

Effects of Temperature and Salinity on *Vibrio cholerae* Growth

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Laboratory microecosystems (microcosms) prepared with a chemically defined sea salt solution were used to study effects of selected environmental parameters on growth and activity of *Vibrio cholerae*. Growth responses under simulated estuarine conditions of 10 strains of *V. cholerae*, including clinical and environmental isolates as well as serovars O1 and non-O1, were compared, and all strains yielded populations of approximately the same final size. Effects of salinity and temperature on extended survival of *V. cholerae* demonstrated that, at an estuarine salinity (25‰) and a temperature of 10°C, *V. cholerae* survived (i.e., was culturable) for less than 4 days. Salinity was also found to influence activity, as measured by uptake of ¹⁴C-amino acids. Studies on the effect of selected ions on growth and activity of *V. cholerae* demonstrated that Na⁺ was required for growth. The results of this study further support the status of *V. cholerae* as an estuarine bacterium.

Vibrio cholerae, causative agent of epidemic cholera, has been isolated from a variety of clinical and environmental samples (1-6, 9, 10, 16, 22, 23). The majority of *V. cholerae* strains isolated from the environment are non-O1 serovars (16, 23), although O1 serovars have also been observed in areas where limited outbreaks of cholera have occurred (2).

The patchy distribution of serovars of *V. cholerae* in coastal and estuarine waters is unrelated to sewage contamination or to the presence of wastes that can serve as potential vehicles of entry into the environment (16). To account for the apparently sporadic distribution of *V. cholerae* in aquatic environments as well as the repeated occurrence of this organism in regions with no reported outbreaks of cholera, Colwell et al. (6) hypothesized that *V. cholerae* is a member of the autochthonous, i.e., naturally occurring, microbial flora of brackish water and estuaries. Many of the results of studies of the occurrence of *V. cholerae* in aquatic environments, as well as sporadic outbreaks of cholera in localities without an apparent source of the organism, can be explained by the autochthonous nature of *V. cholerae* in brackish water and estuarine environments.

Although the organism is known to occur in diverse aquatic systems, the influence of envi-

ronmental parameters on growth of *V. cholerae* has not been examined under controlled conditions. Salinity and nutrient concentrations have been reported to influence the growth and viability of *V. cholerae*, and results of such studies may, in part, explain the apparently selective distribution of *V. cholerae* in estuarine systems (5, 16). However, effects of environmental parameters other than salinity and nutrient concentration have not been considered in detail.

Evaluating the influence of selected environmental parameters on a bacterial population is difficult because of the spatial and temporal heterogeneity of such populations in situ and the constantly changing conditions of the environment, especially in tidal estuaries. Thus, it is difficult to interpret results obtained from in situ studies designed to evaluate the influence of one or more environmental parameters on a given bacterial species. Application of laboratory microecosystems (microcosms) offers a valuable tool for ecological studies, since microcosms enable replication of experimental units. Furthermore, environmental conditions can be simulated in laboratory experiments, and the results can be validated statistically. Thus, a microcosm can be used to define physical, chemical, and biological parameters of a given environment and to control those parameters during experimentation. Results obtained from experiments with microcosms must be interpreted carefully, since in situ activities are simulated but not necessarily precisely reproduced. Microcosm experiments are most useful when run in conjunction with in situ studies, thereby permitting

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tests, both in the laboratory and in the field, of hypotheses concerning activities of bacterial species in the natural environment.

The objective of this study was to evaluate the effects of selected environmental parameters on activity and growth of populations of *V. cholerae* in aquatic microcosms so that the role of this species in aquatic environments can be evaluated.

MATERIALS AND METHODS

Microcosm preparation. Microcosms, each of which consisted of 50 ml of a chemically defined sea salt solution (27) brought to appropriate salinity and contained in an acid-cleaned, 125-ml Erlenmeyer flask, were inoculated with washed cell suspensions of strains of *V. cholerae*. All microcosms were incubated for appropriate intervals at designated temperatures with constant agitation by rotary shakers (125 rpm).

Inoculum preparation. Strains of *V. cholerae* were incubated for 18 h at 30°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.). Cells were collected by centrifugation and washed five times with a sterile 0.85% (wt/vol) NaCl solution. After the final washing, a stock suspension was prepared in sterile 0.85% NaCl. Washed suspensions of test strains were used as inocula at an initial cell concentration of 4×10^7 /ml.

Cells used in experiments designed to establish specific ion requirements for growth were washed and diluted in basal salt solution prepared without NaCl. After stock suspensions were prepared, the cell concentration was determined by acridine orange direct count (AODC) (7, 11, 14), appropriate dilutions were prepared in 0.85% NaCl or sea salt solution (27), and microcosms were inoculated.

Bacterial strains and culture conditions. We used 10 strains of *V. cholerae* obtained from the stock culture collection at the University of Maryland, College Park. Fresh cultures on tryptic soy agar (Difco) slants were prepared, as needed, from stock cultures maintained under liquid nitrogen.

Bacterial enumeration. Samples were collected aseptically with sterile 1-ml pipettes, and total viable i.e., culturable, bacteria were enumerated by spread plating onto tryptic soy agar. Samples for viable counts were diluted in sea salt solution prepared to the same salinity as those of the microcosms being sampled. All culture plates were incubated for 7 days at 25°C before colony-forming units were counted. We established a statistically designed sampling regime based on a preliminary component of variance analysis (18) to estimate accurately the mean number of culturable bacteria. For the experiments reported here, a design consisting of three microcosms per treatment, one sample per microcosm, and three culture plates per sample was used. For total bacterial counts, 1-ml samples were collected aseptically, and counts were obtained by AODC and epifluorescence microscopy (7, 11, 14).

Heterotrophic activities. The heterotrophic activities of *V. cholerae* populations in microcosms were measured by the method of Hobbie and Crawford (13). Sterile, acid-cleaned serum vials, each containing 25 ml of sea salt solution with salinity or specific salt concentration adjusted to a desired level, were inoculated with 25- or 50- μ l samples collected from micro-

cosms after an appropriate incubation period. In some experiments, a washed cell suspension of *V. cholerae* was used as the inoculum. Each vial was supplemented with a ^{14}C -amino acid mixture (57 mCi/mg per atom of carbon; Amersham Corp., Arlington Heights, Ill.) to a final concentration of 50 or 100 $\mu\text{g/liter}$. These concentrations did not result in substrate saturation under the experimental conditions employed.

After addition of amino acids, the vials were sealed with serum stoppers fitted with plastic cups containing folded 1-cm² pieces of filter paper (Whatman no. 1). All vials (including controls, which were each fixed with 0.2 ml of a solution of 30% trichloroacetic acid [wt/vol] and 0.05% mercuric chloride [wt/vol] in 40% Formalin [vol/vol]), unless otherwise stated were incubated at $25 \pm 1^\circ\text{C}$ for 2 h before the reaction was terminated by injection of the trichloroacetic acid-mercuric chloride-Formalin mixture. Each filter wick was saturated with 0.2 ml of phenethylamine to absorb $^{14}\text{CO}_2$ evolved during an additional 1-h incubation period.

After the second incubation period, filter wicks were placed into scintillation vials, and each suspension was filtered through a 0.2- μm membrane filter (type GA-8; Gelman Sciences, Inc., Ann Arbor, Mich.) and rinsed with 10 ml of sterile sea salt solution. After air drying, the membrane filters were placed into scintillation vials. Scintillation cocktail (10 ml; Aqueous Counting Scintillant; Amersham) was added to each vial, and radioactive counts were made with a model LS-3155T liquid scintillation spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Uptake and incorporation of the amino acid mixture (micrograms liter⁻¹ hour⁻¹) were calculated by the procedure of Hobbie and Crawford (13).

Ion requirements. To determine the effect of various concentrations of NaCl on the growth and viability of *V. cholerae*, we prepared selected microcosms with a salt solution containing the following (grams liter⁻¹): $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$, 2.86; KBr, 0.07; H_3BO_3 , 0.019; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 7.74; CaCl_2 , 0.82; SrCl_2 , 0.017; K_2CO_3 , 0.05; and selected concentrations of NaCl. To ensure that the electrolyte solutions were precisely defined, we used reagents of the highest possible grade and purity whenever possible.

To minimize any change in total salinity, we conserved Cl^- and monovalent cation concentrations by substitution of molar equivalents of KCl or LiCl for NaCl as the amount of NaCl was decreased. Microcosms prepared in this way also enabled determination of the extent to which the Na^+ requirement, if there was one, could be satisfied by K^+ or Li^+ (12, 19-21). To investigate further the ion requirements of *V. cholerae*, we used a turbidimetric assay for growth in the presence of different ion concentrations and substrates. Basal salt solution in acid-cleaned, 18-mm culture tubes was supplemented with 1% (wt/vol) tryptone (Difco), 1% (wt/vol) glucose (Sigma Chemical Co., St. Louis, Mo.), or 1% (wt/vol) glucose (Sigma) plus 0.05% yeast extract (Difco). The contents of the culture tubes were amended with selected concentrations of NaCl, NaCl plus KCl or LiCl plus the concentrations of sucrose required to conserve osmotic pressure (12). Culture tubes prepared in this way were inoculated with washed cells to an initial concentration of approximately 10^5 /ml and incubated at 25°C. At 24-h intervals for a period of 14 days, culture tubes were

TABLE 1. Population sizes of *V. cholerae* strains in microcosms with a salinity of 25‰ and a tryptone concentration of 500 µg/liter^a

Strain	Source	Mean colony count ($\times 10^5$)/ml ^b	Mean total cell count ($\times 10^5$)/ml ^b
V-69 ^c	Environmental	6.14 \pm 1.14	6.40 \pm 1.13
N-19	Environmental	6.03 \pm 1.07	8.09 \pm 1.04
N-17	Environmental	5.37 \pm 1.04	5.79 \pm 1.14
N-1400	Environmental	7.00 \pm 1.05	7.19 \pm 1.35
N-1403	Environmental	5.87 \pm 1.12	7.40 \pm 1.16
N-33	Environmental	3.72 \pm 1.15	5.20 \pm 1.20
N-999	Environmental	4.84 \pm 1.06	5.40 \pm 1.29
SGN-7677 ^c	Environmental	5.86 \pm 1.08	5.60 \pm 1.50
LA-4808 ^c	Clinical	6.92 \pm 1.02	8.99 \pm 1.11
CA-401 ^c	Clinical	5.57 \pm 1.08	6.19 \pm 1.12

^a No significant difference between the colony count, obtained by plating on a nutrient medium, and the total direct count, obtained by the AODC method (7, 11, 14), was detected by the Duncan multiple comparison (24).

^b Mean \pm standard error.

^c O1 serovar.

examined for growth, as indicated by visible turbidity. All tubes positive for growth were sampled to verify viability and purity of the cultures. Samples were streaked onto tryptic soy agar and thiosulfate-sucrose-citrate-bile salt (TCBS) agar (Oxoid, Columbia, Md.).

Survival of *V. cholerae* at low temperatures. To determine survival of *V. cholerae* at low temperatures, we prepared a series of microcosms with salinities of 5 and 25‰. These were inoculated with 4×10^2 cells per ml and incubated at 10°C. After appropriate intervals, duplicate microcosms were transferred to 25°C and incubated for an additional 4-day period. Samples for AODCs and viable counts were collected immediately after inoculation, before transfer to 25°C, and after the 4-day incubation period at 25°C.

Salinity measurements. Salinities of the sea salt solutions were measured with a temperature-corrected American Optical refractometer (American Optical Corp., Keene, N.H.).

RESULTS

Comparative growth of *V. cholerae* strains in microcosms. Microcosms with a salinity of 25‰ and a nutrient concentration of 500 µg of tryptone per liter were incubated at 25°C and served as controls (27). Growth and survival in the control microcosms of 10 strains of *V. cholerae* isolated from diverse sources were compared (Table 1). On the average, all isolates, including clinical and environmental isolates of the O1 serovars, grew to approximately the same final cell concentration under the conditions used. Cell numbers, determined by AODC, ranged from 5.2×10^5 to 9.0×10^5 /ml, whereas culturable counts ranged from 3.7×10^5 to 7.0×10^5 /ml.

Bacterial counts, obtained by spread plating, were analyzed statistically. The experimental

design provided sufficient replication of experimental units to permit application of analysis of variance to the microcosm data (24). A comparison by the Duncan multiple comparison (24) of \log_{10} -transformed mean culturable counts for the 10 strains of *V. cholerae* demonstrated that the counts were not significantly different ($P \leq 0.05$; Table 1).

Since the populations of all strains tested developed to approximately the same size in the microcosms, and since corroborative data had been obtained for strain LA-4808 in previous studies (27), strain LA-4808 was selected as a representative strain, and data for this organism are provided in this report. Similar results were obtained for the other strains examined.

Effects of salinity and incubation temperature on *V. cholerae*. Microcosms with salinities of 5, 15, 25, and 35‰ were prepared and incubated at 10, 15, 20, and 25°C. When the microcosms were inoculated with 4×10^2 cells per ml and incubated at 10°C, culturable colony counts declined for all salinities tested (Fig. 1). Changes in total direct cell counts could not be determined, since the number of cells comprising the inoculum was below the practical limit required in the AODC technique. However, in all microcosms examined, culturable *V. cholerae* cells were present throughout the 8-day test period, indicating extended survival at 10°C.

An incubation temperature of 15°C yielded similar results, except for microcosms with a salinity of 15‰, in which both culturable and total cell counts increased (Fig. 2). At salinities of 5 and 35‰, the counts decreased. However, at 25‰ salinity, the culturable counts gradually increased throughout the experiment after day 2 of incubation. In microcosms with 15‰ salinity which were incubated at 15°C, the total cell counts paralleled the culturable counts after day

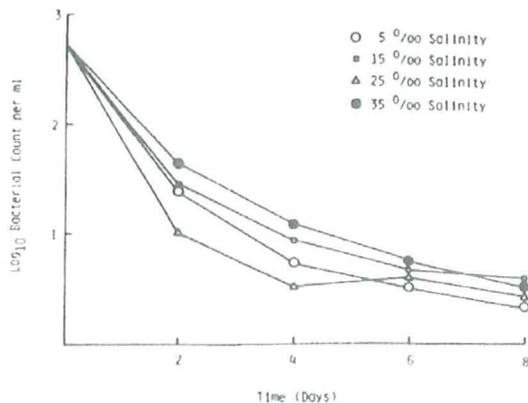


FIG. 1. Growth of *V. cholerae* LA-4808 at 10°C, as measured by plate count.

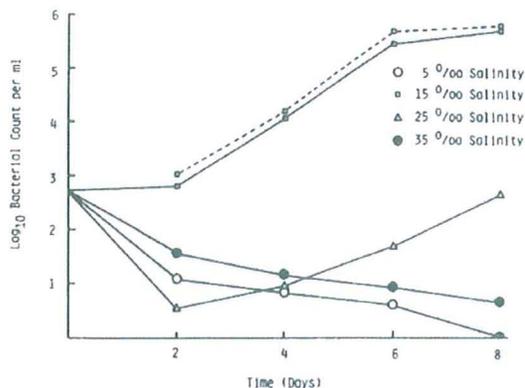


FIG. 2. Influence of salinity on *V. cholerae* LA-4808 grown at 15°C. -----, Total cell counts (AODC); —, total colony counts.

2 of incubation, when the cell concentration became sufficiently high to be monitored accurately by direct counting (Fig. 2).

Growth of *V. cholerae* incubated at 20°C was influenced by salinity, as indicated by the change in total and culturable cell counts (Fig. 3). In microcosms with salinities of 5, 25, and 35‰, culturable colony counts deviated little from the inoculation size 2 days after inoculation. However, *V. cholerae* in microcosms with 15‰ salinity increased by approximately 2.5 log₁₀ cycles. By day 4, populations of *V. cholerae* in all salinities demonstrated a significant increase ($P \leq 0.05$) over the original inoculum size (27). Maximum culturable populations were detected in 25‰ salinity microcosms, although these were not consistently significantly larger ($P \leq 0.05$) than populations in 15‰ salinity

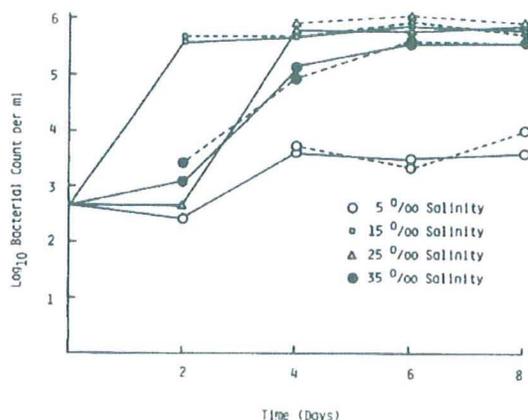


FIG. 3. Effect of salinity on *V. cholerae* LA-4808 incubated at 20°C in microcosms with various salinities. -----, Total cell counts (AODC); —, total colony counts.

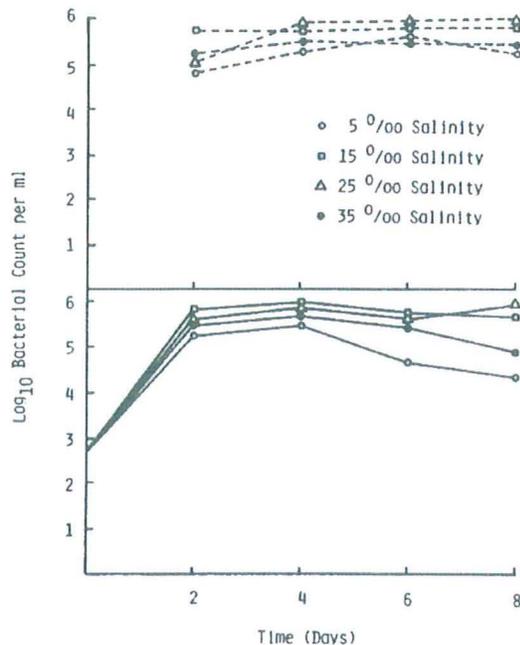


FIG. 4. *V. cholerae* LA-4808 population sizes in microcosms with salinities adjusted to indicated values. Incubation was at 25°C. -----, Total cell counts (AODC); —, total colony counts.

microcosms. The smallest numbers of culturable *V. cholerae* were present in 5‰ salinity microcosms. Total cell populations (determined by AODC) developed according to the same pattern as that of the culturable populations (Fig. 3). Not only were total and culturable population development patterns parallel in the presence of different salinities, but so were population sizes, with 25‰ salinity microcosms containing the maximum populations and 5‰ salinity microcosms containing the minimum populations.

Results obtained for microcosms incubated at 25°C were similar to those obtained for microcosms incubated at 20°C, although no detectable lag in development of *V. cholerae* populations at any of the salinities tested was noted (Fig. 4). Both AODCs and culturable counts increased to maximum by day 4. During the last 4 days of testing, total cell counts were stable at all salinities tested, whereas only the culturable counts remained constant at 15 and 25‰ salinities. Culturable colony counts in microcosms of 5 and 35‰ salinities decreased by at least 0.5 log₁₀ cycle during the last 4 days of testing.

Heterotrophic activity. After the final sampling of microcosms for enumeration in the aforementioned studies, samples were collected to determine heterotrophic uptake of ¹⁴C-amino acids by *V. cholerae* populations incubated at differ-

TABLE 2. Heterotrophic uptake of ^{14}C -amino acids by *V. cholerae* in basal salt solutions of various salinities incubated at selected temperatures^a

Temp (°C)	Salinity (‰)	Uptake rate ($\mu\text{g liter}^{-1} \text{h}^{-1} \times 10^{-2}$) ^b	Uptake rate/culturable cell ($\mu\text{g liter}^{-1} \text{h}^{-1}$) ^c	Uptake rate/total cell no. ($\mu\text{g liter}^{-1} \text{h}^{-1}$) ^d
25	5	2.43	6.93×10^{-7}	1.08×10^{-7}
	15	4.78	1.16×10^{-7}	8.68×10^{-8}
	25	14.40	1.75×10^{-7}	1.81×10^{-7}
	35	2.74	3.38×10^{-7}	8.64×10^{-8}
20	5	2.95	6.82×10^{-6}	3.58×10^{-6}
	15	7.36	9.97×10^{-8}	1.13×10^{-7}
	25	12.9	1.33×10^{-7}	1.66×10^{-7}
	35	4.94	1.14×10^{-7}	1.71×10^{-7}
15	5	ND ^e		
	15	3.36	4.94×10^{-8}	6.87×10^{-8}
	25	ND		
	35	ND		

^a Samples were collected from microcosms and inoculated into reaction vials containing basal salt solution of the same salinity as the microcosms.

^b Mean of duplicate flasks per treatment.

^c Obtained from mean culturable colony count.

^d Obtained from AODC.

^e ND, None detected, as compared with killed controls.

ent salinities and temperatures. However, since the microcosms contained different numbers of bacteria, it was not unexpected that the largest uptake values were obtained for systems which received inocula from microcosms with the largest *V. cholerae* populations (Table 2). At 25 and 20°C, populations of *V. cholerae* from microcosms with a salinity of 25‰ utilized more amino acids than did populations at the other salinities tested. We also observed that at 25 and 20°C, the lowest rates of uptake were obtained for populations from microcosms with 5‰ salinity.

To obtain a representation of the influences of salinity and temperature on heterotrophic activity, we calculated the uptake of the ^{14}C -amino acid mixture on a per-cell basis. When the total uptake of amino acids by *V. cholerae* in the microcosms was compared with the total and culturable population sizes, with uptake per cell calculated, we observed that both temperature and salinity influenced activity (Table 2). In microcosms incubated at 25°C, the largest uptake per cell for the total population was obtained for microcosms of 25‰ salinity. Rates of uptake of amino acids at all other salinities were approximately equivalent and approximately 50% less than that of the 25‰ salinity microcosms. Such a difference was not obvious, however, when uptake rates per culturable cell were compared. Greater uptake per cell was obtained for 5 and 35‰ salinity microcosms.

When microcosms were incubated at 20°C and the activities of the *V. cholerae* populations were compared (Table 2), higher rates of uptake

were obtained for populations in 25‰ salinity. Although uptake rates per total cell number for populations in 15, 25, and 25‰ salinities were between 1.13×10^{-7} and $1.71 \times 10^{-7} \mu\text{g liter}^{-1} \text{h}^{-1}$ the 5‰ salinity microcosms yielded an uptake approximately 25 times greater, which was also the case for uptake per culturable cell.

Prolonged survival of *V. cholerae*. After incubation of 5‰ salinity microcosms for 8 days at 10°C, no culturable cells were detected before or after an additional incubation period of 4 days at 25°C (Table 3). In microcosms of 25‰ salinity, however, culturable cells were detected up to 42 days after incubation at 10°C. After an additional incubation period at 25°C, populations of *V. cholerae* developed to a population size typical for the conditions used (Table 3). *V. cholerae* survived, i.e., remained viable, although at less-than-detectable numbers, after extended incubation (42 days, the maximum period tested) at 10°C in microcosms of 25‰ salinity.

Ionic requirements of *V. cholerae*. To determine whether the effects of salinity on stability and survival of *V. cholerae* were due, in part, to a requirement for a specific ion(s), we prepared a series of microcosms, using selected concentrations of NaCl or MgCl₂, the major salts in seawater, in the sea salt solution (27). Growth of *V. cholerae* at different concentrations of NaCl and MgCl₂ was measured after incubation for 4 days at 25°C. When the concentration of MgCl₂ in the microcosms was varied, little effect was observed on total or culturable cell count (Fig. 5). However, an effect was evident when the NaCl concentration was altered. Both total and

TABLE 3. Survival of *V. cholerae* at 10°C in microcosms with salinities of 5 and 25‰^a

Microcosm salinity (‰)	No. of days at 10°C before 25°C incubation for 4 days	Culturable count before 25°C incubation (log ₁₀ /ml) ^b	Culturable count after 25°C incubation (log ₁₀ /ml) ^b	Total cell count (log ₁₀ /ml) ^b
5	4	ND ^c	ND	ND
25	4	0.836 ± 0.212	5.517 ± 0.065	5.778 ± 0.115
5	8	ND	ND	ND
25	8	0.251 ± 0.207	5.561 ± 0.075	ND
25	25	0.156 ± 0.197	5.533 ± 0.021	5.756 ± 0.040
25	30	ND	ND	5.931 ± 0.020
25	42	ND	5.562 ± 0.041	5.454 ± 0.055

^a All microcosms were inoculated with approximately 4×10^2 cells per ml.

^b Mean ± standard error.

^c ND, None detected.

culturable cell counts followed the same pattern, with maximum populations occurring in control microcosms and minimum populations at lower and higher NaCl concentrations (Fig. 6). When no NaCl was added, a large difference (ca. 3 log₁₀) between total and culturable cell counts was detected.

Uptake of ¹⁴C-amino acids by *V. cholerae* showed that heterotrophic activity was also affected in microcosms with different concentrations of NaCl or MgCl₂ (Table 4). Maximum uptake of the amino acid mixture occurred in the presence of the intermediate, or control, MgCl₂ concentration (7.74 g/liter). When the MgCl₂ concentration was increased or decreased, uptake decreased. A similar uptake pattern was observed in microcosms adjusted to different NaCl concentrations (Table 4). Uptake decreased with increasing or decreasing NaCl concentration, compared with uptake at the intermediate concentrations; the lowest uptake values were detected in the presence of the largest or smallest concentrations.

Uptake values per total cell number were similar in all microcosms amended with NaCl (Table 4). However, it was evident that, as the NaCl concentration increased, the uptake per total cell number decreased. Similar results were, in general, obtained by comparing uptake per culturable cell.

The ratio of the amount of incorporation of amino acid mixture to the amount of respiration was found to be influenced by NaCl and MgCl₂ concentrations (Table 4). In microcosms prepared with NaCl and MgCl₂ concentrations of 17.09 and 7.74 g/liter, respectively, the lowest respiration-to-incorporation ratios were detected. Only in those flasks which received no NaCl was this ratio greater than 1:1.8, indicating the presence of physiologically stressed cells (12).

Since an effect on the *V. cholerae* populations was induced by varying the NaCl concentration, an additional series of microcosms prepared with a chemically defined basal salt solution

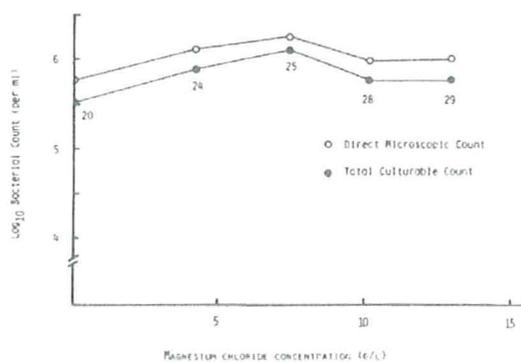


FIG. 5. Influence of magnesium chloride (as MgCl₂ · 6H₂O) concentration on *V. cholerae* LA-4808. Numbers represent salinities of magnesium chloride.

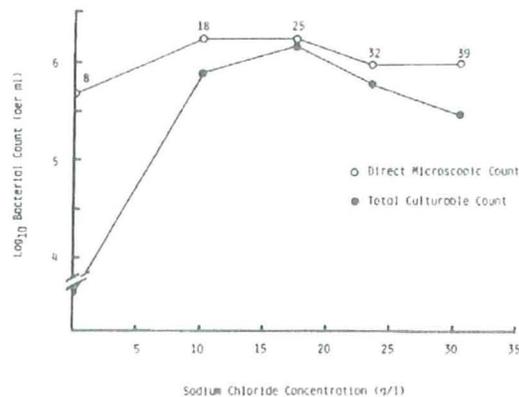


FIG. 6. Effect of sodium chloride concentration on *V. cholerae* LA-4808 in microcosms. Numbers indicate salinities.

TABLE 4. Uptake of ^{14}C -amino acids by *V. cholerae* in basal salt solution amended with selected concentrations of sodium chloride and magnesium chloride

Salt concn (g/liter)	Mean uptake rate ($\mu\text{g liter}^{-1} \text{h}^{-1} \times 10^{-3}$) ^a	Uptake per cell ($\mu\text{g liter}^{-1} \text{h}^{-1} \times 10^{-6}$)	Uptake per culturable cell ($\mu\text{g liter}^{-1} \text{h}^{-1} \times 10^{-6}$)	Respiration/incorporation ratio
NaCl				
0	7.30 \pm 0.40	5.96	0.0146	1:5.3
10.25	16.61 \pm 3.21	3.69	8.29	1:1.7
17.09	12.74 \pm 1.56	4.63	7.45	1:1.8
23.93	10.67 \pm 0.49	3.56	6.53	1:1.5
30.76	5.33 \pm 1.26	1.18	7.03	1:0.6
MgCl₂^b				
0	4.36 \pm 0.39	2.91	4.95	1:1.1
4.641	10.65 \pm 3.62	3.04	5.19	1:1.0
7.74	14.02 \pm 2.41	5.97	9.39	1:3.3
10.83	11.11 \pm 6.19	4.44	7.22	1:1.3
13.92	8.25 \pm 1.65	1.74	5.53	1:1.1

^a Mean \pm standard deviation.^b MgCl₂ added as MgCl₂ · 6H₂O).

containing no added Na⁺ was used to determine whether *V. cholerae* requires Na⁺ for growth. Also, to determine whether an Na⁺ requirement, if there was one, could be satisfied by the presence of another monovalent cation and to minimize change in salinity, we amended the NaCl-basal salt solutions with KCl or LiCl.

After incubation for 4 days at 25°C, population sizes in the microcosms with various NaCl concentrations were compared (Table 5). As the NaCl concentration was decreased from 0.30 to 0.10 M and 0.20 M KCl was added, the *V. cholerae* populations increased, although not significantly ($P \leq 0.5$), as determined by the Duncan multiple comparison (24). As the NaCl concentration was decreased to 0.01 M, the population size also decreased. When no NaCl

was added, no *V. cholerae* could be recovered from any microcosms.

The heterotrophic activity of *V. cholerae* populations was also affected when NaCl concentrations were varied (Table 6). The maximum uptake of ^{14}C -amino acids (Table 6) and the maximum population sizes (Table 5) were detected in samples from microcosms amended with 0.1 M NaCl and 0.2 M KCl. The largest uptake values per culturable cell and per total cell population were detected in samples taken from the 0.3 M NaCl and 0.1 M NaCl microcosms, respectively. Since no populations of *V. cholerae* developed in microcosms amended with LiCl, no uptake rates could be determined.

Since *V. cholerae* did not grow in microcosms amended with LiCl, we evaluated the influence

TABLE 5. *V. cholerae* population sizes in microcosms amended with selected concentrations of NaCl, KCl, and LiCl

Microcosm containing:			Mean log ₁₀ culturable count/ml ^a	Mean log ₁₀ total cell count/ml ^a
NaCl (M)	KCl (M)	LiCl (M)		
0.30	0	0	5.550 \pm 0.045	5.821 \pm 0.030
0.15	0.15	0	5.584 \pm 0.028	5.947 \pm 0.005
0.10	0.20	0	5.778 \pm 0.035	6.000 \pm 0.029
0.05	0.25	0	5.640 \pm 0.041	5.808 \pm 0.028
0.01	0.29	0	5.480 \pm 0.056	5.687 \pm 0.149
0	0.30	0	ND ^b	ND
0.15	0	0.15	ND	ND
0.10	0	0.20	ND	ND
0.05	0	0.75	ND	ND
0.01	0	0.29	ND	ND
0	0	0.30	ND	ND

^a Mean \pm standard error.^b ND, None detected.

TABLE 6. Uptake of ^{14}C -amino acids by *V. cholerae* cultured in microcosms amended with selected concentrations of NaCl and KCl^a

Microcosm containing:		Mean uptake rate ($\mu\text{g liter}^{-1} \text{h}^{-1} \times 10^{-2}$)	Uptake rate/ culturable cell ($\mu\text{g liter}^{-1} \text{h}^{-1}$)	Uptake rate/ total cell no. ($\mu\text{g liter}^{-1} \text{h}^{-1}$)	Respiration/ incorporation ratio
NaCl (M)	KCl (M)				
0.30	0	4.29	1.21×10^{-7}	6.48×10^{-8}	1:0.56
0.15	0.15	1.11	2.89×10^{-7}	1.25×10^{-8}	1:0.81
0.10	0.20	12.60	2.10×10^{-7}	1.25×10^{-7}	1:1.18
0.05	0.25	1.73	3.96×10^{-8}	2.69×10^{-8}	1:8.16
0.01	0.29	1.29	4.27×10^{-8}	2.65×10^{-8}	1:5.16
0	0.30	ND ^b			

^a Samples for uptake studies were collected from the microcosms after the 4-day incubation period and inoculated into reaction vials containing the basal salt solution with salinity adjusted to that of the microcosm from which the inoculum was collected.

^b ND, None detected.

of Na^+ , K^+ , and Li^+ on the activity of *V. cholerae* by determining uptake of the ^{14}C -amino acid mixture by a washed cell suspension (Table 7). The mean uptake of freshly cultured *V. cholerae* cells was found to increase with decreasing NaCl concentration and increasing LiCl concentration until concentrations of 0.20 M NaCl and 0.10 M LiCl were reached. The lowest uptake rate was observed in systems without added NaCl. Similarly, the highest respiration-to-incorporation ratios were detected in NaCl-deficient systems.

When NaCl and KCl were used to amend the basal salt solution, slower uptake rates were obtained (Table 7). In these systems, amino acid uptake typically decreased with decreasing NaCl concentration. Maximum uptake was obtained at 0.01 M NaCl and 0.29 M KCl. In the

TABLE 7. Effects of sodium, lithium, and potassium on uptake of ^{14}C -amino acids by *V. cholerae*^a

NaCl (M)	LiCl (M)	KCl (M)	Mean uptake rate (μg $\text{liter}^{-1} \text{h}^{-1}$ $\times 10^{-2}$)	Respiration/ incorporation ratio
0.30	— ^b	—	4.18	1:1.2
0.295	0.005	—	5.80	1:1.3
0.25	0.05	—	8.83	1:1.2
0.20	0.10	—	9.18	1:1.2
0.15	0.15	—	8.20	1:1.2
0.15	—	0.15	3.76	1:1.0
0.05	—	0.25	3.29	1:0.86
0.01	—	0.29	3.92	1:0.42
0.005	—	0.295	2.34	1:0.34
0.0005	—	0.2995	1.51	1:0.16
—	—	0.30	1.40	1:0.14

^a Uptake was determined with cells cultured in tryptic soy broth and washed five times in basal salt solution containing no added Na^+ .

^b —, 0

presence of 0.15 M NaCl and 0.15 M KCl, the respiration-to-incorporation ratio was 1.0:1. At all other concentrations, however, this ratio was greater than 1.0, indicative of stressed cells (12).

When *V. cholerae* was inoculated into culture tubes containing different substrates and different salt concentrations, both the type of substrate and the salt concentration influenced growth of *V. cholerae* (Table 8). Culture tubes containing 1% (wt/vol) glucose and 0.05% (wt/vol) yeast extract were used to demonstrate the influence of salt concentration on growth of *V. cholerae*. Growth was monitored spectrophotometrically.

Growth occurred in all tubes containing only NaCl, although the lag period increased at the lower NaCl concentrations (Table 8). When 0.01 M NaCl was added, the lag period before detectable growth of *V. cholerae* occurred was between 24 and 48 h. As the NaCl concentration was increased to 0.025 M, the lag period decreased, with growth detected after incubation for 24 h at 25°C. Similarly, at all higher NaCl concentrations tested, growth occurred within 24 h. However, in tubes containing no added NaCl, no growth of *V. cholerae* was evident during the 14-day test period.

Cultures in which NaCl and KCl were present yielded results similar to those of cultures amended with NaCl alone (Table 8). At higher NaCl and lower KCl concentrations, growth of *V. cholerae* occurred within 24 h. Only when the NaCl and KCl concentrations were reduced to 0.025 and 0.275 M, respectively, was the lag period extended. Cultures containing 0.30 M KCl and no added NaCl failed to demonstrate growth of *V. cholerae*.

When cultures were amended with a combination of NaCl and LiCl, the growth pattern of *V. cholerae* was also similar to that obtained when only NaCl was added (Tables 5 and 8). *V. cholerae* grew in all concentrations of LiCl test-

TABLE 8. Growth of *V. cholerae* in basal salt solution with 1% (wt/vol) glucose and 0.05% (wt/vol) yeast extract as the growth substrate and amended with selected concentrations of NaCl, KCl, and LiCl

Added salt(s) (M)	Growth after incubation for (days): ^a					
	1	2	4	8	10	14
NaCl						
0.30	+	+	+	+	+	+
0.20	+	+	+	+	+	+
0.10	+	+	+	+	+	+
0.075	+	+	+	+	+	+
0.05	+	+	+	+	+	+
0.025	+	+	+	+	+	+
0.01	-	+	+	+	+	+
0	-	-	-	-	-	-
NaCl + KCl						
0.03 + 0	+	+	+	+	+	+
0.20 + 0.10	+	+	+	+	+	+
0.10 + 0.20	+	+	+	+	+	+
0.075 + 0.225	+	+	+	+	+	+
0.05 + 0.25	+	+	+	+	+	+
0.025 + 0.275	-	+	+	+	+	+
0.01 + 0.29	-	-	+	+	+	+
0 + 0.30	-	-	-	-	-	-
NaCl + LiCl						
0.30 + 0	+	+	+	+	+	+
0.20 + 0.10	+	+	+	+	+	+
0.10 + 0.20	+	+	+	+	+	+
0.075 + 0.225	+	+	+	+	+	+
0.05 + 0.25	+	+	+	+	+	+
0.025 + 0.274	+	+	+	+	+	+
0.01 + 0.29	-	+	+	+	+	+
0 + 0.30	-	-	-	-	-	-
NaCl + sucrose ^b						
0.13 + 0	+	+	+	+	+	+
0.10 + 0.55	+	+	+	+	+	+
0.06 + 0.125	+	+	+	+	+	+
0.04 + 0.160	+	+	+	+	+	+
0 + 0.190	-	-	-	-	-	-

^a +, Growth as indicated by turbidity; -, no growth.

^b Sucrose was added to maintain osmotic pressure.

ed, provided NaCl was also added. There was, however, an increased period required for visible turbidity to occur when 0.01 M NaCl was added.

In all salt concentrations tested, if 1% (wt/vol) tryptone was used as a substrate, detectable growth occurred within 24 h. Growth did not occur, however, if 1% (wt/vol) glucose or 0.05% (wt/vol) yeast extract was added as the sole substrate. No detectable growth occurred during the 14-day test period without addition of NaCl and both glucose and yeast extract.

To determine if the growth patterns obtained when various concentrations of salts and different substrates were used arose from alteration of

osmotic pressure, we added NaCl and sucrose to the test systems to maintain an osmotic pressure equivalent to that achieved with 0.13 M NaCl (12) (Table 8). As observed in previous experiments, if tryptone was used as the substrate, growth occurred if NaCl was added, regardless of concentration. No growth occurred with 1% (wt/vol) glucose as the substrate. However, with 1% (wt/vol) glucose-0.05% (wt/vol) yeast extract as the substrate, growth occurred within 24 h if NaCl was also added. Without added NaCl, no detectable growth of *V. cholerae* occurred during incubation at 25°C for 14 days.

DISCUSSION

Although *V. cholerae* is known to occur in aquatic environments throughout the world and has been documented as a clinically important organism for nearly a century, surprisingly little is known about its ecology, notably its ecological interaction with abiotic environmental parameters. Such a gap in knowledge, is due, no doubt, to the fact that, in the past, investigations of the biology of *V. cholerae* have been approached with the viewpoint that the species is solely of clinical origin. However, the recent hypothesis of Colwell et al. (5) that *V. cholerae* is an autochthonous member of the bacterial flora of brackish water and estuarine systems offers a new perspective on the ecology of this organism. Results reported here support this hypothesis and emphasize the need for an ecological approach in evaluating the role of *V. cholerae* in nature and in human disease.

Results of a comparison of the growth responses of 10 *V. cholerae* strains of O1 and non-O1 serovars demonstrate that, under stimulated environmental conditions, the population size achieved by a given strain is independent of the source of the strain (Table 1). For example, *V. cholerae* LA-4808, a clinical isolate from a patient who became ill after ingestion of improperly prepared seafood, *V. cholerae* CA-401, a clinical isolate, and *V. cholerae* V-69, an O1 environmental serovar not associated with disease, responded similarly under the conditions used in this study.

A recent investigation into the temporal distribution of *V. cholerae* in aquatic systems in Kent, England, was carried out by West and Lee (28). They observed a growth pattern of *V. cholerae* in situ growth chambers similar to that in the microcosms used in our study. Therefore, on the basis of results presented here and those obtained from our previous work (5, 6, 27), we conclude that under a proper regime of nutrients, temperature, and salinity, *V. cholerae* is capable of growth in an aquatic environment in nature.

In surveys carried out during the winter months, when the temperature is reduced, the occurrence of *V. cholerae* in aquatic environments of the temperature zone is apparently severely restricted (5, 16). A die-off induced by low temperature may, in part, explain the apparent disappearance of allochthonous bacteria, especially human pathogens, from an aquatic system. However, as indicated by results obtained for microcosms incubated at 10°C (Table 3), *V. cholerae* can exist for extended periods of time at low temperatures, although often below detectable levels, as measured by plating or by direct microscopic observation. The few viable cells, i.e., surviving cells, possibly adsorb to surfaces of the container in the laboratory or to natural substrates in the environment (29) and remain viable until favorable growth conditions return. It is also possible that viable organisms simply do not grow on any of the plating media used without pretreatment of the sample, as has been previously suggested for *V. cholerae* (27).

The influence of salinity on survival of *V. cholerae* in microcosms incubated at 10°C is unequivocal: it survived for up to 42 days at a salinity of 25‰ but not 5‰. In the latter case, the bacteria were not detected by culturing within 4 days of the start of incubation (Table 3). In natural aquatic systems in which *V. cholerae* is known to be present, a seasonal distribution of this organism can be explained by "overwintering" in waters with a salinity range that is optimal for *V. cholerae*. In an estuary or area of brackish water, these requirements can easily be met.

Results of experiments designed to detect a requirement for Na⁺ by *V. cholerae* further support the hypothesis that the natural habitat of *V. cholerae* is estuarine or brackish (6) (Tables 5 through 8). The requirement for Na⁺ could not be met by addition of K⁺ or Li⁺, demonstrating a clear specificity of the requirement for Na⁺.

The requirement for Na⁺ demonstrated by many marine bacteria has been well established, notably by the elegant research of MacLeod and co-workers (12, 19–21). The obligate requirement for Na⁺ is considered to be a differentiating characteristic useful in separating marine and estuarine bacteria from freshwater bacteria (19). In a recent report on the effect of Na⁺ on growth patterns of a marine bacterium, Gow et al. (12) demonstrated that as the Na⁺ concentration was decreased to near the minimum required to support growth, the major influence observed on growth was an extended lag period before growth. Also, when no Na⁺ was added, no growth occurred.

In this study, *V. cholerae* was found to respond to various concentrations of NaCl in a manner similar to that of the marine bacterium,

Alteromonas haloplanktis 216, studied by Gow et al. (12). In a substrate containing 1% (wt/vol) glucose and 0.05% (wt/vol) yeast extract, the effects of specific ions on the growth of *V. cholerae* were tested, and, at the lower NaCl concentrations (i.e., <0.01 M), the time required before growth occurred increased (Table 8). Also, in the absence of added NaCl, no growth of *V. cholerae* occurred. In systems with 1% (wt/vol) tryptone as the substrate, growth occurred in the presence or absence of added NaCl, indicating that a sufficient quantity of Na⁺ is available in tryptone to support growth of *V. cholerae*. A quantitative analysis of tryptone (8) demonstrates that, by weight, tryptone contains approximately 2.7% Na⁺. Thus, sufficient Na⁺ is present in a medium prepared with 1% (wt/vol) tryptone (ca. 0.0012 M Na⁺) to support growth of *V. cholerae* (Table 8). Since tryptone alone supported growth of *V. cholerae* and glucose had to be supplemented with yeast extract to support growth, we concluded that *V. cholerae* requires one or more vitamins or other factors for growth, in addition to Na⁺. As indicated by survival at 10°C (Table 3), *V. cholerae* has the capability to survive for extended periods under adverse conditions of low temperature and reduced nutrient concentrations. When conditions become favorable, growth is initiated. Thus, *V. cholerae* possesses an ecological advantage expected and characteristic of an aquatic organism, especially one inhabiting a brackish or estuarine environment.

Growth patterns obtained under conditions simulating those typical of estuarine and marine habitats indicate a preference of *V. cholerae* for moderate salinity, i.e., between 15 and 25‰ (between freshwater and seawater). Also, uptake of ¹⁴C-amino acids by *V. cholerae* under different salinity regimes and within a given temperature range demonstrates an unequivocal effect of salinity on the metabolic state of *V. cholerae* (Table 2). When uptake rates per culturable cell at 20 and 25°C were compared, greater uptake was observed at 5‰ salinity. Since the uptake rate per culturable cell at 5‰ salinity was greater than the uptake rates at 15 and 25‰ salinities, more substrate was required to be metabolized in systems of suboptimum salinity to maintain viability. Such conditions suggest a physiological stress on the cells (D. Kushner, personal communication).

An analysis of the respiration-to-incorporation ratios obtained at different ionic concentrations demonstrated that total salinity, as well as concentration of specific ions, could stress cells physiologically, as indicated by ratios greater than 1.0 (12; Kushner, personal communication; (Tables 4 through 7).

Taking into account the salinity and tempera-

ture data for *V. cholerae* and considering the organism to be an indigenous member of the bacterial community of estuarine systems with a capability for surviving for extended periods under adverse conditions, we can now account for many of the sporadic outbreaks of cholera in geographic areas without a known focus or source of infection. Recent outbreaks of cholera in Louisiana (2) and Texas (4) have been associated with or related to consumption of shellfish. Shellfish may indeed serve as an effective environmental reservoir for *V. cholerae*, either through a nonspecific association, or, as has been hypothesized (6), by interaction with *V. cholerae* in a commensal relationship. Furthermore, the enterotoxin of *V. cholerae* may play a role in salt tolerance, osmoregulation, or both, most probably in crustacea (6). A possible ecological role of *V. cholerae* enterotoxin is the sequestering of Na^+ from its commensal host for growth of the bacterium. When cholera toxin binds to the cell receptors of human intestinal epithelial cells, adenylate cyclase activity increases, which causes the level of cyclic AMP to increase. The increase in cyclic AMP activity results in an efflux of Na^+ and other electrolytes from epithelial cells, along with an efflux of quantities of water, producing the symptoms associated with cholera (15, 17, 25, 26). Should cholera toxin affect epithelial cells of crustacea similarly but less drastically, *V. cholerae*, in association with crustacea, would provide for itself a means for obtaining sufficient quantities of Na^+ from its host should the salinity of the environment fall below that required for its survival and growth.

On the basis of results reported here, we conclude that *V. cholerae* is, as hypothesized by Colwell et al. (6), an indigenous member of the bacterial community of estuarine systems, a conclusion evident not only from the requirement of Na^+ for growth but also from the obvious preference of the organisms for a salinity typical of a brackish water estuary. Distribution of *V. cholerae* in the environment is not necessarily limited to estuaries, however, since the requirement for an optimum salinity can be satisfied by a sufficiently high nutrient concentration (27). Epidemiological surveys for *V. cholerae* would be more effective if they were combined with laboratory-controlled microcosm studies, which would permit a more complete understanding of the role and function of *V. cholerae* in the natural environment.

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