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Pertinence of Indicator Organisms and Sampling Variables to Vibrio Concentrations

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Vibrio-indicator relationships and effects of day, depth, and tidal levels on the density of vibrios enumerated by the most probable number technique were investigated. Counts of vibrios taken monthly from Apalachicola Bay, Fla., were either negatively correlated or showed no correlation with counts of indicator bacteria (*Escherichia coli*, enterococci, fecal coliforms, and total coliforms). Water samples collected on two days from the surface and bottom over a complete tidal cycle on each day were analyzed for differences in vibrio concentrations. Concentrations of vibrios in samples taken on different days, in those taken at different depths, and in those taken at different tidal levels were significantly different, indicating that these factors need to be taken into account in health-related studies.

The pathogenicity of *Vibrio* species has generated considerable impetus for more precise methods of enumeration and for a clearer understanding of factors that affect public health. Presently, the conventional measure for water quality that governs shellfish harvesting is based on the number of total and fecal coliforms. The U.S. Environmental Protection Agency has proposed that *Escherichia coli* and the enterococci also be used as indicator organisms (13). Despite regulations to ensure that shellfish harvesting waters meet acceptable standards of water quality, numerous investigations have shown that these indicators do not necessarily reflect the presence of vibrios in the marine and estuarine environments (3–5, 8–12). The earliest report to cast doubt on the reliability of indicator bacteria in reflecting the presence of vibrios was that of Kaneko and Colwell (5). They found no correlation between *Vibrio parahaemolyticus* and *E. coli* counts in Chesapeake Bay. Subsequent work (3) revealed that the incidence of *Vibrio cholerae* was also not correlated with that of fecal coliforms. Similarly, a recent study (11) in Boston Harbor found no correlation between *V. parahaemolyticus* densities and counts of fecal coliforms, nor was there any correlation with counts of enterococci. Oliver et al. (8) surveyed the East Coast of the United States and reported that there was no correlation of *Vibrio vulnificus* levels with fecal coliform levels. The lack of correlation between *Vibrio* species and conventional indicators has also been detected on the West Coast (4, 9, 10, 12).

Alternatively, significant correlations between *V. parahaemolyticus* densities and levels of *E. coli* and enterococci were found in Narragansett Bay, R.I. (15). Furthermore, a recent study (14) in Fukuyama, Japan, showed that *V. parahaemolyticus* densities were significantly correlated with those of total and fecal coliforms.

Undoubtedly, in any study involving the collection of coastal water samples, the tidal cycle is a potential contributor to the variance of vibrio counts. Likewise, stratification of the water column is a likely source of variation. Watkins and Cabelli (15) found that in Narragansett Bay, *V. parahaemolyticus* densities were highest near the surface of the water. This contrasts with a report (16) that vibrio densities in sediments were nearly 3 orders of magnitude higher than those in the overlying waters of Apalachicola Bay, Fla.

In the interest of public health, it is important to know if indicator organisms provide an adequate measure of vibrio levels in shellfish harvesting waters. Since existing standards of water quality are based on cell counts in water samples, the effect that sampling variables may have on the counts needs to be investigated. The aim of this study was to answer these questions by determining the relationship of vibrios to indicators and examining the effects that tidal level and sampling depth have on the concentration of vibrios in coastal water samples.

The study of the vibrio-indicator relationship was carried out at two stations in Apalachicola Bay, Fla. The first station, East Hole, was an area known to have elevated levels of vibrios (12, 16), and it is approved for shellfish harvesting by the Florida State Department of Natural Resources. The second station, East Point, was directly offshore from a small sewage treatment plant, and it is conditionally approved for shellfish harvesting. The selection of these stations ensured that the various groups of bacteria that were of interest could be detected at either one or the other location. Water samples were collected in clean, air-dried plastic bottles. The bottles were rinsed with water from the surface before the actual samples were collected by holding the bottles approximately 10 cm below the water surface. Samples were collected monthly for one year (April 1990 through March 1991). The recommended method for enumeration of vibrios in water samples is a most probable number (MPN) method with an enrichment step (2). In this method, the samples are diluted serially, and each of the serial dilutions undergoes enrichment in a liquid medium (alkaline peptone broth [APB] [2% peptone, 1% NaCl]) and is then scored on a selective medium (thiosulfate-citrate-bile salt-sucrose agar [TCBS]) that allows vibrios to grow in preference to other bacteria and differentiates the vibrios into sucrose-positive and sucrose-negative subgroups. Vibrios are usually the minority component of the bacteria in these samples, and the enrichment step serves to enhance their detection and yields an MPN estimate of their abundance.

The variability of the MPN technique was assessed by using it to enumerate known concentrations, determined by plate counts on T1N1 agar (1% tryptone, 1% NaCl, 2% agar), of *V.*

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TABLE 1. Correlation coefficients between vibrios and indicator bacteria in Apalachicola Bay

Indicator bacterium or bacteria	Correlation coefficient with vibrios collected at[a]:			
	East Hole (n = 12)		East Point (n = 11)	
	Sucrose +	Sucrose −	Sucrose +	Sucrose −
E. coli	−0.392	−0.403	−0.169	−0.715[b]
Enterococci	0.385	0.330	0.186	−0.342
Fecal coliforms	−0.609[b]	−0.490	−0.012	−0.723[b]
Total coliforms	−0.436	−0.308	0.047	−0.844[b]

[a] Sucrose +, sucrose positive; Sucrose −, sucrose negative.
[b] Significant correlation ($P < 0.05$).

cholerae cultured in the laboratory. To determine the MPN, a series of APB (pH 8.4 to 8.6) tubes were inoculated with serial dilutions of each concentration of *V. cholerae* cell suspension used and incubated at 35°C for 12 to 16 h. One loopful from each APB tube was then streaked on TCBS (Difco Laboratories, Detroit, Mich., and BBL Microbiology Systems, Cockeysville, Md.) plates. The TCBS plates were incubated overnight at 35°C. The enumeration data were log transformed (log [number of cells + 1]) to correct for unequal intervals and the high likelihood of heteroscedasticity. A regression of plate counts on the MPN enumerations of vibrio cell numbers showed that plate counts and MPN values were significantly correlated (Pearson's correlation coefficient, $r = 0.947$; df = 3; $P = 0.014$), and thus the MPN method provides a reasonable assessment of vibrio concentrations relative to plate counts.

The vibrios in environmental samples were enumerated by the MPN method. The enumeration of total coliforms, fecal coliforms, *E. coli*, and enterococci was performed according to standard methods (1) and procedures recommended by the U.S. Environmental Protection Agency (13).

Field experiments to assess the tidal effect and to determine whether there is a difference between samples taken at the water surface and samples taken at the bottom were done on two days (20 March and 9 April 1991) through complete tidal cycles at a site that has a semidiurnal tidal regime. On the first day, the temperatures of the water samples varied from a minimum of 15.5°C to a maximum of 18.5°C, and the salinity values were between 28 and 32 ppt. On the second day, the corresponding physical parameters were 21 to 26°C and 24 to 30 ppt, respectively. Sterile plastic bottles were used to collect water samples from the surface (approximately 10 cm below the air-water interface) and bottom (approximately 10 cm above the water-sediment interface) at four successive tidal stages (outgoing, low, incoming, and high) on each day. The midpoint between high tide and the succeeding low tide was considered an outgoing tide, while the midpoint between low tide and the high tide that followed it was considered an incoming tide. The appropriate times for each tidal level were calculated from tide tables. At each time, five samples were taken at five random spots, roughly 2 mi (ca. 3 km) from shore and along a 3-mi (ca. 6 km) area of the coast between 1.5 and 5 m deep. Care was taken to limit the time interval between collection of the first and last samples for each tidal level to 30 min. The samples were processed immediately at the Florida State University Marine Laboratory adjacent to the area. An incubator was not available at the marine laboratory, and so the APB tubes were incubated at room temperature (approximately 23°C) for 19 h and streaked onto TCBS plates that were then incubated for 12 to 14 h at room temperature before they could be relocated to a 35°C incubator for another 9 to 10 h.

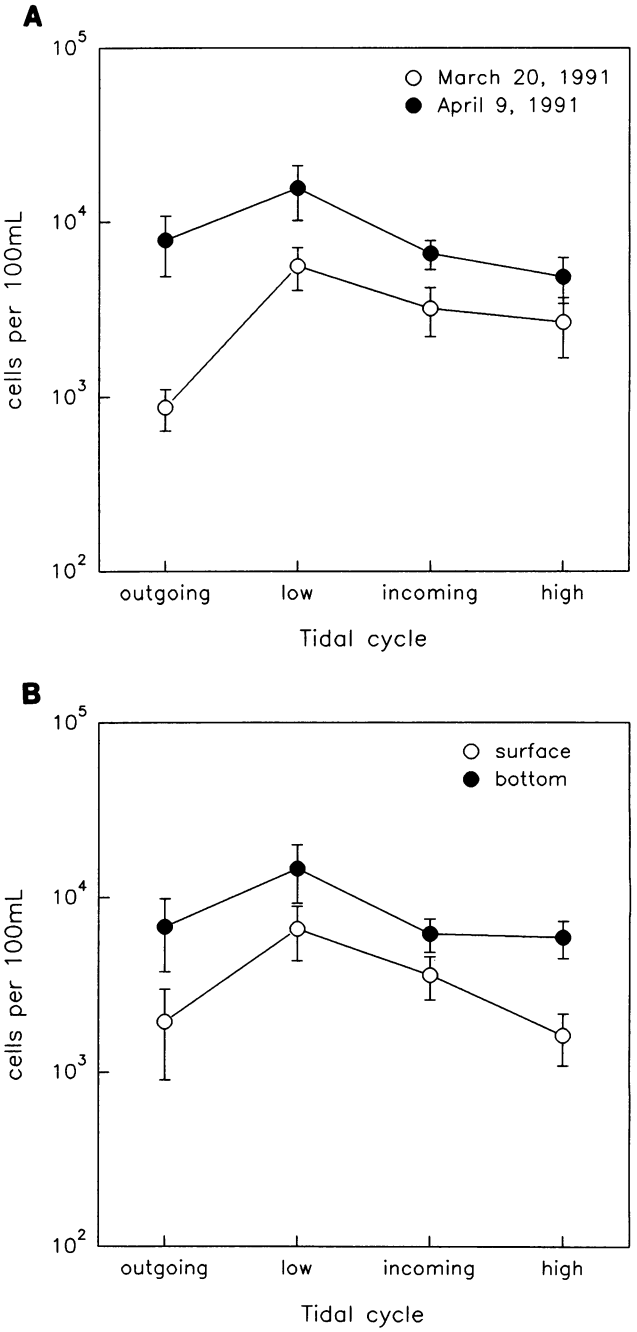

FIG. 1. (A) Variation in sucrose-positive vibrio concentrations (means of values for surface and bottom samples ± standard errors; n = 10) over a single tidal cycle measured on each of two different days (20 March and 9 April 1991). (B) Variation in surface and bottom concentrations of sucrose-positive vibrios over a single tidal cycle (means of values from two days ± standard errors; n = 10).

Results from the study of the vibrio-indicator relationship showed that there were no vibrios detected in samples that were collected when water temperatures were below 10°C or when salinity values dropped below 5 ppt. Concentrations of both sucrose-positive and sucrose-negative vibrios were generally highest in the summer and fall. The correlation coefficients (calculated from log-transformed counts) of the two groups of

TABLE 2. Analysis of variance for sucrose-positive vibrios to assess effects of day, depth, and tidal levels

Source of variation	df	SS[a]	MS[b]	F	P
Factors					
Day	1	3.8148	3.8148	17.50	0.0001
Depth	1	3.7542	3.7542	17.23	0.0001
Tide	3	3.9777	1.3259	6.08	0.0010
Outgoing	1	2.1892	2.1892	10.05	0.0023
Low	1	1.3922	1.3922	6.39	0.0140
Incoming	1	0.3963	0.3963	1.82	0.1821
Interactions					
Day × depth	1	0.3983	0.3983	1.83	0.1812
Day × tide	3	0.6722	0.2241	1.03	0.3861
Depth × tide	3	0.3164	0.1055	0.48	0.6946
Day × depth × tide	3	0.5998	0.1999	0.92	0.4376
Error	64	13.9477	0.2179		
Total	79	27.4811			

[a] SS, sum of squares.
[b] MS, mean of squares.

vibrios relative to the indicator bacteria are presented in Table 1. At East Hole the only significant correlation was a negative one between fecal coliforms and sucrose-positive vibrios ($r = -0.609$; df = 10; $P = 0.036$). In contrast, sucrose-negative vibrios at East Point showed highly significant negative relationships with total coliforms ($r = -0.844$; df = 3; $P = 0.001$), fecal coliforms ($r = -0.723$; df = 9; $P = 0.012$), and *E. coli* ($r = -0.715$; df = 9; $P = 0.013$).

The correlations between vibrios and the indicator bacteria were either negative or not present. Thus, the existing and proposed indicators are not consistently and exclusively associated with the presence of vibrios. In this light, it would be prudent to monitor the levels of vibrios in shellfish harvesting waters or sediments directly instead of relying on the indicators.

Data from the experiments on sampling variables were log transformed and analyzed by three-way analysis of variance (2×2×4 factorial design), with day, depth, and tide as fixed effects. Planned comparisons in which concentrations at each tidal level were compared with those at the subsequent tidal levels were made. The analysis for sucrose-positive vibrios revealed significant effects for all three factors. Consistently higher concentrations were found on 9 April and in samples from the bottom (Fig. 1 and Table 2). Concentrations of sucrose-positive vibrios in the outgoing tide differed significantly from those in the other tides ($F = 10.05$; df = 1, 64; $P = 0.002$), and there was also a significant difference between concentrations in low tide and those in incoming and high tides ($F = 6.39$; df = 1, 64; $P = 0.014$) but not between those in the incoming tide and those in high tide. An a posteriori test showed that the concentrations in outgoing and low tides also differed significantly from each other ($F = 16.38$; df = 1, 64; $P = 0.0001$). The interactions were not significant.

There were also significant differences in sucrose-negative vibrio concentrations between the two sampling days and between depths (higher concentrations were found in bottom samples), but the overall tidal effect was not significant (Fig. 2 and Table 3). Planned comparisons revealed that there was a significant difference between the concentrations at low tide and those at incoming and high tides ($F = 6.28$; df = 1, 64; $P = 0.015$). An a posteriori test showed that the concentrations at low tide were significantly different from those at all the other tidal levels ($F = 5.90$; df = 1, 64; $P = 0.018$). Interactions were not significant.

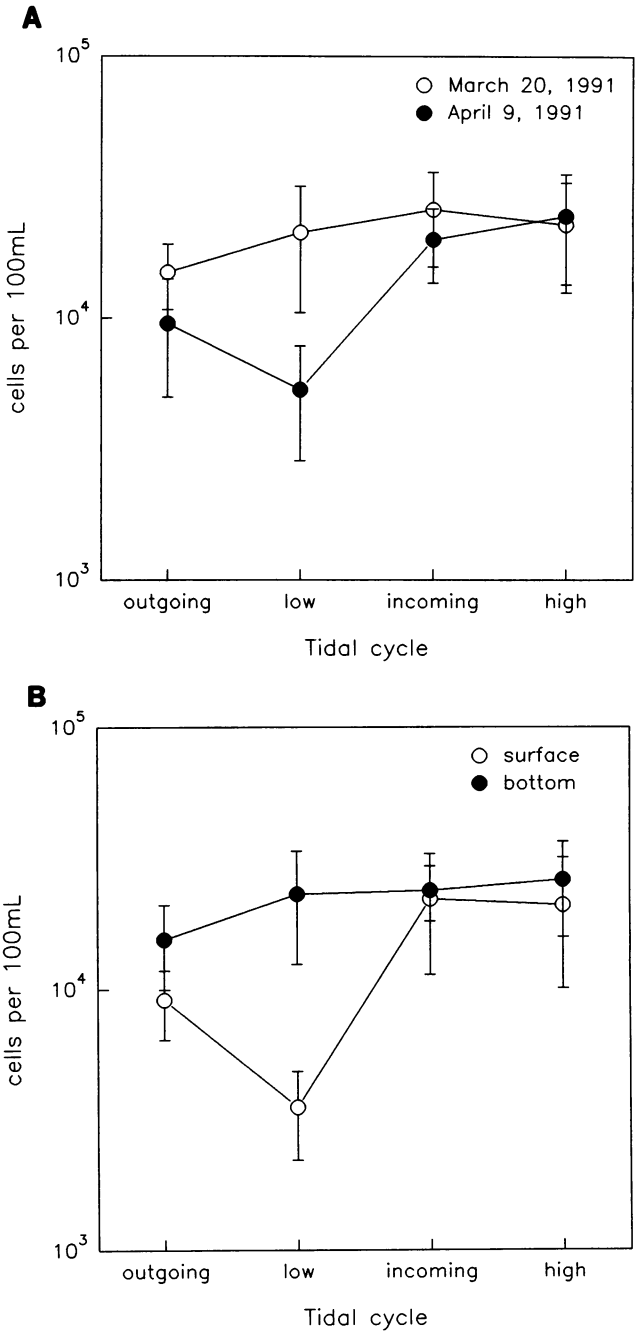

FIG. 2. (A) Variation in sucrose-negative vibrio concentrations (means of values for surface and bottom samples ± standard errors; $n = 10$) over a single tidal cycle measured on each of two different days (20 March and 9 April 1991). (B) Variation in surface and bottom concentrations of sucrose-negative vibrios over a single tidal cycle (means of values from two days ± standard errors; $n = 10$).

The consistently higher concentrations obtained from samples from the bottom (Fig. 1B and 2B) could be the result of resuspension of vibrios from the sediments. A previous study of vibrios in Apalachicola Bay (16) has shown that these organisms occur in sediments at levels that are 2 to 3 orders of magnitude higher than those in the overlying waters, and the possibility that sediments act as a reservoir was suggested.

TABLE 3. Analysis of variance for sucrose-negative vibrios to assess effects of day, depth, and tidal levels

Source of variation	df	SS[a]	MS[b]	F	P
Factor					
Day	1	3.4801	3.4801	8.73	0.0044
Depth	1	2.3995	2.3995	6.02	0.0169
Tide	3	2.5660	0.8553	2.14	0.1032
Outgoing	1	0.0155	0.0155	0.04	0.8421
Low	1	2.5062	2.5062	6.28	0.0148
Incoming	1	0.0444	0.0444	0.11	0.7412
Interactions					
Day × depth	1	0.7408	0.7408	1.86	0.1777
Day × tide	3	1.0943	0.3648	0.91	0.4389
Depth × tide	3	0.9196	0.3065	0.77	0.5158
Day × depth × tide	3	1.0563	0.3521	0.88	0.4547
Error	64	25.5222	0.3988		
Total	79	37.7788			

[a] SS, sum of squares.
[b] MS, mean of squares.

V. parahaemolyticus has been reported to undergo an overwintering process in the sediments of Chesapeake Bay (6). The contradiction of our results with those of Watkins and Cabelli (15) could be the result of the different seasons in which the work was carried out. The distribution of vibrios is known to be affected by water temperatures (3, 4, 6, 7, 9, 12, 16). According to these reports, vibrio concentrations in the water column are generally lowest in the cold winter months and increase to peak densities during the summer. Watkins and Cabelli (15) did their study in the summer, during the peak of vibrio occurrence, whereas the present study was conducted in the spring, when it was cooler, although water temperatures in the Gulf of Mexico are generally higher than those found along the East Coast. Comparing the samples from the two sampling days showed that the concentrations of sucrose-positive vibrios were higher in samples collected later in the season than when the temperatures were warmer (Fig. 1A).

The differences in vibrio concentrations across the tidal levels warrant further research. The reason for this effect is not clear, although variations in temperature and salinity may be factors. The highest concentrations of sucrose-positive vibrios occurred at low tide, and an ebbing tide stirs up the bottom sediments, so that the high concentrations at low tide could be a reflection of the increased numbers of vibrios that were resuspended from the sediments. The significant difference in the concentrations of vibrios at different tidal levels is important. It shows that tidal levels may need to be taken into account when multiple water samples are collected for comparative purposes.

This study has shown that conventional indicators of water quality do not consistently reflect the presence of vibrios and that vibrio concentrations are affected by sampling variables (day, depth, and tidal cycle). The conclusions concerning the effects of sampling variables are restricted to this study. However, if, in the interest of public health, vibrio concentrations are adopted as a measure of water quality for shellfish harvesting, a standard set of procedures (established by further experiments involving a greater number of sampling dates) stipulating the appropriate sampling variables would be necessary.

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