

## Predictability of *Vibrio cholerae* in Chesapeake Bay

Valérie R. Louis,<sup>1</sup> Estelle Russek-Cohen,<sup>2</sup> Nipa Choopun,<sup>1</sup> Irma N. G. Rivera,<sup>1,3</sup> Brian Gangle,<sup>1</sup>  
Sunny C. Jiang,<sup>4</sup> Andrea Rubin,<sup>3</sup> Jonathan A. Patz,<sup>5</sup> Anwar Huq,<sup>1</sup> and Rita R. Colwell<sup>1,6\*</sup>

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202<sup>1</sup>; Department of Animal and Avian Sciences<sup>2</sup> and Department of Cell Biology and Molecular Genetics,<sup>6</sup> University of Maryland, College Park, Maryland 20742; Microbiology Department, Biomedical Science Institute, University of São Paulo, CEP 05508-900 São Paulo, São Paulo, Brazil<sup>3</sup>; Environmental Analysis and Design, University of California, Irvine, California 92697<sup>4</sup>; and Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205<sup>5</sup>

Received 22 August 2002/Accepted 5 February 2003

***Vibrio cholerae* is autochthonous to natural waters and can pose a health risk when it is consumed via untreated water or contaminated shellfish. The correlation between the occurrence of *V. cholerae* in Chesapeake Bay and environmental factors was investigated over a 3-year period. Water and plankton samples were collected monthly from five shore sampling sites in northern Chesapeake Bay (January 1998 to February 2000) and from research cruise stations on a north-south transect (summers of 1999 and 2000). Enrichment was used to detect culturable *V. cholerae*, and 21.1% ( $n = 427$ ) of the samples were positive. As determined by serology tests, the isolates, did not belong to serogroup O1 or O139 associated with cholera epidemics. A direct fluorescent-antibody assay was used to detect *V. cholerae* O1, and 23.8% ( $n = 412$ ) of the samples were positive. *V. cholerae* was more frequently detected during the warmer months and in northern Chesapeake Bay, where the salinity is lower. Statistical models successfully predicted the presence of *V. cholerae* as a function of water temperature and salinity. Temperatures above 19°C and salinities between 2 and 14 ppt yielded at least a fourfold increase in the number of detectable *V. cholerae*. The results suggest that salinity variation in Chesapeake Bay or other parameters associated with Susquehanna River inflow contribute to the variability in the occurrence of *V. cholerae* and that salinity is a useful indicator. Under scenarios of global climate change, increased climate variability, accompanied by higher stream flow rates and warmer temperatures, could favor conditions that increase the occurrence of *V. cholerae* in Chesapeake Bay.**

*Vibrio cholerae* is both the causative agent of cholera and a natural inhabitant of the aquatic environment. Nearly 200 *V. cholerae* serogroups have been identified to date (70), but only two serogroups, serogroups O1 and O139, are associated with epidemic cholera. *V. cholerae* was first isolated from the Chesapeake Bay in the 1970s and was suggested to be an autochthonous member of the aquatic environment (17). Further studies demonstrated clearly that *V. cholerae* is, in fact, autochthonous to the Chesapeake Bay and to the aquatic environment in general (15, 18, 40). *V. cholerae* has since been detected in natural waters worldwide, including areas where clinical cases of cholera did not exist (32, 38, 43, 69). These studies showed that the majority of environmental isolates of *V. cholerae* are members of non-O1, non-O139 serogroups. However, various non-O1, non-O139 *V. cholerae* strains have repeatedly been isolated from patients with diarrhea (20, 59) and have shown a capacity to provoke localized diarrheal outbreaks (2, 19, 53, 56).

Colwell (15) proposed that the natural aquatic environment serves as the reservoir for *V. cholerae* and that it may play a critical role in pandemics of cholera. Horizontal gene transfer, which has been demonstrated in *V. cholerae* by Waldor and Mekalanos (66), has been proposed as a mechanism for the emergence of new pathogenic strains (4), and recent studies

have confirmed such mechanisms (23). Environmental isolates of *V. cholerae* have been shown to harbor genotypes associated with virulence (10, 54), thus supporting the hypothesis that the natural population of *V. cholerae*, including *V. cholerae* non-O1, non-O139 isolates, can serve as a precursor for new pathogenic or epidemic strains. *V. cholerae* can pose a public health risk ranging from limited outbreaks to epidemics when it is ingested via untreated water or contaminated shellfish (49, 51). Because of this inherent risk, it is relevant to understand the mechanisms that affect the natural population of *V. cholerae* in the environment. Studies in coastal and estuarine regions of different parts of the world have shown that temperature and salinity play roles in the occurrence of *V. cholerae* in the environment (3, 39, 65). The objective of the study reported here was to model the occurrence of *V. cholerae* in the Chesapeake Bay as a function of environmental parameters. In particular, the occurrence of *V. cholerae* was investigated with respect to salinity, temperature, association with plankton, the Susquehanna River freshwater influx, and seasonality in order to understand how changing climate conditions might affect the ecology of *V. cholerae* in the environment.

### MATERIALS AND METHODS

**Shore sampling sites.** Sampling was done from January 1998 to February 2000 at coastal sites throughout the upper Chesapeake Bay (Fig. 1). Samples were collected from surface water, about 1 m from the surface. Station F (Susquehanna Flats) is located at the northern tip of the bay at the mouth of the Susquehanna River, where the water salinity is <0.5 ppt. Other sampling sites are located in mesohaline water with salinities ranging from 2 to 15 ppt. Station

\* Corresponding author. Mailing address: Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt St., Baltimore, MD 21202. Phone: (703) 292-8000. Fax: (703) 292-9232. E-mail: colwell@umbi.umd.edu.

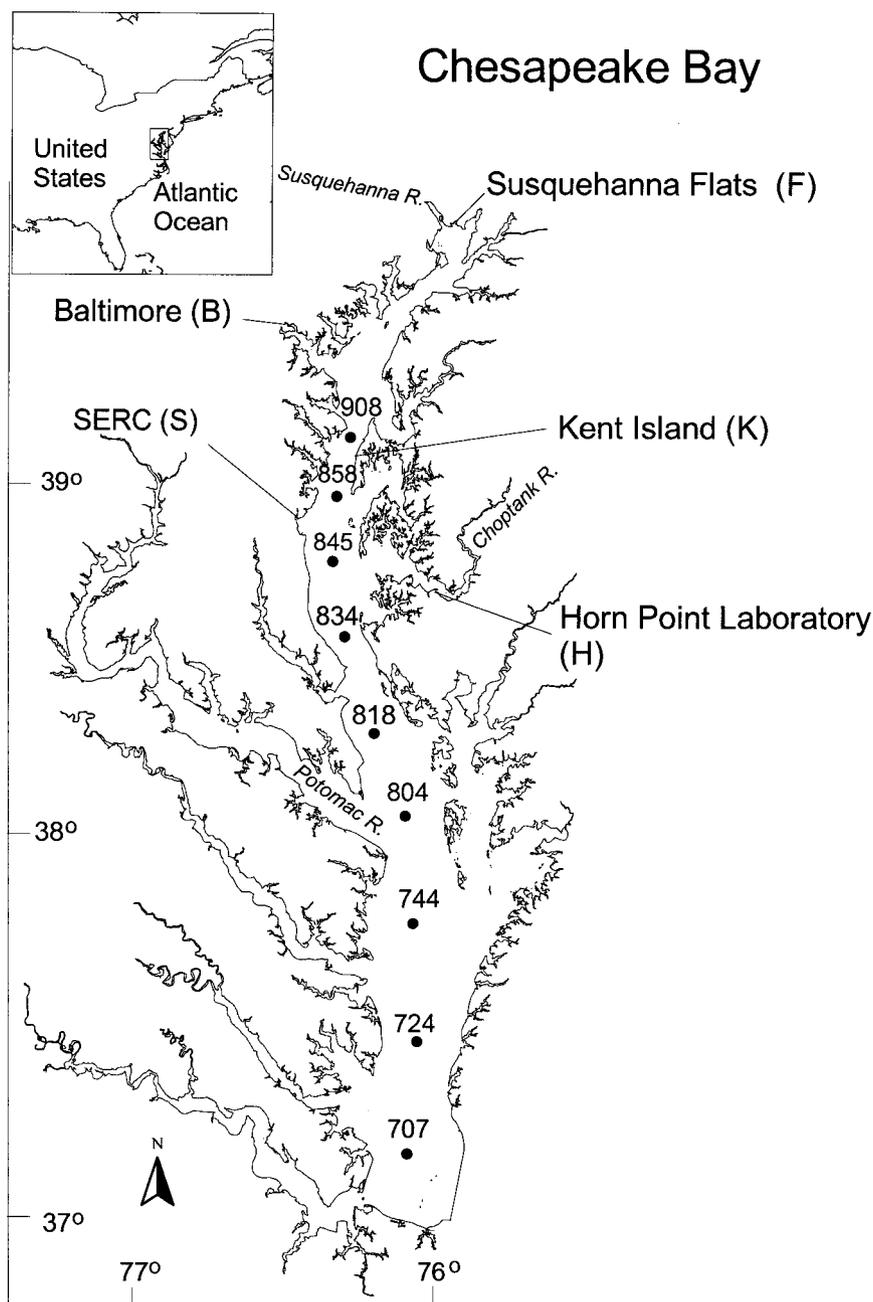


FIG. 1. Sampling sites in Chesapeake Bay. The near-shore sampling sites are as follows: site F, Susquehanna River Flats ( $39^{\circ}33.13'N$ ,  $76^{\circ}02.20'W$ ); site B, Baltimore ( $39^{\circ}17.00'N$ ,  $76^{\circ}36.32'W$ ); site K, Kent Island ( $38^{\circ}58.84'N$ ,  $76^{\circ}20.13'W$ ); site S, Smithsonian Environmental Research Center (SERC) ( $38^{\circ}53.20'N$ ,  $76^{\circ}32.51'W$ ); and site H, Horn Point Laboratory ( $38^{\circ}35.59'N$ ,  $76^{\circ}07.80'W$ ). Cruise sampling sites on a north-south transect were designated as follows: site 908 ( $39^{\circ}08.00'N$ ,  $76^{\circ}20.00'W$ ), site 858 ( $38^{\circ}58'N$ ,  $76^{\circ}23'W$ ), site 845 ( $38^{\circ}45.00'N$ ,  $76^{\circ}26.00'W$ ), site 834 ( $38^{\circ}34.00'N$ ,  $76^{\circ}26.00'W$ ), site 818 ( $38^{\circ}18.00'N$ ,  $76^{\circ}17.00'W$ ), site 804 ( $38^{\circ}04.00'N$ ,  $76^{\circ}13.00'W$ ), site 744 ( $37^{\circ}44.00'N$ ,  $76^{\circ}11.00'W$ ), site 724 ( $37^{\circ}24.00'N$ ,  $76^{\circ}05.00'W$ ), and site 707 ( $37^{\circ}07.00'N$ ,  $76^{\circ}07.00'W$ ).

H, on the eastern shore, is at the Horn Point Laboratory on the Choptank River. Station K is on Kent Island at an open water point of the main stem of Chesapeake Bay. On the western shore, station S is located at the Smithsonian Environmental Research Center on the Rhode River. In January 1999, a site in the inner harbor of Baltimore (station B) was added to the study. Plankton samples could not be collected in January 2000 except in Baltimore harbor, because the sampling sites in Chesapeake Bay were frozen.

Sampling was accomplished, in most cases, within a 2-day period and was done twice monthly during the summer (June, July, August) and monthly the rest of the year. During the entire study, a total of 32 sampling trips were accomplished over 26 months. The seasons were defined as follows: winter, December to

February; spring, March to May; summer, June to August; and autumn, September to November.

**Cruise sampling.** Additional sampling was carried out aboard the research vessel *Cape Henlopen* in August and September 1999 and in June and August 2000. Nine stations on a north-south transect from the upper bay to the mouth of the bay (Fig. 1) were sampled on each cruise within 2 days. Temperature and salinity were recorded by using on-board instruments. Plankton were collected by using a plankton net on a 10-m vertical tow. In 1999 three fractions, water, P20, and P64 (as described below), were collected, and in 2000 two fractions, water and P64, were collected. The enrichment procedure (see below) was used to detect *V. cholerae*.

**Sample collection.** One surface water fraction (fraction W) and two plankton fractions were collected in sterile plastic containers at each of the shore sampling sites. To collect plankton samples, simple 20- and 64- $\mu$ m-mesh, 30-cm-diameter, three-point bridle plankton nets fitted with a polyvinyl chloride collecting bucket (Aquatic Research Instrument, Lehmi, Idaho) were used. The small-plankton fraction (P20) was comprised of phytoplankton and small microzooplankton that passed through the 64- $\mu$ m-mesh net and were captured by the 20- $\mu$ m-mesh net. The large-plankton fraction (P64) included microzooplankton captured by the 64- $\mu$ m-mesh net. Plankton were collected either by towing or by pumping water for 5 min (ca. 500 liters) through the nets. The two methods were used in parallel in the field for 1 month. When the total numbers of bacteria were compared by the method of Bland and Altman (5), the two methods were found to be not significantly different ( $P > 0.55$ ).

**Sample processing.** In the field, 20 ml of each water sample and 10 ml of each concentrated plankton sample were fixed with 2% (final concentration) formaldehyde (Fisher Scientific, Pittsburgh, Pa.) to obtain total bacterial direct counts and for plankton characterization, respectively. Samples for other assays (see below) were transported at the ambient temperature to the laboratory and processed within 6 h of collection.

**Environmental parameters.** Surface water temperature and salinity were measured at the sites by using a field thermometer and a refractometer (American Optical Corporation, Keene, N.H.), respectively. Immediately upon return to the laboratory, the pH was measured with a digital pH/millivolt meter (Orion Research, Inc., Beverly, Calif.).

**Precipitation and Susquehanna River flow.** Daily precipitation data were obtained from the National Climate Data Center (<http://wlf.ncdc.noaa.gov/oa/ncdc.html>) for the weather stations operating closest to the sampling sites during the study, as follows: Conowingo Dam (station 182060; 39°39'N, 76°11'W) for sampling site F, Baltimore-Washington International Airport (station 180465; 39°10'N, 76°41'W) (from January 1998 to April 1998) and Baltimore City (station 185718; 39°17'N, 76°37'W) (from May 1998 to March 2000) for sampling site B, and Annapolis (station 180193; 39°00'N, 76°31'W) for sampling sites S and K. For sampling site H, daily precipitation values were obtained from the rain gauge data of the Horn Point weather station (38°35.4'N, 76°08.0'W) operated by the Chesapeake Bay Observing System ([www.cbos.org](http://www.cbos.org)). Daily Susquehanna River flow data were provided by the U.S. Geological Survey monitoring station at the Conowingo Dam Hydroelectric Power Plant (39°39'N, 76°10'W) ([http://water.usgs.gov/md/nwis/discharge/?site\\_no=01578310](http://water.usgs.gov/md/nwis/discharge/?site_no=01578310)). The station is located approximately 16 km from the river mouth and receives water from approximately 99% of the 71,250-km<sup>2</sup> Susquehanna River basin.

**Total bacterial direct counting.** Ten-milliliter portions of fixed water samples were incubated in the dark with 10  $\mu$ M (final concentration) DAPI (4',6-diamino-2-phenylindole) (Sigma, St. Louis, Mo.) at room temperature for 2 h. After staining, 1 to 3 ml of each sample was filtered through a 25-mm-diameter, 0.2- $\mu$ m-pore-size polycarbonate black membrane (K02BP02500; Osmonics, Inc., Minnetonka, Minn.) in triplicate. Filters were mounted on microscope slides by using low-fluorescence immersion oil (type A or FF) and were examined by using a  $\times 100$  oil immersion lens on a fluorescent microscope fitted with a 420-nm UV filter. A minimum of 400 cells or 10 microscope fields were counted, as recommended by Kepner and Pratt (44). Cell concentration was computed as follows: number of cells per milliliter = (average number of cells per square)  $\times$  (number squares per filter)  $\times$  (dilution factor)/sample volume.

**Plankton identification.** Zooplankton analysis was done for formalin-fixed P64 fractions collected from June 1998 to February 2000 by Nuruddin Mahmood at the Institute of Marine Biology, University of Chittagong, Chittagong, Bangladesh. Organisms were identified to the level of order for adult crustaceans and to the level of subclass for nauplii. For statistical analysis the zooplankton data were log transformed ( $\log[x + 1]$ ) and grouped in the following categories: copepod nauplii, copepodites, adult calanoid copepods, adult cyclopoid copepods, adult harpacticoid copepods, rotifers, barnacle nauplii, and others (cladocerans, polychaetes, ostracods, oligochaetes, amphipods, nematodes, insect larvae, and cumaceans), which when combined represented less than 2.5% of the total number.

**Enrichment, culture, and molecular probes.** *V. cholerae* was isolated by using the alkaline peptone water enrichment procedure described elsewhere (12). Briefly, 250-ml water samples or 25-ml plankton samples were filtered with a 0.22- $\mu$ m-pore-size Supor-200 membrane (Gelman Laboratories, Ann Arbor, Mich.) and incubated for 6 to 18 h at 30°C in 100 ml of alkaline peptone water (1% [wt/vol] peptone, 1% [wt/vol] sodium chloride; pH 8.6). Two loopfuls from each broth culture were streaked onto thiosulfate citrate bile salt sucrose agar (Oxoid Ltd., Basingstoke, England) in triplicate. After overnight incubation at 37°C, presumptive *V. cholerae* colonies were subcultured on Luria-Bertani agar (Difco, Detroit, Mich.) containing 2% (wt/vol) NaCl, a nonselective medium.

TABLE 1. Sequences of oligonucleotides used for molecular analysis

Sequence	Nucleic acid sequence	Amplicon size (bp)
<i>V. cholerae</i> PCR primer <sup>a</sup>	5'-TTA AGC STT TTC RCT GAG AAT G-3' 5'-AGT CAC TTA ACC ATA CAA CCC G-3'	295-310 295-310
<i>V. cholerae</i> O1 PCR primer <sup>b</sup>	5'-CAA CAG AAT AGA CTC AAG AA-3' 5'-TAT CTT CTG ATA CTT TTC TAC-3'	647 647
<i>V. cholerae</i> O139 PCR primer <sup>b</sup>	5'-TTA CCA GTC TAC ATT GCC-3' 5'-CGT TTC GGT AGT TTT TCT GG-3'	741 741
<i>V. cholerae</i> hybridization probe <sup>c</sup>	5'-GCS TTT TCG CTG AGA ATG-3'	

<sup>a</sup> Data from reference 13.

<sup>b</sup> Data from studies of Rivera et al. (I.N.G. Rivera, A. Huq, and R. R. Colwell, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. Q47, 2000; I.N.G. Rivera, E. Lipp, A. Gil, N. Chooopun, A. Huq, and R. R. Colwell, submitted for publication).

<sup>c</sup> Data from a study of Grim et al. (C. J. Grim, I. N. G. Rivera, N. Chooopun, J. Chun, A. Huq, and R. R. Colwell, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. Q33, 2000).

Isolates were screened by using a rapid biochemical test (12) that selected for colonies which were negative for both arginine dihydrolase activity and esculin hydrolysis. Presumptive *V. cholerae* isolates were confirmed by PCR by using *V. cholerae*-specific primers based on the 16S-23S rRNA intergenic spacer regions (13) (Table 1). Alternatively, presumptive colonies were dot blotted onto Luria-Bertani agar, transferred to filter paper (68), and hybridized with a <sup>32</sup>P-labeled *V. cholerae* probe (Table 1). Positive colonies were identified as dark spots by autoradiography. *V. cholerae* O1 and *Vibrio mimicus* were grown on the same petri dish and processed along with presumptive colonies, serving as positive and negative controls, respectively. Enrichment data were collected from January to August 1998 and from March 1999 to February 2000.

**Serology and identification of the O1 and O139 serogroups.** *V. cholerae* isolates were examined to determine if they were members of the O1 and O139 serogroups by slide agglutination by using a polyclonal antibody specific for O1 surface antigen and a monoclonal antibody specific for O139 surface antigen (Centre for Health and Population Research, Dhaka, Bangladesh), respectively. A saline solution was used as a control to identify self-agglutinating isolates that could not be tested by this method. Self-agglutinating strains were tested by PCR by using O1- and O139-specific primers (Table 1).

**DFA O1.** *V. cholerae* O1 was detected by using a direct fluorescent-antibody assay and direct viable counting for *V. cholerae* serogroup O1 (DFA O1) as described elsewhere (9). Briefly, water and plankton samples were incubated overnight at 30°C with 0.002% nalidixic acid and 0.025% yeast extract with shaking (45). Samples were homogenized with a glass tissue grinder, fixed with 2% formaldehyde, and stored at room temperature until they were processed. Samples were processed according to the DFA O1 kit's instructions; 10- $\mu$ l portions of samples were air dried on a microscope slide, fixed with ethanol, and labeled with a *V. cholerae* DFA reagent specific for *V. cholerae* O1 (Cholera DFA; New Horizons Diagnostics Corporation, Columbia, Md.). Slides were examined at a magnification of  $\times 1,000$  by using an epifluorescence microscope (Olympus model AH-2) and an HBO 200W/2 mercury lamp (OSRAM, Munich, Germany).

**Chlorophyll *a* determination.** Water samples (25 ml, in triplicate) were filtered with 47-mm-diameter glass microfiber filters (GF/F; Whatman International Ltd., Maidstone, England). The filters were wrapped in aluminum foil and frozen immediately at  $-80^{\circ}$ C. For extraction of the chlorophyll *a* pigment, the frozen filters were placed in 10 ml of methanol at room temperature for at least 6 h in the dark. The supernatants were transferred to 15-ml centrifuge tubes and centrifuged at 1,200  $\times g$  for 15 min. Fluorescence measurements were obtained with a luminescence spectrophotometer (Perkin-Elmer LS-5B) with the excitation and emission wavelengths set at 663 and 430 nm, respectively. By employing methods described by Holm-Hansen and Rieman (31), fluorescence was measured before and after acidification with 0.1 ml of 0.1 N HCl. Pure chlorophyll *a* extracted from algae (C-6144; Sigma) was used to establish a standard curve. Concentrations of the standard were determined by spectrophotometry (63) by using the specific absorption coefficient for methanol ( $\alpha = 79.95$  liters  $g^{-1} cm^{-1}$ ) (37, 52). Corrected chlorophyll *a* concentrations were computed as described elsewhere (1).

TABLE 2. Occurrence of *V. cholerae* in three sample fractions (water, small plankton, and large plankton) as determined by the enrichment and DFA O1 methods

Season	Enrichment method						DFA O1 method							
	Site F		Site B		Sites K, S, and H		Cruise sites		Site F		Site B		Sites K, S, and H	
	% Positive <sup>a</sup>	n <sup>b</sup>	% Positive	n	% Positive	n	% Positive	n	% Positive	n	% Positive	n	% Positive	n
Winter 1998	0.0	6	ND <sup>c</sup>		0.0	18	ND		0.0	6	ND		0.0	18
Spring 1998	11.1	9	ND		29.6	27	ND		22.2	9	ND		22.2	27
Summer 1998	6.7	15	ND		97.8	45	ND		13.3	15	ND		75.0	44
Autumn 1998	ND		ND		ND		ND		22.2	9	ND		7.4	27
Winter 1999	ND		ND		ND		ND		0.0	9	0.0	5	7.4	27
Spring 1999	0.0	9	22.2	9	0.0	27	ND		0.0	9	0.0	9	26.9	26
Summer 1999	27.8	18	16.7	18	11.1	54	0.0	27	16.7	18	66.7	18	20.4	54
Autumn 1999	0.0	9	22.2	9	7.4	27	18.5	27	0.0	9	77.8	9	22.2	27
Winter 2000	14.3	7	0.0	9	4.8	21	ND		14.3	7	22.2	9	0.0	21
Summer 2000	ND		ND		ND		23.7	36	ND		ND		ND	
Total	11.0	73	15.6	45	27.9	219	15.2	90	11.0	91	42.0	50	24.7	271

<sup>a</sup> Percentage of positive samples.

<sup>b</sup> n is the number of samples collected.

<sup>c</sup> ND, not determined.

**Statistical analyses.** Because the methods used for detection of *V. cholerae* were not quantitative, the statistical analysis was done by using the presence or absence of *V. cholerae* in each sample. Logistic regression (PROC LOGISTIC from the statistical software package SAS, version 8.2; SAS Institute Inc., Cary, N.C.) was used with the presence or absence of *V. cholerae* as the response of interest and provided an odds ratio value. A stepwise selection process was used, in which descriptive variables were entered in turn into the model. Variables remained in the model only if they were significant at each step, when a new variable was added. Two analyses were done; one corresponded to the presence or absence of *V. cholerae* for the water fraction, and the other corresponded to the presence or absence of *V. cholerae* for the combined plankton fraction. In the latter case, the combined plankton sample was considered positive when either plankton fraction (P20 or P64) was positive. A response surface, as described by Carter et al. (7), was used for the analysis with temperature and salinity. The cross-product term between salinity and temperature indicates an interaction, and the cross-product with the same variable indicates a quadratic effect. Cochran's Q statistic for proportions (25) was used to test for significance between proportions in the three sampling fractions (water and two plankton fractions). Binary classification trees were computed by using S-Plus, version 6.0 (Insightful Corp., Seattle, Wash.), as a nonparametric alternative to logistic regression (21). For both the logistics regression and the binary classification tree, model agreement was evaluated by determining whether the model correctly predicted the presence or absence of *V. cholerae* for each sample used in the analysis. Throughout the analysis, a difference was considered significant if the *P* value was <0.05.

## RESULTS

**Detection of *V. cholerae*.** The enrichment method used in this study detects culturable *V. cholerae*, and the DFA O1 method was used to detect both culturable and nonculturable cells of the O1 serogroup. The two methods were in agreement 76% of the time; i.e., samples yielded the same results (both positive or both negative) with both methods. When the two methods did not agree, in 60% of the cases samples that tested positive by DFA O1 were negative as determined by enrichment. Overall, 21.1% of the samples (*n* = 427) were positive for *V. cholerae* as determined by enrichment, including 22.6% of the shore samples (*n* = 337) and 15.6% of the cruise samples (*n* = 90); 23.8% of the shore samples (*n* = 412) were positive for *V. cholerae* as determined by DFA O1 (Table 2). The proportion of positive samples at the freshwater site (sampling site F) was 11.0% for both the enrichment (*n* = 73) and DFA O1 (*n* = 91) analyses. DFA O1-positive samples and culturable *V. cholerae* isolates were obtained at each of the five shore sampling sites.

Combined shore and cruise samples yielded 361 non-O1, non-O139 *V. cholerae* isolates obtained by enrichment. All isolates were tested by PCR with primers for *V. cholerae* (i.e., primers for identifying *V. cholerae* regardless of the serogroup). All isolates were tested by serology to determine whether they were O1 and O139 isolates. However, the self-agglutinating strains (which could not be analyzed by serology) were tested by PCR to determine whether they were O1 and O139 isolates (see Materials and Methods).

The data obtained for shore samples by both the enrichment and DFA O1 methods showed that there was an increased frequency of positive samples during the warmer months (Fig. 2). The enrichment and DFA O1 data showed similar patterns in 1998, whereas the seasonal pattern was not as marked for enrichment data in 1999. A smaller proportion of the samples was positive in 1999 than in 1998, and the reduction was greater when the enrichment method was used than when the DFA O1 method was used (Fig. 2). The enrichment method is not quantitative. However, given a consistent sampling effort, there was a striking difference between the number of isolates collected in 1998 and the number of isolates collected in 1999 for the brackish near-shore samples. At sampling sites K, S, and H, the yield of *V. cholerae* isolates (i.e., the number of isolates) in 1999 was only approximately 5% of that in 1998 (17 and 289 isolates, respectively). As determined by enrichment, the proportion of positive samples decreased from 97.8% in summer 1998 to 11.12% in summer 1999, and as determined by DFA O1 the proportion of positive samples decreased from 75.0 to 20.4% (Table 2). In 1999, sampling site B yielded the largest number of isolates and the highest proportion of positive samples compared to the other shore sites, as determined by both enrichment (17.9% versus 2.6 to 12.8%) and DFA O1 (45.5% versus 8.9 to 35.6%). Sampling site F yielded approximately one-tenth the number of isolates that the other shore sites yielded in 1998. However, the number of isolates at this site was nearly constant in 1998 and 1999, and the proportion of positive samples, unlike the proportion of positive samples at the other shore sites, increased in summer 1999 compared to summer 1998, as determined by both detection methods (Table

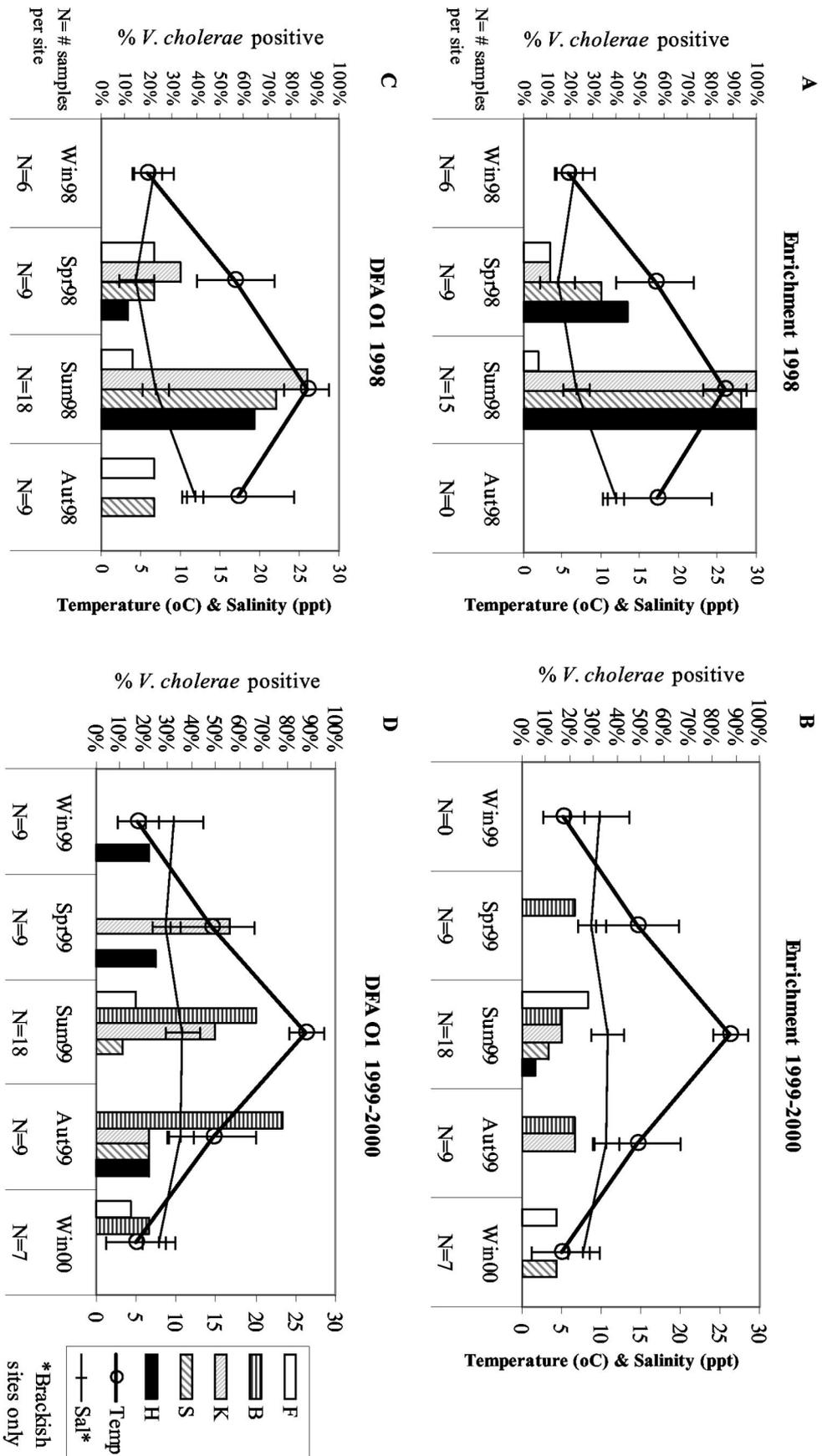


FIG. 2. Percentages of positive samples at the shore sampling sites, by season and by site, as determined by enrichment culturing (A and B) and DFA OI analysis (C and D). The 1998 samples (A and C) included samples from sites F, K, S, and H, and the 1999 and 2000 samples (B and D) included samples from sites F, B, K, S, and H. The average seasonal temperature and salinity are indicated on the right axis. Win, winter; Spr, spring; Sum, summer; Aut, autumn.

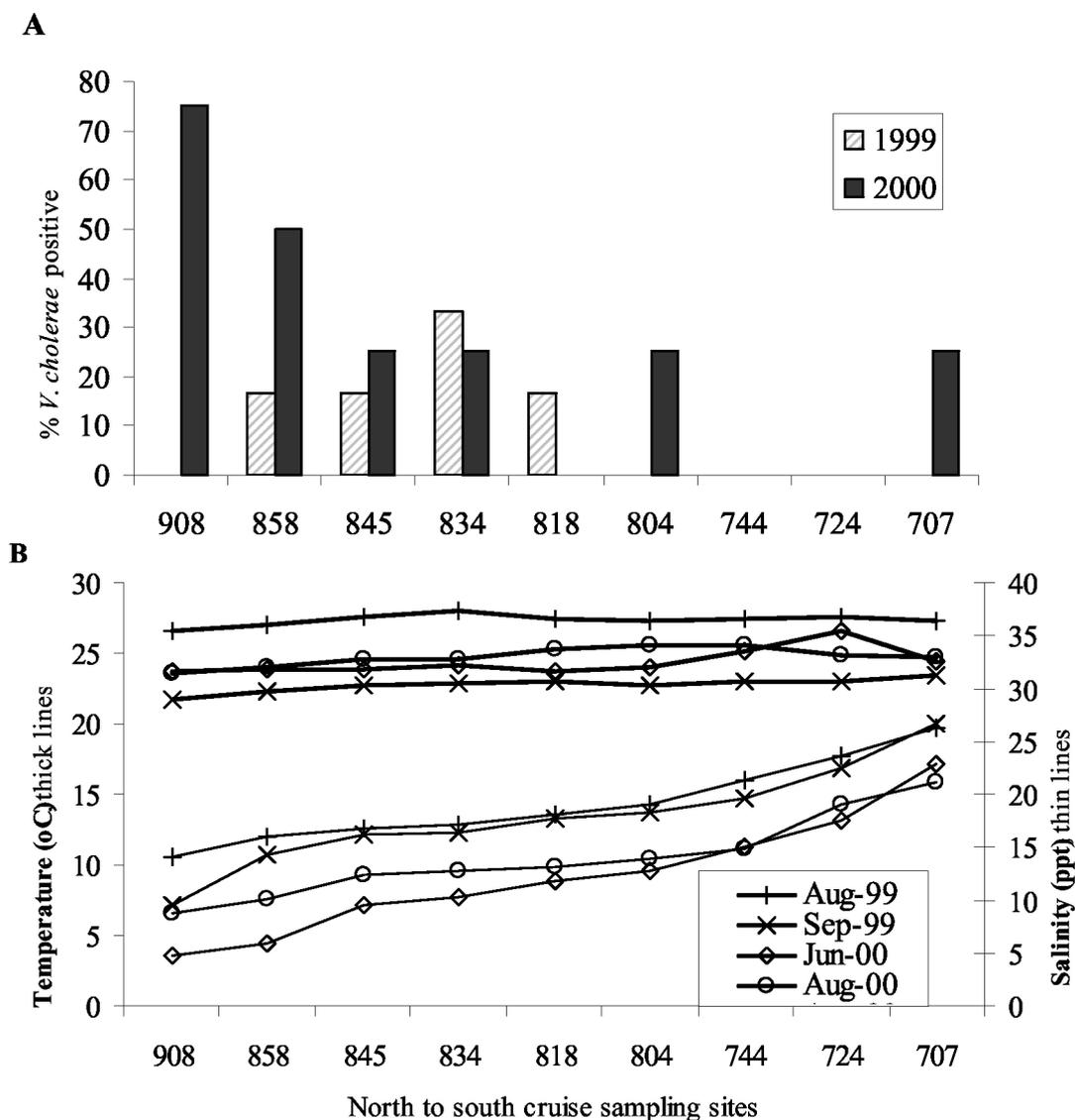


FIG. 3. Results obtained at the cruise sampling sites along a north-south transect. (A) Percentage of *V. cholerae*-positive samples. (B) Temperature and salinity.

2). Furthermore, for site F the typical seasonal pattern observed at the other shore sites was not clearly visible (Fig. 2). For these reasons and because the logistic regression model for freshwater appeared to differ substantially from that for the brackish sites (data not shown), the samples from the freshwater sampling sites were analyzed separately from those from the brackish sites.

For cruise samples, culturable *V. cholerae* isolates were obtained from seven of the nine sampling sites along the north-south transect of Chesapeake Bay (Fig. 3A). In 1999 the percentage of positive samples was significantly lower than the percentage of positive samples in 2000 (9.3 versus 23.7%) (Table 2), and one-half as many isolates were obtained (8 versus 16 strains). The results obtained with the cruise samples also suggest that both in terms of the percentage of positive samples and in terms of the number of isolates, *V. cholerae* occurs more frequently in the northern part of the bay (sites 908 through 834), where the salinity is lower (Fig. 3B). No such

site difference was detectable among the brackish shore sites located in a smaller geographical area, where the salinity and temperature conditions did not vary as much at a given time.

The proportion of positive samples was higher for water samples than for plankton samples, but it was significantly higher only for enrichment samples collected at the brackish sites, at both the shore and the cruise (Fig. 4). In the case of enrichment, the two plankton fractions (P20 and P64) did not differ significantly. In the case of DFA O1, the two plankton fractions differed for the freshwater samples because *V. cholerae* was not detected in the P64 fraction.

**Environmental parameters.** The ranges of the environmental parameters measured in this study are summarized by site in Table 3. The salinity at the brackish sites was inversely correlated with the Susquehanna River flow, with a 3-week lag ( $P < 0.001$ ;  $R = -0.56$ ). Table 4 shows that the measured salinities at brackish sites reflected the general flow pattern of the Susquehanna River compared with the long-term average.

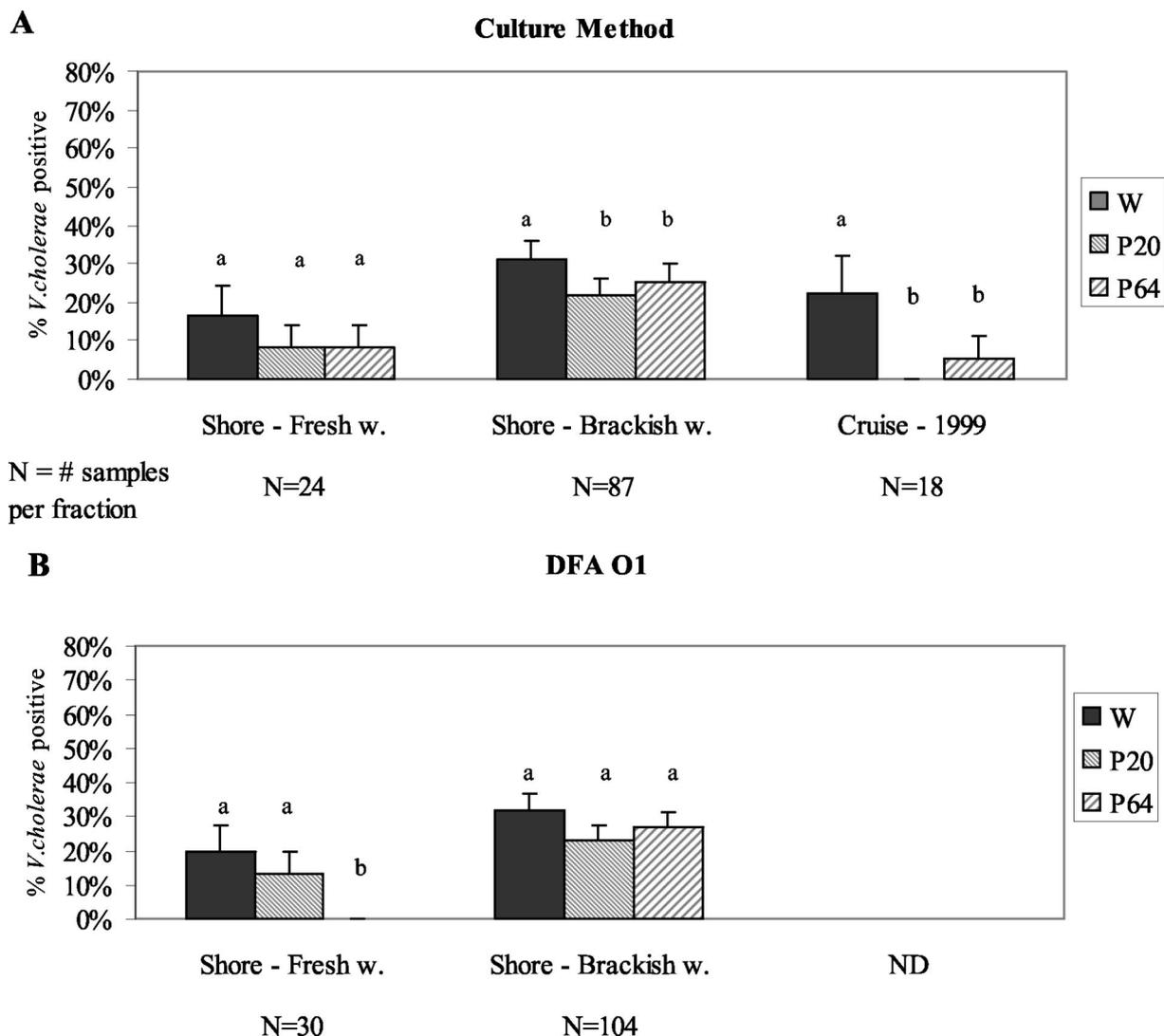


FIG. 4. Percentages of *V. cholerae*-positive samples in fractions for enrichment (A) and DFA O1 (B) analyses. The sites included the shore freshwater site (site F), shore brackish sites (sites B, K, S, and H), and cruise sites in 1999. The Q statistic (25) was used to compare the proportions of *V. cholerae*-positive samples in fractions; for each category, values that are statistically different from one another are indicated by a different letter (a and b). The error bars indicate standard errors (SE), determined as follows:  $SE = \sqrt{[p \cdot (1 - p)/n]}$ , where  $n$  is the number of samples per fraction included and  $p$  is the proportion of positive samples, computed as described by Fleiss et al. (25). Fresh w., freshwater; Brackish w., brackish water; ND, not determined.

Salinity at the brackish sites, however, was not correlated with a lag in precipitation (1 to 21 days) or with 3- or 7-day rain accumulation. Temperature and to a lesser extent salinity displayed annual seasonal patterns (Fig. 2). For all sites, the total

bacterial counts exhibited a seasonal pattern that followed the temperature variation. The log of the total bacterial count (tbc) was positively correlated with temperature ( $T$ ):  $\log(tbc) = 0.0286T + 6.2376$  ( $R = 0.81$ ). The chlorophyll *a* concentra-

TABLE 3. Environmental parameter ranges

Site(s)	Designation	Temp range (°C)	Salinity range (ppt)	pH range	Total bacterial count range ( $10^6$ cells $ml^{-1}$ )	Chlorophyll <i>a</i> concn range ( $mg\ m^{-3}$ )
Susquehanna River Flats	F	0.0–28.5	0	7.2–9.0	0.8–12.8	0.3–128.9
Baltimore	B	1.5–27.0	3–12	7.0–8.2	1.0–12.0	1.2–166.9
Kent Island	K	–0.5–30.0	2–13	7.1–8.5	1.1–11.3	2.3–147.4
SERC <sup>a</sup>	S	–0.5–31.0	2–15	6.7–9.0	1.1–21.5	7.8–64.5
Horn Point Laboratory	H	0.5–30.0	5–15	7.3–8.4	1.6–14.0	2.8–67.5
Cruise		21.1–28.0	4.7–26.6	ND <sup>b</sup>	ND	ND

<sup>a</sup> SERC, Smithsonian Environmental Research Center.

<sup>b</sup> ND, not determined.

TABLE 4. Salinity at brackish sampling sites and Susquehanna River flow measured at Conowingo Dam

Season	Salinity at brackish sites (ppt)		Susquehanna River flow	
	Shore (sites B, K, S, and H) <sup>a</sup>	Cruise	% Difference <sup>b</sup>	Flow
Winter 1998	6.7 ± 2.4	ND <sup>c</sup>	42.0 ± 5.3	Very high
Spring 1998	4.4 ± 2.2	ND	21.3 ± 3.0	High
Summer 1998	6.9 ± 1.7	ND	-18.8 ± 8.4	Avg or low
Autumn 1998	11.9 ± 1.0	ND	-73.9 ± 9.3	Very low
Winter 1999	9.8 ± 3.7	ND	-34.8 ± 7.1	Low
Spring 1999	8.8 ± 1.8	ND	-36.6 ± 4.0	Low
Summer 1999	10.9 ± 2.1	19.1 ± 3.8	-73.0 ± 9.3	Very low
Autumn 1999	10.6 ± 1.7	17.8 ± 4.2	-25.3 ± 3.7	Low
Winter 2000	7.8 ± 2.1	ND	-33.7 ± 9.7	Low
Summer 2000	ND	13.5 ± 4.8	4.4 ± 7.5	Avg

<sup>a</sup> Average ± standard deviation.

<sup>b</sup> Percent difference from 33-year average ± 95% confidence interval.

<sup>c</sup> ND, not determined.

tion exhibited great variability without a seasonal pattern. Similarly, there was no clear pattern for pH, seasonal or otherwise, and sites did not vary together.

Table 5 shows, for the most abundant taxa, the percentages of samples in which a taxon was present or had a relative abundance in the sample that was greater than 20%. For the four brackish water, shore sites (sites B, K, S, and H), copepod nauplii were the most abundant zooplankton in 70.2% of all the samples ( $n = 94$ ). Furthermore, the number of copepod nauplii exceeded 20% of the number of zooplankton in 83.0% of the samples. Adult calanoid copepods, copepodites, and barnacle nauplii were the next most common groups (Table 5). The freshwater site (site F) differed from the other shore sites in that rotifers were considerably more common (73.1 versus 33.0% of the samples) and accounted for a higher proportion of the organisms (50.0% of the samples had a relative abundance greater than 20%, compared to 10.6% of the samples from brackish sites), whereas barnacle nauplii were less common. While adult calanoid copepods were observed more frequently than adult cyclopoid copepods at brackish sites, the trend was reversed for the freshwater site (Table 5).

Table 6 shows the relative distribution of *V. cholerae*-positive samples as a function of temperature and salinity. For shore samples, a higher temperature led to an increase in *V. cholerae* occurrence, except when the salinity was more than 14 ppt. Use

of the odds ratio as a measure of association indicated that occurrence of *V. cholerae* was nearly 20 times more likely at a temperature of 19°C or higher as determined by enrichment culture and nearly 6 times more likely as determined by DFA O1. The salinity range from 2 to 8 ppt yielded the highest percentage of positive samples at temperatures of more than 19°C for both enrichment and DFA O1 analyses. At temperatures below 19°C, *V. cholerae* was detected more often by DFA O1 (10.9%) than by enrichment culturing (5.4%). At temperatures below 19°C, *V. cholerae* was detected more often by enrichment culture at salinities between 2 and 8 ppt, whereas it was detected more often by DFA O1 at salinities between 8 and 14 ppt. At temperatures of 19°C or more and salinities between 2 and 14 ppt, detection of *V. cholerae* was 5.2 times more likely by enrichment culture and 4.1 times more likely by DFA O1 than when the combination of these conditions was otherwise. In freshwater, temperatures higher than 19°C favored *V. cholerae* detection by both methods, but to a lesser extent than for the brackish water samples. Data from the summer cruises showed that elevated temperature alone was not sufficient to yield a high percentage of positive samples, since a temperature above 21°C yielded 15.6% positive samples ( $n = 90$ ), compared to 37.4% positive samples at the shore sites ( $n = 174$ ), and the percentage of positive samples dropped to 9.5% when the salinity was greater than 14 ppt. Even though *V. cholerae* was detected less frequently at a salinity greater than 14 ppt, it was not absent from high-salinity water, since *V. cholerae* was isolated from a site near the mouth of Chesapeake Bay where the salinity was 21.1 ppt.

**Modeling.** Statistical analysis showed that the models performed poorly when the freshwater site was included with the other sites. Consequently, the analysis was done separately for freshwater and brackish water sites. The selected models shown in Table 7 include only data from the brackish water sites, shore sites, and cruise sites. The water fractions and the combined plankton fractions were analyzed separately. For the freshwater sampling site alone, no significant explanatory variables were found. Numbers of adult copepods and copepodites appeared in the stepwise selection model but not at a significant level. For the brackish water sites, the logistic regression showed that temperature and salinity were at the core of a basic model for both the enrichment culture and DFA O1 data. Even when other parameters were allowed to enter the model

TABLE 5. Zooplankton abundance at different sites

Site(s)	<i>n</i>	Copepodites		Copepod nauplii		Calanoid adult copepods		Cyclopoid adult copepods		Barnacle nauplii		Rotifers	
		% of positive samples <sup>a</sup>	>20% samples (%) <sup>b</sup>	% of positive samples	>20% samples (%)	% of positive samples	>20% samples (%)	% of positive samples	>20% samples (%)	% of positive samples	>20% samples (%)	% of positive samples	>20% samples (%)
F	26	61.5	11.5	84.6	42.3	61.5	15.4	69.2	0.0	23.1	0.0	73.1	50.0
B	17	64.7	11.8	100.0	76.5	100.0	35.3	47.1	5.9	70.6	11.8	35.3	5.9
K	25	68.0	12.0	96.0	84.0	88.0	8.0	36.0	0.0	64.0	32.0	40.0	12.0
S	26	69.2	3.8	92.3	92.3	73.1	7.7	42.3	0.0	65.4	11.5	30.8	15.4
H	26	84.6	7.7	100.0	76.9	80.8	11.5	46.2	3.8	65.4	34.6	26.9	7.7
B, K, S, H	94	72.3	8.5	96.8	83.0	84.0	13.8	42.6	2.1	66.0	23.4	33.0	10.6
All	120	70.0	9.2	94.2	74.2	79.2	14.2	48.3	1.7	56.7	18.3	41.7	19.2

<sup>a</sup> Percentage of samples containing the organisms.

<sup>b</sup> Percentage of samples in which the organisms accounted for more than 20% of the organisms present.

TABLE 6. Percentages of *V. cholerae*-positive samples as determined by the enrichment culture and DFA O1 methods at different temperatures and different salinities

Method	% of <i>V. cholerae</i> -positive samples							
	<19°C				≥19°C			
	<0.5 ppt	2–8 ppt	8–14 ppt	>14 ppt	<0.5 ppt	2–8 ppt	8–14 ppt	>14 ppt
Enrichment (cruise sites)	ND <sup>a</sup>	ND	ND	ND	ND	75.0 (4) <sup>b</sup>	21.7 (23)	9.5 (63)
Enrichment (shore sites)	6.5 (31)	9.5 (42)	2.8 (72)	0.0 (3)	14.3 (42)	84.4 (45)	24.2 (99)	0.0 (3)
DFA O1 (shore sites)	8.7 (46)	6.0 (50)	15.4 (104)	0.0 (12)	13.3 (45)	84.1 (44)	29.7 (108)	0.0 (3)

<sup>a</sup> ND, not determined.

<sup>b</sup> The values in parentheses are numbers of samples.

in a stepwise algorithm, they provided only minor improvement over the basic temperature-salinity model. Sometimes the variable total bacterial count, which is strongly correlated with temperature, was substituted for the variable temperature by the stepwise logistic regression. For example, in model 6 (Table 7), the agreement between the observed and predicted responses improved only from 75.5 to 77.8% when the additional variables chlorophyll *a*, rotifer, and barnacle nauplii were entered into the model. Also in this modified model, the variable total bacterial count was substituted for temperature. Similarly in model 1, the agreement between the observed and predicted values increased only from 82.2 to 83.1% when the variable copepod nauplii was added to the model. Analysis with the binary classification tree gave patterns similar to that obtained by the logistic regression analysis with stepwise selection. This method pointed toward a lower threshold for temperature (around 17°C) and toward an upper boundary for salinity (between 8 and 10 ppt). For enrichment cultures, the percentage of agreement between the observed

and predicted values was slightly better with the binary tree (Table 7). For DFA O1, the binary classification tree gave greater improvement over logistic regression. For both modeling methods, the results showed that the combined temperature and salinity conditions predict the presence of *V. cholerae* with an accuracy between 75.5 and 88.5%.

DISCUSSION

**Enrichment culturing versus DFA O1.** The fact that DFA O1 analysis yielded a higher frequency of positive samples than the enrichment culture method suggests that under environmental conditions suboptimal for *V. cholerae* growth, more cells may be nonculturable and, therefore, may not be detected by enrichment culturing (16). This observation is in agreement with previous studies that showed that *V. cholerae* exists in a viable but nonculturable (VBNC) state when environmental conditions such as temperature, salinity, osmotic pressure, pH, and nutrient concentration become less favorable for growth

TABLE 7. Statistical models with logistical regression and binary classification trees<sup>a</sup>

Model	Method	Sites	Fraction	Logistic regression				Classification binary trees	
				Significant variable(s)	Estimate	P	% Agreement <sup>b</sup>	Tree structure (conditions for <i>V. cholerae</i> positive)	% Agreement <sup>b</sup>
1	Enrichment	B, K, S, H	Water	Intercept	0.2073	0.897	82.2 (90)	If temp is >16.8°C and salinity is <7.5 ppt; if temp is >27.5°C and salinity is between 7.5 and 10.8 ppt	85.6 (90)
				Temp	0.1988	0.000			
				Salinity	-0.5351	0.001			
				Salinity · salinity <sup>c</sup>	-0.0791	0.044			
2	Enrichment	B, K, S, H	Plankton	Intercept	-1.1753	0.343	87.4 (87)	If temp is >17.8°C and salinity is <9.8 ppt	88.5 (87)
				Temp	0.2189	0.000			
				Salinity	-0.5281	0.000			
3	Enrichment	B, K, S, H, cruise	Water	Intercept	-4.1340	0.000	82.3 (130)	If temp is >16.8°C and salinity is <8.9 ppt	82.3 (130)
				Temp	0.1713	0.000			
				Temp · salinity	-0.0463	0.000			
4	Enrichment	B, K, S, H, cruise	Plankton	Intercept	-1.7254	0.133	87.4 (127)	If salinity is <7.5 ppt and temp is >17.5°C	88.2 (127)
				Temp	0.1986	0.000			
				Salinity	-0.4329	0.000			
				Salinity · salinity	0.0184	0.035			
5	DFA O1	B, K, S, H	Water	Intercept	-1.1939	0.246	76.9 (108)	If temp is >20.5°C and salinity is <10.3 ppt	81.5 (108)
				Temp	0.1233	0.000			
				Salinity	-0.1997	0.019			
				Temp · salinity	-0.0324	0.005			
6	DFA O1	B, K, S, H	Plankton	Intercept	-0.5009	0.668	75.5 (106)	If temp is >12.8°C and salinity is <7.5 ppt; if temp is >12.8°C and salinity is >7.5 ppt and log (calanoid copepods) is >1.56	83.3 (90)
				Temp	0.1314	0.001			
				Salinity	-0.1964	0.034			
				Temp · salinity	-0.0350	0.020			
				Temp · temp	-0.0121	0.036			

<sup>a</sup> The freshwater site was not included in the analysis. The surface response analysis was performed as described by Carter et al. (7).

<sup>b</sup> Percentage of agreement between predicted and observed values. The numbers in parentheses are numbers of samples.

<sup>c</sup> A chemical point indicates a cross-product.

TABLE 8. Conditions that favor the occurrence of *V. cholerae*, as determined in various studies

Experimental setting	Temp (°C) (study range)	Salinity (study range)	Reference(s)
Laboratory microcosm		5, 10 (0, 5, 10, 30)	8
Laboratory microcosm		15 (5, 10, 15)	36
Laboratory microcosm	20, 25 (10, 15, 20, 25)	15, 25 (5, 15, 25, 35)	60
Chesapeake Bay (estuary)	15–20 (0–20)	4–12 (4–12)	17
Chesapeake Bay (estuary)	>17 (0.2–25.2) <sup>a</sup>	4–17 (0.1–21.4)	40
Southern California (coastal areas)	No preference (13–24)	1–10 (0–34)	39
Louisiana (coastal areas)	Depended on salinity (18–30)	<1 (0–15)	55
Florida (estuary) and laboratory microcosm	20–35 (unspecified)	12–25 (0–34), 10–25 (0–90)	32
England (river and marsh ditch)	>9 (2–19) <sup>b</sup>	3–12 (0–12)	46, 67
Japan (rivers and coastal areas)	ca. 21 (<7 –29) <sup>c</sup>	0.4–32.5 (0–34) <sup>d</sup>	65

<sup>a</sup> *V. cholerae* was detected at temperatures above 10°C.

<sup>b</sup> The temperature was highest in August.

<sup>c</sup> *V. cholerae* was detected at temperatures above 7°C.

<sup>d</sup> The detection range was 0.4 to 32.5 ppt.

and replication (33, 34). Nonetheless, the pattern of detection observed here by enrichment culture corroborated the pattern obtained in a study done in the Choptank River (Chesapeake Bay, sample site H) with a non-culture-based detection method (29, 30). Using fluorescent-oligonucleotide direct counting, Heidelberg et al. (30) showed that the abundance of the *V. cholerae*-*V. mimicus* subgroup was greater in the summer and that the relative proportion of this subgroup within the *Vibrio-Photobacterium* group increased with higher temperatures.

In this study, *V. cholerae* isolates were *V. cholerae* non-O1 and non-O139. In contrast, detection by direct fluorescence by using the O1 antibody (DFA O1) gave positive results for the *V. cholerae* O1 population. The fact that the two methods did not always agree can be explained by the fact that the fluorescent-antibody assay principally assesses the nonculturable subpopulation of *V. cholerae* O1, whereas the enrichment procedure detects only culturable cells. This suggests that *V. cholerae* O1 in Chesapeake Bay is mostly VBNC. The assay, by itself, does not necessarily measure the public health risk associated with *V. cholerae* O1, since not all O1 strains may be pathogenic (47) and the genetic characteristics of nonculturable *V. cholerae* in Chesapeake Bay have yet to be determined. Nevertheless, *V. cholerae* O1 has previously been isolated from Chesapeake Bay (18), and VBNC strains have been shown to conserve their antigenic determinants (9), suggesting that a subpopulation of potentially pathogenic *V. cholerae*, either VBNC *V. cholerae* or *V. cholerae* that is difficult to culture, is present in Chesapeake Bay. Furthermore, because of the possibility of horizontal gene transfer and the presence in the environment of *V. cholerae* variants that potentially carry toxigenic genetic elements (24), an increase in the *V. cholerae* population or VBNC *V. cholerae* O1 in the environment may lead to an increase in public health risk.

**Roles of zooplankton, temperature, and salinity.** While all of the brackish sites had similar patterns of dominant zooplankton groups, the freshwater site differed from the brackish sites. This may be one reason why the detection pattern of *V. cholerae* was very different at the freshwater site (detection was infrequent and erratic). In this study, *V. cholerae* was detected more often in water samples than in plankton samples, and no clear association could be established between detection of *V. cholerae* and the presence of a specific group of zooplankton. These observations are similar to those reported in 1989

by Venkateswaran et al. (65) for non-O1 *V. cholerae* in the coastal waters of Japan.

In this study, *V. cholerae* showed a seasonal pattern of occurrence, which was correlated with higher temperatures. The data indicate that there is a temperature threshold between 17 and 19°C, and the frequency of occurrence of *V. cholerae* is significantly greater at temperatures above 19°C ( $P < 0.001$ ). Furthermore, the results indicate that there is an optimal salinity range for *V. cholerae* detection (namely, between 2 and 14 ppt, especially at salinities below 8 ppt). These observations are in agreement with previous studies done in Chesapeake Bay, where *V. cholerae* was isolated only at salinities ranging from 4 to 17 ppt and only at temperatures above 10°C (40) and was more frequently detected when temperatures were greater than 17°C (14). In recent studies carried out in Southern California (39), Jiang found that high concentrations of *V. cholerae* were more frequently detected at salinities below 10 ppt but above 0 ppt. Laboratory microcosm studies also suggested that optimum growth of *V. cholerae* occurs at a range of salinities and that below or above this range growth decreases. For example, Cavari and Colwell (8) showed that there was rapid *V. cholerae* growth in microcosms at salinities of 5 and 10 ppt compared to the growth at a salinity of 0 or 30 ppt. Singleton et al. (60) documented that there was an absolute requirement for NaCl in the 10 strains of *V. cholerae* which they tested and showed that growth was optimum in microcosms at salinities of 15 and 25 ppt. Huq et al. (36) found little difference in the growth of *V. cholerae* in microcosms at salinities of 5, 10, and 15 ppt, except that at a salinity of 5 ppt *V. cholerae* culturability was reduced in the absence of copepods. Results of previous studies describing conditions that favor detection of *V. cholerae* are summarized in Table 8.

These results offer insight into cholera epidemiology both in developed countries and in developing countries, such as Bangladesh, where cholera is endemic. The seasonality of cholera in regions where cholera is endemic suggests that environmental conditions are linked to cholera epidemics (57). Recent studies (6, 48) further support the role of environmental factors in endemic cholera, indicating that the ecology of *V. cholerae* in natural waters is influenced by environmental conditions, such as sea surface temperature. Along these lines, a study of diarrheal diseases, including cholera, in Peru indicated that there was a link with temperature and that there was a

19°C threshold, above which the number of cases increased (11).

**Role of the Susquehanna River.** In the upper Chesapeake Bay, defined as the section north of the mouth of the Potomac River, which includes the shore sampling sites used in this study, the Susquehanna River provides nearly 90% of the overall freshwater influx (58). In this area, salinity is coupled to the freshwater flow from the Susquehanna River (28, 58). Salinity averaged by season at the brackish sites was inversely related to the general flow trend of the Susquehanna River (Table 4). The first half of 1998 was marked by higher-than-usual river flow, and consequently, the average salinities at the near-shore sites in the winter, spring, and summer of 1998 were significantly lower ( $P < 0.005$ ) than the values during the corresponding times of year in 1999, a low-flow year.

In contrast, the period from August to December 1998 was marked by very low flow, and the average autumn salinity turned out to be higher in 1998 than in 1999, but the values were not statistically different. At the cruise sites, the average salinity along the north-south transect was significantly higher ( $P < 0.02$ ) in 1999, a year of low flow, than in 2000, a year of average flow. These observations illustrate (i) that salinity varies seasonally (for example, a spring freshet [i.e., lower salinity] was apparent in both 1998 and 1999) and (ii) that there is annual variability due to the magnitude of the Susquehanna River inflow, as described by Schubel and Pritchard (58).

Due to variation in the flux of freshwater and, along with it, nutrients, there is significant year-to-year variability in salinity, turbidity, and dissolved organic and inorganic matter throughout the bay, especially in the northern part. For example, the timing, location, duration, and amplitude of the chlorophyll peak, which depends on nutrient input, can vary up to 80% in certain parts of the bay from one year to the next (28). The average salinities in spring and summer were significantly lower in 1998 than in 1999, while the temperature patterns were similar. The periods of lower salinity, given similar temperature conditions, resulted in increased *V. cholerae* occurrence and much higher numbers of isolates. In contrast, little year-to-year variability was observed at the freshwater sampling site. Interestingly, *V. cholerae* was also detected much less frequently at that site, suggesting that the dynamics that regulate *V. cholerae* are very different in freshwater. The cruise samples collected in the summers of 1999 and 2000 also confirmed that 1999 was a year in which low *V. cholerae* yields were obtained. In this analysis, salinity may be the effective parameter per se, as laboratory studies have determined, but it may also be a proxy for other factors, such as turbidity or nutrient load, that were not measured in this study. Singleton et al. (61) studied the combined effects of nutrients and salinity on *V. cholerae* growth and showed that when the nutrient concentration was elevated, the *V. cholerae* concentration increased and the range of salinity tolerated was larger. Thus, an increase in the riverine influx, laden with nutrients, may favor the growth of *V. cholerae* by increasing the growth potential and by making the bacterium able to thrive over a wider salinity range. Whatever the mechanisms, these results suggest that year-to-year salinity variability or other factors associated with freshwater influx may generate large variations in the *V. cholerae* populations. For example, lower salinities in the upper bay due to increased Susquehanna River flow, a possible outcome of cli-

mate change scenarios that include a doubling of atmospheric carbon dioxide (27), may favor increased occurrence of *V. cholerae* in Chesapeake Bay. Conversely, drought conditions, such as those experienced in recent years in the Chesapeake Bay region, may produce conditions less conducive to *V. cholerae* proliferation in the estuary. In the past century, precipitation has been changing in the United States, and there have been increases in extreme precipitation events (41, 42). These changing rainfall patterns, most evident in the mid-Atlantic region, are consistent with expectations of a more vigorous hydrological cycle expected to accompany global warming (26, 50, 64). Based on the salinity findings of this study, expected changes in stream flows could modulate *V. cholerae* populations in Chesapeake Bay. Furthermore, the effects of sea level rise (a potential consequence of global warming) on the salinity regimen of an estuary such as Chesapeake Bay may also be important for long-term assessment.

**Predictability.** The statistical models used in this study described the occurrence of *V. cholerae* in Chesapeake Bay mostly as a function of temperature and salinity. This knowledge is valuable in itself because it provides an understanding of the large-scale processes that dominate the ecology of *V. cholerae* in Chesapeake Bay. Inclusion of the variables for plankton resulted in only marginal improvement to the models. Since the distribution of plankton in Chesapeake Bay is known to be patchy (28), an adequate model may require much higher temporal resolution and spatial resolution (on the order of days and kilometers, respectively) at the data acquisition step to properly describe the role of plankton. Thus, the association between plankton and *V. cholerae*, which has been well documented (22, 35, 62), may come into play at a small scale within large-scale processes.

Both the logistic regression and the binary classification tree proved to be useful modeling methods for describing *V. cholerae* occurrence and pointed out the most relevant parameters. As discussed by De'ath and Fabricius (21), binary classification trees are well suited for describing complex ecological data. In this study, binary classification trees dealt effectively with the threshold situation and outperformed logistic regression models. Such modeling techniques could be useful for describing *V. cholerae* occurrence in other estuarine or coastal regions. The analysis showed that freshwater and brackish sites should be analyzed separately. Even though this study did not yield significant results for the freshwater site, models could be applied to freshwater or nearly freshwater conditions if several freshwater sampling sites are included in order to provide enough power for analysis. To become a more accurate predictive tool, the models need to be developed over a period of time that is longer than 2 years, so that year-to-year variability can effectively be taken into account.

In conclusion, the results provided by this study showed that there is clear seasonality in the occurrence of *V. cholerae* in the temperate estuarine environment of Chesapeake Bay, as detected both by enrichment culturing and direct fluorescent-antibody detection for *V. cholerae* O1. Even though *V. cholerae* was found to occur at a wide range of salinities (from freshwater to a salinity of 21 ppt), the salinity most favorable for *V. cholerae* was between 2 and 14 ppt, and the optimal salinity appeared to be between 2 and 8 to 10 ppt. In brackish water, temperature and salinity are the two major parameters deter-

mining the frequency of occurrence and geographical distribution of *V. cholerae*. However, in freshwater or at a high salinity (conditions that may be suboptimal for growth of *V. cholerae*), another factor, such as zooplankton, may be the dominant variable that explains the occurrence of *V. cholerae*. In the upper Chesapeake Bay, where *V. cholerae* was most frequently detected, salinity was linked to freshwater input from the Susquehanna River. Climate and weather conditions greatly contribute to river discharge, and there is great year-to-year variability. In this study, lower-than-normal salinity resulted in high *V. cholerae* occurrence in the spring and summer, suggesting that climate patterns have the potential to shift the estuarine ecosystem toward circumstances for which very favorable conditions for *V. cholerae* can occur. Since the salinity of Chesapeake Bay can affect the occurrence of *V. cholerae*, the predictability of *V. cholerae* in Chesapeake Bay depends on large-scale phenomena that regulate parameters such as temperature and salinity. Statistical models provided a useful tool for describing such phenomena and can be used in other estuarine regions of the world, like the Bay of Bengal, where cholera is endemic.

#### ACKNOWLEDGMENTS

This research was supported by U.S. Environmental Protection Agency STAR grant R824995 and by NIH grant RO1 NRO4257-01A1. We thank Wayne Coats for providing the opportunity to conduct research aboard the R/V *Cape Henlopen* (NSF grant OCE-9730695).

#### REFERENCES

- American Public Health Association. 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, D.C.
- Bagchi, K., P. Echeverria, J. D. Arthur, O. Sethabutr, O. Serichantalergs, and C. W. Hoge. 1993. Epidemic of diarrhea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. *J. Clin. Microbiol.* **31**:1315–1317.
- Barbieri, E., L. Fladanza, C. Fiorentini, A. Pianetti, W. Affone, A. Fabbri, P. Matarrese, A. Casiere, M. Katouli, I. Kuhn, R. Mollby, F. Bruscolini, and G. Donelli. 1999. Occurrence, diversity, and pathogenicity of halophilic *Vibrio* spp. and non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic coast. *Appl. Environ. Microbiol.* **65**:2748–2753.
- Bik, E. M., A. E. Bunschoten, R. D. Gouw, and F. R. Mooi. 1995. Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. *EMBO J.* **14**:209–216.
- Bland, J. M., and D. G. Altman. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **i**:307–310.
- Bouma, M. J., and M. Pascual. 2001. Seasonal and interannual cycles of endemic cholera in Bengal 1891–1940 in relation to climate and geography. *Hydrobiologia* **460**:147–156.
- Carter, W. H., Jr., G. L. Wampler, and D. M. Stablein. 1983. Regression analysis of survival data in cancer chemotherapy. Marcel Dekker, New York, N.Y.
- Cavari, B. Z., and R. R. Colwell. 1981. Effect of salt on survival and growth of *Vibrio cholerae*, p. 227–231. In H. Shuval (ed.), *Developments in arid zone ecology and environmental quality*. Balaban ISS, Philadelphia, Pa.
- Chaiyanan, S., S. Chaiyanan, A. Huq, T. Mauget, and R. R. Colwell. 2001. Viability of the nonculturable *Vibrio cholerae* O1 and O139. *Syst. Appl. Microbiol.* **24**:331–341.
- Chakraborty, S., A. K. Mukhopadhyay, R. K. Bhadra, A. N. Ghosh, R. Mitra, T. Shimada, S. Yamasaki, S. M. Faruque, Y. Takeda, R. R. Colwell, and G. B. Nair. 2000. Virulence genes in environmental strains of *Vibrio cholerae*. *Appl. Environ. Microbiol.* **66**:4022–4028.
- Checkley, W., L. D. Epstein, R. H. Gilman, R. I. Figueroa, J. A. Cama, J. A. Patz, and R. E. Black. 2000. Effect of El Niño and ambient temperature on hospital admissions and diarrhoeal diseases in Peruvian children. *Lancet* **355**:442–450.
- Choopun, N., V. Louis, A. Huq, and R. R. Colwell. 2002. Simple procedure for rapid identification of *Vibrio cholerae* from the aquatic environment. *Appl. Environ. Microbiol.* **68**:995–998.
- Chun, J., A. Huq, and R. R. Colwell. 1999. Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus*. *Appl. Environ. Microbiol.* **65**:2202–2208.
- Colwell, R. R., P. A. West, D. Maneval, E. F. Remmers, E. L. Elliot, and N. E. Carlson. 1984. Ecology of pathogenic vibrios in Chesapeake Bay, p. 367–387. In R. R. Colwell (ed.), *Vibrios in the environment*. John Wiley & Sons, Inc., New York, N.Y.
- Colwell, R. R. 1996. Global climate and infectious disease: the cholera paradigm. *Science* **274**:2025–2031.
- Colwell, R. R., and A. Huq. 1994. Vibrios in the environment: viable but nonculturable *Vibrio cholerae*, p. 117–133. In I. K. Wachsmuth, P. A. Blake, and Ø. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. American Society for Microbiology, Washington, D.C.
- Colwell, R. R., J. Kaper, and S. W. Joseph. 1977. *Vibrio cholerae*, *Vibrio parahaemolyticus*, and other vibrios: occurrence and distribution in Chesapeake Bay. *Science* **198**:394–396.
- Colwell, R. R., R. J. Seidler, J. Kaper, S. W. Joseph, S. Garges, H. Lockman, D. Maneval, H. Bradford, N. Roberts, E. Remmers, I. Huq, and A. Huq. 1981. Occurrence of *Vibrio cholerae* serotype O1 in Maryland and Louisiana estuaries. *Appl. Environ. Microbiol.* **41**:555–558.
- Dalsgaard, A., M. Albert, D. Taylor, T. Shimada, R. Meza, O. Serichantalergs, and P. Echeverria. 1995. Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. *J. Clin. Microbiol.* **33**:2715–2722.
- Dalsgaard, A., A. Forslund, L. Bodhidatta, O. Serichantalergs, C. Pitarangsi, L. Pang, T. Shimada, and P. Echeverria. 1999. A high proportion of *Vibrio cholerae* strains isolated from children with diarrhoea in Bangkok, Thailand are multiple antibiotic resistant and belong to heterogeneous non-O1, non-O139 O-serotypes. *Epidemiol. Infect.* **122**:217–226.
- De'ath, G., and K. E. Fabricius. 2000. Classification and regression trees: a powerful yet simple technique for ecological data analysis. *Ecology* **81**:3178–3192.
- Dumontet, S., K. Krovacek, S. B. Baloda, R. Grottolli, V. Pascuale, and S. Vanucci. 1996. Ecological relationship between *Aeromonas* and *Vibrio* spp. and planktonic copepods in the coastal marine environment in southern Italy. *Comp. Immunol. Microbiol. Infect. Dis.* **19**:245–254.
- Faruque, S., Asadulghani, M. Kamruzzaman, R. Nandi, A. Ghosh, G. Nair, J. Mekalanos, and D. Sack. 2002. RS1 element of *Vibrio cholerae* can propagate horizontally as a filamentous phage exploiting the morphogenesis genes of CTX $\phi$ . *Infect. Immun.* **70**:163–170.
- Faruque, S., and G. Nair. 2002. Molecular ecology of toxigenic *Vibrio cholerae*. *Microbiol. Immunol.* **46**:59–66.
- Fleiss, J. L. 1981. *Statistical methods for rates and proportions*, 2nd ed., p. 112–137. John Wiley & Sons, New York, N.Y.
- Fowler, A., and K. Hennessey. 1995. Potential impacts of global warming on the frequency and magnitude of heavy precipitation. *Nat. Hazards* **11**:283–303.
- Gibson, J. R., and R. G. Najjar. 2000. The response of Chesapeake Bay salinity to climate-induced changes in streamflow. *Limnol. Oceanogr.* **45**:1764–1772.
- Harding, L. W., Jr. 1994. Long-term trends in the distribution of phytoplankton in Chesapeake Bay: role of light, nutrients and streamflow. *Mar. Ecol. Prog. Ser.* **104**:267–291.
- Heidelberg, J. F., K. B. Heidelberg, and R. R. Colwell. 2002. Bacteria of the  $\gamma$ -subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. *Appl. Environ. Microbiol.* **68**:5498–5507.
- Heidelberg, J. F., K. B. Heidelberg, and R. R. Colwell. 2002. Seasonality of Chesapeake Bay bacterioplankton species. *Appl. Environ. Microbiol.* **68**:5488–5497.
- Holm-Hansen, O., and B. Rieman. 1978. Chlorophyll *a* determination: improvement in methodology. *Oikos* **30**:438–447.
- Hood, M., G. Ness, G. Rodrick, and N. Blake. 1983. Distribution of *Vibrio cholerae* in two Florida estuaries. *Microb. Ecol.* **9**:65–75.
- Huq, A., R. R. Colwell, R. Rahman, A. Ali, M. A. Chowdhury, S. Parveen, D. A. Sack, and E. Russek-Cohen. 1990. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. *Appl. Environ. Microbiol.* **56**:2370–2373.
- Huq, A., I. N. G. Rivera, and R. R. Colwell. 2000. Epidemiological significance of viable but nonculturable microorganisms, p. 301–323. In R. R. Colwell and D. J. Grimes (ed.), *Nonculturable microorganisms in the environment*. ASM Press, Washington, D.C.
- Huq, A., E. B. Small, P. A. West, M. I. Huq, R. Rahman, and R. R. Colwell. 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Appl. Environ. Microbiol.* **45**:275–283.
- Huq, A., P. A. West, E. B. Small, M. I. Huq, and R. R. Colwell. 1984. Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar O1 associated with live copepods in laboratory microcosms. *Appl. Environ. Microbiol.* **48**:420–424.
- Jeffrey, S. W., and N. A. Welschmeyer. 1997. Spectrophotometric and fluorometric equations in common use in oceanography, p. 597–615. In S. W. Jeffrey, R. F. C. Mantoura, and S. W. Wright (ed.), *Phytoplankton pigment in oceanography: guidelines to modern methods*. UNESCO monogram. UNESCO, Paris, France.
- Jesudason, M. V., V. Balaji, U. Mukundan, and C. J. Thomas. 2000. Ecological study of *Vibrio cholerae* in Vellore. *Epidemiol. Infect.* **124**:201–206.

39. Jiang, S. C. 2001. *Vibrio cholerae* in recreational beach waters and tributaries of Southern California. *Hydrobiologia* **460**:157–164.
40. Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph. 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Appl. Environ. Microbiol.* **37**:91–103.
41. Karl, T., and R. Knight. 1998. Secular trends of precipitation amount, frequency, and intensity in the USA. *Bull. Am. Meteorol. Soc.* **79**:231–241.
42. Karl, T., R. Knight, and N. Plummer. 1995. Trends in high-frequency climate variability in the twentieth century. *Nature* **377**:217–220.
43. Kaysner, C. A., C. Abeyta, Jr., M. M. Wekell, A. DePaola, Jr., R. F. Stott, and J. M. Leitch. 1987. Incidence of *Vibrio cholerae* from estuaries of the United States west coast. *Appl. Environ. Microbiol.* **53**:1344–1348.
44. Kepner, R. L., Jr., and J. R. Pratt. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiol. Rev.* **58**:603–615.
45. Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. *Can. J. Microbiol.* **25**:415–420.
46. Lee, J. V., D. J. Bashford, T. J. Donovan, A. L. Furniss, and P. A. West. 1984. The incidence and distribution of *Vibrio cholerae* in England, p. 399–409. *In* R. R. Colwell (ed.), *Vibrios in the environment*. John Wiley & Sons, Inc., New York, N.Y.
47. Levine, M., R. Black, M. Clements, L. Cisneros, A. Saah, D. Nalin, D. Gill, J. Craig, C. Young, and P. Ristaino. 1982. The pathogenicity of nonenterotoxigenic *Vibrio cholerae* serogroup O1 biotype El Tor isolated from sewage water in Brazil. *J. Infect. Dis.* **145**:296–299.
48. Lobitz, B., L. Beck, A. Huq, B. Wood, G. Fuchs, A. S. G. Faruque, and R. R. Colwell. 2000. Climate and infectious disease: use of remote sensing for detection of *Vibrio cholerae* by indirect measurement. *Proc. Natl. Acad. Sci. USA* **97**:1438–1443.
49. Mahon, B., E. Mintz, K. Greene, J. Wells, and R. Tauxe. 1996. Reported cholera in the United States, 1992–1994: a reflection of global changes in cholera epidemiology. *JAMA* **276**:307–312.
50. Mearns, L., F. Giorgi, L. McDaniel, and C. Shields. 1995. Analysis of daily variability of precipitation in a nested regional climate model: comparison with observations and doubled CO<sub>2</sub> results. *Global Planet. Change* **10**:55–78.
51. Morris, J., and R. Black. 1985. Cholera and other vibrios in the United States. *N. Engl. J. Med.* **312**:343–350.
52. Porra, R. J., W. A. Thompson, and P. E. Kriedemann. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* **975**:384–394.
53. Rivas, M., M. Cacace, L. Ayala, A. Baschkier, E. Miliwebsky, and M. Caffer. 1996. Cases of gastroenteritis associated to *Vibrio cholerae* non O1 in Oran, Salta. *Rev. Argent. Microbiol.* **28**:163–169. (In Spanish.)
54. Rivera, I. N. G., J. Chun, A. Huq, B. Sack, and R. R. Colwell. 2001. Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. *Appl. Environ. Microbiol.* **67**:2421–2429.
55. Roberts, N. C., J. H. B. Bradford, and J. R. Barbay. 1984. Ecology of *Vibrio cholerae* in Louisiana coastal areas, p. 389–398. *In* R. R. Colwell (ed.), *Vibrios in the environment*. John Wiley & Sons, Inc., New York, N.Y.
56. Rudra, S., R. Mahajan, M. Mathur, K. Kathuria, and V. Talwar. 1996. Cluster of cases of clinical cholera due to *Vibrio cholerae* O10 in east Delhi. *Indian J. Med. Res.* **103**:71–73.
57. Russell, A. J. H. 1925. The epidemiology of cholera. *Indian J. Med. Res.* **13**:427–439.
58. Schubel, J. R., and D. W. Pritchard. 1986. Response of upper Chesapeake Bay to variation in discharge of the Susquehanna River. *Estuaries* **9**:236–249.
59. Sharma, C., M. Thungapathra, A. Ghosh, A. Mukhopadhyay, A. Basu, R. Mitra, I. Basu, S. Bhattacharya, T. Shimada, T. Ramamurthy, T. Takeda, S. Yamasaki, Y. Takeda, and G. Nair. 1998. Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. *J. Clin. Microbiol.* **36**:756–763.
60. Singleton, F. L., R. Attwell, S. Jangi, and R. R. Colwell. 1982. Effects of temperature and salinity on *Vibrio cholerae* growth. *Appl. Environ. Microbiol.* **44**:1047–1058.
61. Singleton, F. L., R. W. Attwell, M. S. Jangi, and R. R. Colwell. 1982. Influence of salinity and organic nutrient concentration on survival and growth of *Vibrio cholerae* in aquatic microcosms. *Appl. Environ. Microbiol.* **43**:1080–1085.
62. Tamplin, M. L., A. L. Glauzens, A. Huq, D. A. Sack, and R. R. Colwell. 1990. Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. *Appl. Environ. Microbiol.* **56**:1977–1980.
63. Trees, C. C., R. R. Bidigare, D. M. Karl, and L. V. Heukelem. 2000. Fluorometric chlorophyll *a*: sampling, laboratory methods, and data analysis protocols, p. 162–169. *In* G. S. Fargion and J. L. Mueller (ed.), *Ocean optics protocols for satellite ocean color sensor validation*, 2nd ed. Publication NASA/TM-2000–209966. Goddard Space Flight Center, Greenbelt, Md.
64. Trenberth, K. 1999. Conceptual framework for changes of extremes of the hydrologic cycle with climate change. *Clim. Change* **42**:327–339.
65. Venkateswaran, K., T. Takai, I. Navarro, H. Nakano, H. Hashimoto, and R. Siebeling. 1989. Ecology of *Vibrio cholerae* non-O1 and *Salmonella* spp. and role of zooplankton in their seasonal distribution in Fukuyama coastal waters, Japan. *Appl. Environ. Microbiol.* **55**:1591–1598.
66. Waldor, M. K., and J. J. Mekalanos. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**:1910–1914.
67. West, P. A., and J. V. Lee. 1982. Ecology of *Vibrio* species, including *Vibrio cholerae* in natural waters of Kent, England. *J. Appl. Bacteriol.* **52**:435–448.
68. Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. J. Morris. 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl. Environ. Microbiol.* **59**:541–546.
69. Yamai, S., T. Okitsu, and Y. Katsube. 1996. Isolation and incidence of *Vibrio cholerae* from river water. *J. Jpn. Assoc. Infect. Dis.* **70**:1234–1241. (In Japanese.)
70. Yamai, S., T. Okitsu, T. Shimada, and Y. Katsube. 1997. Distribution of serogroups of *Vibrio cholerae* non-O1 non-O139 with specific reference to their ability to produce cholera toxin, and addition of novel serogroups. *J. Jpn. Assoc. Infect. Dis.* **71**:1037–1045. (In Japanese.)