surface waters. There is some evidence that *Y. enterocolitica* may survive longer in the environment during the colder months of the year in temperate areas because the numbers of background organisms are lower [20, 21]. In a study on the Aterno River, L'Aquila, Italy [19], high densities of *Y. enterocolitica* were attributed, in part, to the cold climate at water temperatures of 6.0–6.5°C, 20°C colder than our study. They also suggested a life-competition phenomenon at those sites where the organism was not detected. Since total bacterial densities were high in the rain forest, this suggested that competition could be a major factor in keeping densities of *Y.*

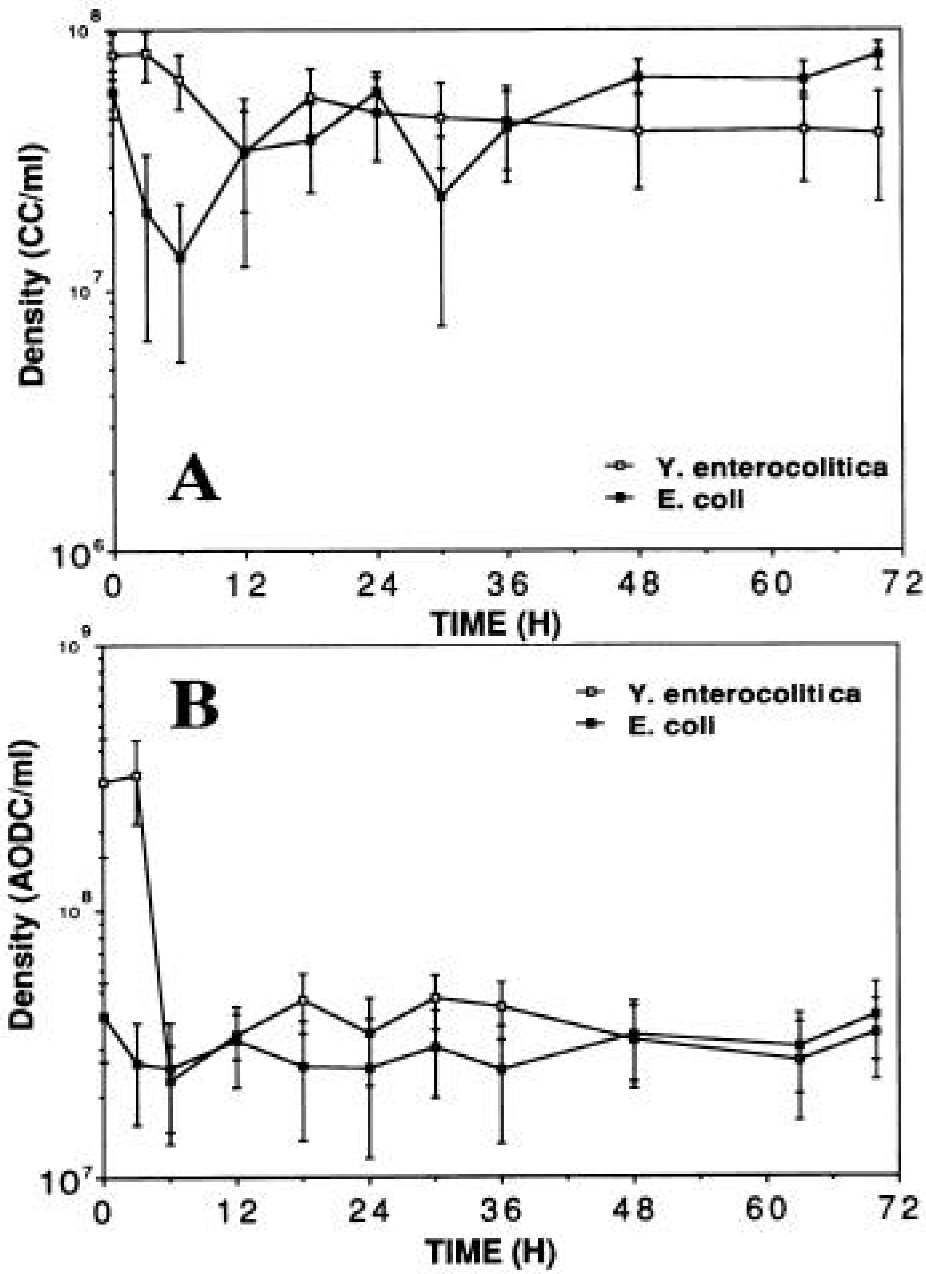

Fig. 2. Survival of *Yersinia enterocolitica* and *Escherichia coli* at Mameyes River sites 1 and 4, as determined by Coulter Counter (A) and AODC (B) (mean ± one standard error, n = 8).

enterocolitica low, if it could be demonstrated that the organism survived.

In situ survival. *Y. enterocolitica* has been recognized as a psychrotroph with very simple nutritional requirements and, therefore, would appear to have the capability of adapting well to aquatic environments [26]. The results obtained from the survival study demonstrated that *Y. enterocolitica* was a survivor in tropical freshwater. After the first 6 h, *Y. enterocolitica* cell densities showed an increase and stabilization as determined by both direct count methods (Fig. 2). However, the *Y. enterocolitica* population was metabolically more active, after 6 h, and more stable than *E. coli*, as shown by their higher respiration rate (up to 85%) (Fig. 3). The ability to survive and regrow was observed for *E. coli* by AODC and Coulter Counter direct counts (Fig. 2). This behavior has been previously observed in studies in which *E. coli* was capable of growth at temperatures greater than 13°C during a 5-day exposure period in situ [2]. The high AODC

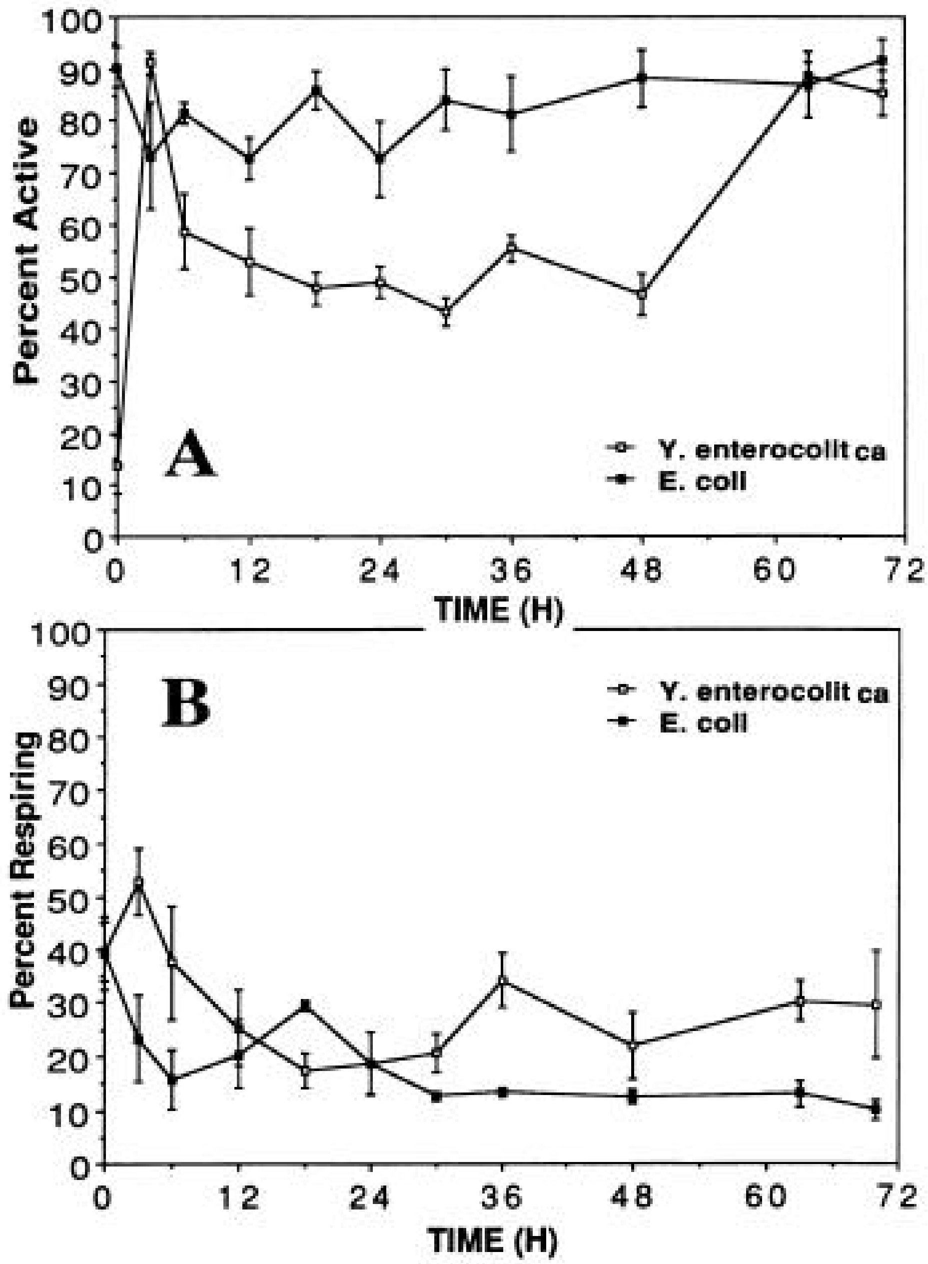

Fig. 3. Activity of *Y. enterocolitica* and *E. coli* at Mameyes River sites 1 and 4, as determined by AODC (A) and percentage respiration (B) (mean ± one standard error, n = 8).

activity and respiration observed in both bacteria confirmed that they were indeed active and growing or capable of growing. Thus, as shown by other studies, *E. coli* is not considered to be an appropriate indicator of recent fecal contamination in tropical waters [6, 12].

The survival study suggests that interspecies competition in the environment with the indigenous microflora influenced our isolation results more than did temperature. The survival of *Y. enterocolitica* in the diffusion chambers can be explained in terms of an absence of competition or antagonistic effects between these cells and any other microbial populations present in the natural environment.

Martin et al. [20] found that *Y. enterocolitica* at environmental temperatures survived for 7 days in unfiltered surface waters obtained in the summer, whereas the same water after being filtered allowed its survival for 184 days. This same behavior has been observed for *E. coli*, which was capable of extended survival even in estuarine water over the seasonal range of 1–25°C, if autochthonous microbiota were excluded from the diffusion chambers [2]. Some investigators have suggested that the effects of cold temperatures and cold enrichment techniques are to equalize the metabolic rates of the rapidly growing, Gram-negative flora, allowing *Y. enterocolitica* to reach higher population densities in mesophilic environments [22]. Schiemann and Olson [27] believed that the inability of *Y. enterocolitica* to attain its potential maximum population in mixed cultures appears to result from "metabolic crowding," which occurred when the faster-growing antagonistic organisms reached a stationary phase.

Yersinia spp., to be detectable with media, must be in densities higher than 10^3 cells ml^{-1}. Mehlman et al. [22] could recover the organism from pork samples only when the total standard plate counts were 10^8 cells g^{-1} of meat and the *Y. enterocolitica* initial input was at least 10^4 cells ml^{-1}. In this study, no culturable isolated could be obtained after filtering of samples of 1–3 liters. Fliermans and Schmidt [10] suggested that culturing alone was an indirect approach, which isolates a particular bacterium from its habitat in a prescribed set of culture conditions unlike those found in the natural habitat. Thus our lack of isolation of *Y. enterocolitica* may be due to densities of the bacterium below 10^3 cells ml^{-1}, as indicated by the immunofluorescent counts and not because a high number of background organisms growing on the isolation medium hindered its growth. However, high numbers of other microbes could be keeping the ambient densities of *Y. enterocolitica* low.

This is the first study to demonstrate the survival of *Y. enterocolitica* in tropical aquatic systems; *Y. enterocolitica* showed great adaptability to the environment but did not survive as well as *E. coli*. Interspecies competition probably keeps densities of *Y. enterocolitica* in water low. *Y. enterocolitica* may be more common in tropical environments when conditions are more favorable, since *Yersinia*-like cells were detected by immunofluorescence. The low incidence of Yersiniosis in the tropics [5] is corroborated by the low densities of *Y. enterocolitica* observed in water in this study.

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