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# Influence of Water Temperature, Salinity, and pH on Survival and Growth of Toxigenic *Vibrio cholerae* Serovar O1 Associated with Live Copepods in Laboratory Microcosms

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**The influence of water temperature, salinity, and pH on the multiplication of toxigenic *Vibrio cholerae* serovar O1 cells and their attachment to live planktonic crustaceans, i.e., copepods, was investigated by using laboratory microcosms. By increasing water temperatures up to 30°C, a pronounced effect on the multiplication of *V. cholerae* was demonstrated, as was attachment of the cells to live copepods. These were measured by culturable counts on agar plates and direct observation by scanning electron microscopy, respectively. Of the three salinities examined (5, 10, and 15‰), maximum growth of *V. cholerae* and attachment to copepods occurred at 15‰. An alkaline pH (8.5) was optimal both for attachment and multiplication of *V. cholerae*, as compared with pH 6.5 and 7.5. It is concluded that conditions affecting attachment of *V. cholerae* serovar O1 to live copepods observed under laboratory conditions may also occur in the natural estuarine environment and, thereby, are significant in the epidemiology of cholera.**

The widespread occurrence of *Vibrio cholerae* in the natural aquatic environment in the absence of cholera and *V. cholerae*-associated gastroenteritis cases strongly suggests that this species is an autochthonous member of brackish water and estuarine microbial communities (3, 6–8). The epidemiology of cholera may, therefore, be closely linked to the microbial ecology of the aquatic environment and to the association of toxigenic *V. cholerae* serovar O1 cells with resident micro- and macroflora (3). An association between toxigenic cells of *V. cholerae* serovar O1, as well as nontoxigenic, serovar non-O1 cells, and live planktonic copepods has been established (4).

We report here the results of studies showing the influence of three abiotic parameters, water temperature, salinity, and pH, on the association of *V. cholerae* cells with live copepods maintained in laboratory microcosms.

## MATERIALS AND METHODS

**Sample Collection.** Both adult and immature copepods were collected at different times of the year with a hand trawl, or mechanical trawl from the Patuxent River at Dunkirk, Md., and the Chesapeake Bay at Love Point, Md., by previously described methods (4). The predominant copepod species used were *Acartia tonsa* and *Eurytemora* species.

**Bacterial strain.** A toxigenic strain of *V. cholerae* serovar O1, biovar classical, serovar Inaba (CA401) was used in these studies. This strain was originally isolated from a cholera patient at Calcutta, India, in 1953.

**Preparation of laboratory microcosms.** Copepods and water samples were collected from different locations (Table 1). Microcosms were prepared by using 2-liter Erlenmeyer flasks, in one of which the parameters under study were adjusted.

To determine the effect of temperature, the flasks were incubated at temperatures of 5, 15, 25, and 30°C, with the pH (7.2) and salinity (1‰, parts per thousand) being that of the water from which the copepods were originally collected. Copepods were acclimatized in water at each temperature over a period of 48 h before the addition of *V. cholerae*.

Three salinities, 5, 10 and 15‰, were examined at the pH of the original water sample, and in these cases, room temperature, i.e., ca. 22°C, was employed. The salinity was raised or lowered for each flask by the addition of NaCl or distilled water in sterile solution. The effect of pH was measured by using pH 6.5, 7.5, and 8.5 at a salinity (3‰) of the original water sample and by incubating at room temperature. Different pH values were tested by adding 1 M NaOH or 1 M HCl to the microcosms to adjust the pH, as desired, before the addition of the copepods.

For each microcosm, copepods were washed by the method of Huq et al. (4) to remove surface bacteria. Approximately 500 copepods were placed in 500 ml of filter-sterilized (pore size, 0.2 μm) water in flasks with a 2-liter volume. Cells of *V. cholerae* CA401, grown in alkaline peptone water broth at 30°C for 18 h and washed three times in phosphate-buffered saline, were added to each flask, so the final concentration of cells in each microcosm was ca. $10^4$ CFU per ml (4).

**Bacteriological sampling.** Five copepods were randomly selected and removed from each flask at various time intervals along with 2 ml of water. The copepods were homogenized, and counts of *V. cholerae* were determined by using thiosulfate citrate bile salt sucrose agar plates (Difco Laboratories, Detroit, Mich.) after appropriate dilution (4).

**Scanning Electron Microscopy.** The extent of bacterial attachment to live copepods was assessed by scanning electron microscopy. Samples were removed, fixed in Bouin's solution, and examined by a previously described method (4).

## RESULTS AND DISCUSSION

The effect of water temperature on multiplication and attachment of *V. cholerae* onto live copepods is presented in Fig. 1 and 2. Live copepods and *V. cholerae* were added to filter-sterilized river water (pH 7.2; salinity 1‰). After 48 h,

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TABLE 1. Selected chemical and physical variables measured for water samples taken at sites of copepod collection

| Date of sample collection | Source | Water temp (°C) | Salinity (‰) | pH |
|---|---|---|---|---|
| 13 February 1982 | Patuxent River | 10 | 1.0 | 7.2 |
| 13 August 1982 | Chesapeake Bay | Not recorded | 10.0 | 7.6 |
| 17 August 1983 | Patuxent River | 22 | 3.0 | 7.2 |

the bacterial count in the flask with the live copepods held at 30°C increased to $1.09 \times 10^7$ cells per ml from an initial count of $6.04 \times 10^4$ cells per ml. In the absence of copepods, the number of *V. cholerae* cells showed only a slight increase within the first 12 h and gradually decreased thereafter (Fig. 2). At 5 and 15°C, an effect of the presence of copepods on growth and survival of *V. cholerae* was not apparent. Clearly, however, *V. cholerae* cells survived longer in the presence of live copepods when incubated at higher temperatures.

The effect of salinity was not as pronounced as the effect of temperature (Fig. 3). At all salinity values, a linear increase or decrease in bacterial count with and without copepods, respectively, was observed, except at 5‰; at this salinity value, *V. cholerae* survived longer in the presence of live copepods (Fig. 3).

Variation in water pH had a slight effect on the growth of *V. cholerae*, depending on whether live copepods were present or absent. At 23°C, increase in bacterial numbers, expressed as CFU, was less at pH 6.5 and decreased to the initial inoculum size by 72 h. At higher pH, the decrease in bacterial count (CFU) was less (Fig. 4).

Examination of copepods by scanning electron microscopy showed maximum attachment of bacterial cells to copepods at 30°C. Significantly less attachment occurred at 5 and 15°C. A gradual increase in the number of cells attached to the copepods was observed with increased salinity. A significantly larger number of cells attached to copepods at 5‰ (Fig. 5), with most of the surface of the copepods being covered with bacteria at 15‰ (Fig. 6). The highest concentration of attached cells was around the oral region and on the feeding appendages or "mouth parts." Fewer bacterial cells were observed to attach to the copepods at pH 6.5, as compared with microcosms adjusted to pH 7.5 and 8.5.

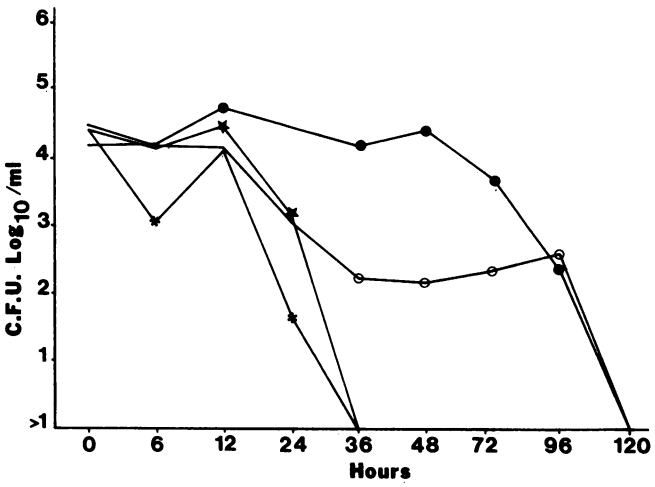


FIG. 2. Effect of different temperatures on the multiplication of *V. cholerae* at pH 7.5 and salinity 1‰ without copepods. Symbols: ★, 5°C; *, 15°C; ○, 25°C; ●, 30°C.

In earlier studies, water temperature, salinity, and nutrient concentration were identified, by using laboratory microcosms, as abiotic parameters affecting growth and survival of *V. cholerae* in chemically defined aquatic environments (14, 15). These environmental parameters also were shown to influence the temporal and spatial distribution of *V. cholerae* in freshwater and estuarine environments in nature (5, 6, 8, 17).

Salinity alone did not influence growth of *V. cholerae* at temperatures as low as 10°C, but when the temperature was increased to 15°C, the influence of salinity was significant (16). West and Lee (17) have found in in situ studies on the seasonality of *V. cholerae* that until the water temperature rises above 9°C, survival of the organisms remains critical. High temperatures during summer months appear to be favorable for survival of *V. cholerae* in water in the environment (6, 9, 12).

An association between *V. cholerae* and another member of the brackish water and estuarine aquatic environment, the copepod, has been reported (4). Because water temperature and salinity were demonstrated to be significant factors in

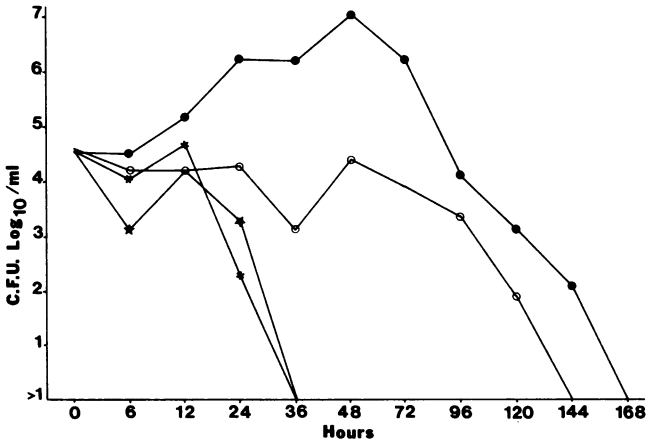


FIG. 1. Effect of different temperatures on the multiplication of *V. cholerae* at pH 7.5 and salinity 1‰ in the presence of live copepods. Symbols: ★, 5°C; *, 15°C; ○, 25°C; ●, 30°C.

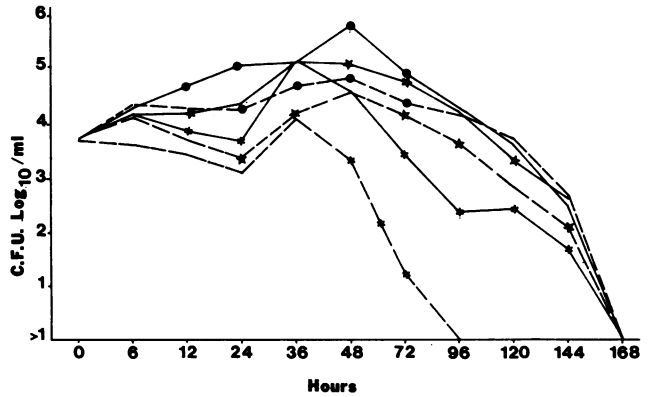


FIG. 3. Effect of salinity of multiplication of *V. cholerae* at temperatures of 22 to 25°C, pH 7.5, and with and without copepods. Symbols: ——, with copepods; – –, without copepods; *, salinity 5‰; ★, salinity 10‰; ●, salinity 15‰.

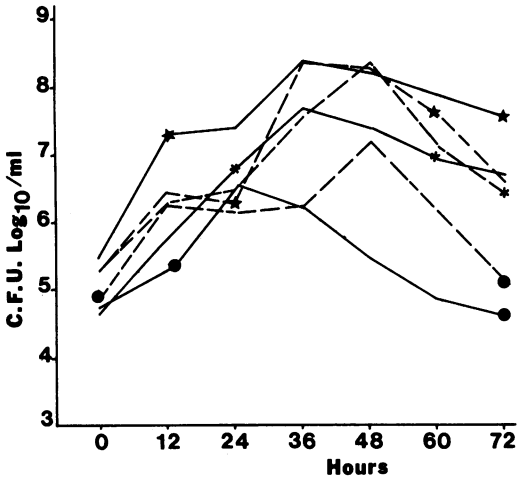


FIG. 4. Effect of pH on the multiplication of *V. cholerae* at temperatures of 20 to 25°C and 3‰ salinity. Symbols: ——, with copepods; – –, without copepods; ●, pH 6.5; *, pH 7.5; ★, pH 8.5.

the ecology of *V. cholerae*, the effect of these parameters, along with pH, on attachment of *V. cholerae* cells to live copepods was measured. We conclude that water temperature influences significantly the attachment of *V. cholerae* cells to, and multiplication on, copepods. The largest increase in cell numbers of *V. cholerae* associated with copepods was observed at 25 and 30°C. *V. cholerae* cells survived longest at these temperatures, as compared with 15 and 5°C. Growth and survival of *V. cholerae* cells with live copepods were similar at all salinities. However, cells of *V. cholerae* at a salinity of 5‰ demonstrated a shorter survival time in the absence of copepods.

An alkaline pH (8.5) was found to be optimal for rapid multiplication of *V. cholerae* on copepods and also for their attachment to these planktonic crustaceans. An acidic pH (6.5), however, did not allow the same degree of multiplication. Rapid decline in the plate count for *V. cholerae* may be explained by the early death of copepods, the onset of which was at 12 h after inoculation. The *V. cholerae* cell count decreased in all the flasks when the copepods in those flasks began to die off.

The observations reported here, along with those published earlier (4) on the attachment of *V. cholerae* to copepods, can be used to explain, in part, the epidemiology of cholera and the non-O1 serovar of *V. cholerae* gastroenteritis. In certain geographical areas, such as Bangladesh, in which cholera is endemic, seasonal, explosive outbreaks of disease occur. In recent years, sporadic cholera outbreaks have also been reported in nonendemic areas, such as Louisiana (1). A feature of these types of outbreaks is an inability to link the index case with prior contact with other

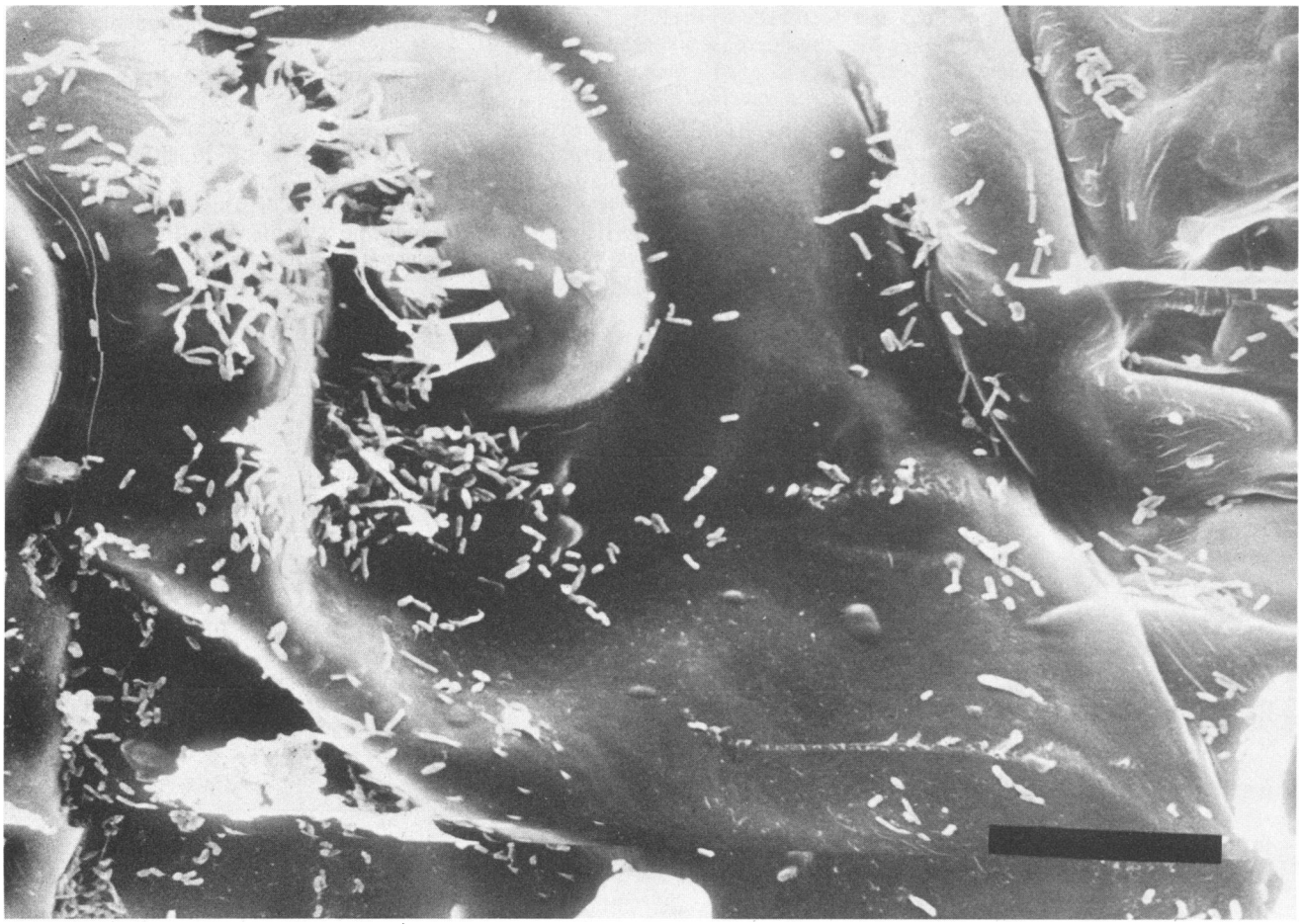

FIG. 5. Scanning electron micrographs showing bacterial attachment around the copepod oral region at 5‰ salinity after incubation for 36 h with *V. cholerae* CA401. Bar, 10 μm.

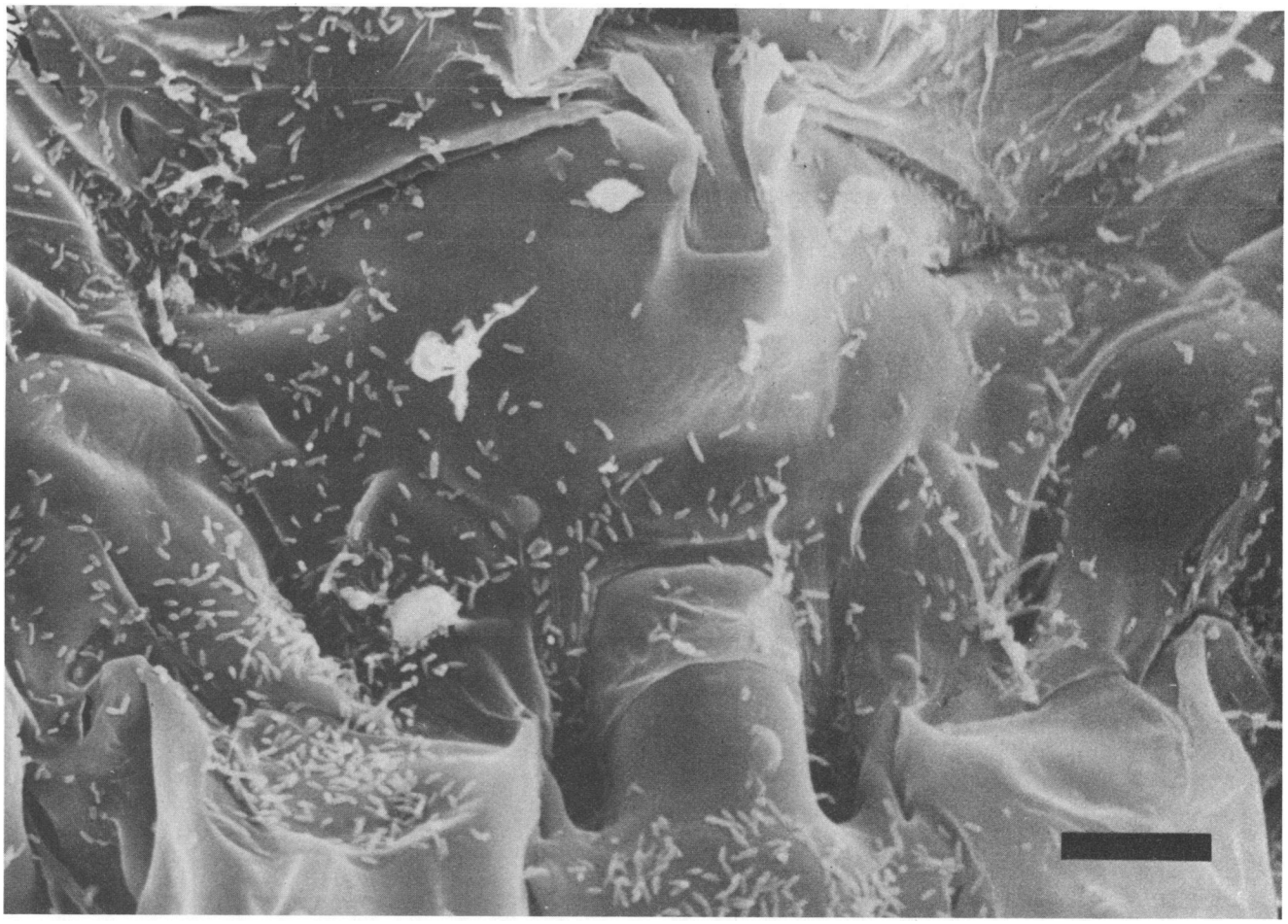

FIG. 6. Attachment of *V. cholerae* CA401 to a copepod oral region after incubation for 36 h at 15‰ salinity. Bar, 10 μm.

cholera victims (1, 13). As the microbial ecology of the causative agent of cholera becomes better understood, ecological explanations for the epidemiological characteristics of this disease begin to emerge (3).

In Bangladesh, seasonal cholera outbreaks begin around November, after the monsoon season. The water temperature often exceeds 25°C for prolonged periods during the summer, and zooplankton blooms occur after the monsoons (11). Based on data collected in our laboratory on the ecology of *V. cholerae*, a combination of high water temperature and an abundance of live copepods is concluded to be optimum for the survival and multiplication of *V. cholerae* in the aquatic environment, despite low salinities (1 to 5‰) found in much of the delta regions of Bangladesh. Furthermore, extensive attachment of *V. cholerae* cells to copepods would be expected to occur at these times. Thus, ingestion of raw, unpurified water containing copepods heavily contaminated with *V. cholerae* may offer an inoculum size sufficient to cause the disease in humans, as well as to initiate the disease within a community (4).

Sporadic outbreaks of cholera in developed countries have been associated with seafood consumption in warmer regions of the globe, such as the Gulf coast of America (2). It is likely that in these regions, where water temperatures are usually above 10°C for most of the year and the water is shallow, *V. cholerae* can survive in the aquatic environment in numbers below that required for an infective dose for humans (3, 17). Infection in humans by these organisms in warm waters of such geographical localities is, therefore, fortuitous, occurring only when several epidemiological factors join together, for example, a zooplankton-associated increase in the number of *V. cholerae* in the aquatic environment, inadvertent contamination of seafood with *V. cholerae*, and failure to prevent transmission of *V. cholerae* to susceptible human hosts via seafood or other aquatic vectors.

The public health risk of contracting cholera from consumption of shellfish harvested and processed in temperate global regions, such as those surrounding the Chesapeake Bay or the Oregon coast, is significantly, if not vanishingly, less probable than in warmer regions. In the former region, the inability of *V. cholerae* to multiply and survive for extended periods at the low temperatures of the local water (5°C), i.e., from late fall through early spring, would reduce the risk, even if large populations of copepods were to occur.

In our experiments, we used ca. $10^4$ *V. cholerae* cells per ml, which is somewhat high compared with the number found in the natural environment in cholera-endemic areas. Roberts et al. (12) have reported $1.1 \times 10^2$ cells per ml in sewer water in Louisiana, and Lee et al. (9) have reported $7.0 \times 10^2$ *V. cholerae* cells per ml in environmental water in England. However, an area in which cholera is endemic, e.g., Bangladesh, may have higher numbers of *V. cholerae* cells in water in the environment. Observations made in this

study with pure cultures may, therefore, aid in understanding the ecology of *V. cholerae* in experiments designed to replicate conditions in nature. Studies of competition with other bacteria of marine origin would also be interesting.

The epidemiology and ecology of *V. cholerae* are fascinatingly interwoven. Environmental data now being accumulated for this organism should provide not only an explanation for the outbreaks and endemic focus of cholera, but also offer new ideas for prevention and control.

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