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SURVIVAL AND DISTRIBUTION OF VIBRIO CHOLERAE IN A TROPICAL RAIN FOREST STREAM

by

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ABSTRACT

For 12 months Vibrio cholerae and fecal coliforms were monitored along with 9 other water quality parameters at 12 sites in a rain forest watershed in Puerto Rico. Densities of V. cholerae and fecal coliforms were not significantly correlated even though the highest densities of both bacteria were found at a sewage outfall. High densities of V. cholerae were also found at pristine sites high in the watershed. V. cholerae and Escherichia coli were inoculated into membrane diffusion chambers, placed at two sites and monitored for 5 days on two different occasions. Two different direct count methods indicated that the density of E. coli and V. cholerae did not change significantly during the course of either study. Physiological activity, as measured by INT-reduction and relative nucleic acid composition declined for E. coli during the first 12 h then increased and remained variable during the remainder of the study. V. cholerae activity, as measured by relative nucleic acid concentrations, remained high and unchanged for the entire study. INT-reduction in V. cholerae declined initially but regained nearly all of it's original activity within 48 h. This study suggests that V. cholerae is an indigenous organism in tropical freshwaters and that assays other than fecal coliforms or E. coli must be used for assessing public health risk in tropical waters.

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INTRODUCTION

Vibrio cholerae is generally accepted as having an autochthonous origin in brackish estuaries (9, 18, 21, 22, 33). However, there is some disagreement as to the normal range of salinities where V. cholerae is most abundant. Colwell et al. (9) found that 2 - 20 ppt is optimal in the Chesapeake Bay. Roberts et al. (33) reported high densities of V. cholerae when salinities were below 2 ppt in Louisiana. Lee et al. (22) also found high densities of V. cholerae in England when salinities were very low. Hood et al. (19) found higher densities of V. cholerae when salinities were between 12 and 25 ppt. Using V. cholerae data from Maryland, Oregon, and Louisiana, Seidler and Evans (36) developed a predictive mathematical model based on temperature and salinity. The model suggested that as salinity decreased that densities of V. cholerae increased. The model also suggested that increases in density of V. cholerae were greatest when temperatures were elevated and salinities were low.

Since all of the above studies were done using viable count methods, plate counts or MPN, their applicability to public health has recently been questioned. Several studies have shown that at high salinities V. cholerae decreases in culturability on standard media, yet retains its virulence and infectivity (2, 8, 17, 34, 41). Thus, failing to isolate V. cholerae in a stressful environment does not indicate that this environment is free from public health concern. These findings suggest that the effects of ambient water on densities of V. cholerae must be

reexamined using direct count techniques that do not rely on culturability.

Recent **in situ** studies by our laboratory in Puerto Rico using direct enumeration and activity measurements have shown that V. cholerae can survive and remain moderately active on a tropical coral reef (30). In Puerto Rico and other parts of the Caribbean, V. cholerae has even been isolated from near shore coastal waters when the salinity was 35 ppt and the temperature was 25°C (3, 13, 14, 38). Freshwater in India was also shown recently to have very high densities of V. cholerae (27). These findings have suggested that tropical rain forest environments should provide ideal conditions for V. cholerae survival, yet few if any studies have examined the survival of this pathogen in this environment. Indeed, considering the importance of this pathogen in underdeveloped tropical nations an understanding of the survival of V. cholerae in this environment is vital to tropical public health. In the tropics, coliforms and fecal coliforms are universally used as indicators of pathogens (16). The target enteric organism in both of these assays is Escherichia coli. Thus, it is also essential that we examine the survival of E. coli simultaneously with V. cholerae. The present study examines the distribution and abundance of V. cholerae and fecal coliforms in a tropical rain forest watershed. Membrane diffusion chambers were used to determine the **in situ** survival and activity of V. cholerae and E. coli, using direct measurements of density and activity.

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University of Puerto Rico, Río Piedras, Puerto Rico, 1983.)

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MATERIALS AND METHODS

Study site. The Mameyes River watershed is located on the northeastern coast of the island of Puerto Rico at 18° 15' N, 65° 45' W (Fig. 1). This watershed has a drainage area of 27.27 km^2 and a total length of 17.1 km (7). The annual average precipitation in the upper third of the estuary is 395 cm; this area is classified as a cloud rain forest and is part of the Luquillo Experimental Forest of the U. S. Forest Service. For detailed descriptions of the study area see Carrillo et al. (7), López-Torres et al. (23), and Valdés-Collazo et al. (39).

Water analysis. Measurements were taken **in situ** for conductivity, salinity, pH, dissolved oxygen, light intensity, and temperature. The pH was measured with a digital pH meter (model 201, Orion Research, Inc., Cambridge, Mass.) and dissolved oxygen was measured with a DO meter (model 57, Yellow Springs Instruments Co., Yellow Springs, Ohio). An S-C-T meter (model 33, Yellow Springs) was used to measure conductivity and salinity. Turbidity, alkalinity, hardness, and ammonia measurements were done in the field by using a spectrophotometer (Mini Spectronic 20, Bausch & Lomb, Inc., Rochester, N.Y.). Light intensity was measured in the field with an underwater photometer (Protomatic, Dexter, Mich.). For chlorophyll *a* determination, water samples were placed in amber colored plastic bottles and analyzed at the laboratory by the trichromatic extraction method (1). Other samples were fixed with mercuric chloride, sulfuric acid and zinc acetate before being transported to the laboratory, where

they were analyzed for nitrate plus nitrite, sulfate, total phosphorus, and orthophosphate, according to procedures in standard methods for water and wastewater analysis (1).

Bacteriological analysis. For V. cholerae enumeration water samples were filtered through 0.45-µm-pore-size membrane filters (Millipore Corp., Bedford, Mass). Filters were then placed on Thiosulfate-Citrate-Bile salts-Sucrose agar (TCBS; Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 24 h. All round, yellow colonies were considered V. cholerae-like (1). Identifications of random isolates were confirmed using the biochemical tests of the API 20E strips (Analytab Products, Plainview, N.Y.). Further corroboration of V. cholerae identifications were done using ploy valent antiserum against V. cholerae (Difco). Fecal coliform densities were determined by filtering samples with type HC, 0.7-µm-pore-size membrane filters (Millipore). Filters were placed on mFC media (Difco) in tight-fitting petri dishes (Millipore) and incubated at 44.5 ± 0.1°C in a block type FC incubator (Millipore) for 24 h. All blue colonies were enumerated as fecal coliform positive (1).

Direct cell counts for V. cholerae and E. coli in diffusion chambers were done using a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla) and acridine orange staining (AODC). Red fluorescing cells are assumed to be active in protein synthesis since the red fluorescence is caused by a dominance in RNA content. Cells with more DNA than RNA will fluoresce green (10). Studies in our laboratory have shown that by careful preparation of reagents E. coli and other bacteria can be

measured for their relative activity in this way (7, 23, 24). Total number of bacteria and the number involved in respiration were determined by the technique of Zimmermann et al. (43).

Survival studies. For survival studies, plexiglas diffusion chambers, a modification of McFeters and Stuart (26), with 100 ml capacity were used with a 0.45-μm-pore-size, nylon-reinforced Versapor membrane filters (Gelman Instrument Co., Ann Arbor, Mich) as diffusion surface (3, 15). Pure cultures of V. cholerae and E. coli were grown in 5% tryptic soy broth at 37°C for 24 h (30). The cells were then harvested by centrifugation (10,000 x g for 10 min) and suspended in filter-sterilized phosphate-buffered saline (pH 7). Cell density was determined with a model ZF Coulter Counter and adjusted to a concentration of 10^7 cells per ml. The bacterial suspension was placed into the sterile diffusion chamber just before it was placed at the study site. At each study site, a total of four chambers were placed strategically at a depth of 30 cm. Periodically, 1.0-ml samples were taken from each chamber with a sterile syringe. Of each sample, 0.5 ml was fixed with 1.5 ml of phosphate-buffered Formalin for later counting at the laboratory with a Coulter Counter as described by Hazen and Esch (15). The other 0.5 ml was incubated with INT and fixed according to Zimmermann et al. (43). The preserved sample was then stored on ice for membrane filtration at the laboratory and subsequent total direct counts and activity measurements as described above.

Data analysis. The data were analyzed by using prepared programs for Apple II, Macintosh, and IBM 4321 computers. Factorial

analyses of variance were used to test for differences among sites and collection times. Data were subjected to the appropriate transformation before statistical analysis by the method of Zar (42). Any probability less than or equal to 0.05 was considered significant.

RESULTS

Water quality. During the sampling period, the physicochemical parameters measured showed small variability within the same sites (Table 1). Generally, higher measurements for temperature, sulfates, phosphates and total phosphorus were obtained from the lower parts of the watershed (Table 1). Site 12 was the only site with any detectable salinity.

Distribution of V. cholerae and fecal coliforms. Densities of fecal coliforms were highest at sites 9 and 12 (Fig. 2). Average densities of fecal coliforms at all sampling sites ranged from 12 ± 2 colony forming units (CFU) per 100 ml to 21,960 ± 2,748 CFU per 100 ml. The highest densities of V. cholerae were observed at site 1 and site 9 (Fig. 2). V. cholerae was isolated and identity confirmed from all sites sampled. Concentrations ranged from 31 ± 2 CFU/100 ml to 52,000 ± 2,000 CFU/100 ml. Densities of both V. cholerae and fecal coliforms were significantly different by site. Densities of V. cholerae and fecal coliforms were not significantly correlated when compared by site, by time, or by site and time.

Survival of bacteria in situ. Densities of V. cholerae and E. coli in the diffusion chambers were not significantly different between sites for any of the density or activity measurements, thus measurements for both sites were pooled for each study. Densities of V. cholerae as measured by both direct count methods remained relatively the same varying less than 20% during both studies (Fig. 3 and 4). In study A,

densities of V. cholerae declined significantly from 48 to 90 h ($F = 47$, df = 17 & 102, $P < 0.001$); however, a significant increase was observed at 102 h (Fig. 3A). The overall decline in Coulter Counter densities during study A was less than 75%. Densities of E. coli, as measured by AODC were significantly different over time for study A ($F = 157$, df = 17 &108, $P < 0.0001$), reaching a high at 30 h and a low at 60 h (Fig. 4A). Densities varied as much as 1.5 log units in less than 30 h. Survival of E. coli was still 75% after 90 h. Densities of E. coli, as determined by AODC, in study B did not change significantly over time (Fig. 4B). Coulter Counter determined densities of E. coli did not change significantly over time for either study; however, densities in study B showed more variability than study A (Fig. 3). The calculated 90% decrease time (T_{90}) or 1 log decrease time using AODC for V. cholerae, for both studies was infinite. The calculated T_{90} for E. coli for study A was 108 h; however, given the variability observed and that densities of E. coli increased significantly twice during the study, 6 - 30 h and 66 - 84 h, this calculation probably underestimates the survival time of E. coli in this environment. The calculated T_{90} for E. coli for study B was infinite.

Activity of V. cholerae as indicated by acridine orange (AO) fluorescence did not change significantly over time (Fig. 5). Activity of V. cholerae cells during the entire study period never went below 90%. Activity of E. coli as indicated by acridine orange fluorescence was significantly different over time ($F = 64$, df = 17 & 104, $P < 0.0001$); decreasing more than 60% in the first 6 h (Fig. 6). Variability of AO

activity increased with time; even though average activities for E. coli cells from 6 - 90 h remained between 15 to 45%.

The percentage of respiring V. cholerae cells declined significantly during the first 18 h from 91 to 11% ($F = 2.18$, $df = 17$ &104, $P < 0.05$, Fig. 7). However, by the end of the study the percentage of respiring cells had increased to 72%. The percentage of E. coli respiring cells also declined significantly during the first 18 h of exposure from 92 to 32% ($F = 126$, $df = 17$ & 104, $P < 0.0001$, Fig. 8). The proportion of respiring E. coli cells was much more variable than V. cholerae. By the end of the study the percentage of E. coli cells that were respiring had decreased to 10%. Activity and respiration measurements for V. cholerae and E. coli were not significantly correlated.

DISCUSSION

The Mameyes River watershed is relatively oligotrophic despite the fact that the source of the watershed is a cloud type tropical rain forest. For a complete description of all sites and a thorough discussion of trophic status of this watershed see Carrillo et al. (7), López-Torres et al. (23), Valdés-Collazo et al. (39) and C. F. Aranda, M. S. thesis, University of Puerto Rico, Río Piedras, 1982. Concentrations of total phosphorus, alkalinity, phosphates, and nitrates + nitrites are within the range of values for oligotrophic-mesotrophic freshwaters (20). Sites 1, 9 and 12 were the only sites that were not low in nutrients, eg. phosphates, total phosphorus, nitrates + nitrites. Site 9 is the point source for a primary sewage treatment plant effluent and site 12 is a river mouth estuary surrounded by mangroves, both very productive environments. Site 1; however, is the highest point sampled in the watershed in a very pristine part of the rain forest. The increased concentrations of some nutrients at this site may be explained by the higher algal densities observed. Higher algal densities at this site could be the result of a thinner forest canopy in the upper part of the rain forest allowing sunlight to reach the river. Further down in the watershed the forest canopy is dense enough to inhibit algal growth (7). Another possibility is that slightly more nutrients are leached into the river at the top of the watershed because of higher levels of biodegradation of leaf litter and lower levels of nutrient adsorption by the forest standing crop (28). This is indicated by the dwarf nature of

the forest, trees that normally reach > 20 m are 5 m or less in the dwarf forest at the top of the watershed.

Torrential rainfalls (>10 cm h^{-1}) which can cause the river level to change more than 2 m in less than an hour are the principal cause of the low densities of resident flora and fauna and the lack of seasonality in all parameters (C. F. Aranda, M. S. Thesis, University of Puerto Rico, Río Piedras, 1982). Temperature is quite constant year-round and rainfall does not exhibit any consistent pattern in the area.

Bacteria distribution and abundance. The highest densities of fecal coliforms and V. cholerae, as expected, were recorded at site 9, the sewage outfall. However, densities of fecal coliforms reported in this study at other sites were lower than those reported by Evison and James (11) for river samples taken in two countries in tropical Africa and much higher than those reported for river samples taken in England. The densities of fecal coliforms at all sites exceeded recommended coliform maximum contaminant levels (MCL) for potable waters, 0.04 CFU ml^{-1} (12). Site 9 also exceeded the recommended fecal coliform MCL for primary contact recreational waters, 4 CFU ml^{-1} (5, 6).

Densities of V. cholerae were also high at site 1, a pristine area high in the rain forest. As noted in other studies; however, this locale has higher densities of fecal coliforms, total anaerobes, and Candida albicans than sites immediately below it in the watershed (7, 29, 35, 39). Recent studies have even demonstrated that supposedly anthropogenic microbes like E. coli may even be indigenous in this environment (4, 32). Bermúdez and Hazen (4) showed, using DNA

hybridization, that E. coli could be found 10 m off the ground in the rain forest trees. Thus, it is not surprising that microbes like V. cholerae are also in high densities in the rain forest and in the water in general. High densities of V. cholerae have also been reported in tropical freshwaters in India (27). Indeed, several investigators have reported the isolation of V. cholerae in cold, uncontaminated, temperate freshwaters (25, 31).

Survival of bacteria in situ. Direct count density estimates for both V. cholerae and E. coli changed little during the course of two studies in the upper part of the watershed. Despite subtle differences in water quality between the study sites, no significant difference was observed in survival rates for either direct count method at any of the sites. In fact the calculated T_{90} for both bacteria for both studies was infinite, except for AODC densities of E. coli in study A (T_{90} = 108 h). This one anomaly is readily explained by the variability in density observed over time and the density increases observed from 6 - 30 h and from 66 - 84 h. Certainly, if the study had continued longer the calculated T_{90} for E. coli in this study would also have been infinite. West and Lee (40) in England also observed stable survival of V. cholerae in diffusion chambers during the summer months when river water was at it's warmest.

The survivability of V. cholerae and E. coli in this environment is also borne out by the activity measurements taken during study A. Though the percentage of respiring V. cholerae cells declined during the first 18 h more than 80%, they increased during the next 48 h and then

stabilized at 78%, this is indicative of a very active population. As reported by Zimmermann et al. (43), natural populations of bacteria in cold waters rarely exceed 10% activity in INT-reduction. This finding is confirmed by the differential fluorescence of acridine orange which indicated that more than 90% of the cells were active at all times of the study. It is assumed that INT reduction indicates cells actively respiring and AO fluorescence indicates proportions of RNA and DNA, i.e. protein synthesis. Thus V. cholerae cells in the chambers initially declined and subsequently acclimated their respiring ability in response to the new environment they were exposed to; however, the stress of the new environment was not sufficient to reduce protein synthesis during the acclimation period. This suggests that fermentative metabolism may have been able to compensate for the loss in oxidative metabolism. Klebsiella pneumoniae shows a similar pattern of activity response in this environment (23).

The activity of E. coli as measured by both AO fluorescence and INT-reduction was significantly lower than V. cholerae, indicating a more stressful environment for E. coli. The variability for both activity measurements was also much greater for E. coli. However, even at these lower measurements of activity for E. coli, the activities observed were greater than those observed for natural populations of bacteria in both warm (37) and cold freshwaters (43). Indeed, three other studies at these same sites have also demonstrated that E. coli can survive and remain active in this environment for extended periods of time (7, 23, 39).

The diffusion chamber study of West and Lee (40) in an English river, the mathematical model of Seidler and Evans (36), and the surveys of Roberts et al. (33) in Louisiana all indicate that warm temperatures and low salinities are conducive to V. cholerae survival. The present study confirms that tropical freshwaters are nearly ideal for V. cholerae and may represent one of it's natural habitats. The lack of correlation between fecal coliforms and V. cholerae found in this study and by several others suggest that assays for fecal coliforms are not reliable indicators of pathogens like V. cholerae (22, 33) The ability of both V. cholerae and E. coli to survive and remain active in tropical environments further suggests the unreliability of such assays for indicating recent human fecal contamination and thus public health risk. The increased survival and possible indigenous nature of pathogens like V. cholerae further emphasize the need for direct enumeration standards and health risk assessments for underdeveloped tropical countries where these diseases exact a horrible toll.

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Figure Legends

Figure 1. Location of study sites in Mameyes River watershed, Puerto Rico.

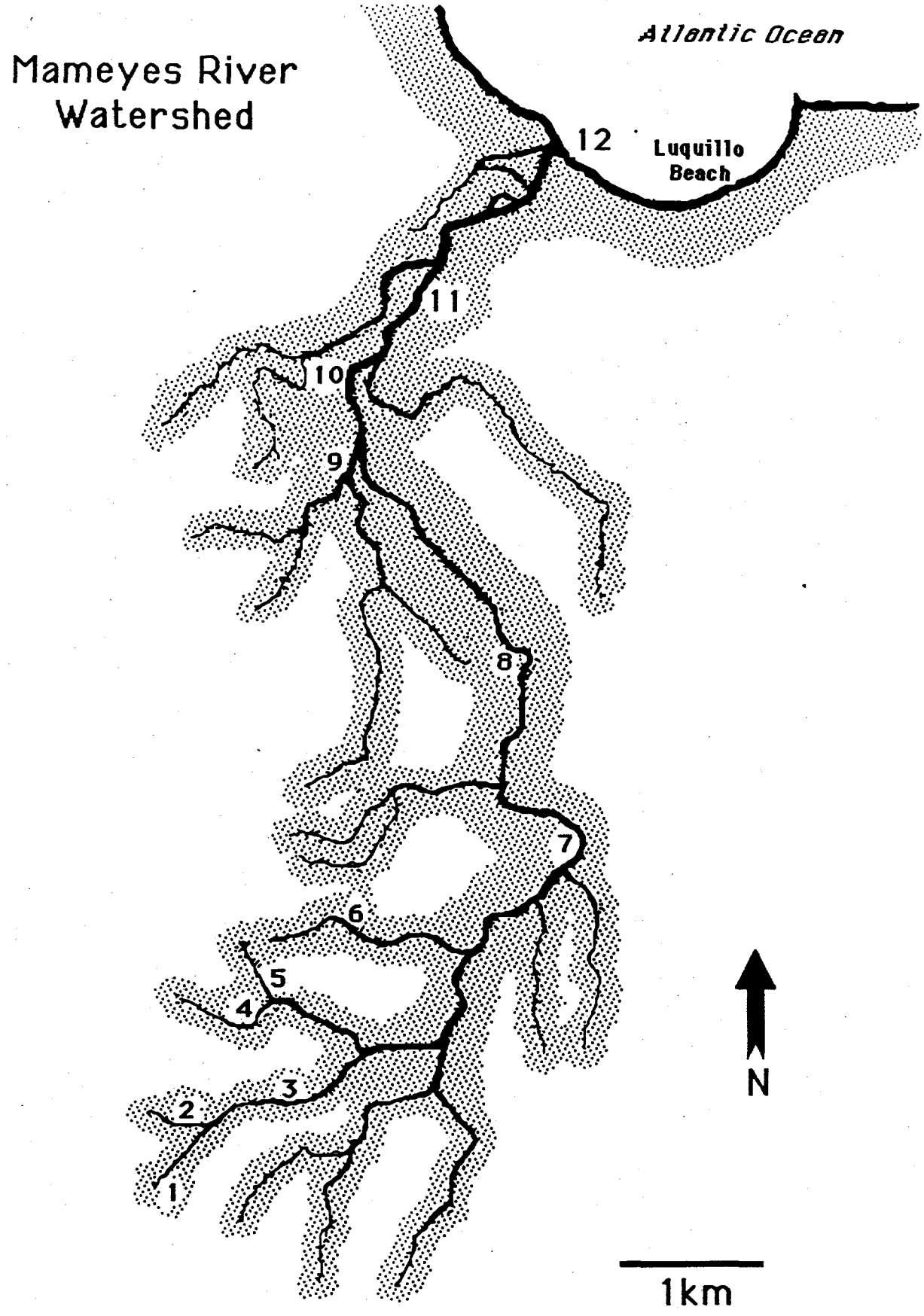
Figure 2. Densities of V. cholerae and fecal coliforms by site in the Mameyes River watershed.

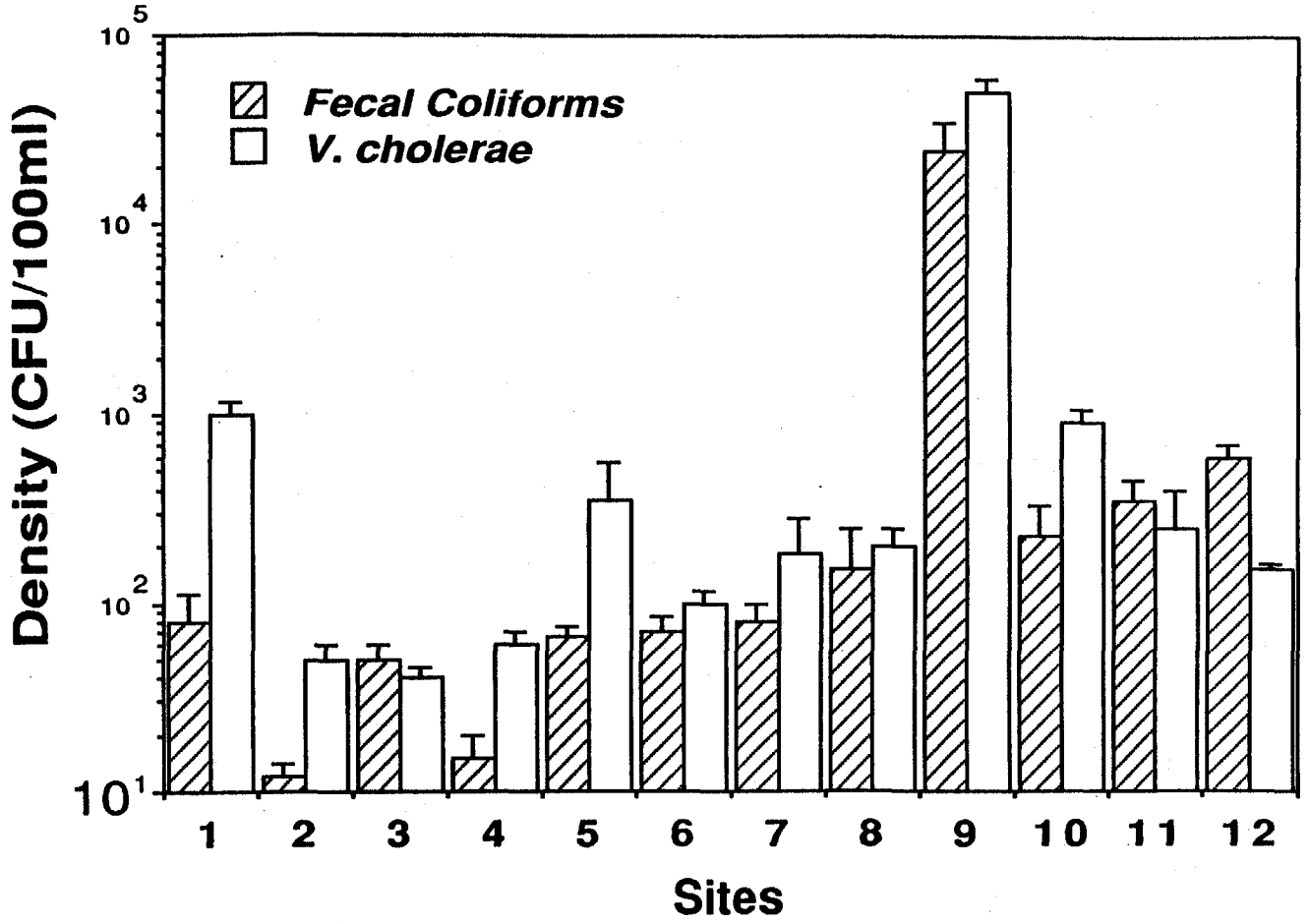
Figure 3. Changes in total density as measured by Coulter Counter for V. cholerae and E. coli for Study A (sites 4 and 5) and Study B (sites 1 and 4) (mean ± one standard error, n=8).

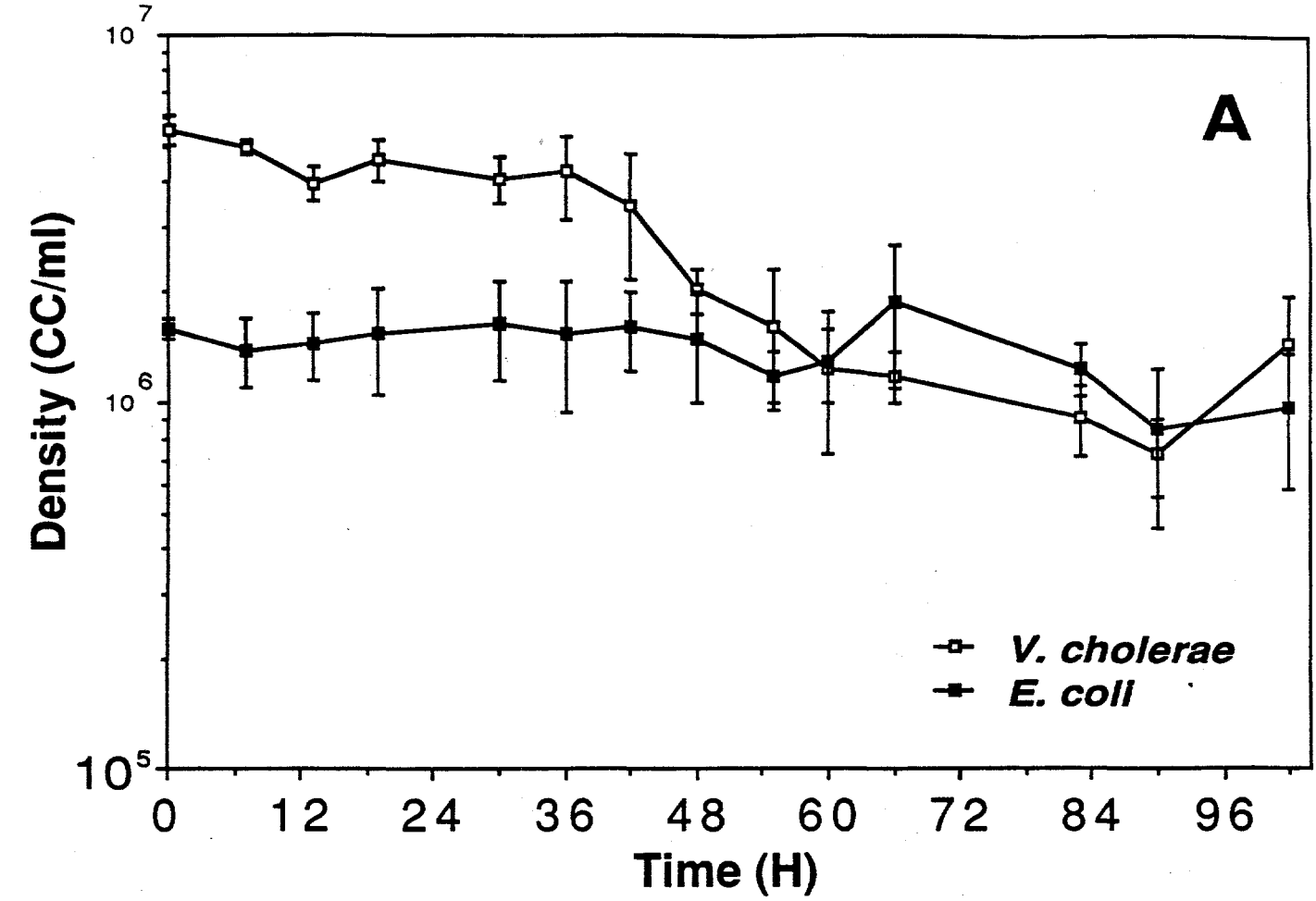
Figure 4. Changes in total density as measured by AODC for V. cholerae and E. coli for Study A (sites 4 and 5) and Study B (sites 1 and 4) (mean ± one standard error, n=8).

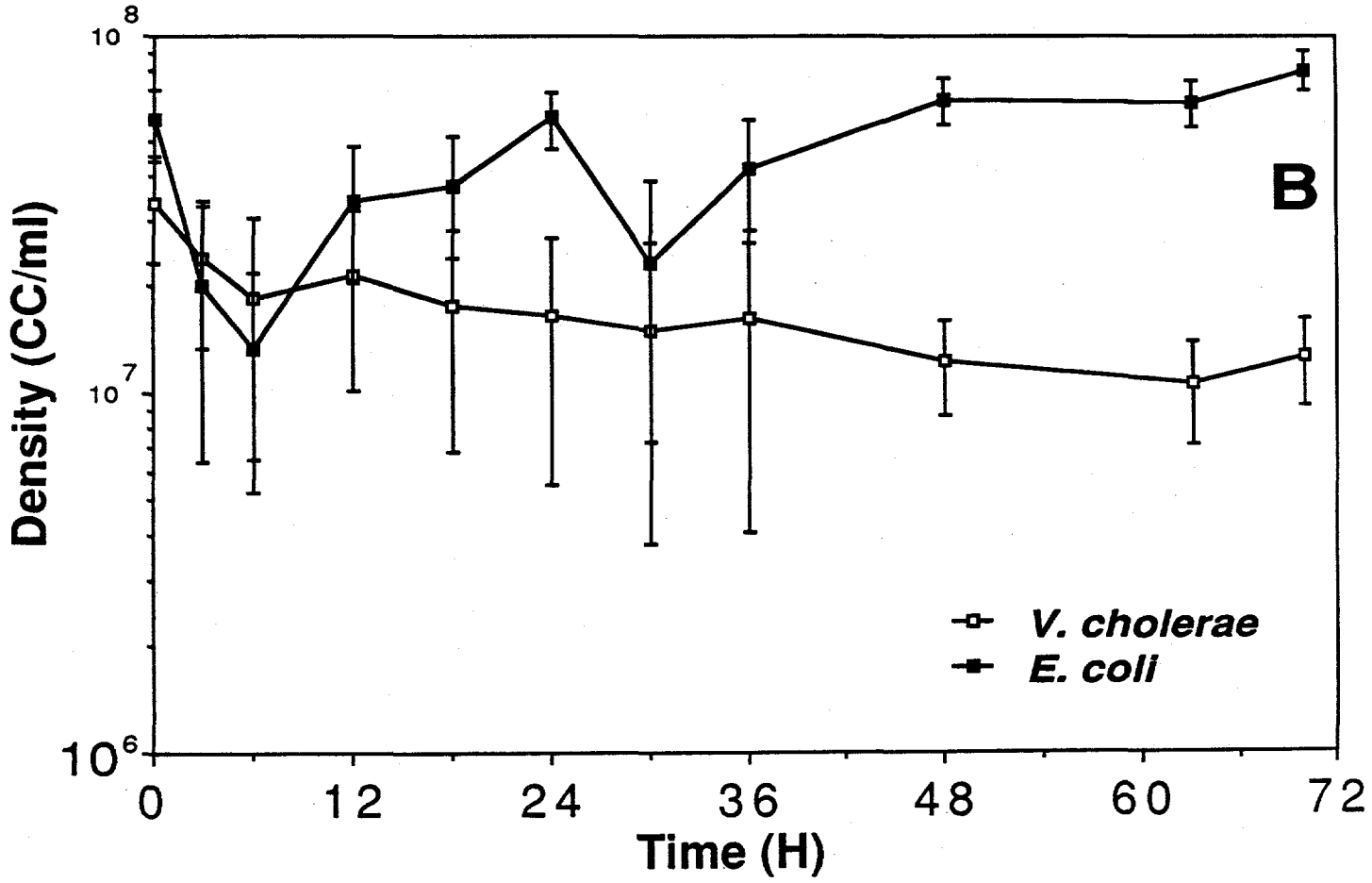
Figure 5. Changes in percent activity as measured by AODC for V. cholerae and E. coli for Study A (sites 4 and 5) (mean ± one standard error, n=8).

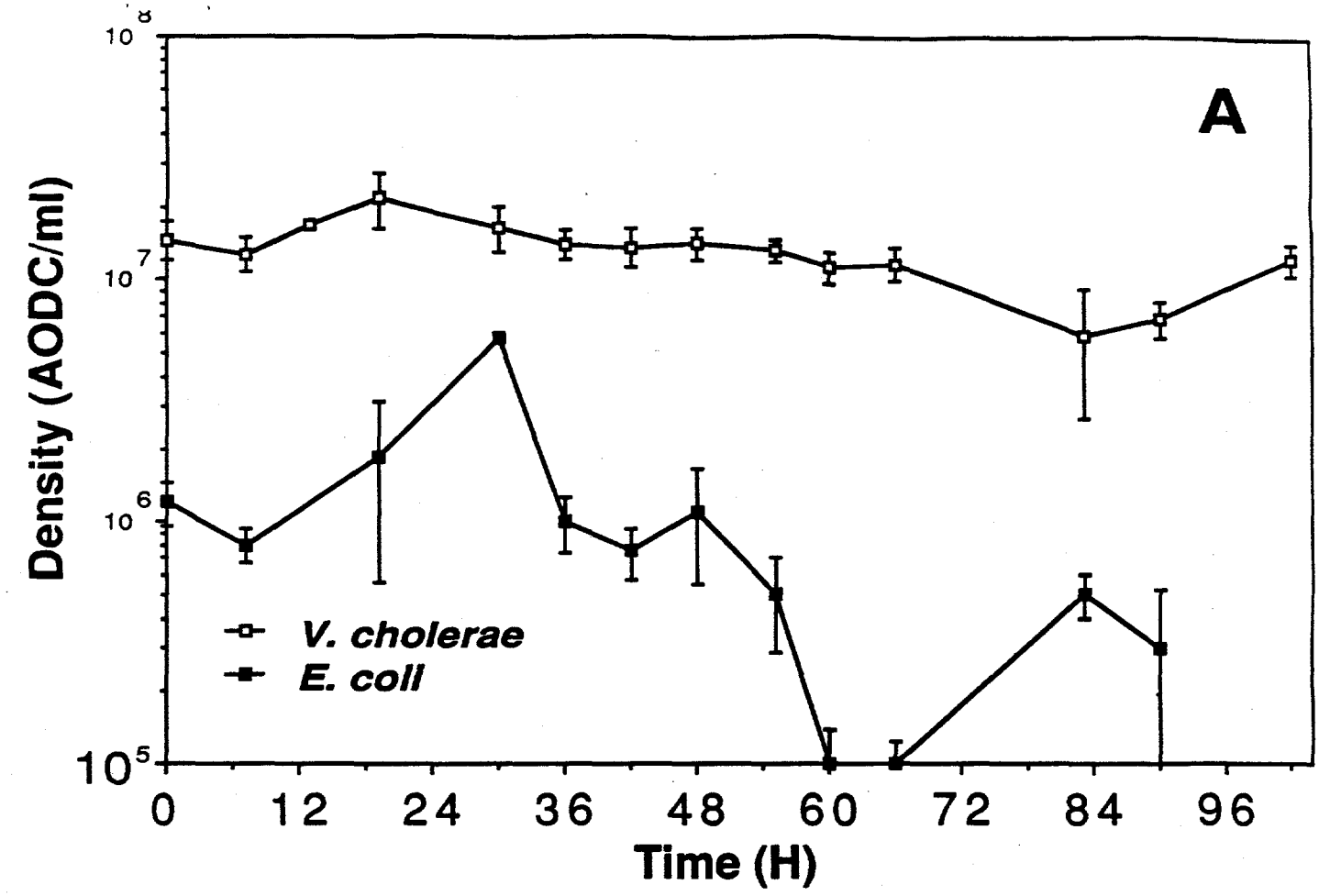
Figure 6. Changes in percent INT-positive cells as measured by INT-reduction for V. cholerae and E. coli for Study A (sites 4 and 5) (mean ± one standard error, n=8).

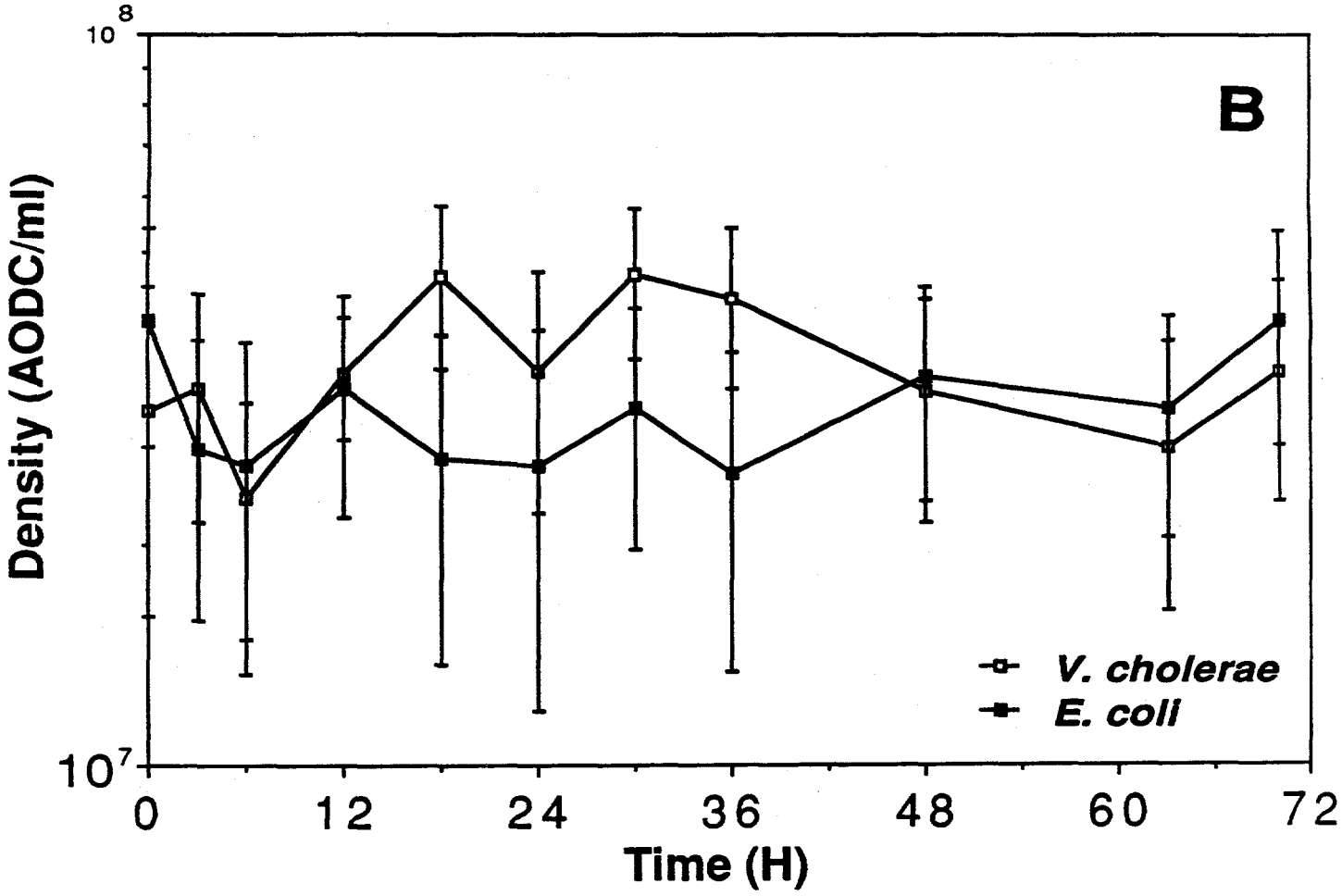

Mameyes River
Watershed
Atlantic Ocean
12
Luquillo
Beach
11
10
9
8
7
6
5
4
3
2
1
N
1km

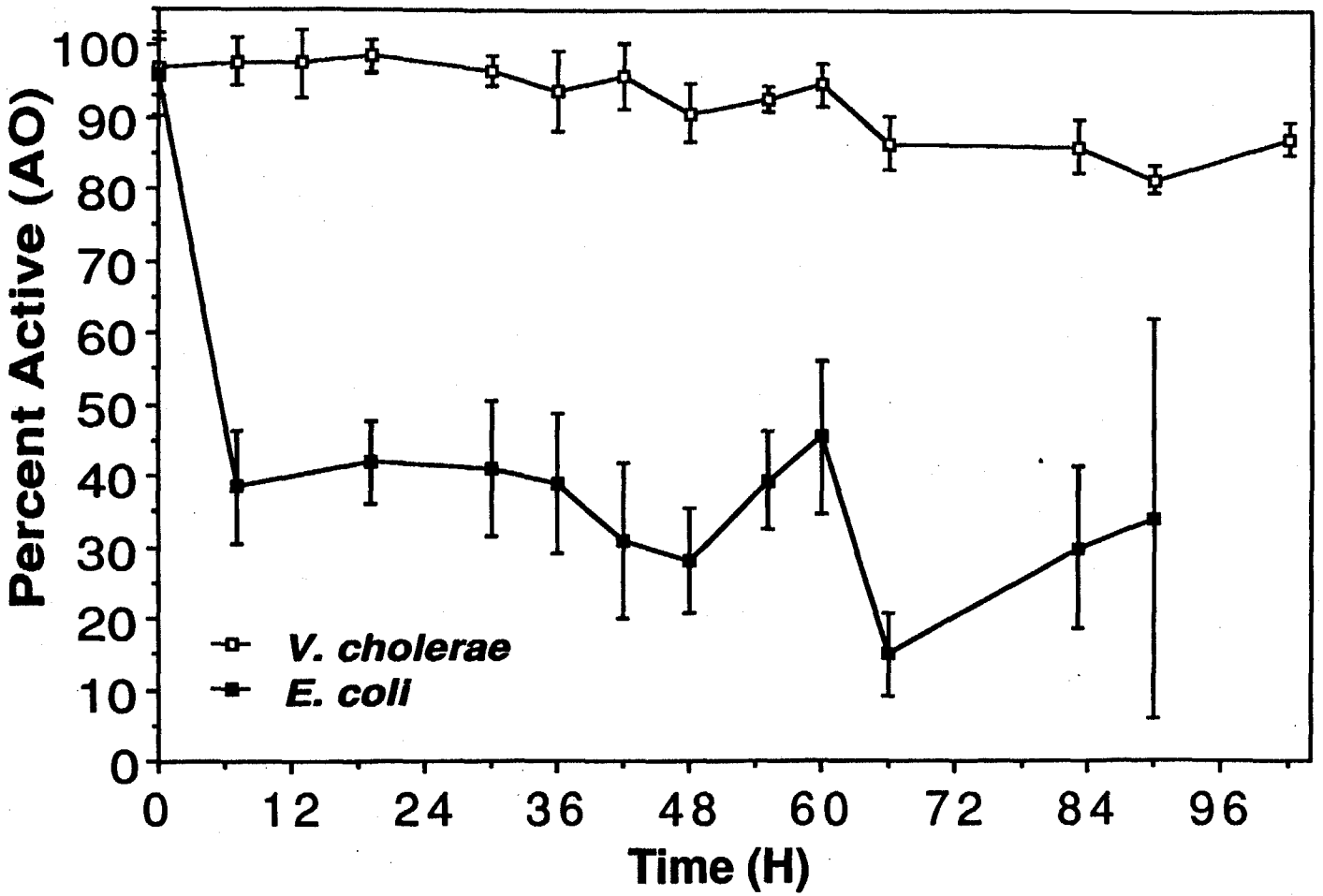

Fecal Coliforms
V. cholerae
Density (CFU/100ml)
10^5
10^4
10^3
10^2
10^1
1
2
3
4
5
6
7
8
9
10
11
12
Sites


A
Density (CC/ml)
10^7
10^6
10^5
0
12
24
36
48
60
72
84
96
Time (H)
V. cholerae
E. coli


B
Density (CC/ml)
10^8
10^7
10^6
0
12
24
36
48
60
72
Time (H)
V. cholerae
E. coli


A
Density (AODC/ml)
10^8
10^7
10^6
10^5
V. cholerae
E. coli
0
12
24
36
48
60
72
84
96
Time (H)


B
Density (AODC/ml)
10^8
10^7
V. cholerae
E. coli
0
12
24
36
48
60
72
Time (H)


Percent Active (AO)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
84
96
Time (H)
V. cholerae
E. coli

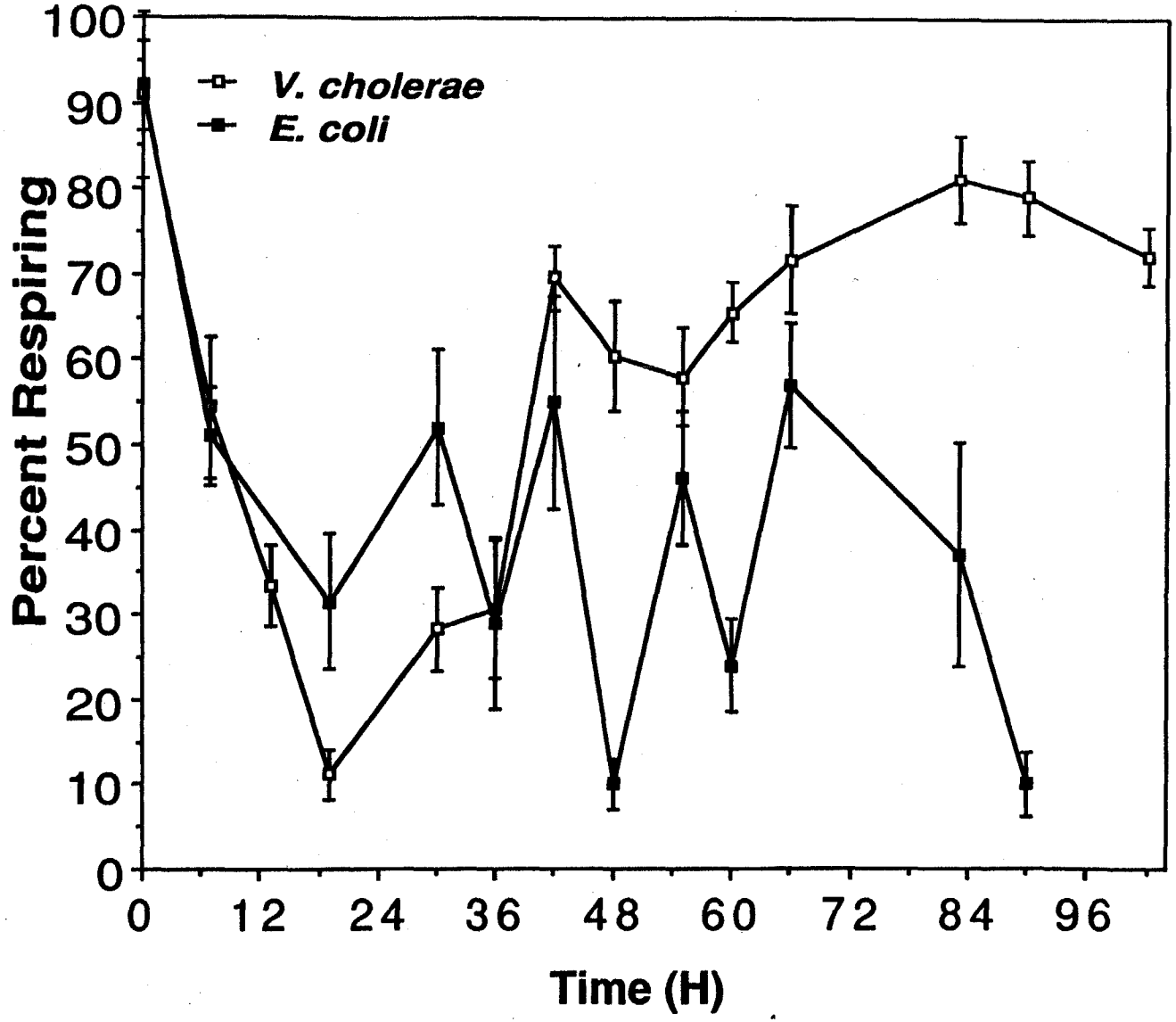

V. cholerae
E. coli
Percent Respiring
Time (H)
0
10
20
30
40
50
60
70
80
90
100
0
12
24
36
48
60
72
84
96

Table 1. Water quality of Río Mameyes, Puerto Rico

Sites	ATEMP	WTEMP	DO	pH	Sal	NO_{2+3}	PO_4	TP	ChlA
1	23 ± 1.0	21 ± 0.5	7.9 ± 0.2	6.2 ± 0.1	0	0.4 ± 0.1	2.4 ± 0.9	3.5 ± 1.2	148 ± 71
2	23 ± 1.0	20 ± 0.5	6.9 ± 1.1	6.3 ± 0.1	0	0.4 ± 0.4	0.9 ± 0.3	2.8 ± 1.8	22 ± 21
3	23 ± 0.9	20 ± 0.5	7.8 ± 0.5	6.5 ± 0.2	0	0.4 ± 0.4	1.47 ± 0.5	4.8 ± 2.5	9 ± 7
4	23 ± 0.9	22 ± 0.9	8.1 ± 0.2	6.8 ± 0.1	0	0.7 ± 0.2	1.0 ± 0.7	5.7 ± 2.8	47 ± 19
5	24 ± 0.9	22 ± 0.4	8.2 ± 0.2	7.2 ± 0.1	0	0.5 ± 0.1	1.4 ± 0.9	3.0 ± 1.2	77 ± 29
6	25 ± 1.0	21 ± 0.3	7.5 ± 0.7	7.0 ± 0.2	0	0.2 ± 0.1	2.3 ± 0.7	6.9 ± 6.0	18 ± 17
7	26 ± 1.0	23 ± 0.5	7.3 ± 1.2	7.0 ± 0.1	0	0.4 ± 0.3	6.7 ± 3.5	5.3 ± 3.8	30 ± 29
8	27 ± 1.0	24 ± 0.8	7.2 ± 0.6	7.1 ± 0.1	0	0.5 ± 0.4	3.0 ± 0.8	2.9 ± 0.6	63 ± 61
9	28 ± 1.4	25 ± 0.9	6.9 ± 0.5	7.0 ± 0.1	0	1.4 ± 0.7	6.8 ± 2.3	9.0 ± 2.6	106 ± 63
10	29 ± 2.0	25 ± 1.0	7.4 ± 1.0	7.1 ± 0.2	0	0.1 ± 0.0	4.8 ± 0.9	10 ± 1.4	44 ± 42
11	29 ± 2.0	26 ± 1.0	7.2 ± 1.0	7.2 ± 0.2	0	0.3 ± 0.2	6.8 ± 0.9	2.3 ± 1.0	63 ± 22
12	30 ± 2.0	25 ± 0.3	6.2 ± 1.0	7.4 ± 0.2	5.0 ± 3.0	0.2 ± 0.1	3.6 ± 1.9	8.6 ± 3.9	54 ± 53

*All values are mean ± one standard error (n = 6), ATEMP = air temperature (°C), WTEMP = water temperature (°C), DO = dissolved oxygen (mg/L), Sal = salinity (ppt), NO_{2+3} = nitrites plus nitrates (mg/L), PO_4 = orthophosphate (µg/L), TP = total phosphorus (µg/L), ChlA = chlorophyll *A* (mg/L).