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In Situ Survival of *Vibrio cholerae* and *Escherichia coli* in Tropical Coral Reefs

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***Vibrio cholerae* and *Escherichia coli* were inoculated into membrane diffusion chambers and placed around two small coral reef islands in Puerto Rico and monitored for 5 days. Several chambers were also buried in the sands of one of the reefs. Both *E. coli* and *V. cholerae* densities declined by 2 orders of magnitude, as measured by direct particle counts with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). However, the density of neither bacteria changed dramatically when the same samples were analyzed by epifluorescent direct counts. Differences in the two direct count methods were accounted for by changes in cell morphology that occurred in both bacteria after exposure to seawater. Morphological changes occurred more rapidly in *E. coli* compared with those in *V. cholerae*. Bacteria in chambers exposed to sediment did not show significant changes in morphology and had only a slight decline in density. Physiological activity declined by more than 40% for both bacteria within 24 h. The decline in activity was less severe in the sediments. Tropical coral reef sands and turtle grass beds were shown to be less stressful environments for *V. cholerae* and *E. coli* than would have been predicted from temperate and microcosm studies. *V. cholerae* can survive the in situ conditions of a tropical coral reef and could become a source of bacterial contamination for fish and shellfish in this environment. The simultaneous monitoring of *E. coli* levels established that this bacteria can not be used as an indicator of *V. cholerae* or other fecal-borne pathogens in coral reef environments because of the greater stress these environments put on *E. coli*. Both bacteria could be of greater public health importance in tropical marine areas than previously imagined.**

It is known that *Vibrio cholerae* and other vibrios are part of the indigenous microflora of most estuaries (8, 18). Considering the reports of recent outbreaks of cholera traced to shellfish from warm water environments, it has become increasingly important to understand the ecology of these bacteria. Many studies have suggested that *V. cholerae* is incapable of surviving in seawater at salinities of 35‰ and that high temperatures, i.e., >25°C, are stressful (17, 22). However, recent studies have suggested that *V. cholerae* and *Escherichia coli*, its indicator, may be able to survive under these conditions or under starvation conditions and be unculturable on standard media (3, 8, 26). It has also been well documented that the survival of *E. coli* can be dramatically decreased by high solar radiation (11, 20). Since all of these conditions are normal and constant in tropical marine areas like Puerto Rico, it was surprising to us that our laboratory (4, 10, 15, 16, 24) and others (12, 13, 22) were able to isolate *V. cholerae* and *E. coli* from tropical coastal waters. Other work by our group had shown that *Aeromonas hydrophila* was capable of growing at densities above 10^7 cells per ml when diffusion chambers with the bacteria were suspended in marine coastal areas of Puerto Rico contaminated with rum distillery effluent (4). We also observed that rum distillery effluent served as a chemoattractant for *V. cholerae* (10). Thus, we undertook the present study to determine *V. cholerae* and *E. coli* survival by using in situ diffusion chambers at several marine sites and by employing two direct count methods and two population activity measurements. Previous studies had used laboratory microcosms and had not measured physiological activity (18). The present study was undertaken to establish whether *V. cholerae* could survive the in situ conditions of a tropical coral reef and thus whether this environment could become a reservoir source for fish and shellfish in coral reefs. The simultaneous monitoring of *E. coli* established whether this bacterium could be used as an indicator of *V. cholerae* in the coral reef environment.

(This study was part of the M.S. thesis of N. Pérez-Rosas at the University of Puerto Rico, Rio Piedras, Puerto Rico, 1983.)

MATERIALS AND METHODS

Study sites. Palominitos is a coral sand island off the northeastern shore of Puerto Rico, 18° 20′ N, 65° 25′ W (Fig. 1). It has a well-developed coral reef dominated by *Acropora palmata* and *Montastrea annularis* along the east side, with turtle grass beds (*Thalassia testudinum*) dominating the west side. This island is frequented for recreation on weekends. La Gata Island is located on the inner shelf of the southwest coast of Puerto Rico at 17° 57′ N and 67° 02′ W near the small fishing village of Parguera (Fig. 1). The island was created by a patch reef, with subsequent colonization by the red mangrove *Rhizophora mangle*. The south part of the island is dominated by a coral reef dominated by *M. annularis* and *A. palmata* (1). The north side is dominated by extensive beds of turtle grass. During 1980, La Gata was inaugurated as a recreational area by the Department of Natural Resources. During 1981, a total of 85,909 persons visited the island for

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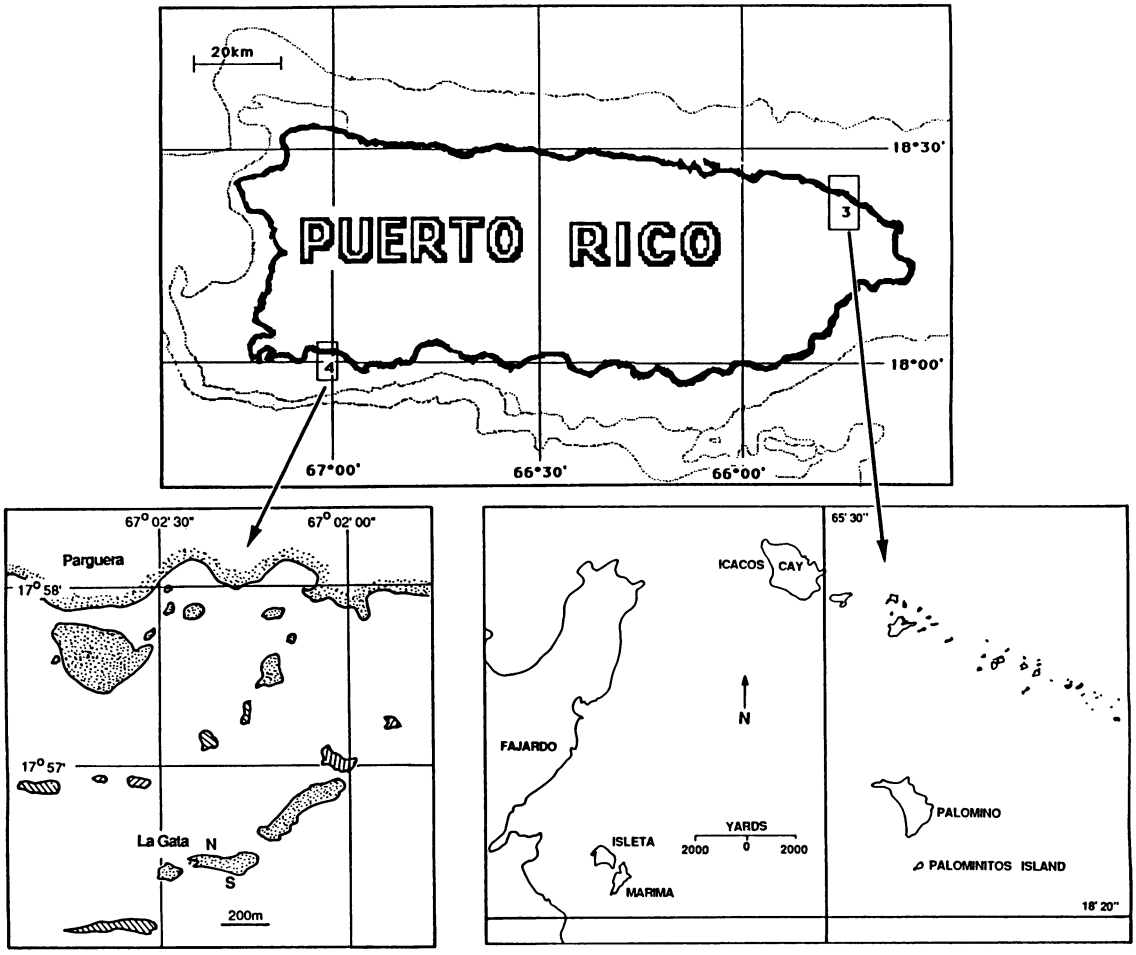

FIG. 1. Map of study sites at La Gata and Palominitos Islands, Puerto Rico.

an average of 7,160 persons monthly and 239 persons daily (Department of Natural Resources, personal communication). The installation of two toilets created a raw sewage outfall on the island. Dye studies by our laboratory showed that 20 min after a dye cone was flushed dye could be detected in the surrounding waters. After 1 h, the dye had spread to 50% of the water along the north coast of the island, and within 2 h it covered the water along the entire north coast, including the area reserved for swimming.

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 (2). 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 the procedures in *Standard Methods for Water and Wastewater Analysis* (2).

Bacteriological analysis. Direct cell counts for *V. cholerae* and *E. coli* in diffusion chambers were done by 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 (9). 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, 19). Total number of bacteria and the number involved in respiration were determined by the technique of Zimmerman et al. (28).

Survival studies. For survival studies, Plexiglas (Rohm & Haas Co., Philadelphia, Pa.) diffusion chambers, a modification of those of McFeters and Stuart (21), with 100-ml capacities were used with 0.45-μm-pore-size, nylon-reinforced Versapor membrane filters (Gelman Instrument Co., Ann Arbor, Mich.) as diffusion surfaces (4, 14). O-rings were added to the chambers to reduce leakage and contamination. Pure cultures of *V. cholerae* and *E. coli* were grown in 5% tryptic soy broth at 37°C for 24 h. The cells were then harvested by centrifugation (10,000 × *g* for 10 min) and

TABLE 1. Water quality of La Gata Island and Palominitos Island, Puerto Rico[a]

Site[b]	Temp (°C)		Dissolved oxygen (mg/liter)	pH	Salinity (‰)	Nitrites plus nitrates (mg/liter)	Ortho-phosphate (μg/liter)	Total phosphorus (μg/liter)	Chlorophyll *a* (mg/liter)
	Air	Water							
LG-N	24.3 ± 0.6	26.2 ± 0.2	7.1 ± 0.3	7.2 ± 0.2	34.2 ± 0.5	0.2 ± 0.0	0.7 ± 0.5	0.9 ± 0.7	0.63 ± 0.60
LG-S	24.9 ± 0.4	26.1 ± 0.2	7.5 ± 0.2	7.7 ± 0.1	33.6 ± 0.5	0.3 ± 0.0	0.7 ± 0.1	0.8 ± 0.8	0.80 ± 0.04
PI-T	21.4 ± 0.5	20.5 ± 0.5	8.2 ± 0.1	7.1 ± 0.1	34.0 ± 0.0	0.1 ± 0.0	<0.1	0.1 ± 0.0	0.19 ± 0.03
PI-C	21.4 ± 0.5	19.8 ± 0.6	7.9 ± 0.1	7.4 ± 0.2	34.0 ± 0.0	0.1 ± 0.0	<0.1	0.1 ± 0.0	0.27 ± 0.08

[a] All values are mean ± 1 standard error (*n*, >5).
[b] LG-N, La Gata north; LG-S, La Gata south; PI-T, Palominitos Island, *Thallassia* site; PI-C, Palominitos Island, coral.

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 the study site, a total of five chambers were placed strategically at a depth of 1 m. 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 (14). The other 0.5 ml was incubated with INT and fixed by the method of Zimmerman et al. (28). 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. Five chambers were also carefully buried in the sandy sediment near the chambers suspended in the water column. To avoid unnecessary disturbance, two chambers were

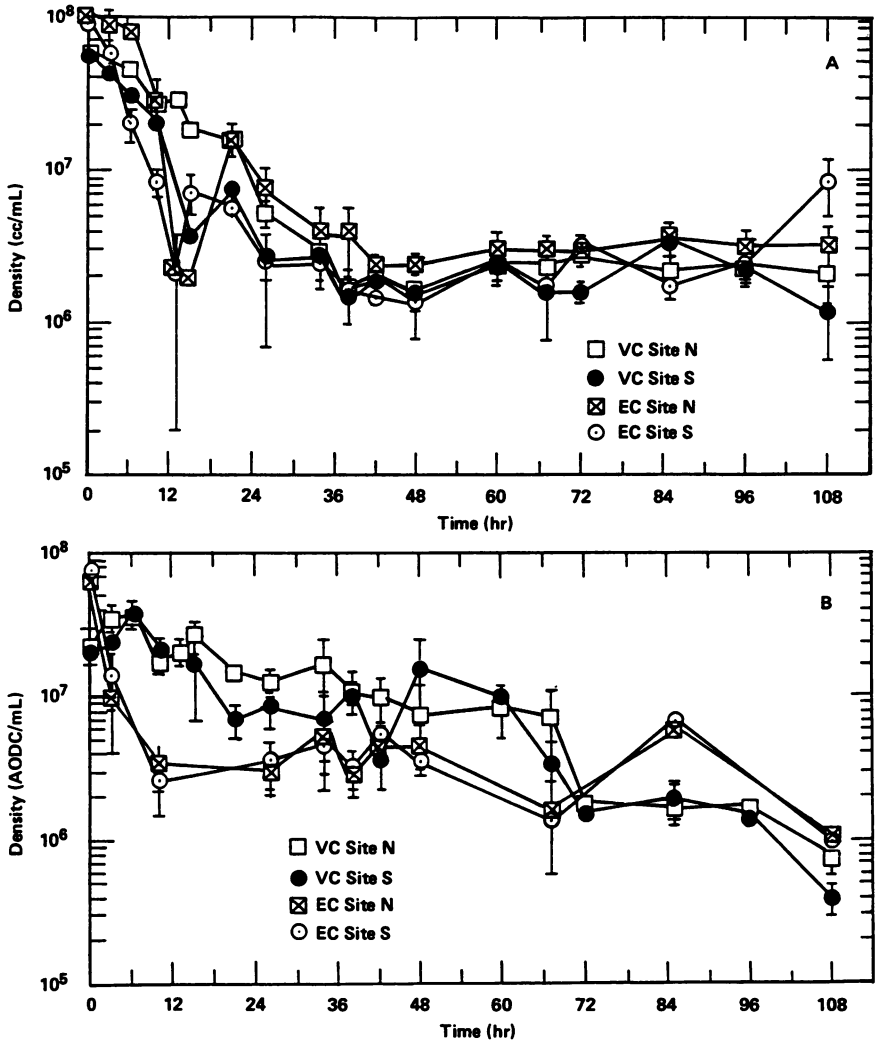

FIG. 2. Changes in total density for *V. cholerae* (VC) and *E. coli* (EC), as measured by Coulter Counter (A) and AODC (B), at La Gata Island by site (mean ± 1 standard error, $n = 4$). Sites are defined in Table 1, footnote *b*.

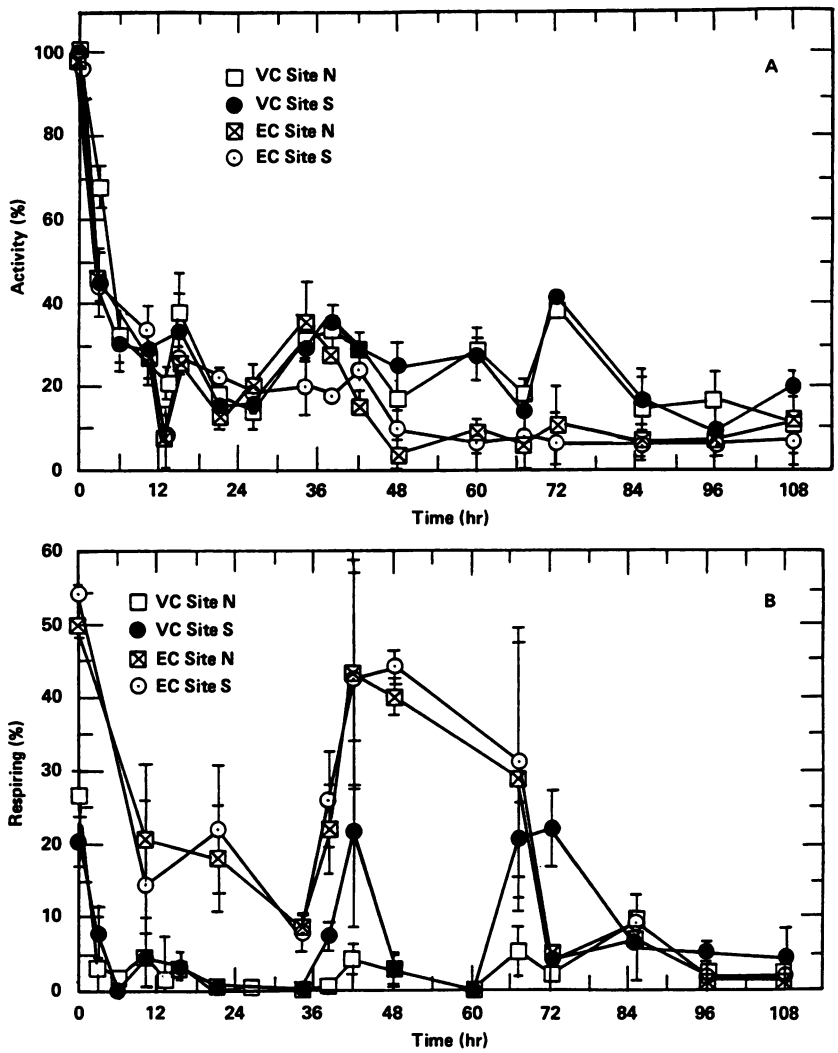

FIG. 3. Changes in percent activity for *V. cholerae* (VC) and *E. coli* (EC), as measured by AODC (A), and in percent respiration, as measured by INT (B), at La Gata Island by site (mean ± 1 standard error, $n = 4$). Sites are defined in Table 1, footnote *b*.

removed and sampled at 24 h, and the remaining chambers were removed and sampled at 42 h. All sampling and measurements were 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 (27). Any probability less than or equal to 0.05 was considered significant.

RESULTS

Water quality. The two sites at La Gata Island were not significantly different in water quality (Table 1). All parameters measured were stable during the 108-h sampling period. The same observations were made at the two sites at Palominitos Island (Table 1).

Survival of bacteria in situ. At La Gata Island, the densities of *V. cholerae* as determined by Coulter Counter counts (CC counts) in the diffusion chambers decreased significantly over time ($F = 47.0$, df = 17 and 30, $P = 0.001$), decreasing by 80% during the first 12 h, and were significantly higher at the north site ($F = 9.91$, df = 1 and 30, $P = 0.001$) (Fig. 2), i.e., more than 50% higher after 12 h. Densities of *V. cholerae*, as determined by AODC, were also significantly different over time ($F = 22.0$, df = 17 and 30, $P < 0.0001$) but not by site (Fig. 2). However, densities of *V. cholerae*, as measured by AODC, decreased by only 10% after 12 h. The proportion of the *V. cholerae* population that was active, as measured by AODC, declined significantly during the first 9 h ($F = 51.0$, df = 17 and 30, $P < 0.0001$) and then remained low; the north site had a significantly higher proportion of active cells ($F = 5.63$, df = 17 and 30, $P < 0.05$) (Fig. 3). The proportion of the *V. cholerae* population that was respiring, as determined by INT, also declined significantly during the first 9 h ($F = 2.17$, df = 17 and 30, $P < 0.05$) but was not significantly different by site (Fig. 3). The initial decline (first 3 h) in activity and respiration was greater than 70%, after this both measurements fluctuated between 1 to 30% without apparent pattern.

Densities of *E. coli* in the diffusion chambers at La Gata

Island, as determined by CC counts, declined significantly over time (F = 15.0, df = 17 and 34, $P < 0.0001$) (Fig. 2), with less than 1% remaining after only 12 h. The same was also true when densities of *E. coli* were determined by AODC, i.e., over time differences were significant (F = 157, df = 10 and 24, $P < 0.0001$) (Fig. 2). The activity of the *E. coli* population declined by 70% in the first 3 h and then fluctuated between 5 to 40%, with a large variability among samples (Fig. 3). The percentage of the *E. coli* population that was respiring changed significantly over time (F = 126, df = 10 and 24, $P < 0.005$) (Fig. 3). Respiration rate of the *E. coli* cells declined significantly only during the first 3 h, i.e., more than 30%. During subsequent samplings, the variability in these measurements was even greater for *E. coli* than it was for *V. cholerae*. In comparison, *V. cholerae* had a similar survival rate as did *E. coli* at La Gata Island. However, unlike *E. coli*, *V. cholerae* had a significantly higher survival rate and activity at the north site.

At Palominitos Island, the densities of *V. cholerae*, as determined by CC counts, in the diffusion chambers decreased significantly over time (F = 5.16, df = 6 and 18, P = 0.001); there were no differences by site (Fig. 4). After 12 h, densities of *V. cholerae* in the water had declined 80%. Chambers buried in the sediments at these sites showed no significant changes over time (Fig. 5). Densities of *V. cholerae* in the water, as determined by AODC, showed a significant increase, >20% after 42 h (F = 3.50, df = 6 and 18, $P < 0.05$); however, chambers buried in the sediments showed a slight, though insignificant, reduction (Fig. 4 and 5). The proportion of the *V. cholerae* population that was active, as measured by AODC, declined significantly during the first 18 h (F = 3.62, df = 6 and 18, $P < 0.05$) but then increased briefly to 80% (Fig. 6). Activity in the chambers that were buried in the sediments decreased slowly by only 30% during the entire period (Fig.7). The proportion of the *V. cholerae* population that was respiring, as determined by INT, did not decline significantly at the *Thalassia* site but declined by more than 40% at the other site (Fig. 6).

Densities of *E. coli* in the diffusion chambers at Palominitos Island, as determined by CC counts, remained unchanged both in the water and the sediments (Fig. 4). However, when densities of *E. coli* were determined by AODC a 20% increase was observed during the first 24 h, followed by a 90% decrease. In the sediments, the AODC densities of *E. coli* decreased by only 50% after 42 h (Fig. 5). The activity of the *E. coli* population declined by 80% in the

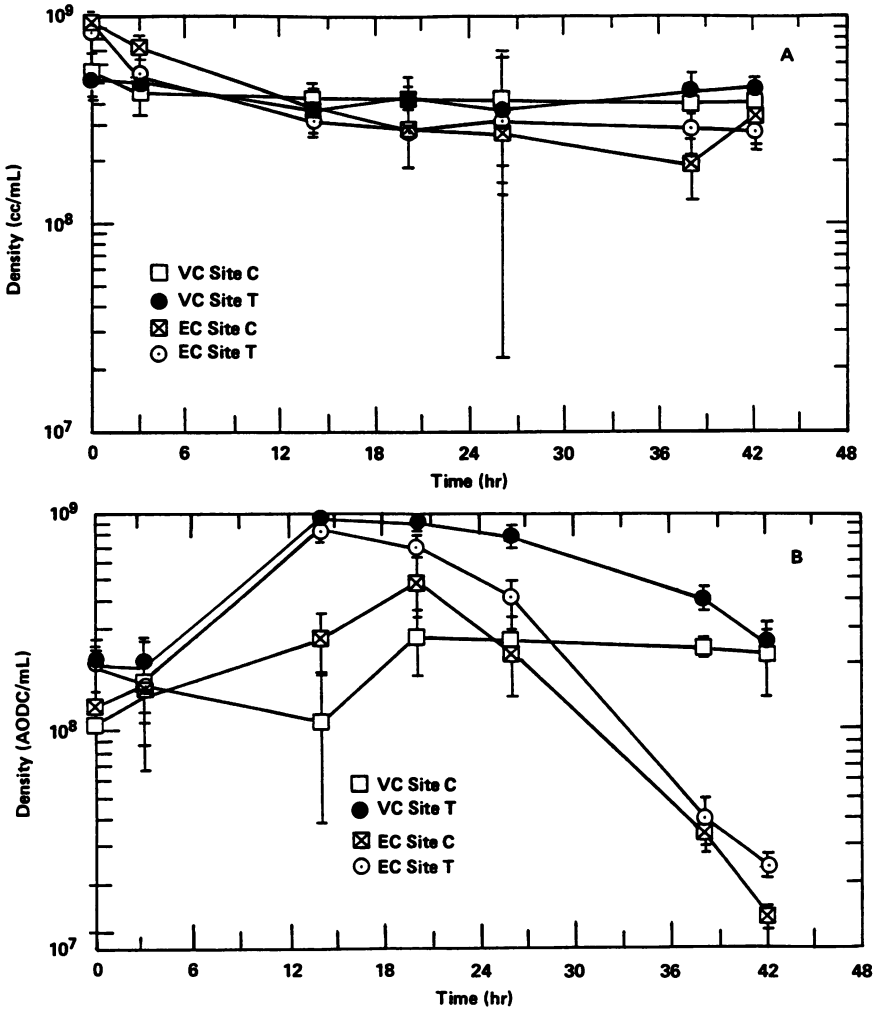

FIG. 4. Changes in total density for *V. cholerae* (VC) and *E. coli* (EC), as measured by Coulter Counter (A) and AODC (B), for chambers in water at Palominitos Island by site (mean ± 1 standard error, n = 4). Sites are defined in Table 1, footnote *b*.

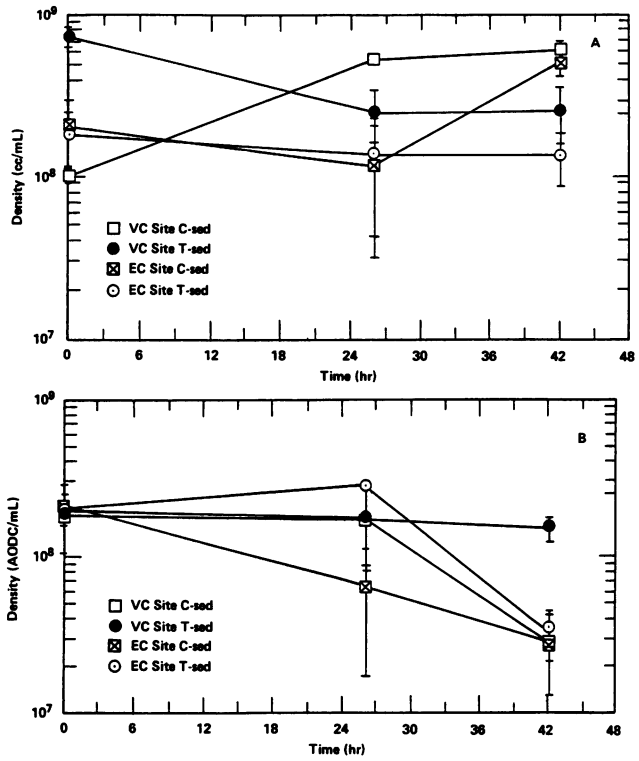

FIG. 5. Changes in total density for *V. cholerae* (VC) and *E. coli* (EC), as measured by Coulter Counter (A) and AODC (B), for chambers in sediments (sed) at Palominitos Island by site (mean ± 1 standard error, $n = 3$). Sites are defined in Table 1, footnote *b*.

first 18 h and remained less than 5% thereafter (Fig. 6). *E. coli* activity in the chambers in the sediments also declined, but 30% of the cells were still active after 42 h (Fig.7). The percentage of the *E. coli* population that was respiring also changed significantly over time ($F = 3.53$, df = 6 and 18, $P < 0.05$) (Fig. 6). Respiration rate of the *E. coli* cells declined significantly only during the first 12 h, i.e., more than 40%. In comparison, *V. cholerae* had a significantly better survival rate than did *E. coli* at Palominitos Island. However, unlike *E. coli*, *V. cholerae* had significantly higher survival and respiration rates at the *Thalassia* site. Both bacteria survived and maintained higher levels of activity and respiration in the sediments than in the overlying waters.

DISCUSSION

Both direct count methods showed the largest decline in *V. cholerae* densities at La Gata during the first 24 h. Simultaneously, densities of *E. coli* in adjacent chambers showed a very precipitous decline to less than 0.1% of the initial density in only 12 h. At Palominitos, densities of neither bacteria declined more than 50% after 42 h of exposure for either direct count method; however, *E. coli* density again declined more rapidly than *V. cholerae*. Chambers buried in the sediment at Palominitos showed no change in density for either direct count method during the entire 42-h period. Xu et al. (26), using microcosms of artificial seawater at a salinity of 5‰ and 25°C for 96 h, showed that *E. coli* AODC densities decreased by 50%, while *V. cholerae* increased by 33%. They also observed that population activity and culturable densities decreased for *E. coli* and increased for *V. cholerae*. When they used even higher salinities (25‰), the *E. coli* AODC densities and activity levels increased slightly, while *V. cholerae* densities and activity levels decreased slightly. For both bacteria they observed decreases in culturability at this higher salinity. In our study using in situ exposure to higher salinities and higher water temperatures, changes in *E. coli* population density and activity were always greater than the changes that occurred in *V. cholerae*. Although our Palominitos results paralleled those of Xu et al. (26), the La Gata sites had a much greater effect on survival and activity of both *E. coli* and *V. cholerae*. The only other similar studies reported from in situ chambers in subtropical waters are those of Colwell et al. (8) which were done at Bimini Lagoon with *E. coli*. They reported that little change in AODC densities occurred over an 18-day period, but population activity and culturability declined dramatically after only 4 days. Thus, as the mathematical model of Seidler and Evans (22) predicted, higher salinities and temperatures should decrease the probability of finding *V. cholerae* in tropical environments. Hood and Ness (18) also reported that *V. cholerae* in Florida survived better than *E. coli* in microcosms containing sterile sediment or water at 20°C and 20‰ salinity. Contrary to the results of our study, they found that *V. cholerae* could not survive in laboratory microcosms in nonsterile sediment. Since our chambers did not allow direct sediment contact with the bacteria, this might account for the differences observed. Thus, sediment interaction and other factors can apparently have a significant impact on *V. cholerae* survival as observed at Palominitos.

The differences between the AODC and CC counts for *V. cholerae* and *E. coli* were greater than 1 order of magnitude for La Gata Island. This observation is undoubtedly because of the morphological changes that occur in *E. coli* in the absence of culturable changes. As reported by others (3, 18, 26), we observed shortening and condensation of the bacterial cells at both La Gata sites, although change occurred at a slower rate for Palominitos and at an even slower rate for chambers buried in sediment at Palominitos. After 24 h of in situ exposure at La Gata, 50% of the cells had transformed to micrococci, i.e., <1 μm. Since the CC counts were done with a 10-μm aperture, the micrococci could not be detected with the Coulter Counter unlike results obtained with the AODC method.

Physiological activity of the bacteria in the diffusion chambers at La Gata Island, as measured by AODC and INT, declined by more than 40% after only 3 h of exposure for both bacteria. The present study showed that *V. cholerae* activity was affected less rapidly than that of *E. coli* and could maintain a level of activity that was higher than that of *E. coli* under some conditions, e.g., in sediments and tropical sea grass areas. Since *E. coli* is generally classified as a nonsurvivor in marine environments (5, 6, 23), it is not surprising that it is rapidly and severely stressed in tropical marine environments. Other studies have also shown that sediment interaction will greatly decrease the stressing effect that the marine environment has on *E. coli* (18). The differences observed for different tropical marine environments are surprising since most studies would indicate that both *E. coli* and *V. cholerae* should be rapidly inactivated and thus not be found in these environments. Indeed, it appears that coral sands and sea grass beds could harbor *V. cholerae* and even *E. coli* for much longer periods than previously thought. This suggests that the fecal coliform and fecal streptococci maximum contaminant levels used for marine recreational waters (6) are inadequate for tropical areas.

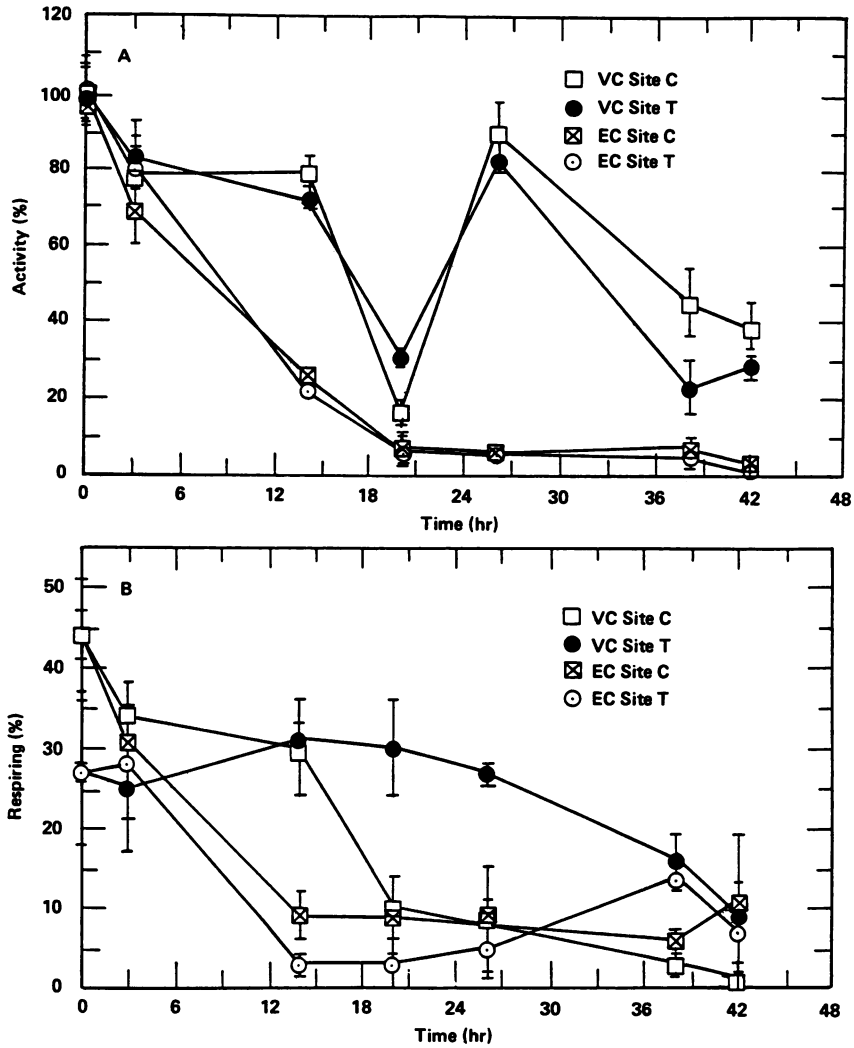

FIG. 6. Changes in percent activity for *V. cholerae* (VC) and *E. coli* (EC), as measured by AODC (A), and in percent respiration, as measured by INT (B), for chambers in water at Palominitos Island by site (mean ± 1 standard error, $n = 4$). Sites are defined in Table 1, footnote *b*.

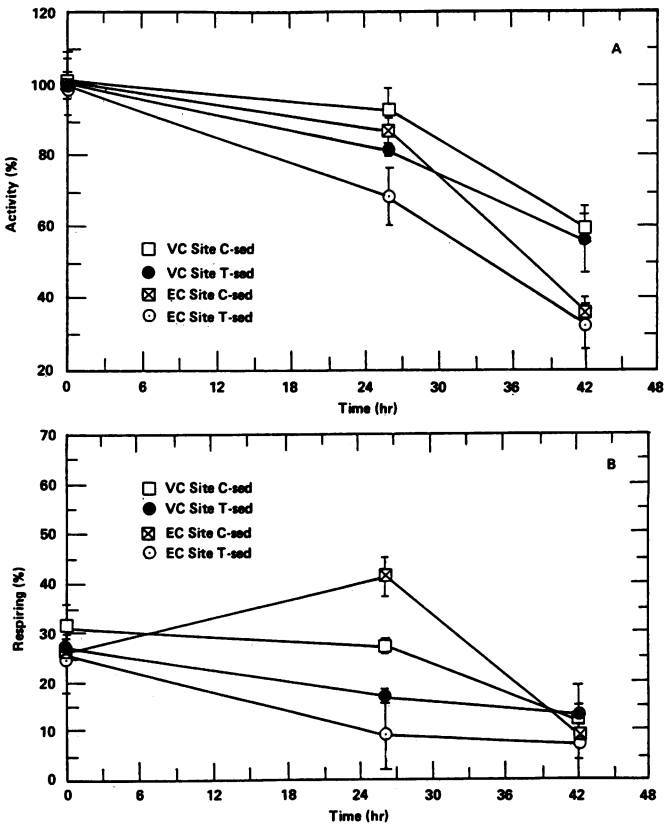

FIG. 7. Changes in percent activity for *V. cholerae* (VC) and *E. coli* (EC), as measured by AODC (A), and in percent respiration, as measured by INT (B), for chambers in sediments (sed) at Palominitos Island by site (mean ± 1 standard error, n = 3). Sites are defined in Table 1, footnote *b*.

Since most of these same habitats are also shellfish and fish harvesting areas, the results further emphasize the need to monitor contaminant levels of pathogens in specific tropical habitats.

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