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Temporal Occurrence of *Vibrio* Species and *Aeromonas hydrophila* in Estuarine Sediments

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Marine sediments were assayed for their concentration of *Vibrio* spp. and *Aeromonas hydrophila* over 1 year. A temporal variation was observed in which *A. hydrophila*, and to a lesser degree *V. fluvialis*, were found in the winter months, *V. parahaemolyticus* and *V. vulnificus* predominated in the spring and summer, with non-O-1 *V. cholerae* and *V. alginolyticus* detected in the late summer and fall. These organisms were found in greatest numbers in the top 5 cm of sediment, but were detectable down to 15 cm. Epidemiological data revealed a predominance of non-O-1 *V. cholerae* infections at the time the organisms were observed to flourish in the sediments.

Apalachicola Bay, Florida, produces approximately 90% of the commercially available shellfish in Florida, and the revenues account for nearly 50% of the income of Franklin County, Florida. The recovery of pathogenic *Vibrio* spp. from shellfishing regions has become a great concern to state officials. Among the most active oystering areas within Apalachicola Bay are Cat Point and East Hole oyster bars adjacent to George's Island, a barrier island off of the Florida Gulf Coast. The Apalachicola River flows into the bay, and the stage of the river influences salinity. Between 8 November and 17 December 1979, six shellfish-associated *Vibrio cholerae* (non-O-1 serotype) infections were reported by the Florida Department of Health and Rehabilitative Services from oysters harvested in Apalachicola Bay and prompted a number of studies to identify the source of the problem. Investigations of the distribution of *V. cholerae* in the bay (3, 7, 13) identified both O-1 and non-O-1 serotypes in waters and oyster meats throughout the bay. Interestingly, *V. cholerae* was recovered from areas where no apparent sources of human-associated pollution existed and where the fecal coliform densities were within acceptable National Shellfish Sanitation Program waters standards for shellfish.

Published reports concerning the association of *V. cholerae* and other pathogenic vibrios with estuarine sediments are limited. Hood et al. (8) quantitatively examined 10 sediment samples taken by a grab sampler from Apalachicola Bay and confirmed the presence of *V. cholerae* in 3 of these samples. In vitro survival studies of *V. cholerae* and *Escherichia coli* were conducted by Hood and Ness (6) in water and water-sediment chambers. They found that *V. cholerae* survived equally well in sterile water, nonsterile water, and sterile sediment but not as well in nonsterile sediment. Rapid die-off occurred after 5 h of incubation, which suggested intraspecific competition in the nonsterile sediment environment.

At present, *V. cholerae* is thought to be a ubiquitous, autochthonous bacterium in the coastal marine environment (1, 11, 18, 19). The ecology of this organism, its reservoirs, dispersal mechanisms, and substrates have not been clearly defined, although reports have provided clues suggesting potential vectors of transmission (19) and seasonality in water samples (14).

East Hole is one of the most actively harvested oyster bars in the Apalachicola Bay and was identified as having high recoveries of *Vibrio* organisms in an earlier study (13). The number of shellfish-associated illnesses traced to oysters harvested from this area suggested a local reservoir. Information on the role of the sediments as a potential reservoir is lacking. In this paper we report on the temporal variation of *Vibrio* species and *Aeromonas hydrophila* in marine sediments and their relationship to epidemiological data reported for shellfish-associated human illnesses in Florida.

MATERIALS AND METHODS

Sample collection. Samples were collected monthly from April 1983 through March 1984, with additional collections in June and August 1984. Sediment samples were taken with a hand-held coring device that could be forced into the bay bottom through both shell material and sand and maintain sediment integrity. The sampler was an aluminum cylinder 30 cm long and 4 cm in diameter, with a check valve fastened to the top end and a removable stainless steel cutting edge attached to the bottom end. Just before use, a sterilized plastic core liner (3.75-cm diameter) approximately 25 cm long was placed inside the core barrel and secured with the cutting edge when it was reattached to the barrel. To retain the coarse sediment in the liner, a core catcher consisting of a 15 cm long rubber cylinder cut from a condom was put over the bottom edge of the core liner and then folded up into the cylinder. The sandy material freely passes up into the core when inserted into bottom material, but when the core is withdrawn, the rubber core retainer collapses and holds the core intact.

The coring device was forced into the bay bottom by means of a 1/2-in diameter galvanized pipe attached to the top of the check valve. The handle had drains at the bottom and also served as an air vent when the corer was submersed. The check valve allowed entrained air and water to escape from the core during insertion but closed on withdrawal to isolate the sediment sample. After retrieval, cores were capped and held at ambient temperature and processed within 2.5 h of collection.

Water trapped over the sediment in the top portion of the core tube was carefully pipetted off and transferred to a sterile glass container for subsequent dilution. Occasionally, a flocculent material was found as a distinct layer just above the sediments, and when observed, this layer was carefully removed and analyzed as was the water. The sediment core

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was aseptically cut into segments, each 2.5 cm thick, which were placed into separate sterile plastic containers for homogenization and serial dilution. Generally, only the top 7.5 cm were analyzed, but occasionally the entire core was sectioned and assayed. The methodology used for the enumeration of the various bacterial species was essentially that of the Food and Drug Administration incorporating the changes introduced by Hood et al. (7). Modifications included a three-tube-series most probable number dilution scheme in which 100 g of sediment taken from each core segment was slurried with 100 ml of phosphate-buffered saline, and the slurry was then further diluted with phosphate-buffered saline to cover a dilution range between 1- and 10^6-fold. Next, 1 ml of each dilution was added to three tubes of alkaline peptone broth, and incubated at room temperature for 18 h as recommended by Furniss and Donovan (4). After incubation, portions of each enrichment were streaked onto thiosulfate-citrate-bile salt-sucrose agar (Difco Laboratories, Detroit, Mich.). Both the green and yellow colonies were taken and streaked onto trypticase-sodium chloride agar (Trypticase, 10 g/liter; NaCl, 10 g/liter; agar, 20 g/liter [pH 7.5]) and screened for the presence of cytochrome oxidase with Pathotec strips (General Diagnostics). Oxidase-positive cultures were tested for H_2S production with triple sugar-iron agar (Difco Laboratories), and those cultures that tested H_2S negative were characterized by using the API-20E system (Analytab Products, Inc., Plainview, N.Y.). By translating the identification information back to the alkaline-peptone enrichment dilution series, a three-tube most probable number estimate was determined for each of the *Vibrio* spp. and *A. hydrophila*. Presumptive *V. cholerae* cultures were serologically tested with *V. cholerae* Polyvalent O Antisera (Difco).

Water temperature and salinity were determined with a Beckman RS 5-3 electrodeless induction salinometer. Dissolved oxygen was measured with a YSI model 58 digital dissolved oxygen meter. Rainfall and Apalachicola River stage data were supplied by the Florida Department of Natural Resources.

RESULTS AND DISCUSSION

Two locations were chosen for our study; station 1 was on East Hole Bar, where active oyster harvesting is ongoing, and station 2 was several hundred meters from the oyster bar in an area of slower water movement. The two stations are relatively shallow, having a depth over the survey period of between 0.6 and 1.0 meter. The river stage is given (Tables 1 and 2) to illustrate the effects of freshwater flow on the respective salinities of each station. The two stations differ in that station 1 was directly affected by tidal exchange, while station 2 was closer to the barrier island and as such was protected from the factors affecting the more centrally located oyster bar.

Salinities recorded during this study ranged from 20 to 30‰ with the exception of April. Water temperatures ranged between 11.1 and 31.0°C, with temperatures in excess of 20°C occurring from mid May to mid October. River stage records show that the Apalachicola River declined from approximately 6.1 m in May to approximately 1.2 m in November. River stage for this area is important since the river is directly affected by rainfall and thus reflects the amount of freshwater being introduced in this bay.

The principal bacteriological findings are: (i) there appears to be a seasonality or temporal order in which the various species of target bacteria were detected; (ii) the presence of an organic floc greatly influenced the bacterial counts of some of the species; and (iii) the various species of bacteria are frequently found down to 5 cm or more in the sediment. In one experiment, *V. parahaemolyticus* was recovered at a sediment depth of 12 cm (Tables 1 and 2; August data), but generally the greatest cell densities were above 5 cm and usually confined to the top 2.5 cm of sediment. The data in Tables 1 and 2 essentially show the same temporal displacement of the various bacterial species monitored, but there are some differences in the cell densities detected at each of the stations for a given species at any time.

We detected *V. cholerae* at two times, first in June when the organism was primarily associated with the floc, and second in the fall between September and December when the organism was found only in the sediments.

It is interesting to note that a chi-square analysis of 5 years of epidemiological data collected from 1979 to 1983 by the Department of Health and Rehabilitative Services of the State of Florida, indicated that more *V. cholerae*-mediated cases of gastroenteritis were reported in the fourth quarter of the year (October, November, December) than in the other three quarters combined ($P < 0.01$) (Spencer Lieb, personal communication). These coincide in time with the increased cell density of this organism in the sediments.

In the spring we found that most of the six species of bacteria we looked for were detected, although in most cases the cell density was low. When the temperature and salinity both were high in the months of May through August, *V. parahaemolyticus* attained its highest cell densities in the water, floc, and sediments. *V. parahaemolyticus* was, however, detected in the sediments from April to November, excluding October. *V. vulnificus* occurred early in the year, predominately between April and July. This organism was generally associated with the sediments, but the greatest cell density was found in July in the floc. *V. alginolyticus* was detected at one or the other stations over the entire year, although the greatest cell density was found in the floc at station 2 in August and in the sediments of station 1 in September. *V. fluvialis* was only detected in the late fall and winter months, and in this sense was the only vibrio to be present at low water temperatures. *A. hydrophila* was consistently detected year round, but achieved high cell densities at two times, first in July and August and then between December and January. Station 1 had consistently higher cell densities of *A. hydrophila*, which were found in all segments of the sediment column.

Of the organisms recovered throughout this study, *V. parahaemolyticus* and *A. hydrophila* were the two that attained the greatest cell density. In terms of numbers, *V. parahaemolyticus* dominated the earlier months of the survey, although it was detected as late as November, while *A. hydrophila* predominated in the latter months. In similar fashion, although the cell densities were considerably lower, *V. vulnificus* and *V. fluvialis* were present in the earlier and final phases of the survey period, during the spring and winter seasons, respectively. *V. alginolyticus* and *V. cholerae*, non-O-1 serotype, were recovered in the middle of the survey during the summer and fall months, with *V. alginolyticus* attaining consistently higher densities.

The sporadic appearance of the floc was enigmatic. This floc was a definite feature of the cores between May and September, and contained high concentrations of *Vibrio* spp. and *A. hydrophila*. We have no clear indication, however, of the source of the material or its composition. Microscopically, the floc appeared amorphous, with masses of bacteria associated with it. The floc first appeared in May, after the water temperature had become fairly high, and could reflect

TABLE 1. Bacteriological and environmental parameters at station 1

Date	Core segment[a]	Bacterial species[b] (cells per ml or per g [wet wt])						Temp (°C)	Salinity (‰)	River stage (m)
		V. vulnificus	*V. parahaemolyticus*	*V. alginolyticus*	*V. cholerae*	*V. fluvialis*	*A. hydrophila*			
4/22/83	Water						11	18.4	10.1	5.15
	0–2.5	24								
	2.5–5	2.4	24				150			
5/20/83	Water	1,100	2,400	9.3				27.8	29.6	3.42
	Floc		240	240						
	0–2.5	24	4,600	7,500						
	2.5–5		24							
6/23/83	Water	1	1,100				15	27.8	21.9	2.42
	Floc		2,400		2,400					
	0–2.5	100	2,400							
	2.5–5		460							
7/22/83	Water		24,000				24,000			1.76
	Floc	46,000	46,000				46,000			
	0–2.5		2,400				4,600			
	2.5–5	110	110							
8/26/83	Water		24,000	0.4			460	28.9	30.2	1.68
	Floc		2,400	1,000			15,000			
	0–2.5		1,100	460			460			
	2.5–5		2,400	4,600	460		4,600			
	5–7.5		46	1,100			110			
	7.5–10		24							
	10–12.5		2.3				0.7			
	12.5–15		0.4							
9/17/83	Water		24	11,000				26.3	21.9	2.07
	Floc			2,000			46			
	0–2.5		2,400	11,000	24					
	2.5–5		2.3	150						
	5–7.5			1.5		210				
	5.7–10		14							
10/15/83	Water			7.5	2.3	7.5		21.8	31.6	1.71
	0–2.5			20	2.3					
	2.5–5									
	5–7.5									
11/19/83	Water			24	2.3		460	17.1	22.7	1.19
	0–2.5	240	240	2.3	2,400		240			
	2.5–5		2,400	2,400						
	5–7.5			75	2.3					
12/17/83	Water	2.3				24	2,400	12.1	9.8	5.61
	0–2.5			24,000			24,000			
	2.5–5	240					24,000			
	5–7.5					24,000	24,000			
1/28/84	Water						2,100	12.4	10.1	5.98
	0–2.5						24,000			
	2.5–5				24		4,600			
	5–7.5					46	24			
3/10/84	Water					110	24	14.3	4.2	6.18
	0–2.5					24	24,000			
	2.5–5					2,400	11,000			
	5–7.5					2.3	24			
3/31/84	Water		1,600	800	0.8		1,600	12.4	17.6	5.55
	0–2.5		1,600				2,400			
	2.5–5		1,320	1,600			1,320			
	5–7.5		0.3	800			1,600			
6/18/84	Water		46	153			800	28.8	27.0	1.92
	0–2.5		2.3	8,000			16,000			
	2.5–5		24	400			8,000			
	5–7.5		24	463						
8/24/84	Water			153			800	30.0	18.0	3.42
	0–2.5	8,000	13	8,000			16,000			
	2.5–5	31		400			8,000			
	5–7.5	668	80	463						

[a] Core segment notation is as follows: water, water immediately over the sediment in the core tube; floc, a particulate layer that was occasionally apparent between the sediment and the water; 0–2.5, the sediment in the interval between 2.5 cm of core depth; 2.5–5, 5–7.5, etc., similarly refer to the depth interval within the sediment core.

TABLE 2. Bacteriological and environmental parameters at station 2

Date	Core segment[a]	Bacterial species[b] (cells per ml or per g [wet wt])						Temp (°C)	Salinity (‰)	River stage (m)
		V. vulnificus	*V. parahaemolyticus*	*V. alginolyticus*	*V. cholerae*	*V. fluvialis*	*A. hydrophila*			
4/22/83	Water							18.8	6.8	5.17
	0–2.5	4.3	2.3	2.3	24		21			
	2.5–5					24				
5/20/83	Water		46					26.9	29.2	3.43
	Floc		2,400							
	0–2.5	1,100	24	24						
	2.5–5		46	11,000						
6/23/83	Water		10					28.9	20.0	2.42
	Floc		93		2,400					
	0–2.5		4,600		110		1			
	2.5–5		240	24	21					
7/22/83	Water	1,500	46		4.3					1.76
	Floc									
	0–2.5	24,000	11,000				4,600			
	2.5–5	460	460				2,400			
	5–7.5	4.3	2.3							
8/26/83	Water		2,400	240				28.5	27.7	1.68
	Floc		1,100	24,000						
	0–2.5		2,400	24			1,500			
	2.5–5		240	460						
	5–7.5		150				460			
	7.5–10		150	7.5						
	10–12.5	0.7	0.4	0.7						
	12.5–15	0.4					0.4			
9/17/83	Water			2.3	24			26.7	21.3	2.07
	Floc		1,100	46						
	0–2.5			9.3						
	2.5–5		9.3	9.3						
	5–7.5		24							
	5.7–10		24	0.3						
	10–12.5									
10/15/83	Water			0.4				19.6	30.3	1.71
	0–2.5				2.3					
	2.5–5				4.3					
	5–7.5				0.4					
11/19/83	Water						0.9	17.1	21.9	1.19
	0–2.5			2,400	2,400	2.3				
	2.5–5				240					
	5–7.5				24					
12/17/83	Water						24	11.1	10.1	5.61
	0–2.5						110			
	2.5–5			2.3	24		240			
	5–7.5			2.3			46			
1/28/84	Water			2.3			24	13.2	7.7	5.98
	0–2.5						24,000			
	2.5–5						2,400			
	5–7.5						2.3			
3/10/84	Water					2.3	9.3	13.0	4.6	6.18
	0–2.5					24	120			
	2.5–5						46			
	5–7.5						24			
3/31/84	Water							11.9	18.2	5.55
	0–2.5		0.9	0.3						
	2.5–5					0.8				
	5–7.5				800					
6/18/84	Water	240	240					27.5	31.5	1.92
	0–2.5		2,400	24	2,400					
	2.5–5		24		2,400					
	5–7.5		2.3	240						
8/24/84	Water		13	277				31.0	16.0	3.42
	0–2.5		3,820	3,820						
	2.5–5	3,600		14						
	5–7.5									

[a] Core segment notation is the same as in Table 1, footnote *a*.

or be the remains of the extensive plankton production which seasonally occurs in this bay. Livingston (12) indicated that phytoplankton productivity is minimal in February, reaches a seasonal maximum in April, and then falls to a summer minimum in August. Zooplankton biomass follows a similar pattern, with a minimum in February, increasing toward a yearly maximum in May, and followed by a slow, but steady, decline for the remainder of the year. It was after the maximum and during this period of decline that we observed the floc.

The State of Florida gives careful attention to shellfish-associated illnesses. During September 1983 all illnesses resulting from *Vibrio* spp. became reportable, not just those associated with *V. cholerae* O-1, as had been the case in the past. The number of reported cases of gastroenteritis related to *Vibrio* spp. in 1983 for Florida is shown in Table 3 (epidemiological data are available from 1979, although we have only presented the 1983 data that corresponds to our survey). It must be emphasized that the number of cases reported in the first half of the year may be an underestimate of the actual number of illnesses occurring because of the change in the reporting requirements. The sediment enumeration data for bacteriological species (Tables 1 and 2) suggest that the greatest potential for human infections would occur during the summer months of June, July, and August. Fortunately most of the bay is closed to shellfish harvesting by the regulatory agency from 1 June through 31 August to allow for spawning.

The occurrence of *V. cholerae* infections begins in May, but 70% of the reported cases occur between August and December, most notably in October, November, and December. Our enumeration data (Tables 1 and 2) indicate *V. cholerae* non-O-1 was most prevalent in the sediments during the time when most of the illnesses were reported within the state. Furthermore, the cases earlier in the year coincided with the detection of *V. cholerae* in the water or sediments, suggesting that sediment analyses may be a useful monitoring tool in the absence of a suitable indicator. Eight cases of gastroenteritis tentatively identified as being caused by *A. hydrophila* were reported to the state in December (Spencer Lieb, personal communication). The oysters implicated were harvested in Louisiana, but sold in Florida. The sediment data of Tables 1 and 2 indicate that December through March was a period when *A. hydrophila* would have been most prevalent.

TABLE 3. Reported cases of gastroenteritis attributable to *Vibrio* species in 1983 in Florida[a]

Month	Causative organism	
	V. cholerae	*Vibrio* spp. (other)[b]
January		
February		1
March		1
April		1
May	1	2
June	2	
July		1
August	1	1
September	1	
October	1	1
November	2	
December	2	1

[a] Cases with multiple organisms isolated are not included in this table.

[b] Two cases are still under investigation for the months of October and November. In December, eight cases of gastroenteritis were caused by *A. hydrophila* in oysters harvested from Louisiana, and they are not included in the table.

The factors of organic enrichment, temperature, and salinity present a complex inter-relationship, each of which has been shown to aid in the development or survival of vibrios in the marine environment (18, 19). The nutritional role of chitin has also been implicated in the development of the *Vibrio* organisms. It has been suggested that outbreaks of cholera that occurred in Bangladesh were tied to the development of the copepod population, upon whose chitinous carapace the bacteria adhere and feed (10). Apalachicola Bay is somewhat unique in that there exists a vast reservoir of chitin in the molts of the blue crab (*Callinectes sapidus*) and the white shrimp (*Penias seteferis*) and the spring zooplankton bloom (12). In addition, herpacticoid copepods inhabiting the sediments serve as an additional source of chitin to the sediments. Together, these resources should serve as a substantial nutritional reservoir to the vibrio population, even if no other factors were involved. Since chitin is added continuously to the bay from February to November from several sources, we would not have expected to find the seasonal pattern that was observed if chitin alone were the driving force regulating the survival and recovery of vibrios. Chitin per se may be overstated in importance relative to other factors such as the association with a particular organism (2, 9, 20). In fact, it is tempting to speculate that the expression of a particular bacterial species may be tied to the development of some host organism as well as to seasonal parameters.

Temperature and salinity appear to exert considerable influence on the recoverability of the vibrios. *V. parahaemolyticus* grows well at salinities between 10 and 30‰, but low temperatures, as found in winter, severely restrict the growth of the organism (10). The trend for *V. parahaemolyticus*, therefore, appears to be low cell densities or no detection in the colder winter months, with a rapid increase in numbers as the temperature increases. In our work *V. parahaemolyticus* was not detected until the water temperature rose to 27°C, and it reached its maximum density when the temperature was between 27 and 29°C.

The temperature and salinity range for *V. cholerae* growth appear to be more critical. Seidler and Evans (17) used computer analysis to evaluate the cell densities of *V. cholerae* non-O-1 recovered from the Gulf of Mexico, Chesapeake Bay, and the Oregon Coast. They found that the highest *V. cholerae* counts were found in the temperature range of 21 to 28°C, with few organisms detected below 14 or above 35°C. Approximately one-half of the *V. cholerae* organisms were recovered in the temperature interval between 16 and 24°C. Roberts et al. (15) showed increased *V. cholerae* densities with increased temperatures at salinities of less than 1‰, but at high temperatures (28°C) and high salinities (15‰), *V. cholerae* densities decreased. Seidler and Evans (17) similarly reported that high cell densities of *V. cholerae* were not found at salinities of greater than 14‰. Singleton et al. (19) in microcosm experiments on *V. cholerae* growth and survival, reported a salinity optimum greater than 14 to 15‰, but more importantly, they found that by adding organics in the form of tryptone, the need for an optimum salinity was spared. Singleton and co-workers further suggested that *V. cholerae* may exist in a resting state under unfavorable conditions of temperature and salinity. Sporadic detection or illnesses, such as observed in June, may be the positive effect of added nutrients (the floc) in an otherwise unfavorable temperature or salinity regime. The combination of high temperature and high salinity found in

Apalachicola Bay may actually serve to control the cell density of this organism.

A. hydrophila is an organism which has been associated with food spoilage, clinical infections, and the environment. The medical literature suggests a wide range of growth temperatures, with a maximum of 45°C and an optimum of 37°C. One study concerned with the temperature optima of 33 *Aeromonas* species indicated that the psychrophilic strains were primarily *A. hydrophila* (16). Studies on the natural prevalence of *A. hydrophila* can also shed light on the temperature and salinity regime under which this organism proliferates. Hazen et al. (5) examined 147 natural aquatic environments in 30 states and Puerto Rico to determine the cell density of the organism. On closer examination of the data, one finds that the greatest density of *A. hydrophila* occurred over the temperature range between 13 to 20°C and at near zero salinity. *A. hydrophila* was recovered from samples collected at 30‰ salinity although much less frequently. The general conclusions to be drawn suggest that *A. hydrophila* is able to proliferate at relatively low temperatures and salinities comparable to those occurring in the winter months of our survey.

The temporal sequence of the *Vibrio* spp. and *A. hydrophila* that we observed can be explained on the basis of temperature variation, given the information from the literature and the relatively high and constant salinity documented during most of the winter months. Nutritional parameters may contribute to the development of a given species, but we are unable at this time to assess the impacts of these resources, particularly the floc. Although chitin may serve as a potential nutritional reservoir, the prevailing conditions in Apalachicola Bay would tend to minimize variations in the chitin pool, and thus, the temporal sequence observed is probably driven by some other factor. The results of this study indicate that the observed sequence in the Apalachicola Bay sediments of *V. parahaemolyticus*, *V. cholerae* non O-1 serotype, and *A. hydrophila* is most probably temperature driven.

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