ACKU

ACC# 249986

DP-MS-88-77

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

by

N. Pérez-Rosas
Microbial Ecology Laboratory
College of Natural Sciences
University of Puerto Rico
Rio Piedras, Puerto Rico 00931

and

T. C. Hazen
E. I. du Pont de Nemours & Co.
Savannah River Laboratory
Aiken, SC 29808

INFORMATION ONLY

SRL
RECORD COPYRECEIVED
MAY 26 1998
OSTI

This paper was prepared in connection with work done under Contract No. DE-AC09-76SR00001 with the U.S. Department of Energy. By acceptance of this paper, the publisher and/or recipient acknowledges the U.S. Government's right to retain a nonexclusive, royalty-free license in and to any copyright covering this paper, along with the right to reproduce and to authorize others to reproduce all or part of the copyrighted paper.

MASTER

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, make any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

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 its 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.

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

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

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

25 (This study was part of the M.S. thesis of N. Pérez-Rosas at the

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

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

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 km² 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

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

4 **Bacteriological analysis.** For V. cholerae enumeration water
5 samples were filtered through 0.45- μ m-pore-size membrane filters
6 (Millipore Corp., Bedford, Mass). Filters were then placed on
7 Thiosulfate-Citrate-Bile salts-Sucrose agar (TCBS; Difco Laboratories,
8 Detroit, Mich.) and incubated at 37°C for 24 h. All round, yellow
9 colonies were considered V. cholerae-like (1). Identifications of random
10 isolates were confirmed using the biochemical tests of the API 20E .
11 strips (Analytab Products, Plainview, N.Y.). Further corroboration of
12 V. cholerae identifications were done using polyvalent antiserum
13 against V. cholerae (Difco). Fecal coliform densities were determined by
14 filtering samples with type HC, 0.7- μ m-pore-size membrane filters
15 (Millipore). Filters were placed on mFC media (Difco) in tight-fitting
16 petri dishes (Millipore) and incubated at $44.5 \pm 0.1^\circ\text{C}$ in a block type FC
17 incubator (Millipore) for 24 h. All blue colonies were enumerated as
18 fecal coliform positive (1).

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

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

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

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

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

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

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 $21,960 \pm 2,748$ 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 $52,000 \pm 2,000$ 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,

1 densities of V. cholerae declined significantly from 48 to 90 h ($F = 47$, df
2 $= 17$ & 102 , $P < 0.001$); however, a significant increase was observed at
3 102 h (Fig. 3A). The overall decline in Coulter Counter densities during
4 study A was less than 75%. Densities of E. coli, as measured by AODC
5 were significantly different over time for study A ($F = 157$, $df = 17$
6 & 108 , $P < 0.0001$), reaching a high at 30 h and a low at 60 h (Fig. 4A).
7 Densities varied as much as 1.5 log units in less than 30 h. Survival of
8 E. coli was still 75% after 90 h. Densities of E. coli, as determined by
9 AODC, in study B did not change significantly over time (Fig. 4B).
10 Coulter Counter determined densities of E. coli did not change
11 significantly over time for either study; however, densities in study B
12 showed more variability than study A (Fig. 3). The calculated 90%
13 decrease time (T_{90}) or 1 log decrease time using AODC for V. cholerae,
14 for both studies was infinite. The calculated T_{90} for E. coli for study A
15 was 108 h; however, given the variability observed and that densities
16 of E. coli increased significantly twice during the study, 6 - 30 h and 66
17 - 84 h, this calculation probably underestimates the survival time of
18 E. coli in this environment. The calculated T_{90} for E. coli for study B
19 was infinite.

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

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

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

13

14

15

16

17

18

19

20

21

22

23

24

25

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

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

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

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

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

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

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

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

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

16 The activity of E. coli as measured by both AO fluorescence and
17 INT-reduction was significantly lower than V. cholerae, indicating a
18 more stressful environment for E. coli. The variability for both activity
19 measurements was also much greater for E. coli. However, even at
20 these lower measurements of activity for E. coli, the activities observed
21 were greater than those observed for natural populations of bacteria in
22 both warm (37) and cold freshwaters (43). Indeed, three other studies
23 at these same sites have also demonstrated that E. coli can survive and
24 remain active in this environment for extended periods of time (7, 23,
25 39).

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

17

18

19

20

21

22

23

24

25

ACKNOWLEDGMENTS

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

We are grateful for the technical assistance of Enid Elías, Francisco A. Fuentes, Carmen Ortiz-Roque, María Guerra, Emilia Medina, Carlos Aranda and Laura Valdés-Collazo. We are indebted to Ivette García-Castro for the use of her Coulter Counter. The U. S. Forest Service was also cooperative in this study.

This work was supported by Sea Grant R/LR-08-87-THA1 and in part by the Water Resources Research Institute of the University of Puerto Rico at Mayagüez and by Public Health Service grants RR-2657 and RR-8102 from the National Institutes of Health. In addition, portions of the information contained in this article were developed during the course of work under Contract No. DE-AC09-76SR00001 with the U. S. Department of Energy.

LITERATURE CITED

1. American Public Health Association. 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. Baker, R. M., F. L. Singleton, and M. A. Hood. 1983. Effects of nutrient deprivation on Vibrio cholerae. Appl. Environ. Microbiol. 46:930-940.
3. Biamón, E. J., and T. C. Hazen. 1983. The distribution and survival of Aeromonas hydrophila in tropical near-shore coastal waters receiving rum distillery effluents. Water Res. 17:319-326.
4. Bermúdez, M., and T. C. Hazen. 1988. Phenotypic and genotypic comparison of Escherichia coli from pristine tropical waters. Appl. Environ. Microbiol. 54:0000-0000.
5. Bonde, G. J. 1977. Bacterial indicators of water pollution. Adv. Aquat. Microbiol. 1:273-364.
6. Cabelli, V. J., A. P. Dufour, L. J. McCabe, and M. A. Levin. 1983. A marine recreational water quality criterion consistent with indicator concepts and risk analysis. J. Water Pollut. Control Fed. 55:1306-1314.
7. Carrillo, M., E. Estrada, and T. C. Hazen. 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.
8. Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L.

- 1 M. Palmer. 1985. Viable but non-culturable Vibrio cholerae and
2 related pathogens in the environment: implications for release of
3 genetically engineered microorganisms. Bio/Technology 3:817-820.
- 4 9. Colwell, R. R., P. A. West, D. Maneval, E. F. Remmers, E. L. Elliot, and N.
5 E. Carlson. 1984. Ecology of pathogenic vibrios in Chesapeake Bay, p.
6 367-387. In: R. R. Colwell (ed.), Vibrios in the Environment. John
7 Wiley & Sons, New York.
- 8 10. Daley, R. J. 1979. Direct epifluorescence enumeration of native
9 aquatic bacteria: uses, limitations, and comparative accuracy, p. 29-
10 45. In: T. W. Costerton and R. R. Colwell (ed.), Native Aquatic
11 Bacteria: Enumeration, Activity, and Ecology. American Society for
12 Testing and Materials, Philadelphia.
- 13 11. Evison, L. M., and A. James. 1973. A comparison of the distribution
14 of intestinal bacteria in British and East African water sources. J.
15 Appl. Bacteriol. 36:109-118.
- 16 12. Federal Register. 1983. Fed. Regist. 48:45502-45521.
- 17 13. Fuentes, F. A., E. J. Biamón, and T. C. Hazen. 1983. Bacterial
18 chemotaxis to effluent from a rum distillery in tropical near-shore
19 coastal waters. Appl. Environ. Microbiol. 46:1438-1441.
- 20 14. Grimes, D. J., F. L. Singleton, J. Stemmler, L. M. Palmer, P. Brayton,
21 and R. R. Colwell. 1984. Microbiological effects of wastewater
22 effluent discharge into coastal waters of Puerto Rico. Water Res.
23 18:613-619.
- 24 15. Hazen, T. C., and G. W. Esch. 1983. The effect of nitrogen fertilizer
25 factory and pulp mill effluent on the distribution and abundance of

- 1 Aeromonas hydrophila in Albemarle Sound, North Carolina. Appl.
2 Environ. Microbiol. 54:31-42.
- 3 16. Hazen, T. C., J. Santiago-Mercado, G. A. Toranzos, and M. Bermúdez.
4 1987. What do water fecal coliforms indicate in Puerto Rico ? A
5 review. Bul. PR Med. Assoc. 79:189-193.
- 6 17. Hood, M. A., J. B. Guckert, D. C. White, and F. Deck. 1986. Effect of
7 nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein
8 levels in Vibrio cholerae. Appl. Environ. Microbiol. 52:788-793.
- 9 18. Hood, M. A., and G. E. Ness. 1982. Survival of Vibrio cholerae and
10 Escherichia coli in estuarine waters and sediments. Appl. Environ.
11 Microbiol. 43:578-584.
- 12 19. Hood, M. A., G. E. Ness, G. E. Rodrick, and N. J. Blake. 1984. The
13 ecology of Vibrio cholerae in two Florida estuaries, p. 399-410. In:
14 R. R. Colwell (ed.), Vibrios in the Environment. John Wiley & Sons,
15 New York.
- 16 20. Hutchinson, G. E. 1975. A Treatise on Limnology. Vol. 1, Part 2.
17 John Wiley & Sons. New York.
- 18 21. Kaper J., H. Lockman, R. R. Colwell, and S. W. Joseph. 1979 Ecology,
19 serology, and enterotoxin production of Vibrio cholerae in
20 Chesapeake Bay. Appl. Environ. Microbiol. 37:91-103.
- 21 22. Lee, J. V., D. J. Bashford, T. J. Donovan, A. L. Furniss, and P. A. West.
22 1984. The incidence and distribution of Vibrio cholerae in England,
23 p. 427-450. In: R. R. Colwell (ed.), Vibrios in the Environment. John
24 Wiley & Sons, New York.
- 25 23. López-Torres, A. J., T. C. Hazen, and G. A. Toranzos. 1987.

- 1 Distribution and in situ survival and activity of Klebsiella
2 pneumoniae in a tropical rain forest watershed. Curr. Microbiol.
3 15:213-218.
- 4 24. López, A. J., L. Prieto, and T. C. Hazen. 1988. Comparison of the in
5 situ survival and activity of Klebsiella pneumoniae and Escherichia
6 coli in tropical marine environments. Microb. Ecol. 15:41-57.
- 7 25. McFeters, G. A., A. K. Bissonnette, J. J. Jezeski, C. A. Thomson, and D.
8 G. Stuart. 1974. Comparative survival of indicator bacteria and
9 enteric pathogens in well water. Appl. Microbiol. 27:823-829.
- 10 26. McFeters, G. A., and D. G. Stuart. 1972. Survival of coliform
11 bacteria in natural waters: field and laboratory studies with
12 membrane filter chambers. Appl. Microbiol. 24:805-811.
- 13 27. Nair, G. B., B. L. Sarkar, S. P. De, M. K. Chakrabarti, R. K. Bhadra, and
14 S. C. Pal. 1988. Ecology of Vibrio cholerae in the freshwater
15 environs of Calcutta, India. Microb. Ecol. 15:203-215.
- 16 28. Odum, H. T. 1970. Rain forest structure and mineral-cycling
17 homeostasis, p. H3-52. In H. T. Odum and R. F. Pigeon (ed.). A
18 tropical rain forest, a study of irradiation and ecology at El Verde,
19 Puerto Rico. U. S. Atomic Energy Commission. National Technical
20 Information Service, Springfield, Va.
- 21 29. Ortiz-Roque, C., and T. C. Hazen. 1987. Abundance and distribution
22 of Legionellaceae in Puerto Rican waters. Appl. Environ. Microbiol.
23 53:2231-2236.
- 24 30. Pérez-Rosas, N., and T. C. Hazen. 1988. In situ survival of Vibrio
25 cholerae and Escherichia coli in tropical coral reefs. Appl. Environ.

- 1 Microbiol. 54:1-9.
- 2 31. Rhodes, J. B., H. L. Smith jr, and J. E. Ogg. 1986. Isolation of non-O1
3 Vibrio cholerae serovars from surface waters in western Colorado.
4 Appl. Environ. Microbiol. 51:1216-1219.
- 5 32. Rivera, S., T. C. Hazen, and G. A. Toranzos. 1988. Isolation of fecal
6 coliforms from pristine sites in a tropical rain forest. Appl. Environ.
7 Microbiol. 54:513-517.
- 8 33. Roberts, N. C., H. B. Bradford jr, and J. R. Barbay. 1984. Ecology of
9 Vibrio cholerae in Louisiana coastal areas, p. 389-398. In: R. R.
10 Colwell (ed.), Vibrios in the Environment. John Wiley & Sons, New
11 York.
- 12 34. Roszak, D. B., and R. R. Colwell. 1987. Survival strategies of bacteria
13 in the natural environment. Microbiol. Rev. 51:365-379.
- 14 35. Santiago-Mercado, J. and T. C. Hazen. 1987. Comparison of four
15 membrane filtration and MPN methods for fecal coliform
16 enumeration in tropical waters. Appl. Environ. Microbiol. 53:2922-
17 2928.
- 18 36. Seidler, R. J., and T. M. Evans. 1984. Computer-assisted analysis of
19 Vibrio field data: four coastal areas, p. 411-426. In: R. R. Colwell
20 (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New
21 York.
- 22 37. Tabor, P., and J. Neihof. 1984. Direct determination of activities for
23 microorganisms of Chesapeake Bay populations. Appl. Environ.
24 Microbiol. 48:1012-1019.
- 25 38. Tamplin, M. L., G. E. Rodrick, N. J. Blake, D. A. P. Bundy, and L.

- 1 Alexander. 1983. Public health aspects of halophilic vibrios in
2 Jamaica. West Indian Med. J. 32:147-151.
- 3 39. Valdés-Collazo, L., A. J. Schultz, and T. C. Hazen. 1987. Survival of
4 Candida albicans in tropical marine and freshwaters. Appl. Environ.
5 Microbiol. 53:1762-1767.
- 6 40. West, P. A., and J. V. Lee. 1984. Distribution and growth of Vibrio
7 cholerae in a natural brackish water system, p. 451-461. In: R. R.
8 Colwell (ed.), Vibrios in the Environment. John Wiley & Sons, Inc.,
9 New York.
- 10 41. Xu, H-S., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R.
11 R. Colwell. 1982. Survival and viability of nonculturable
12 Escherichia coli and Vibrio cholerae in the estuarine and marine
13 environment. Microb. Ecol. 8:313-323.
- 14 42. Zar, J. H. 1984. Biostatistical analysis. Prentice-Hall, Inc.,
15 Englewood Cliffs, N.J.
- 16 43. Zimmermann, R., R. Iturriaga, and J. Becker-Birck. 1978.
17 Simultaneous determination of the total number of aquatic bacteria
18 and the number thereof involved in respiration. Appl. Environ.
19 Microbiol. 36:926-934.
- 20
21
22
23
24
25

Figure Legends

- 1
2
3 Figure 1. Location of study sites in Mameyes River watershed, Puerto
4 Rico.
- 5 Figure 2. Densities of V. cholerae and fecal coliforms by site in the
6 Mameyes River watershed.
- 7 Figure 3. Changes in total density as measured by Coulter Counter for
8 V. cholerae and E. coli for Study A (sites 4 and 5) and Study B
9 (sites 1 and 4) (mean $\pm$ one standard error, n=8).
- 10 Figure 4. Changes in total density as measured by AODC for V. cholerae
11 and E. coli for Study A (sites 4 and 5) and Study B (sites 1
12 and 4) (mean $\pm$ one standard error, n=8).
- 13 Figure 5. Changes in percent activity as measured by AODC for
14 V. cholerae and E. coli for Study A (sites 4 and 5) (mean $\pm$
15 one standard error, n=8).
- 16 Figure 6. Changes in percent INT-positive cells as measured by INT-
17 reduction for V. cholerae and E. coli for Study A (sites 4 and
18 5) (mean $\pm$ one standard error, n=8).
- 19
20
21
22
23
24
25

Mameyes River Watershed

A **B**

Table 1. Water quality of Río Mameyes, Puerto Rico

Sites	ATEMP	WTEMP	DO	pH	Sal	NO ₂₊₃	PO ₄	TP	ChIA
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), NO₂₊₃ = nitrites plus nitrates (mg/L), PO₄ = orthophosphate (µg/L), TP = total phosphorus (µg/L), ChIA = chlorophyll A (mg/L).