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Incidence of *Vibrio cholerae* and Related Vibrios in a Coastal Lagoon and Seawater Influenced by Lake Discharges along an Annual Cycle

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Most probable numbers of *Vibrio cholerae* and related vibrios were determined in Albufera Lake, Valencia, Spain, and in coastal waters under the influence of the lake discharges over the course of an annual cycle. The influence of temperature, kind of water, and characteristics of the different sampling sites on the numbers of vibrios recovered was evaluated. Maximum recovery of vibrios reached 10^3/ml in both types of waters analyzed. *V. cholerae* numbers reached 10^3/ml in the lake and 10^2 in one of the coastal sites. Frequently during the warm season, all vibrios isolated were identified as *V. cholerae*. Occasionally, no *V. cholerae* was recovered. The recovery of vibrios was significantly influenced by the temperature of the water and the type of water analyzed. Most of the *V. cholerae* isolates were included in Heiberg groups I and II, and nearly 50% of the strains used chitin as sole carbon source. Indole was not produced by 100% of the strains. All strains tested were non-O1 serovars.

The members of the family *Vibrionaceae* constitute a predominant heterotrophic bacterial group in aquatic environments (22). Among them, *Vibrio cholerae* has been isolated with increasing frequency from continental water, as well as from estuarine water and seawater (2, 4, 5, 11, 13, 15–17, 26), in many different geographic areas. Although the majority of the isolates are non-O1 serovars, there are recent descriptions of O1 isolates from natural waters (2, 5, 13, 19), along with many non-O1 strains. The ability of such environmental strains to produce enterotoxin has been extensively demonstrated by different assays (5, 6, 11, 18, 27). Presently, *V. cholerae* is considered a member of the autochthonous bacterial flora of these habitats (5, 9, 10, 11, 13), and its presence is not correlated with the commonly used coliforms as fecal indicators (8, 11). This lack of correlation and the fact that *V. cholerae* can survive longer than *Escherichia coli* in estuarine water and seawater (8) have important consequences from a public health standpoint for water and shellfish quality when that quality is assessed only on a basis of fecal coliforms, as is generally the case.

The objective of this study was to evaluate the incidence of *V. cholerae* and related vibrios in a lake and in coastal waters south of Valencia influenced by the lake discharges over the course of a year. The effect of water temperature, type of water, and load of vibrios discharged by the lake into the sea was also evaluated.

MATERIALS AND METHODS

Sampling. Four sampling sites with different characteristics were chosen at two communication channels between Albufera Lake and the Mediterranean Sea (Fig. 1). Albufera Lake is a very shallow hypereutrophic coastal lagoon, close to the sea, from which it is separated by a small litoral bar. Three channels connect the lake with the sea, and the water level, regulated for fishing and agricultural purposes (rice fields surround the lake), is controlled by means of gates

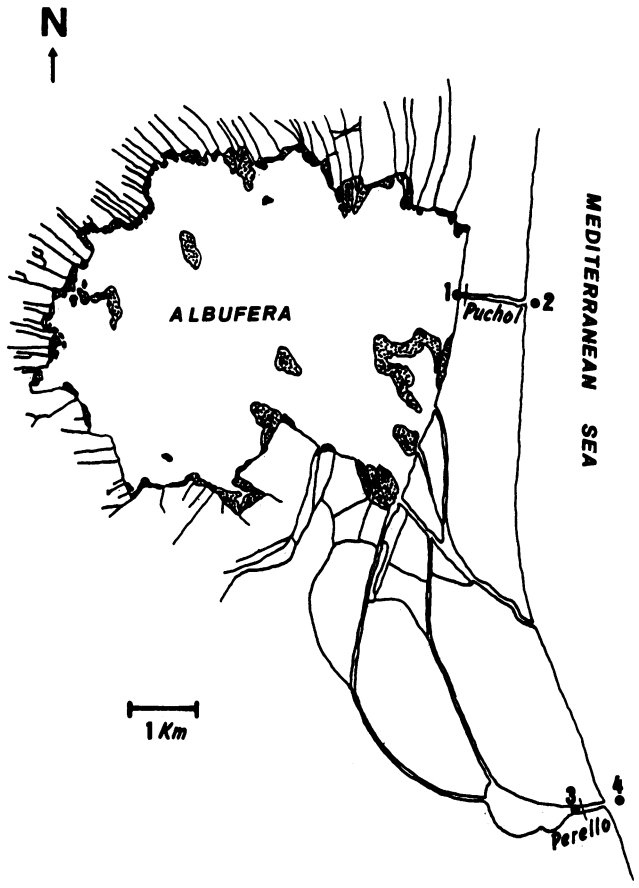

FIG. 1. Map of Albufera Lake and communication channels with the sea, indicating the sampling sites.

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TABLE 1. Physicochemical parameters for sites 1 and 2

Sampling event	Date	Temp (°C)		pH		Salinity (‰)		Gates[a]
		Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	
1	1/11/82	15	13	8.5	8	ND[b]	ND[b]	Open (1)
2	2/1/82	13	12	9.1	8.3	0.56	21.26	Open (3)
3	2/24/82	15	12	9.2	7.9	0.64	21.8	Open; B
4	5/3/82	20	18	8.2	8	0.7	32.7	Closed
5	6/1/82	23	22	9.1	8.2	ND	11.87	Open (4)
6	6/21/82	28	25	9	7.9	1.4	30.38	Closed
7	7/26/82	30.5	29	8.7	7.9	1.43	21.88	Closed
8	9/6/82	25.5	25	8.9	8.3	0.57	12.03	Open (6)
9	9/27/82	23	24	8.7	8	0.58	34.78	Open (3); B
10	12/7/82	10.5	14.5	8.2	8	0.34	28.5	Closed

[a] Values in parentheses represent the number of open gates. B, Sand bar between channel and sea.
[b] ND, Not determined.

located in each channel. The lake receives a heavy load of untreated agricultural, industrial, and urban effluents through more than 50 runaways and irrigation ditches.

Site 1 was located in the lake just before the gates of Puchol channel. Site 3 corresponded to lake water after a long way (7 to 8 Km) through several irrigation ditches and was located also just before the gates of Perelló channel, where a sporting pier has recently been developed. The urban effluents of Perelló village (1,000 to 3,000 inhabitants) also are discharged into this channel. Sites 2 and 4 were located in the sea under the influence of the Puchol and Perelló channel mouths, respectively.

Water samples were collected in 500-ml sterile screw-capped Pyrex bottles at ca. 15 cm below the surface. Enough air space was left in the bottles to allow thorough mixing. The samples were always processed within 3 h of collection. All samples were taken between 9 and 11 a.m. A total of 40 samples corresponding to 20 samplings were analyzed during 1982.

The samplings were organized to obtain an equal number of samples for each range of temperatures in the two channels (10 samplings below and 10 above 20°C). The sampling dates are shown (see Tables 1 and 2).

Environmental parameters. Water temperature, salinity, and pH were measured by using, respectively, a mercury-in-glass thermometer, a titration method (1), and a Crison 74 pH meter.

Bacteriological parameters. (i) Enumeration of *Vibrio* spp. Series of 100, 10, 1, and 0.1 ml of water as inocula were used for the most probable number (MPN) procedure. Single-strength alkaline peptone water was used for enrichment after incubation at 28°C for 18 h (1, 7, 13). Water (100 ml) was filtered through membrane filters (0.45 μm; Millipore Corp.), which were placed in flasks containing 100 ml of single-strength alkaline peptone water. Inocula (10 ml) were added to flasks containing 90 ml of single-strength peptone water. Finally, 1 and 0.1 ml of inocula were added to tubes containing, respectively, 9 and 9.9 ml of single-strength alkaline peptone water. After incubation, the tubes or flasks showing turbidity were spread onto Oxoid thiosulphate citrate bile salts sucrose agar and Monsur agar (7, 14) and incubated for 24 h at 35°C. The plates were examined for vibrio-like colonies, and at least three of each colony type present were subcultured onto tryptic soy agar (Difco Laboratories) for purification. The presumptive MPN obtained from the incubation in APW was confirmed biochemically on Kligler iron agar (Difco) and SIM medium (Difco) and by the oxidase test.

(ii) Enumeration and identification of *V. cholerae*. The MPN of *V. cholerae* was obtained from the confirmed *Vibrio* spp. by means of decarboxylase tests (arginine, lysine, ornithine) and growth in the absence of added NaCl: 1% (wt/vol) tryptone (Difco) and 0.3% (wt/vol) yeast extract (Difco). Two additional tests were performed on the *V. cholerae* confirmed strains: use of chitin as sole carbon source and fermentation of arabinose, mannose, and sucrose (Heiberg groups). For chitin utilization, the medium of Kaneko and Colwell (10) was used. Serological analysis was

TABLE 2. Physicochemical parameters for sites 3 and 4

Sampling event	Date	Temp (°C)		pH		Salinity (‰)		Gates[a]
		Site 3	Site 4	Site 3	Site 4	Site 3	Site 4	
1	1/25/82	10	11	8.3	8.1	0.47	8.27	Open (1)
2	2/8/82	12.5	13.5	8	7.9	0.6	28.09	Open (1)
3	5/17/82	20	19	7.8	7.4	0.8	ND[b]	Closed
4	6/14/82	25.5	24	8.3	7.9	ND[b]	ND[b]	Closed
5	7/7/82	29	27.5	7.4	7.4	1.37	12.79	Open (1)
6	8/2/82	27	28	7.9	7.9	ND[b]	13.45	Open (1)
7	9/13/82	26.5	25.5	7.4	7.5	2.5	14.03	Open (2)
8	9/29/82	23	22.5	8.1	8.3	0.81	21.98	Open (2)
9	11/23/82	14	16	7.9	8	0.27	30.22	Closed
10	12/13/82	11.5	13	7.9	7.9	0.24	27.51	Closed

[a] Values in parentheses represent the number of open gates.
[b] ND, Not determined.

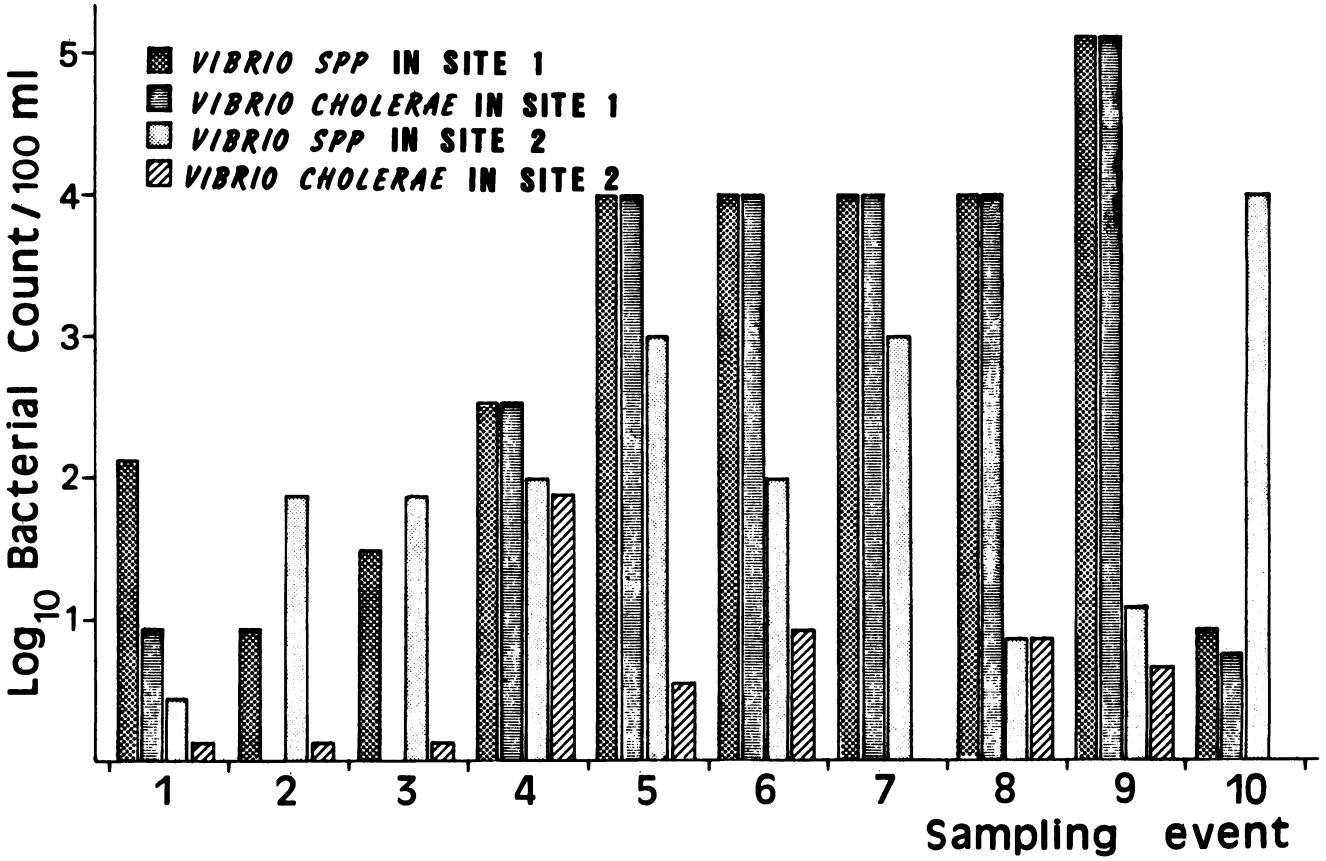

FIG. 2. MPNs of *Vibrio* spp. and *V. cholerae* in sites 1 and 2.

carried out by using polyvalent *V. cholerae* O1 antiserum kindly provided by H. Smith (Vibrio Reference Library, Philadelphia, Pa.).

An analysis of variance by using $\log_{10}$ transformed MPN was performed to learn the influence of the temperature, type of water analyzed, and characteristics of the sampling site on the recovery of *V. cholerae*.

The decrease of salinity at the sea sites was used to determine indirectly the load of *V. cholerae* discharged by the lake into the sea when the gates were open, since it was impossible to quantify the flux of water allowed to pass through each of the six gates located in the channels.

RESULTS AND DISCUSSION

This is the first report on the incidence of *V. cholerae* and related vibrios in Albufera Lake, Valencia, Spain, and in coastal waters under the influence of the lake discharges.

Vibrios are common inhabitants of aquatic environments, including estuarine water and seawater (4, 5, 13, 20–24, 26). In this work, *Vibrio* spp. and *V. cholerae* were isolated from all sites analyzed throughout the year, covering a salinity range from 0.24 to 34.78‰, a temperature range from 10°C (January) to 30.5°C (July), and a pH range from 7.4 to 9.2 (Tables 1 and 2).

The influence of temperature on the numbers of recoverable *V. cholerae* in water has been established in several studies (4, 19, 20, 23). In our study, all sites analyzed showed very similar evolution in regard to this parameter, which significantly influenced the recovery of *V. cholerae*. Although the effect of temperatures below 10°C could not be studied, since this is a temperate zone, temperatures above 20°C were clearly favorable for the recovery of *V. cholerae*. This finding is in complete accordance with similar studies by Seidler and Evans (20), who found the highest counts of this species at temperatures from 21 to 28°C.

The characteristics of the analyzed water also significantly influenced the recovery of *V. cholerae*. Figures 2 and 3 show the incidence of *Vibrio* spp. and *V. cholerae* at the four sites. Although maximum *Vibrio* numbers reached 10^5/100 ml both in the lake and the sea, their levels were usually higher in the former, with very few exceptions. From the two sampling points located in the sea, site 4, more influenced by the lake discharges, showed higher levels of *Vibrio* spp. and *V. cholerae* than site 2.

The pH values for sites 3 and 4 were very similar, always between 7.4 and 8.2. At site 1 the pH values corresponded to the typical alkaline ones of an eutrophic lake and ranged from 8.2 to 9.2. Site 2, less influenced by the lake, showed pH values between 7.9 and 8.3. All these values were most adequate for the recovery of vibrios.

The salinity has also a marked influence on the recovery of *V. cholerae* from aquatic environments (8, 11, 20, 23, 24). In our study, the salinity at sites 1 and 3 (lake water) varied only from 0.2 to 1.43‰. On the other hand, the sea sites (2 and 4) showed great fluctuations, from 8.27 to 34.78‰, which reflected the load of lake water discharged into the sea by open gates (Tables 1 and 2). *V. cholerae* was isolated over the entire salinity range. Site 2 showed higher salinities and lower *V. cholerae* numbers (Table 1; Fig. 2) than site 4, markedly influenced by the discharges of Perello channel,

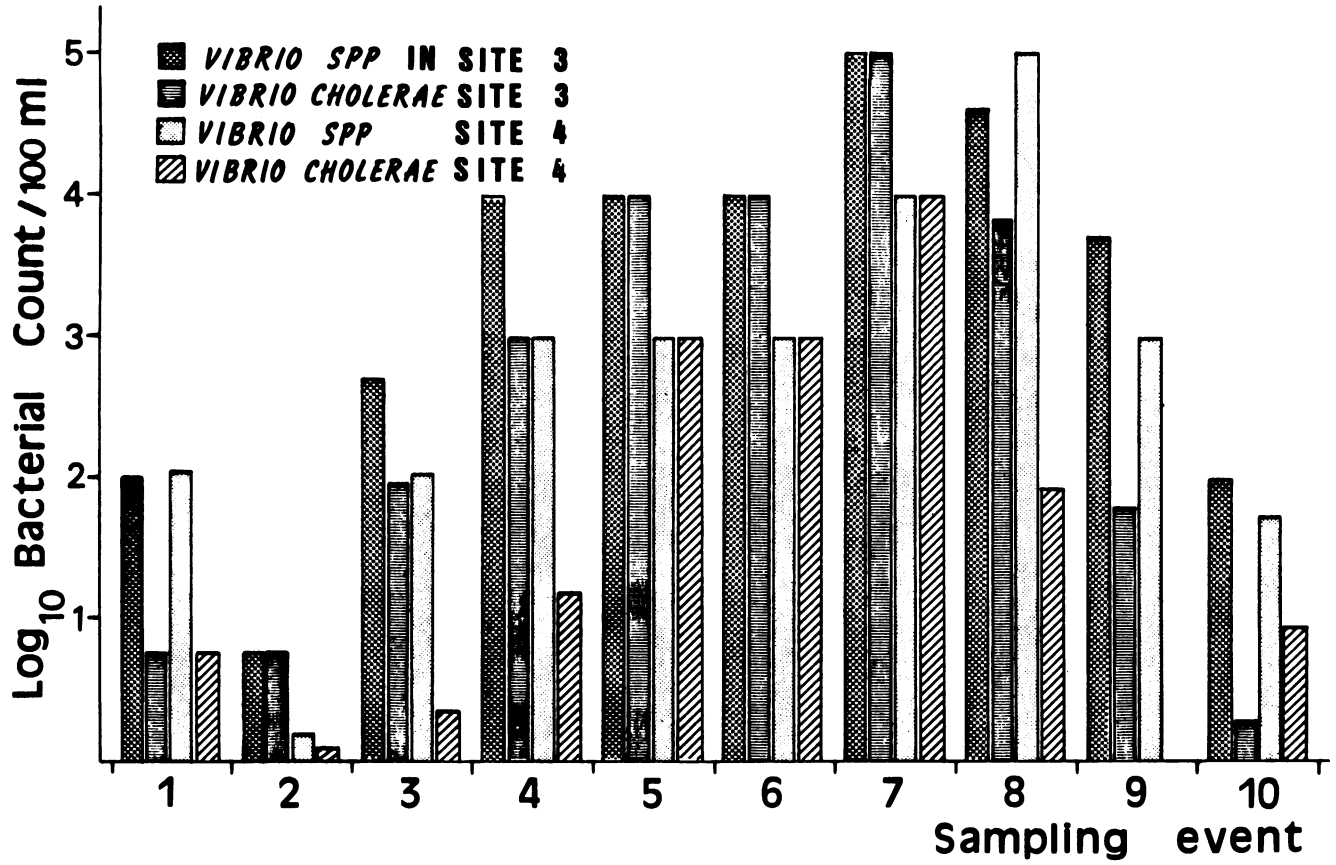

FIG. 3. MPNs of *Vibrio* spp. and *V. cholerae* in sites 3 and 4.

which is periodically dredged to maintain an adequate depth for boating purposes (Table 2; Fig. 3). Although the elevated *Vibrio* counts at lower salinities correlated with the highest incidence of cholera isolations ($P < 0.001$), this species was recovered at salinities as high as 32.7‰ and 34.78‰, with counts around 100/100 ml and 7/100 ml, respectively (Table 1; Fig. 2). Similar studies found much lower levels of *V. cholerae* at these salinities (20).

As the decrease of salinity at the sea sites was used to determine indirectly the load of *V. cholerae* discharged into the sea, these results and the higher numbers of this species found always in the lake water indicate the periodical seeding of *V. cholerae* from Albufera Lake into the Mediterranean Sea.

A total of 420 selected isolates were identified as *V. cholerae* on the basis of biochemical characterizations. Their numbers in the lake reached 10^5/100 ml in sites 1 and 3 and only 100/100 ml and 10^4/100 ml in sites 2 and 4, respectively (Fig. 2 and 3). Occasionally no *V. cholerae* could be recovered with the methodology employed. Low numbers always corresponded to low water temperatures in all sites. In the lake water, especially during the warm season, all vibrios selected from the plates were very often identified as *V. cholerae*.

Indole production was shown by only 79% of the lake water strains and by 68% of the seawater strains. Most of the isolates were identified as Heiberg groups I and II, and a very low percentage were included in the rest of the groups (III, IV, V, and VI). In our study, there was a poor reproductibility of groupings of strains based on the Heiberg groups, except for the sucrose results. This finding is in agreement with recent extensive phenotypic characterizations (12). These groupings were useful in the past but do not seem to be very reliable for the future.

Chitin as sole carbon source was used by 24.5% of the strains from site 1, 59.5% from site 2, 49% from site 3, and 58% from site 4. The fact that nearly 50% of the *V. cholerae* strains produced chitinase points towards an extrahuman ecological niche associated with chitinous organisms. Studies are in progress to evaluate the incidence of zooplankton-associated vibrios in the same environment.

In a first screening, with Difco antisera, a few O1 strains were found (2.5%). After confirmation with the polyvalent *V. cholerae* O1 antiserum provided by H. Smith, none of these strains showed agglutination. Of the strains, 26% were autoagglutinable. As is generally recognized, the overwhelming majority of the environmental *V. cholerae* strains are non-O1 (11, 13, 15–17). No taxonomic separation is presently made in this species on the basis on serology, confirmed by DNA/DNA hybridization (3, 12, 25).

The results obtained in this study and in previous studies covering other lake zones (unpublished data) revealed a constant presence of *V. cholerae* in Albufera Lake near Valencia throughout the year, with high levels of this pathogenic species during the warm season (June to September). The many different uses of the lake water (fishing, recreational, and irrigation purposes) and the fact that these microorganisms are periodically discharged into the Mediterranean Sea, where the salinity is not a limiting factor as it is for coliforms, represent a constant risk for the exposed

population. Further studies will investigate the possible enterotoxigenicity of these environmental non-O1 strains.

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