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Ecology of *Vibrio cholerae* Non-O1 and *Salmonella* spp. and Role of Zooplankton in Their Seasonal Distribution in Fukuyama Coastal Waters, Japan

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Seasonal variation of human pathogens such as *Vibrio cholerae* non-O1 and *Salmonella* spp. in Fukuyama coastal waters and the role of zooplankton in their distribution were studied for a period of 1 year. Comparison of two established methods, viz., the elevated temperature method and the two-step enrichment method of enumerating *V. cholerae*, showed that the former is superior in the recoveries of *V. cholerae* non-O1. Isolation of this pathogen on a wider range of salinities (0.4 to 32.5‰) revealed that these organisms are apparently an autochthonous component of the aquatic environment. Temperature appears to be the most crucial element in governing the distribution of *V. cholerae* non-O1. Among the 69 isolates serotyped, 22 different serovars were identified, while one isolate failed to react with any of the known Louisiana State University antisera tested. Zooplankton samples did not harbor more *V. cholerae* non-O1 than the water column did. Better isolation of an allochthonous pathogen, viz., *Salmonella* spp., was noticed from the water samples when swabs were employed. Of the 251 isolates serotyped, 18 serotypes with three variants of *Salmonella* spp. were identified. A high amount of nutrients in the water column increased the survival rate of these pathogens in saline waters as evidenced by a higher incidence of various serotypes in polluted Fukuyama port than in clean marine waters. *Salmonella* spp. association with zooplankton remained below detectable levels in most of the sampling periods. No significant association between *V. cholerae* non-O1 or *Salmonella* spp. with zooplankton could be noticed as influencing their seasonal distribution.

Vibrio cholerae non-O1 has been widely distributed in marine environments, especially bays, estuaries, and other brackish waters around the world (6, 7, 10, 15, 19, 22). The occurrence and ecology of *V. cholerae* was extensively studied for the past 4 years in the coastal belts of the United States (15, 17, 24, 29) and the United Kingdom (20, 34). The ecology of *V. cholerae* non-O1 is not well understood (35), and little significance has been attached to the occurrence unless associated with disease in Japan.

Enteric pathogens frequently become associated with aquatic animals ranging from microscopic invertebrates to marine mammals. These associations can be accidental and transient, as seems to be the case with fish and *Salmonella* spp. (18), or they can be very specific and long lasting, as demonstrated for copepods and *Vibrio parahaemolyticus* (13, 14). The association of *V. parahaemolyticus* with copepods is dictating the seasonal variation in Chesapeake Bay (14) and in Fukuyama coastal waters (K. Venkateswaran, S. W. Kim, H. Nakano, T. Onbé and H. Hashimoto, Syst. Appl. Microbiol., in press). Experimental studies by Huq et al. (12) concluded that adhesion of cells of *V. cholerae* onto the surfaces of live copepods may contribute significantly to the survival and distribution of *V. cholerae* in the aquatic environment. However, the adsorption of *Salmonella* spp. onto zooplankton has not been hitherto ascertained (K. Ramamurthy, Ph.D. thesis, Annamalai University, India, 1987).

Thus, this ecological study is carried out to examine the association between zooplankton and enteric pathogens like *V. cholerae* and *Salmonella* spp. in an aquatic environment. In addition, we have correlated various environmental parameters and the pollution indicator bacterial population with the isolation of *V. cholerae* and *Salmonella* spp.

MATERIALS AND METHODS

Study area. Samples were collected in five stations with two in riverine (Takaya River and Ashida River) and three in marine (Fukuyama port, Tajiri port, and Abuto) areas (Fig. 1). The Takaya River (station 1) is a branch stream which sheds the effluents of the slaughterhouse situated upstream into the Ashida River (station 2) and subsequently flows into the Seto Inland Sea of Japan at Fukuyama City, Hiroshima Prefecture. Fukuyama port is an engulfed eutrophic area which experiences heavy traffic of vessels used for unloading iron ore, etc., and there is an alarming level of pollution by industrial and domestic effluents (station 3). Tajiri port (station 4) is a fishing area with a jetty for local fishermen. Abuto (station 5) is a clean marine environment, seldom disturbed by human activities.

Surface water samples were collected from each of the five stations once a month from March 1987 to February 1988. Swabs for *Salmonella* spp. examination were prepared by wrapping them in sterile cotton gauze, sandwiching them between wire mesh, and allowing them to float in the water column for 2 days. Zooplankton samples from Fukuyama port and Abuto were collected by towing a standard Kitahara type NXX 13 plankton net with a mesh size of 100 μm horizontally at subsurface level. The plankton samples were washed twice with the water sampled on the same site and concentrated by passing through a net with a 100-μm pore

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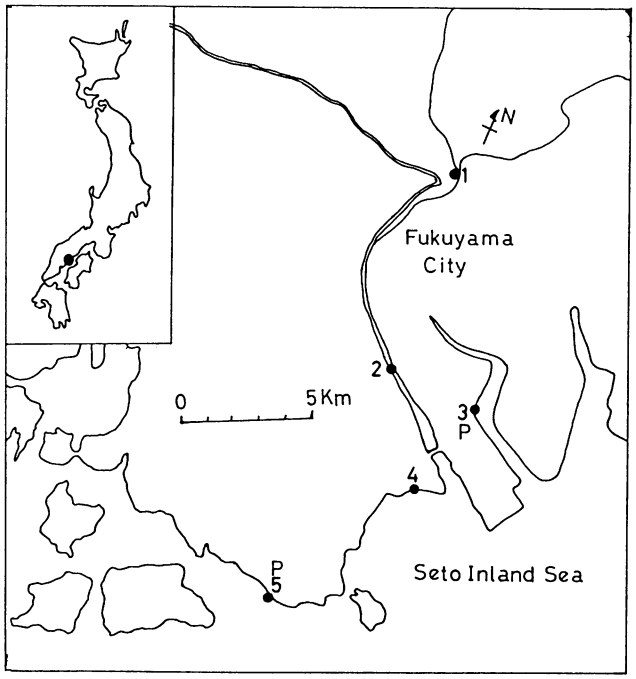

FIG. 1. Sampling stations. 1, Takaya River; 2, Ashida River; 3, Fukuyama port; 4, Tajiri port; 5, Abuto; P, Plankton.

size. The concentrated plankton samples were homogenized in an all-glass sterile homogenizer with 50% seawater ([vol/vol] actual aged seawater and distilled water) and subjected to various bacteriological examinations. For determining the characteristics of the zooplankton, samples were transferred into a wide-mouthed bottle, to which neutralized Formalin was added to give a final concentration of 4%. The samples were then examined with a stereoscopic binocular microscope for identification of the components.

Bacteriological examination. Appropriate decimal dilutions of water and homogenized plankton samples were serially prepared with sterile 50% seawater. Total viable counts (TVC) were done by spread plating onto 2% NaCl-enriched nutrient agar. The standard five-tube, most-probable-number (MPN) procedures combining lactose broth, brilliant green lactose bile broth, and *Escherichia coli* broth (Eiken, Japan) were followed for the enumeration of total coliform and fecal coliform (FC) counts as described elsewhere (2). For the enumeration of fecal streptococci, the method described by Horie et al. (11) was followed.

V. cholerae. *V. cholerae* non-O1 was enumerated qualitatively (alkaline peptone water [APW] was incubated at 37°C) from March 1987 to June 1987 in Takaya River water, Fukuyama port water, and plankton samples. Higher recovery of this pathogen during this period continued quantitatively from July to February 1988. Two methods of the three-tube MPN procedure were compared. In one method, APW with 1% NaCl was incubated at 42°C (5), and in the other, a two-step enrichment method as described by Rhodes et al. (22) was followed. Thiosulfate-citrate-bile salts agar (Eiken) was used as plating medium in both methods. Presumptive *V. cholerae* colonies were initially screened on the modified multitest medium (21). The tubes showing alkaline slant and acid butt were subjected to a series of biochemical tests to confirm the isolates (33). Serotyping was performed by slide agglutination with commercial O antiserum (Seiken, Japan) and further *V. cholerae* non-O1 serotyping was conducted at Louisiana State University (LSU), Baton Rouge, La. (1, 26).

***Salmonella* spp.** Qualitative enumeration of *Salmonella* spp. was performed by placing swabs onto selenite brilliant green sulfa broth (Eiken) or Rappaport-Vassilidis 10 broth (30) at 42.5 ± 0.5°C for 18 h. The enriched broths were then streaked onto novobiocin glucose brilliant green (30), xylose lysine brilliant green (30), and bismuth sulfite agars (Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 24 to 48 h. Homogenized plankton samples were preenriched in dulcitol broth (16) and incubated at 42.5 ± 0.5°C before being enriched and streaked as described above. Typical *Salmonella*-like colonies were picked onto lysine iron agar and subjected to various biochemical tests. Isolates which biochemically resembled salmonellae were further screened with a slide agglutination test with *Salmonella* poly-O antiserum (Seiken). Confirmation of *Salmonella* spp. was performed at National Salmonella and Escherichia Centre, Kasauli, India.

Water temperature and pH were measured in a pH meter (YEW PH 51), and salinity was measured with a salinometer (YSI 33). Simple correlation coefficient and analysis of variance were computed (27).

RESULTS

Bacterial indicators of pollution. Differences in the biotic and abiotic characteristics of the sampling sites are presented in Table 1. The monthly variation in the populations of TVC, FC, total coliform, and fecal streptococci is depicted in Fig. 2. The results of this study suggested that the riverine and polluted marine environments have high incidences of TVC and bacterial indicators of pollution, com-

TABLE 1. Biotic and abiotic characteristics of various stations

Sample	Yearly mean ± SD of:						
	Temp (°C)	pH	Salinity (‰)	Total no. of vibrios[a]	FC/TC (%)	FC/FS ratio	Total plankton[b]
Takaya River water	15.4 ± 8.7	7.4 ± 0.3	1.4 ± 0.6	2.76 ± 0.62	30.6 ± 32.7	7.8 ± 16.9	
Ashida River water	16.2 ± 8.8	7.6 ± 0.4	1.1 ± 0.6	1.01 ± 1.39	31.2 ± 29.3	14.3 ± 16.4	
Fukuyama port							
Water	18.3 ± 6.2	8.0 ± 0.2	28.0 ± 3.9	3.21 ± 0.74	42.2 ± 37.4	538.5 ± 1,720.1	
Plankton	17.7 ± 5.8	8.0 ± 0.2	28.6 ± 3.5	4.58 ± 1.21	37.3 ± 41.3	153.7 ± 348.8	4.47 ± 0.40
Tajiri port water	17.5 ± 6.5	8.2 ± 0.2	31.8 ± 1.9	2.25 ± 0.83	38.4 ± 37.9	15.3 ± 45.7	
Abuto							
Water	17.3 ± 5.6	8.2 ± 0.1	31.6 ± 1.8	1.20 ± 0.86	26.2 ± 43.3	0.02 ± 0.07	
Plankton	16.3 ± 5.0	8.2 ± 0.1	32.5 ± 0.9	3.61 ± 1.06	28.6 ± 38.4	1.5 ± 2.7	4.55 ± 0.34

[a] Log_{10} CFU/100 ml; thiosulfate-citrate-bile salts agar plates were incubated for 24 h at 37°C.
[b] Log_{10} individuals per cubic meter.

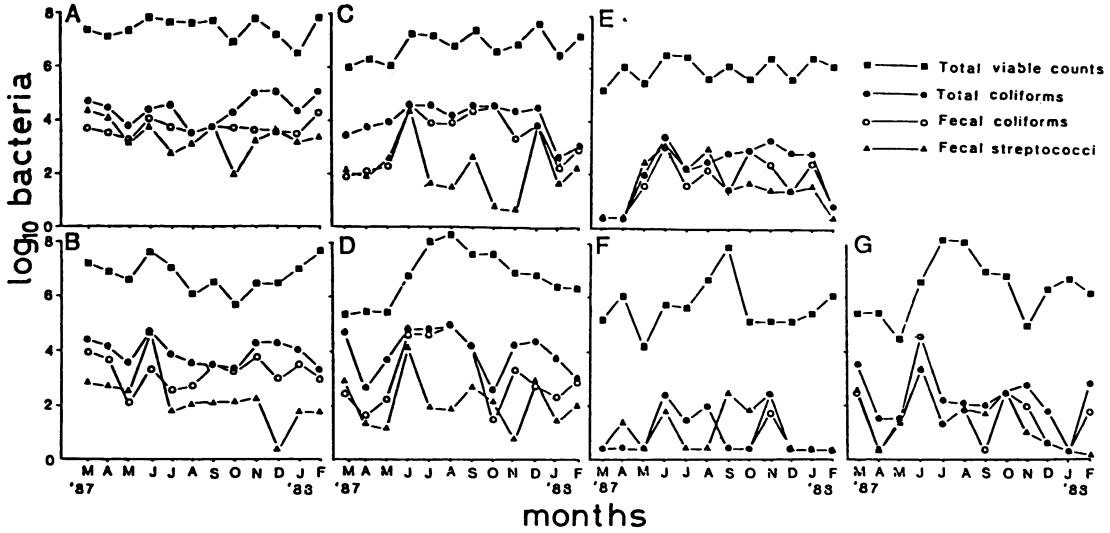

FIG. 2. Microbial characteristics of various biotopes in Takaya River (A), Ashida River (B), Fukuyama port (C), Fukuyama port plankton (D), Tajiri port (E), Abuto (F), and Abuto plankton (G). The bacterial counts (log_{10} bacteria) are expressed as number per 100 ml by the MPN procedure for water (A to C, E, and F), except for TVC (CFU/100 ml), and as number per gram by the MPN procedure for plankton (D and G), except for TVC (CFU/g).

pared with unpolluted marine realms. The Takaya River appears to be polluted more in terms of these bacterial populations. In general, a high population of all the bacterial indices except that of fecal streptococci was recorded in summer. The peak in the bacterial counts of the plankton samples during the summer months corresponded with the abundance of zooplankton. Among the marine samples, Fukuyama port water and plankton samples were the most highly contaminated, followed by samples of Tajiri port water and those from Abuto.

Zooplankton characteristics. The yearly mean in the abundance and percentage of species composition of zooplankton are presented in Table 2. Fukuyama port water samples had high zooplankton numbers, and copepods accounted for 81.1% of the zooplankton. In the Abuto samples, dinoflagellates like *Noctiluca miliaris* constituted 40% of the total plankton collected and copepods constituted only 45.8%. *N. miliaris* blooms were apparent in June and January in the Abuto area.

***V. cholerae* non-O1.** We compared two recently developed methods of isolation for efficiency in the isolation of *V. cholerae* non-O1 (Table 3). Results of this study suggested that APW enrichment incubated at elevated temperature (42°C) allowed recovery of more population of *V. cholerae* non-O1 than did the two-step enrichment method. The established enrichment procedures are reasonably effective in their selectivities, which include a preference for alkaline conditions and resistance to bile salts and sodium tellurite, etc. (28). However, this study suggested that APW incubated at 42°C, which is recommended for the oyster samples (5), is more efficient in detecting *V. cholerae* non-O1 in the water samples. The *V. cholerae* population present in enrichment step 1 was either absent or reduced in step 2 of the two-step enrichment method, and this might be due to the fact that the lincomycin in 96S4YL medium (22) is inordinately selective in inhibiting the *V. cholerae* population.

Although *V. cholerae* non-O1 was isolated from fresh waters (salinity, 0.4 to 2.6‰) and from absolute marine

TABLE 2. Yearly mean of the abundance of zooplankton in Fukuyama port and Abuto[a]

Zooplankton	Fukuyama port			Abuto		
	Yearly mean	%	Peak period	Yearly mean	%	Peak period
Copepoda						
Copepodid I-VI	38,177	81.1	July, September	22,069	45.8	September, December
Nauplius	1,710	3.6	July	1,685	3.5	July
Noctiluca miliaris	1,422	3.0	July	19,262	40.0	June, January
Cladocera	1,815	3.9	July	1,722	3.4	July
Chaetognatha	70	0.2	July	28	0.1	
Polychaeta	530	1.1	July	300	0.6	August
Appendicularia	2,392	5.1	July, December	1,972	4.2	July
Bivalve larvae	77	0.2	May	230	0.5	June
Amphipoda	46	0.1	August	62	0.1	October
Ostracoda	142	0.3	May	19	0.1	
Cirriped nauplius	88	0.2	July	191	0.3	June
Zoea	21	0.1	August			
Other	540	1.1		654	1.4	
Total	47,030	100.0	July, December	48,194	100.0	June

[a] Determined as number of individuals per cubic meter.

TABLE 3. Comparison of APW and 96S4YL methods for the isolation of *V. cholerae* non-O1

Month	Results of indicated methods with:					
	Takaya River water		Fukuyama port sample			
			Water		Plankton	
	APW[a]	96S4YL[b]	APW	96S4YL	APW	96S4YL
March 1987	−[c]	ND[d]	+[c]	ND	−	ND
April 1987	−	ND	+	ND	−	ND
May 1987	−	ND	+	ND	−	ND
June 1987	−	ND	+	ND	−	ND
July 1987	Nil	Nil	1.5[f]	Nil	20.0[g]	Nil
August 1987	3.0	Nil	21.0	Nil	183.0	Nil
September 1987	15.0	0.4	11.0	0.4	Nil	Nil
October 1987	9.0	9.0	110.0	0.4	90.3	1.94
November 1987	0.4	0.4	0.4	Nil	9.6	Nil
December 1987	Nil	Nil	0.4	Nil	Nil	Nil
January 1988	Nil	Nil	Nil	Nil	Nil	Nil
February 1988	Nil	Nil	Nil	Nil	Nil	Nil

[a] APW elevated temperature method (5).
[b] Method of Rhodes et al. (22).
[c] Qualitative method negative.
[d] Abbreviations: ND, not determined; Nil, absent with quantitative method.
[e] Positive.
[f] Number per 100 ml by the MPN procedure.
[g] Number per gram by the MPN procedure.

waters (salinity, 18.9 to 32.5‰), quantitative examination from July 1987 to February 1988 revealed higher recoveries in Fukuyama port water. This bacterium was found to be undetectable in colder months, when the temperature fell below 11°C in marine and 7°C in riverine environments. *V. cholerae* non-O1 was isolated throughout the period of the sampling except in January and February 1988 in water samples of Fukuyama port. The maximum number of *V. cholerae* non-O1 was 110/100 ml by the MPN procedure. *V. cholerae* non-O1 was isolated from the plankton samples in July through November 1987 but not from the samples examined during that winter, nor from the samples taken between March and June 1987. It was not possible to isolate *V. cholerae* non-O1 from the plankton material when it was not present in the water column. The highest population of *V. cholerae* non-O1 was observed during August 1987 (183/g by the MPN procedure) in plankton samples. The population of *V. cholerae* non-O1 in 1 gram (wet weight) of the plankton materials could not be compared with that in 100 ml of water. This suggested that plankton samples harbored a smaller population, compared with that in water samples. In fresh water, strains of *V. cholerae* non-O1 were isolated from August through November, and the maximum number of strains was 15/100 ml during September 1987 by the MPN procedure.

The distribution of various serotypes of *V. cholerae* non-O1 is summarized in Table 4. Of the 138 isolates, serotyping was carried out for 69 strains which agglutinated in 22 different serovar-specific antisera with one nontypeable strain (Table 4). Among the various serotypes identified most were *V. cholerae* LSU serovar J (eight strains); others identified were Z and AA (six strains), TT, YY, and CAL V (six strains), and Ie (five strains). Seven strains agglutinated in two anti-O sera of two kinds (one strain agglutinated in Ia and Id and six strains agglutinated in Z and AA), and six more strains agglutinated in three anti-O sera of one kind (TT, YY, and CAL V). In general, no serovar predominated throughout the sampling period. It is not possible, however,

TABLE 4. Distribution of various serotypes of *V. cholerae* non-O1

Month	*V. cholerae* non-O1 serotypes isolated from:		
	Takaya River water	Fukuyama port (Marine samples)	
		Water	Plankton
March 1987	−[a]	CAL VI	−
April 1987	−	H	−
May 1987	−	YY	−
June 1987	−	D	−
July 1987	−	CAL II[b]	AA
			CAL II
			TT, YY, CAL V[c]
August 1987	TT, YY, CAL V	A	D
		J	AA
		AA	JJ
		Ia, Id[d]	TT, YY, CAL V
		TT, YY, CAL V	Non-typ[e]
September 1987	J	D	−
	HH	H	
	JJ	Ie	
	CAL VI	FF	
		II	
		LL	
		YY	
		BBB	
October 1987	HH	U	C
	Ie	K	D
	CAL VI	Z, AA	Ie
		H	CAL VI
November 1987	J	M	YY
		J	C
December 1987	−	+[f]	−
January 1988	−	−	−
February 1988	−	−	−

[a] Negative; no serotypes were found.
[b] New LSU serovar.
[c] Agglutinated in three different anti-serovar O sera.
[d] Agglutinated in two different anti-serovar O sera.
[e] Nontypeable in LSU antiserum.
[f] Serotyping is still under way.

to make quantitative conclusions concerning the distribution of serovars because the serotyping was performed retrospectively only on selected strains.

***Salmonella* spp.** Though this pathogen could be isolated irrespective of saline conditions except in Tajiri port water, the highest frequency of occurrence was observed in Takaya River water, Ashida River water (second highest frequency), Fukuyama port water (third highest), and Abuto water (lowest frequency). *Salmonella* spp. association with zooplankton samples remained below detectable levels throughout the sampling periods except that of June and October. The distribution of different serotypes of salmonellae is summarized (Table 5). Among 251 strains isolated, 18 serotypes with three variants were recorded. Various serotypes of *Salmonella* spp. were found to be abundant in Takaya River samples (fourteen in all), with a well-marked monthly fluctuation in their occurrence. *Salmonella hadar* was found to be abundant in the earlier period (March to June), and *Salmonella infantis* was abundant in the subsequent months (May to September). A high incidence of various serotypes was found in August (six serotypes), and four were found in March, in Takaya River water. June and September were the

TABLE 5. Distribution of *Salmonella* serovars

Month	Serovars found in samples from indicated area						
	River		Marine area				
	Takaya (water)	Ashida (water)	Fukuyama port		Tajiri port	Abuto	
			Water	Plankton	Water	Water	Plankton
March 1987	*S. hadar* *S. agona* *Salmonella typhimurium* *Salmonella litchfield*	–[a]	–	–	–	–	–
April 1987	–	–	–	–	–	–	–
May 1987	*S. infantis*	–	–	–	–	–	–
June 1987	–	*S. hadar* *S. thompson* *Salmonella* II 4,12:b:- *Salmonella* I monophasic variant	–	*S. hadar* *Salmonella heidelberg*	–	–	*S. hadar*
July 1987	–	–	–	–	–	–	–
August 1987	*Salmonella mbandaka* *Salmonella weltevreden* *S. infantis* *S. thompson* *S. paratyphi* B *Salmonella* I rough strain	–	–	–	–	–	–
September 1987	*Salmonella infantis* *Salmonella* I rough strain	*S. agona* *S. typhimurium*	*S. agona* *S. montevideo* *S. infantis* *Salmonella* I nonmotile variant	*Salmonella* I nonmotile variant	–	*S. infantis*	–
October 1987	*Salmonella muenchen* *Salmonella* III 38: z_{10}:z_{53} *Salmonella* IV 1,40:z_4,z_{23}	–	*Salmonella muenchen* *Salmonella* III 48:l,v:1,5,7 *Salmonella* IV 1,40:z_4,z_{23}	*Salmonella* IV 1,40:z_4,z_{23}	–	–	*Salmonella* IV 1,40:z_4,z_{23}
November 1987	*Salmonella braenderup*	–		–	–	–	–
December 1987	–	–	*Salmonella cerro*	–	–	–	–
January 1988	*Salmonella montevideo*	–	–	–	–	–	–
February 1988	*S. montevideo*	–	–	–	–	–	–

[a] Negative; no serovars were found.

months in which more serotypes (four in each month) could be recorded for Ashida River and Fukuyama port water, respectively. *S. agona* and *S. hadar* were distributed in all the biotopes. Serotypes found that were associated with zooplankton were not isolated in the water column of this area. It should, however, be stated that the detection of *Salmonella* strains of subgenus IV (*Salmonella* IV 1, 40:z_4, z_{23}) in all the October 1987 samples appears to be influenced by an unknown vehicle in this area.

DISCUSSION

The widespread distribution of *V. cholerae* non-O1 was comparable to those reported for the Atlantic coast (15), Louisiana Gulf coast (24), and estuaries of the United States (17) and the United Kingdom (19). Roberts et al. (23) are of the view that *V. cholerae* numbers rose with the temperature but dropped dramatically at a temperature of 28°C. Present investigations also support the findings that, during October when the temperature of the water column was around 21°C, *V. cholerae* numbers reached maximum and that the hottest month (August, when the water column temperature was 28.7°C) witnessed low population. Moreover, the computer-assisted analysis of field data in four coastal areas of the United States (25) showed that the peak in *V. cholerae* counts was observed in water samples at 21 to 28°C.

During this study, 18 different serotypes were found in

TABLE 6. Analysis of variance in various microbial and environmental characteristics

Characteristic of samples	Analysis of variance value for bacterial population from:			
	All samples[a]		Fukuyama port samples	
	Seasonal	Stations	Seasonal	Stations
TVC	26.39[b]	6.42[c]	1.00	9.21[b]
Total coliforms	12.50[b]	2.48	1.88	0
FC	2.50	2.40	3.30	3.51
Fecal streptococci	4.30[c]	1.63	6.61[c]	3.51
V. cholerae non-O1	1.15	3.81	7.41[c]	6.40[c]
Temperature	31.36[b]	6.33[c]	58.00[b]	1.90
pH	2.80	3.60		
Salinity	2.94	497.50[b]	3.84	2.81
Total no. of vibrios	1.97	17.98[b]	2.31	1.90

[a] Takaya River, Fukuyama port water, and plankton samples.
[b] Significant at 1% level.
[c] Significant at 5% level.

marine water while the riverine runoff had eight serotypes only. This shows that *V. cholerae* non-O1 is ubiquitous in the aquatic environment and that these organisms are not introduced significantly into the marine realms by sewage or the adjacent fresh waters. Grimes et al. (8) pointed out that many investigators erroneously interpret the wastewater discharge as the source of the pathogens (7) rather than as a source of nutrients which may stimulate growth of the autochthonous pathogens. Furthermore, Hood et al. (10) showed that the bacterium was most abundant when salinities were 10 to 25‰ in Florida estuaries.

Analysis of variance (Table 6) showed that *V. cholerae* non-O1 was found to be distributed heterogeneously, but significant 5% level homogeneous distribution could be noticed among the samples of Fukuyama port water in which water samples were found to have a larger population, compared with that in zooplankton samples.

Isolation of this bacterium below detectable levels in zooplankton samples during colder months did not support the hypothesis reported by Huq et al. (12) that zooplankton might be associated with this pathogen during unfavorable conditions. Though other studies have shown that *V. cholerae* lives in an epibiotic stage (3, 34), the results of this study are contrary to those findings. However, all the published results pertaining to *V. cholerae* (12) were found under laboratory conditions, unlike the ecological studies of *V. parahaemolyticus* (14; K. Venkateswaran, S. W. Kim, H. Nakano, T. Onbé, and H. Hasimoto, Syst. Appl. Microbiol., in press). This study is not denying the established association between copepods and *V. cholerae* or the assertion that these microscopic crustaceans dictate the seasonal distribution of *V. cholerae* as in the case of *V. parahaemolyticus*.

TABLE 8. Relationship between FCs and *V. cholerae* non-O1

Sample	No. of samples with FC and *V. cholerae* incidences of:			
	0–200[a]	201–2,000[a]	2,001–20,000[a]	20,000 and above[a]
Takaya River water			8[b]	
			4[c] (50)[d]	
Fukuyama port water	1	2	4	1
	0	1 (50)	4 (100)	1 (100)
Fukuyama port plankton	2	3	0	3
	1 (50)	0	0	3 (100)

[a] Values are given as number of FCs per 100 ml by the MPN procedure.
[b] Incidence of FCs.
[c] Incidence of *V. cholerae* non-O1.
[d] Percentage of *V. cholerae* non-O1 incidence among FCs.

Further research on association with other suspended matter is under way to understand the overwintering process of *V. cholerae* (K. Venkateswaran, C. Kiiyukia, K. Nakanishi, H. Nakano, O. Matsuda, and H. Hashimoto, manuscript in preparation). In addition, a survival model for *V. cholerae* in Gulf Coast estuaries (10) was proposed in which *V. cholerae* would survive in a free-living state and, under certain conditions like nutrient depletion, would develop into a small, cellular reduced form called the microvibrio. Such a phenomenon should also be considered.

High-nutrient conditions seem to be ideal for proliferation, as evidenced by the high population in Fukuyama port water samples, irrespective of the saline conditions. Watkins and Cabelli (32) agreed that nutrient supplied by waste, surface runoff, or both would influence the survival of enteric pathogens in coastal zones. *V. cholerae* non-O1 could not be correlated with any of the bacterial population investigated in Takaya River water, but FC was found to be significantly correlated in Fukuyama port water samples (Table 7). The frequency of *V. cholerae* isolation from different samples grouped into FC MPN ranges (Table 8) also suggested the existence of a relationship between *V. cholerae* non-O1 and FC densities, with the increase in isolation of *V. cholerae* non-O1 corresponding to the increase in FC densities. The explanation for this may be that riverine discharge rich in nutrients with high indices of pollution indicators perhaps influenced the proliferation of *V. cholerae* non-O1.

Isolated *V. cholerae* non-O1 serotypes are of wide geographic occurrence. The 68 typeable strains represented serotypes previously isolated from a variety of specimens throughout the world. CAL II, V, and VI and BBB—new LSU serovars and Sakazaki or VRL designations were unknown—were found to be isolated in this study. In addition to these, isolation of one nontypeable serotype with the available LSU sera warranted more detailed study. Agglutination of *V. cholerae* non-O1 in two (Ia and Id; Z and AA) or more (TT, YY, and CAL V) serovar-specific antisera

TABLE 7. Relationship between *V. cholerae* non-O1 and other characteristics

Sample	Correlation coefficients between no. of *V. cholerae* non-O1 and:								
	TVC	TC[a]	FC	FS[a]	TV[a]	Temperature	pH	Salinity	Plankton
Takaya River water	−0.01	−0.61	−0.13	−0.42	−0.53	0.58	0.01	0.15	
Fukuyama port water	−0.24	0.63	0.82[b]	−0.38	0.74[c]	0.60	−0.79[c]	−0.33	
Fukuyama port plankton	0.32	−0.39	−0.40	−0.29	0.31	0.45	−0.60	−0.10	−0.50

[a] TC, total number of coliforms; FS, fecal streptococci; TV, total number of vibrios.
[b] Significant at 1% level.
[c] Significant at 5% level.

TABLE 9. Relationship between FCs and *Salmonella* spp.

Sample	No. of samples with FC and *Salmonella* sp. incidences of:			
	0–200[a]	201–2,000[a]	2,001–20,000[a]	20,000 and above[a]
Takaya River		1[b]	11	
		1[c] (100)[d]	7 (63.6)	
Ashida River	1	5	6	
		2 (40)	1 (16)	
Fukuyama port				
Water	4	2	4	2
	0	0	2 (50)	1 (50)
Plankton	4	4	1	3
	1 (25)	0	0	1 (33)
Tajiri port	9	3		
	0	0		
Abuto				
Water	12			
	1 (8.3)			
Plankton	9	2	1	
	0	1 (50)	1 (100)	

[a] Number of FCs per 100 ml by the MPN procedure.
[b] Incidence of FCs.
[c] Incidence of *Salmonella* spp.
[d] Percentage of *Salmonella* incidence among FCs.

showed that *V. cholerae* non-O1 may express more than two O antigen determinants like that of *V. cholerae* O1 (1).

The high incidence of *Salmonella* spp. in river water suggested that salinity is the most crucial factor in governing the distribution (4), but the occurrence of *Salmonella* spp. in Fukuyama port marine samples might stem from an increased discharge of polluted waters from an adjacent land or coastal area. Temperature exerted no pressure on the magnitude of fecal bacteria as evidenced by the presence of *Salmonella* spp. in both summer and winter months during this investigation. This was supported by previous studies carried out in this area (30). However, Hendricks and Morrison (9) showed that there was no multiplication of *Salmonella* spp. in polluted or unpolluted river water when the temperature dropped below 10°C. Further study is required.

Data summarized from numerous studies on stream and estuarine pollution showed low isolation of salmonellae when FC densities were below 200/100 ml. The result (Table 9) suggested that the polluted Takaya River, as well as the unpolluted Ashida River, had higher *Salmonella* incidence when the level of FC densities were 200 to 2,000/100 ml by the MPN procedure, but an increase in FC densities does not correspond to an increase in *Salmonella* incidence (30, 31). Also, recovery of *Salmonella* could be noticed when even FC was below detectable levels in clear marine (Abuto) waters in September. The higher incidence of various serotypes in the Takaya River leads to the assumption that the slaughterhouse effluents released into its upstream ensued their isolation. The occurrences of *Salmonella agona*, *S. hadar*, *S. infantis*, *Salmonella thompson*, and *Salmonella paratyphi* B, which are responsible for causing salmonellosis in humans, are pointers to human sources of pollution.

Incidences of *Salmonella* spp. associated with zooplankton at both sampling localities were found to be fewer, compared with incidences in water samples. Also, occurrences of salmonellae did not coincide with zooplankton peaks during this study. It is interesting to note that the serotypes found which were associated with zooplankton were not isolated in the water column; further study of this is required. The existence of different kinds of mechanisms in *Salmonella* spp. for withstanding the higher saline conditions is possible. Research on this is under way in our laboratory.

It is concluded from the results of this study that *V. cholerae* non-O1 is ubiquitous in aquatic environments as proven in water samples from the United States, and its distribution is influenced by the degree of nutrients rather than by the association with zooplankton. Furthermore, allochthonous pathogens have also not been associated with these crustacean communities. However, the isolation methods employed should be suitable (8) in enumerating the viable but unculturable cells of these pathogens.

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