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AUTECOLOGY OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* IN TROPICAL WATERS

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Abstract—Water and shellfish samples collected from estuaries, mangroves and beaches along the coast of Puerto Rico were examined for *Vibrio vulnificus* and *Vibrio parahaemolyticus*. An array of water quality parameters were also measured simultaneous with bacteria sampling. Both species of vibrio were associated with estuary and mangrove locations, and neither was isolated from sandy beaches. Densities of *V. vulnificus* were negatively correlated with high salinities, 10–15 ppt being optimal. *Vibrio parahaemolyticus* was isolated from sites with salinities between 20 and 35 ppt, the highest densities occurring at 20 ppt. Densities of *Vibrio* spp and *V. parahaemolyticus* for a tropical estuary surpassed those reported for temperate estuaries by several orders of magnitude. Both densities of total *Vibrio* spp and *V. parahaemolyticus* in the water were directly related to densities of fecal coliforms, unlike *V. vulnificus*. The incidence of ONPG(+) strains among sucrose(−) *Vibrio* spp served as an indicator of the frequency of *V. vulnificus* in this group. More than 63% of the *V. vulnificus* isolated were pathogenic. *Vibrio vulnificus* and *V. parahaemolyticus* occupy clearly separate niches within the tropical estuarine-marine ecosystem.

Key words—*Vibrio*, *V. parahaemolyticus*, *V. vulnificus*, tropical, marine, estuary, shellfish

INTRODUCTION

The importance of *Vibrio* spp in recent seafood poisoning cases has been well established. Blake *et al.* (1979) reported that 24 of 39 cases of disease caused by *V. vulnificus* were associated with food ingestion. Forty-six percent of these food ingestion cases were fatal. The source of contamination in 83% of these cases was identified as raw oysters. Outbreaks of gastroenteritis caused by *V. parahaemolyticus* are also invariably associated with the consumption of seafood (CDC, 1971).

Temperature and salinity seem to play important roles in regulating densities of *V. vulnificus* and *V. parahaemolyticus* (Kaneko and Colwell, 1973; Kelly, 1982; Roberts *et al.*, 1982; Tamplin *et al.*, 1982). Increased water temperature and salinity appears to favor the survival and growth of *V. vulnificus* and *V. parahaemolyticus* in the environment. The tropical climate of Puerto Rico, where year-round temperature averages 28°C, would seem ideal for *Vibrio* spp and therefore Vibriosis. In temperate areas, 85% of *V. vulnificus* infections occur during the warm months of the year (Blake *et al.*, 1979). High evaporation rates and low rainfall increase estuary and coastal salinities in shellfish harvesting waters. Thus, higher salinities and temperature should be optimal for *V. vulnificus* and *V. parahaemolyticus* growth in tropical areas. In addition, raw oysters are quite often consumed at roadside stands in Puerto Rico where refrigeration is nonexistent. As observed by Oliver (1981), the bacterium grows quite rapidly in unchilled raw oysters. Considering that for 1986, Puerto Rico had 54,569 municipal clinic and hospital reported cases of gastroenteritis with a specific attack rate of more than 200/100,000 population (Rigau, 1986), it is conceivable that *Vibrio* spp are responsible for many of these cases [see Hazen (1988) for a more thorough review]. This study examines the distribution, and pathogenicity of *V. parahaemolyticus* and *V. vulnificus* in shellfish and near shore coastal waters of Puerto Rico.

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MATERIALS AND METHODS

Study sites

Luquillo Beach (LB) and the Río Mameyes estuary are on the northeast coast of the island (18°15′N, 65°45′W), see Carrillo *et al.* (1985) and Pérez-Rosas and Hazen (1989) for details (Fig. 1). Torrecilla Lagoon (TL) (18°20′N, 66°00′W) is a recreational center and a shellfish harvesting area near San Juan. It receives incoming currents from the Atlantic Ocean and is surrounded by mangroves. Palo Seco Channel (PSC) (18°20′N, 66°10′W) is on the northern coast of the island and drains into the Atlantic Ocean. Bayamon River Channel estuary (BRC) (18°25′N, 66°09′W) receives sewage treatment plant effluent, has a total length of 6.9 km and drains into Ensenada de Boca Vieja cove. Ensenada de Boca Vieja (EBV) (18°27′N, 66°45′W) is a protected cove adjacent to San Juan Bay [see Biamón and Hazen (1983), Rojas and Hazen (1989) and Valdés-Collazo *et al.* (1987) for details]. Bayamon River estuary (BR) (18°25′N, 66°10′W) drains into San Juan Bay and is surrounded by mangroves. It is also a site of limited shellfish harvesting. Mandry Channel (MC) (18°9′N, 65°46;W) near Humacao flows across low

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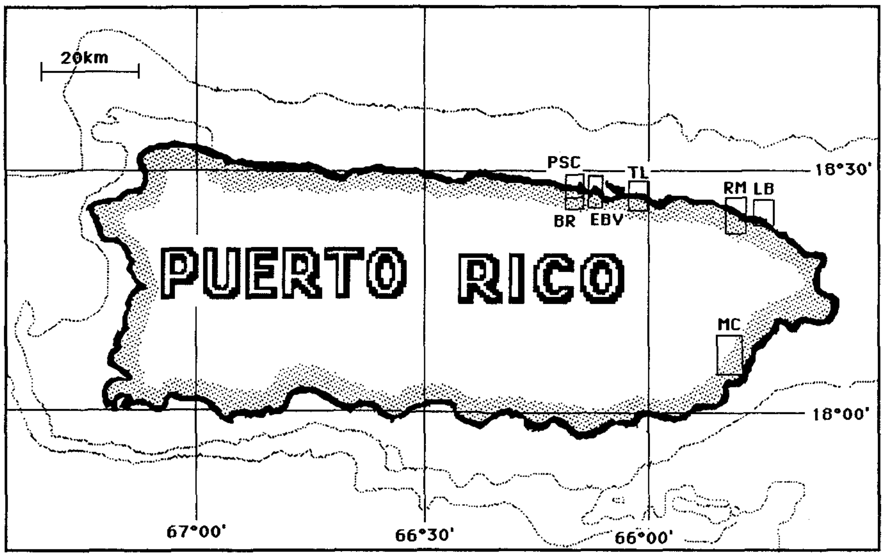

Fig. 1. Map of study sites around Puerto Rico.

coastal lands receiving runoff from farming and pasture lands. All sites were sampled 3–4 times during the course of 2 yr.

Water quality

In situ measurements were taken of salinity and both air and water temperature. Salinity was measured by using a hand refractometer (model 10419, American Optical, Buffalo, N.Y.). Collected samples were analyzed in the laboratory for turbidity, pH, chlorophyll *a*, nitrites plus nitrates, phosphates, total phosphorus, and dissolved oxygen. These were determined using *Standard Methods* (APHA, 1985).

Bacteriological procedures

Sterile, 1 liter bottles were filled with water for bacteria counts. Collected shellfish were placed in sterile Whirl-Pak bags (Nasco Int., Fort Wilkinson, Wis.). All samples were transported on ice to the laboratory for analysis within 3–5 h. Total cell counts were determined by direct count (AODC) methods using acridine orange (Hobbie *et al.*, 1977). Percent activity was established by calculating the ratio of red cells to the total cell number (López-Torres *et al.*, 1988). Density of actively respiring cells was determined using the INT reduction technique of Zimmermann *et al.* (1978). All techniques are as described previously (Biamón and Hazen, 1983; Carrillo *et al.*, 1985; Hazen *et al.*, 1987; López-Torres *et al.*, 1987; Ortiz-Roque and Hazen, 1987). Densities of fecal coliforms were estimated by membrane filtration (APHA, 1985).

Densities of *Vibrio* spp were determined by filtering with a 0.45-μm pore size, 47-mm-dia, HA-type membrane filter (Millipore Corp., Bedford, Mass.), previous studies by our laboratory (unpublished data) have shown that this pore size is adequate, unlike temperature areas where smaller pore size is more appropriate. After filtration, filters were placed on TCBS medium (Difco, Detroit, Mich.) in sterile tight fitting Petri dishes and incubated at 35°C for 24 h. When incubation was completed, total *Vibrio* spp were estimated by counting all colonies. Sucrose positive *Vibrio* spp were counted as colonies appearing yellow. Sucrose negative *Vibrio* spp were counted as colonies appearing blue or green. Random sucrose negative colonies were picked and transferred to marine agar medium (Difco). All isolates were tested for oxidase production using the API Oxidase kit (Analytab Products, Plainview, N.Y.), and ONPG hydrolysis using ONPG diffusion disks (Difco) or API-20E strips (Analytab). All oxidase positive organisms were subjected to a battery of biochemical tests using API-20E strips (Analytab) with 20 ppt marine salts diluent (Instant Ocean, Aquarium Systems, Eastlake, Ohio) and incubation at 22°C (MacDonell *et al.*, 1982). Isolates with typical reactions were identified as presumptive *V. vulnificus* and *V. parahaemolyticus* and subjected to further tests to confirm their identity. Sensitivity to 2-4 diamino 6-7 di-isopropyl pteridine phosphate (O/129) was determined using the disk diffusion method. Presumptive *V. vulnificus* sensitive to both 150 and 10 μg of O/129 were tested further as were presumptive *V. parahaemolyticus* isolates sensitive to 150 μg but resistant to 10 μg of O/129. Salt tolerance tests were conducted by adding 0, 7 and 10% NaCl to modified salt water yeast extract agar MSWYE (Poole and Oliver, 1978). Isolates growing in 7% NaCl but not 10% NaCl, with typical biochemical reactions for *V. parahaemolyticus* were identified accordingly. Those isolates unable to grow in either 7 or 10% NaCl with typical biochemical reactions for *V. vulnificus* were tested for sensitivity to penicillin (10 U) and colistin (10 μg). Isolates resistant to colistin and sensitive to penicillin were identified as *V. vulnificus*. *Vibrio vulnificus* (ATCC 27562) and *V. parahaemolyticus* (ATCC 17802) were used as controls for all tests and media.

Identification of *V. parahaemolyticus* and *V. vulnificus* was further confirmed with a slide flocculation procedure using core flagellar antiserum against *V. vulnificus* and both flagellar and core flagellar antiserum against *V. parahaemolyticus* donated by Dr R. Siebeling, Louisiana State University (Simonson and Siebeling, 1986).

Pathogenicity

Isolates positively identified as *V. vulnificus* were used to prepare an active inoculum containing 10^9 cells ml^{-1} grown in Brain Heart Infusion broth (Difco) 1.5% NaCl and incubated for 18 h at 35°C. One-half ml of this inoculum was injected intraperitoneally to 6–8 week old AKR/J female white mice to determine strain pathogenicity (Poole and Oliver, 1978). Pathogenicity of *V. parahaemolyticus* isolates was determined by the Kanagawa test (Miyamoto *et al.*, 1969). Fresh human blood was used with Wagatsuma's agar (Cherwonogrodzky and Clark, 1982; Wagatsuma, 1974) to determine the isolates ability to cause β-hemolysis of erithrocytes.

Data analysis

One factor analysis of variance (ANOVA) without replication was used to test differences between sites using programs developed for a Macintosh computer. Multiple correlation was used to determine relationships between density and water quality parameters. Any statistical probability <0.05 was considered significant (Zar, 1984).

RESULTS

Representative water quality data for each site is given in Table 1. A total of 409 sucrose negative isolates were examined (Table 2). The nine study sites examined ranged in AODC density from 9.6×10^5 to 1.7×10^7 cells ml^{-1} (Fig. 2). The AODC measurement correlated positively with viable count densities of both *Vibrio* spp and fecal coliforms (Table 3). Total bacterial densities also held strong positive correlations with concentrations of phosphate and total phosphorus in the environment. The percent activity of the bacterial population at the various sites ranged from 14.4 to 74.7 (Fig. 2). Bacterial densities as measured by both direct count and all viable count methods were negatively correlated with percent activity (Table 3). Although the percentage of respiring cells in the bacterial community was much lower than the percent activity for all sites examined (Fig. 2), both measurements were significantly positively correlated.

The percentage that *Vibrio* spp represented in the total bacterial community was very small for all sites (Table 2). Yet, *Vibrio* spp share with the entire bacterial community a significant positive correlation with phosphates and total phosphorus concentrations in the water. When densities of fecal coliforms increased so did the density of *Vibrio* spp as did the proportion of *Vibrio* spp in the total bacterial community (Fig. 2). Densities of *Vibrio* spp and the percentage of *Vibrio* spp in the bacterial community was negatively correlated with dissolved oxygen. Densities of *Vibrio* spp by site ranged from 16.9 to 1.5×10^6 CFU ml^{-1} (Table 2). For shellfish, densities of *Vibrio* spp by site, ranged from 5.2×10^3 to 1.5×10^4 CFU g^{-1}. Densities of *Vibrio* spp were not correlated with salinity while both sucrose(−) *Vibrio* spp ml^{-1} and the percentage of sucrose(−) *Vibrio* spp were negatively correlated with salinity. The percentage of sucrose(−) *Vibrio* spp making up the vibrio population decreased with increasing salinity of the sites (Table 2). The percentage of sucrose(+) *Vibrio* spp was not correlated with salinity and was generally higher than that of sucrose(−) *Vibrio* spp. Densities of sucrose(−) *Vibrio* spp at the various sites ranged from 3.24 to 12.76×10^5 CFU ml^{-1} (Table 2). In shellfish, densities of sucrose(−) *Vibrio* spp by site ranged from 1.3×10^3 to 2.7×10^3 CFU g^{-1}.

The densities of sucrose(−) *Vibrio* spp showed a highly significant positive correlation with densities of ONPG(+) *Vibrio* spp (Tables 3 and 4). Both the density of ONPG(+) *Vibrio* spp and the percentage of sucrose(−) *Vibrio* spp made up of ONPG(+) vibrios were significantly negatively correlated with salinity (Table 3). A significant difference by site was observed for ONPG(+) *Vibrio* spp ml^{-1}. Sites with increasing salinity showed decreasing percentages of ONPG(+) *Vibrio* spp. Densities of ONPG(+) *Vibrio* spp for the various sites ranged from 0.83 to 5.94×10^5 CFU ml^{-1} (Table 2). Densities of ONPG(+) *Vibrio* spp in shellfish ranged from 208 to 449 CFU g^{-1} by site.

As shown in Table 2, densities of *V. vulnificus* by site ranged from 38 to 4124 CFU 100 ml^{-1}. Both the highest densities and the highest frequencies of isolation of *V. vulnificus* were obtained at salinities of 10 and 15 ppt. *Vibrio vulnificus* was never isolated from sandy beach, seawater samples (sites LB and EBV). Bayamon River estuary (BR) and the upper Río Mameyes estuary (URM) possess extreme salinities of 32.2 and 1.7 ppt respectively (Table 1). At these sites the lowest frequencies of isolation were observed, representing <4% of sucrose negative *Vibrio* spp. In Torrecilla Lagoon (TL), for both water and shellfish, *V. vulnificus* was isolated only when salinities were between 20 and 25 ppt. The percentage of *V. vulnificus* isolates which proved lethal to mice showed an even higher significant negative correlation with salinity than did all *V. vulnificus* isolates (Table 3). At sites TL and BR where salinities were highest, none of the *V. vulnificus* isolated proved pathogenic (Table 4). Overall, 46% of *V. vulnificus* isolates were pathogenic. It is interesting to note that densities of fecal coliforms were not significantly correlated with densities of *V. vulnificus*. Significant negative correlations were observed between densities of *V. vulnificus* and phosphates, total phosphorus, and pH.

The proportion of sucrose(−) *Vibrio* spp were confirmed as *V. parahaemolyticus*, were significantly positively correlated with salinity (Table 4). Sites yielding *V. parahaemolyticus* isolates ranged in salinity from a mean of 20.2 to 35.0 ppt. *Vibrio parahaemolyticus*, like *V. vulnificus*, was never isolated from LB or EBV coastal sites far removed from marsh lands and estuaries. The highest density of *V. parahaemolyticus* was observed in BRC with a mean salinity of 20.2 ppt, a site which never yielded *V. vulnificus*. The densities of *V. parahaemolyticus* for the various sites ranged from 315 to 3.2×10^5 CFU 100 ml^{-1}. In shellfish the densities of *V. parahaemolyticus* for TL and BR were 37.4 and 207.6 CFU g^{-1} respectively (Table 2). Significant positive correlations were observed between the percentage of *V. parahaemolyticus* among sucrose(−) *Vibrio* spp and concentrations of phosphates and total phosphorus. Fecal coliform densities in the water column showed a significant positive correlation with densities of *V. parahaemolyticus* (Table 3). The Kanagawa pathogenicity test for 94% of *V. parahaemolyticus* isolates resulted in a Kanagawa negative assay. These results were confirmed on isolates sent to C. A. Kaysner, Food and Drug Administration, Seattle. The percent *V. vulnificus* and *V. parahaemolyticus* among sucrose negative *Vibrio* spp was negatively correlated (Table 3).

Table 1. Physical–chemical water quality by site

Site	ATEMP	WTEMP	SAL	DO	pH	CHLA	TURB	NO_3	PO_4	TP
URM	27.0 ± 0.5	24.5 ± 0.6	1.7 ± 0.0	8.0 ± 0.2	7.2 ± 0.1	8.4 ± 2.2	96.3 ± 1.4	1.09 ± 0.14	0.030 ± 0.003	0.045 ± 0.006
MC	30.1 ± 0.0	29.2 ± 0.4	8.0 ± 1.3	2.8 ± 0.4	7.3 ± 0.1	27.0 ± 12.6	95.9 ± 0.8	0.76 ± 0.55	0.029 ± 0.011	0.075 ± 0.003
LRM	29.3 ± 0.2	27.8 ± 0.5	15.0 ± 0.0	5.1 ± 0.7	7.7 ± 0.2	1.7 ± 0.4	97.3 ± 0.5	0.53 ± 0.12	0.045 ± 0.019	0.051 ± 0.019
BRC	30.7 ± 0.9	28.8 ± 0.4	20.2 ± 0.4	2.0 ± 0.7	7.9 ± 0.1	122.4 ± 117.9	96.0 ± 0.9	0.86 ± 0.29	0.423 ± 0.086	0.479 ± 0.078
TL	28.6 ± 0.6	26.9 ± 0.6	29.3 ± 1.3	7.7 ± 2.0	7.9 ± 0.2	30.1 ± 6.9	93.8 ± 0.9	0.59 ± 0.18	0.152 ± 0.017	0.219 ± 0.016
BR	28.3 ± 0.6	28.7 ± 0.4	32.2 ± 0.7	5.7 ± 0.6	7.8 ± 0.2	7.5 ± 1.2	95.3 ± 1.1	0.30 ± 0.06	0.052 ± 0.006	0.085 ± 0.012
EBV	25.7 ± 0.6	25.2 ± 1.4	34.8 ± 0.5	5.8 ± 0.8	7.0 ± 0.5	18.8 ± 12.4	94.5 ± 1.4	0.37 ± 0.03	0.048 ± 0.013	0.071 ± 0.015
PSC	28.2 ± 0.8	32.5 ± 1.0	35.0 ± 0.7	6.3 ± 0.5	7.4 ± 0.2	3.7 ± 1.1	96.1 ± 1.3	0.53 ± 1.25	0.038 ± 0.004	0.053 ± 0.004
LB	26.8 ± 0.7	25.7 ± 0.6	36.2 ± 0.7	6.9 ± 0.4	7.7 ± 0.0	11.8 ± 2.4	91.9 ± 1.9	1.75 ± 0.80	0.013 ± 0.003	0.018 ± 0.005

All values are mean ± 1SE ($n = 7$), ATEMP = air temperature (°C), WTEMP = water temperature (°C), DO = dissolved oxygen ($mg l^{-1}$), SAL = salinity (ppt), NO_{2+3} = nitrites ($mg l^{-1}$), PO_4 = orthophosphate ($\mu g l^{-1}$), TP = total phosphorus ($\mu g l^{-1}$), CHLA = chlorophyll *a* ($mg l^{-1}$), TURB = turbidity (% transmittance).

Table 2. Densities of bacteria by site

Site	V	S(+)	S(−)	AODC	FC	Vp	(O+)	Vv	VvP
URM	213.3 ± 92.2	181.4 ± 88.9	27.00 ± 5.26	46.3 ± 7.9	5.98 ± 1.15	0.00	20.54 ± 4.00	0.38 ± 0.07	0.38 ± 0.07
MC	321.7 ± 161.7	235.9 ± 147.6	73.75 ± 21.44	49.6 ± 19.1	10.03 ± 5.27	0.00	65.12 ± 18.92	41.24 ± 11.98	26.06 ± 7.57
LRM	63.3 ± 13.6	40.5 ± 9.8	20.63 ± 4.67	111.0 ± 33.0	3.20 ± 0.78	0.00	14.14 ± 3.21	3.23 ± 0.73	1.21 ± 0.27
BRC	15.2 ± 14.4	2.4 ± 1.8	12.76 ± 12.62	173.0 ± 39.6	0.03 ± 0.02	3.20 ± 3.16	5.94 ± 5.86	0.00	0.00
TL	189.0 ± 56.5	175.9 ± 54.1	10.51 ± 4.61	103.0 ± 20.9	21.16 ± 12.60	3.15 ± 1.38	1.58 ± 0.69	0.53 ± 0.23	0.00
TL shellfish	5169 ± 1521	4011 ± 1506	1271 ± 725	ND	205.3 ± 125.3	37.4 ± 21.3	449 ± 256	225 ± 128	37.6 ± 21.4
BR	210.0 ± 103.6	173.9 ± 78.3	35.82 ± 28.66	90.3 ± 26.1	0.62 ± 0.12	6.39 ± 5.13	10.23 ± 8.19	1.28 ± 1.02	0.00
BR shellfish	$14{,}625 \pm 25$	6550 ± 4550	2700 ± 800	ND	18.5 ± 5.5	207.6 ± 61.5	208 ± 62	0.00	0.00
EBV	746.3 ± 386.5	735.0 ± 392.6	16.67 ± 8.70	63.3 ± 19.2	2.13 ± 0.97	0.00	3.85 ± 2.01	0.00	0.00
PSC	126.7 ± 14.5	85.0 ± 14.4	32.50 ± 6.29	115.0 ± 59.7	39.70 ± 30.14	4.33 ± 0.84	2.16 ± 0.42	0.00	0.00
LB	16.9 ± 2.6	13.8 ± 2.7	3.24 ± 0.72	9.6 ± 2.1	0.20 ± 0.07	0.00	0.83 ± 0.19	0.00	0.00

All units are in CFU ml^{-1}, except AODC which is $\times 10^5$ cells ml^{-1}, shellfish values are CFU g^{-1}, V = total *Vibrio* spp, S(+) = sucrose positive, S(−) = sucrose negative, AODC = Acridine Orange direct counts, FC = fecal coliforms, Vp = *V. parahaemolyticus*, O(+) = ONPG positive, Vv = *Vibrio vulnificus*, VvP = pathogenic *Vibrio vulnificus*.
ND = not detected.

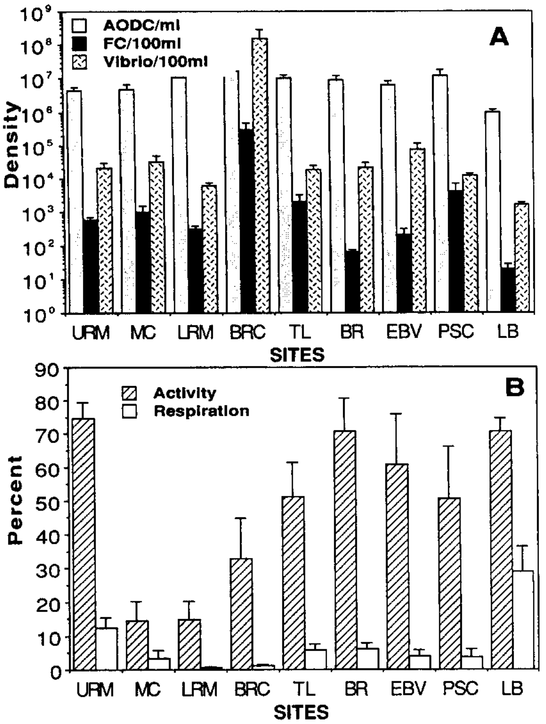

Fig. 2. (A) Density for *Vibrio*, fecal coliforms (FC), and total bacteria (AODC) by site (mean $\pm$ 1 SE, $n = 7$); (B) percent activity of total bacteria as measured by AODC (activity) and percent respiration as measured by INT (respiration) by site (mean $\pm$ 1 SE, $n = 7$).

DISCUSSION

The density of fecal coliforms and *Vibrio* spp in the water column was positively correlated with other pollution indicators in Peurto Rico waters, like phosphates, total phosphorus, and total bacterial counts, and significantly negatively correlated with dissolved oxygen, and salinity. A highly significant positive correlation was observed between densities of *Vibrio* spp and densities of fecal coliforms. Oliver *et al.* (1983) also observed a significant correlation between *Vibrio* spp and fecal contamination in marine environments along the east coast of the United States.

Prior to this study the maximum density of *Vibrio* spp reported in natural waters was in a temperate estuary, 10^2 MPN ml^{-1} (Kaneko and Colwell, 1973). The highest densities of *Vibrio* spp observed in this study, a tropical estuary, were 1.5×10^6 CFU ml^{-1}. The constant optimum growth temperature offered by a tropical climate may allow *Vibrio* spp to stabilize at higher densities. The periodic drastic reduction in *Vibrio* spp densities caused by winter (Kaneko and Colwell, 1973; Kelly, 1982; Tamplin *et al.*, 1982), would not be a regulating factor in a tropical estuary. The sucrose($-$) vibrio population showed the same high density as did the total *Vibrio* spp population. Kaneko and Colwell (1978) report a sucrose($-$) vibrio maximum density of 62.0 CFU ml^{-1} in Chesapeake Bay. Bayamon River Channel estuary (BRC) had mean densities of sucrose($-$) *Vibrio* spp of 1.3×10^6 CFU ml^{-1}. This could be a combination of both favorable temperature and an allochthonous source, e.g. sewage. Fecal coliform densities at this site averaged 3.0×10^3 CFU ml^{-1}. The significant positive correlation between *Vibrio* spp and fecal coliforms observed for all sites would support this observation. Other studies by our laboratory (Biamón and Hazen, 1983; Carrillo *et al.*, 1985; Hazen, 1988; Hazen *et al.*, 1987; López-Torres *et al.*, 1987, 1988; Pérez-Rosas and Hazen, 1988, 1989; Rojas and Hazen, 1989; Valdés-Collazo *et al.*, 1987) indicate that the survival of *Vibrio* spp and other enteric bacteria in natural waters is much greater in the tropics.

Salinity appears to play a role in regulating sucrose($-$) *Vibrio* spp. Densities of sucrose($-$) *Vibrio* spp and proportion of sucrose negative vibrios, were significantly negatively correlated with salinity. Oliver *et al.* (1983) made similar observations for salinity and sucrose($-$) vibrios from oysters. The negative effect that salinity has on densities of *V. vulnificus* follows the same pattern as that observed for sucrose($-$) vibrios and sucrose($-$) ONPG(+) vibrios. The frequency of ONPG(+) spp among sucrose($-$) *Vibrio* spp may serve as an indicator of the presence of *V. vulnificus*. The highly significant negative correlation that this bacteria has with salinity is also suggested by a markedly reduced frequency of isolation from sites with increased salinity. Sites having salinities of 10 and 15 ppt had both the highest densities of *V. vulnificus* and the highest frequency of isolation among sucrose($-$) vibrios. Kelly (1982) also found that *V. vulnificus* was most frequently isolated from sites where salinities ranged between 7 and 16 ppt. Tamplin *et al.* (1982) reported the isolation of *V. vulnificus* more frequently in waters with a salinity >17 ppt and in a higher proportion of samples >23 ppt. The results of the present study do not corroborate those findings. *Vibrio vulnificus* was isolated only from estuaries and mangroves. Sandy beaches such as LB and EBV did not yield *V. vulnificus*. Bayamon River estuary and the upper Río Mameyes estuary which have extreme differences in salinities were sites of lowest isolation. Less than 4% of sucrose($-$) isolates tested from these sites resulted in positive identification. Considering that *in vitro* experiments have shown the optimum salinity ranges for *V. vulnificus* between 10 and 20 ppt (Kelly, 1982), it is understandable that these sites would not arbor this bacteria. The isolation of *V. vulnificus* in Torrecilla Lagoon from water and shellfish only when salinities ranged between 20 and 25 ppt also indicates its low salinity requirements.

When estimating the frequency of isolation of *V. vulnificus* based on sucrose($-$) vibrio isolates which were also ONPG(+) the frequency of isolation increases. Estimated in this manner, the percentage of *V. vulnificus* obtained from all sites averaged 23%. These results are comparable to those of Oliver *et al.*

Table 3. Water quality and bacteria correlations

	WTEMP	ATEMP	SAL	DO	pH	CHLA	TURB	NO_3	PO_4	TP	V	S(-)	S(+)	FC	AODC	Vv	Vp	O(+)	%S(-)	%V	%A	%R	%O(+)	%Vv	%VvP
WTEMP	1.000																								
ATEMP	0.465	1.000																							
SAL	0.227	-0.138	1.000																						
DO	-0.052	0.138	0.288	1.000																					
pH	-0.036	0.020	0.243	-0.301	1.000																				
CHLA	-0.125	-0.097	0.120	0.323	-0.088	1.000																			
TURB	-0.211	-0.233	-0.488	-0.216	-0.113	-0.365	1.000																		
NO_3	-0.012	0.098	-0.137	-0.252	-0.028	-0.047	0.386	1.000																	
PO_4	-0.318	0.046	0.368	-0.096	0.322	-0.048	-0.186	0.196	1.000																
TP	-0.256	0.132	0.299	-0.193	0.396	0.232	-0.403	0.095	0.885	1.000															
V	-0.029	0.150	-0.072	-0.552	0.226	-0.179	-0.134	0.215	0.511	0.542	1.000														
S(-)	0.018	-0.112	-0.358	-0.211	-0.088	-0.047	0.184	-0.155	-0.125	-0.077	0.467	1.000													
S(+)	-0.040	0.143	0.028	-0.546	0.250	-0.234	-0.125	0.296	0.617	0.601	0.979	0.321	1.000												
FC	-0.168	0.064	0.191	-0.476	0.410	0.131	-0.134	0.022	0.330	0.460	0.766	0.501	0.686	1.000											
AODC	-0.174	-0.215	0.384	0.021	0.184	-0.047	-0.479	-0.387	0.140	0.127	0.161	-0.003	0.150	0.299	1.000										
Vv	0.046	0.108	-0.761	-0.217	-0.412	0.026	0.488	0.212	-0.536	-0.420	-0.165	0.326	-0.248	-0.100	-0.492	1.000									
Vp	0.059	-0.110	0.111	-0.169	0.153	-0.020	-0.167	-0.258	0.217	0.245	0.646	0.821	0.548	0.613	0.278	-0.241	1.000								
O(+)	-0.048	-0.056	-0.471	-0.269	-0.114	-0.105	0.200	-0.171	-0.156	-0.080	0.494	0.974	0.344	0.508	-0.013	0.400	0.759	1.000							
%S(-)	0.062	0.041	-0.598	0.021	-0.152	0.148	0.174	-0.313	-0.594	-0.439	-0.005	0.745	-0.199	0.278	-0.075	0.545	0.401	0.755	1.000						
%V	-0.001	0.081	-0.060	-0.376	0.212	-0.132	-0.137	-0.050	0.368	0.419	0.889	0.720	0.811	0.742	0.164	-0.175	0.882	0.722	0.286	1.000					
%A	-0.246	-0.528	0.234	0.120	-0.040	0.332	0.343	0.236	0.147	0.008	-0.240	-0.236	-0.175	-0.151	-0.185	-0.210	-0.147	-0.345	-0.290	-0.259	1.000				
%R	0.166	-0.088	0.050	0.265	-0.578	0.246	0.090	0.188	-0.094	-0.123	-0.181	-0.075	-0.156	-0.445	-0.493	0.079	0.110	-0.112	-0.100	-0.147	0.446	1.000			
%O(+)	-0.136	0.269	-0.823	-0.150	-0.044	-0.239	0.454	-0.054	-0.359	-0.291	0.023	0.389	-0.175	0.101	-0.412	0.724	0.064	0.519	0.616	0.102	-0.425	-0.206	1.000		
%Vv	0.026	0.066	-0.722	-0.235	-0.257	-0.011	0.537	0.122	-0.565	-0.435	-0.240	0.271	-0.320	-0.125	-0.548	0.942	-0.269	0.352	0.529	-0.216	-0.207	0.005	0.759	1.000	
%VvP	-0.119	-0.025	-0.838	-0.086	-0.391	-0.084	0.476	0.101	-0.614	-0.602	-0.198	0.270	-0.282	-0.130	-0.378	0.752	-0.210	0.353	0.558	-0.156	-0.077	0.159	0.683	0.668	1.000
%Vp	0.138	0.136	0.656	0.192	0.092	0.251	-0.561	-0.089	0.472	0.467	0.176	-0.195	0.229	0.074	0.364	-0.689	0.284	-0.289	-0.405	0.180	0.226	0.179	-0.762	-0.767	-0.603

Where $P < 0.05$ when $r > 0.381$, underlined values are significant, see previous tables for abbreviations.

Table 4. Isolate identification by site

Site	S(−)	O(+)	Vv	VvP	Vp
URM (water)	26.6 (71)*	76.1 (54)	1.41 (1)	100 (1)	0 (0)
MC (water)	46.4 (34)	88.2 (30)	55.9 (19)	63.2 (12)	0 (0)
LRM (water)	46.5 (51)	68.6 (35)	15.7 (8)	37.5 (3)	0 (0)
BRC (water)	37.1 (56)	46.4 (26)	0 (0)	0 (0)	25.0 (14)
TL (water)	8.0 (40)	15.0 (6)	5.0 (2)	0 (0)	30.0 (12)
(shellfish)	30.6 (34)	35.3 (12)	17.7 (6)	16.7 (1)	2.9 (1)
BR (water)	16.3 (28)	28.6 (8)	3.6 (1)	0 (0)	17.9 (5)
(shellfish)	18.5 (13)	7.7 (1)	0 (0)	0 (0)	7.7 (1)
EBV (water)	9.9 (13)	23.1 (3)	0 (0)	0 (0)	0 (0)
PSC (water)	26.8 (15)	6.7 (1)	0 (0)	0 (0)	13.3 (2)
LB (water)	21.8 (54)	25.9 (14)	0 (0)	0 (0)	0 (0)
Total	409	190	37	17	35

*Percent positive (No. of positive isolates). See previous tables for abbreviations.

(1982) who found *V. vulnificus* represented 20% of all lactose(+) sucrose(−) vibrios.

The highest densities of *V. vulnificus* were obtained from Mandry Channel. Densities at this site averaged 4.1×10^3 CFU 100 ml^{-1} with 63% testing positive for pathogenicity. The detection of a *V. vulnificus* mean density of 225 CFU g^{-1} shellfish in Torrecilla Lagoon further suggests the importance of this bacteria as a probable agent of foodborne disease in Puerto Rico. In Torrecilla Lagoon, the percentage of sucrose(−) vibrios that were *V. vulnificus* was over three times greater in shellfish than in the over lying water column. The incidence of *V. vulnificus*; however, was not connected to sewage contamination, since no correlation was observed with fecal coliforms. This organism appears an inhabitant of marine aquatic systems that are totally unaffected by sewage effluent. This lack of association between *V. vulnificus* and fecal coliforms has also been noted in temperate areas (Oliver *et al.*, 1982, 1983).

Vibrio parahaemolyticus, unlike sucrose(−) vibrios, was positively correlated with salinity. This bacteria was found at sites with salinities between 20 and 35 ppt and was never isolated from sites with salinities <20 ppt. This would indicate that in the tropics higher salinities favor *V. parahaemolyticus*; however, the highest density of this bacteria (3.2×10^3 CFU 100 ml^{-1}) was detected at BRC, a site with a salinity of only 20.2 ppt. In contrast, PSC, 35 ppt salinity, harbored only 433 CFU 100 ml^{-1}. An increase in salinity was also accompanied by a general decrease in the percentage of sucrose(−) vibrios that were confirmed as *V. parahaemolyticus*. Intermediate salinities appear more favorable to this bacteria. The fact that the organism was never isolated from sandy beaches indicates that although it can tolerate high salinity environments, it is an estuary and marsh inhabitant.

On the coast of West Africa (Bockemühl and Triemer, 1974), the lagoon system proved the most important reservoir of *V. parahaemolyticus*. The seasonality observed in the incidence of this bacterium for West Africa was closely related to salinity. During the dry season, when isolation was most frequent, salinity of the lagoons was between 15 and 21 ppt. The rainy season which rendered lagoon salinity between 1.6 and 4.2 ppt had the lowest incidence of *V. parahaemolyticus*. These findings are in close agreement to the present study.

Maximum densities of *V. parahaemolyticus* in this study were observed for Bayamon River Channel estuary (3.2×10^7 CFU 100 ml^{-1}). These elevated densities contrast markedly with those obtained for temperate estuaries. Kaneko and Colwell (1973) report maximum densities of 400 CFU 100 ml^{-1} in Chesapeake Bay. They observed that *V. parahaemolyticus* were undetectable until early June, when the water temperature was 19°C. Watkins and Cabelli (1985) also report far lower densities for Narragansett Bay, R.I. (495 CFU 100 ml^{-1}), than those recorded in this study. As in the case of total vibrios, high densities of *V. parahaemolyticus* may be due to the high constant temperature of a tropical climate and associated increased survival.

Although previous studies conducted in the tropics did not quantify *V. parahaemolyticus*, they did establish the presence of this bacterium in tropical waters and shellfish (Molitoris *et al.*, 1985). In our study, densities in shellfish were 37.4 and 207.6 CFU g^{-1}. Although these levels are low in terms of the 10^6 cell dose required to trigger gastroenteritis (Sakazaki *et al.*, 1968), they do bring to light the presence of this bacterium in shellfish harvested for local consumption. The possible health hazard that these shellfish may represent is aggravated by the typical handling they receive upon harvesting. The local practice of selling shellfish at road side stands where there is no refrigeration would favor a marked increase in numbers of *V. parahaemolyticus* present (Blake, 1984).

The results of the present study demonstrated a significant positive correlation between fecal coliform levels and density of *V. parahaemolyticus* in the water column. Watkins and Cabelli (1985) also reported a significant positive correlation between the level of fecal pollution and density of *V. parahaemolyticus*. These authors observed that its densities decreased sharply with distance from the source of fecal pollution. Maximum density of *E. coli* recorded in their study was 2.3×10^3 100 ml^{-1}. Maximum density of fecal coliforms in the present study was recorded for Bayamon River Channel estuary, 3.0×10^5 CFU 100 ml^{-1}. Thus, the difference in densities of *V. parahaemolyticus* between the tropical and temperate estuaries may be attributed not only to temperature differences but also to differences in levels of fecal contamination.

The present study indicates that *V. vulnificus* and *V. parahaemolyticus* behave distinctly in tropical waters. While one species is strongly associated with fecal contamination the other is not. In addition, both species appear to be strongly influenced by some of the same environmental factors, but with opposite effects. While highest densities of *V. vulnificus* were obtained at low salinities, *V. parahaemolyticus* densities were greatest at high salinities. Phosphate and total phosphorus levels both were significantly correlated with densities of *V. vulnificus* and *V. parahaemolyticus*; however, like salinity these relationships were inverse. The differences observed indicate that these two organisms, although very similar, occupy clearly separate niches in the tropical aquatic ecosystems.

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