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Seasonal Incidence of *Vibrio vulnificus* in the Great Bay Estuary of New Hampshire and Maine†

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***Vibrio vulnificus*, a normal bacterial inhabitant of estuaries, is of concern because it can be a potent human pathogen, causing septicemia, wound infections, and gastrointestinal disease in susceptible hosts. From May 1989 through December 1990, oysters and/or water were obtained from six areas in the Great Bay estuary of New Hampshire and Maine. Water was also sampled from three freshwater sites that lead into these areas. *V. vulnificus* was first detected in the estuary in early July and remained present through September. *V. vulnificus* was isolated routinely during this period from oysters and water of the Squamscott, Piscataqua, and Oyster Rivers but was only isolated twice from the oysters or water of the Great Bay itself. This study determined that there was a strong correlation (by analysis of variance) between temperature, salinity, and the presence of *V. vulnificus* in water and oysters. However, other unidentified factors appear to influence its presence in certain areas of the estuary.**

Vibrio vulnificus has been identified as a causative agent in three disease syndromes: septicemia and gastroenteritis, both linked to ingestion of shellfish, and wound infections derived from contact with shellfish or the marine environment (2, 5, 6). The recent focus on this indigenous estuarine organism stems from disease incidence caused primarily by the ingestion of oysters. From 1975 to 1989, there were 115 cases of shellfish-associated *V. vulnificus* infections (22). Over 80% of these cases either occurred in southern Gulf coast states or were associated with shellfish from these areas. There have also been two cases of *V. vulnificus* septicemia in Connecticut, both of which were fatal (28). It was not determined whether shellfish from the area (e.g., Long Island Sound) were implicated in these cases. To date, no infections associated with northern New England water or shellfish have been reported.

V. vulnificus can be isolated from a wide variety of ecosystems. In the United States, *V. vulnificus* has been isolated from coastal and estuarine waters along the Gulf Coast; the Atlantic Coast as far north as southern Maine; and the West Coast, from southern California to British Columbia (9–11, 18, 20, 21, 23, 26–28). *V. vulnificus* has been isolated from oysters, clams, mussels, and fish, as well as from sediment and plankton (9, 11, 20, 21, 23, 27–29). *V. vulnificus* has been isolated from waters with temperatures of 13 to 31°C and salinities of 0.8 to 34‰ (9–11, 18, 20). Despite its apparent tolerance of wide ranges of salinity and temperature, *V. vulnificus* is more frequently observed and concentrations are higher in water with temperatures of 17 to 31°C and salinities of 15 to 25‰.

The Great Bay estuary of New Hampshire and Maine is a shallow, north temperate embayment subject to great extremes of temperature (−1.0 to 29.0°C) and salinity (0 to 28‰). Consequently, this estuary was deemed advantageous for determining the effects of natural extremes of salinity and temperature on the incidence of *V. vulnificus*. This study summarizes two years of research on *V. vulnificus* in the Great Bay estuary of New Hampshire and Maine.

MATERIALS AND METHODS

Collection and sampling procedures. Oysters (*Crassostrea virginica*) and water samples were obtained during 1989 and 1990 from several sites in the Great Bay estuary of New Hampshire and Maine (Fig. 1). Samples were collected biweekly during summer and autumn and monthly during spring and most winter months. Oysters were collected at the same time as water samples at each site. Water and oyster samples were collected from Great Bay (site 1), from two sites on the Oyster River (sites 2 and 3), and from the Piscataqua River (site 4). Most sampling in Great Bay took place at Adams Point, as indicated in Fig. 1, with a small amount of oyster and water sampling at sites further south into the Bay. Because there are no oysters present, only water samples were obtained from the Squamscott River (site 5) and from freshwater sites (sites 6 to 8) located above dams on three tributaries that lead into the estuary. These areas have always been included in the State of New Hampshire monitoring program for shellfish-growing waters and have been sampling sites for previous microbial research in this area (1, 7, 16, 17, 21, 25). Oysters were also obtained from a company that harvests from the Maine area of the Piscataqua and Salmon Falls rivers (site 9).

Oysters were collected from boats by using oyster tongs or a dredge or hand collected from shallow subtidal areas at low tide when the beds were accessible. Oysters were kept refrigerated on ice packs for up to 45 min during transport to the Jackson Estuarine Laboratory, cleaned under running water with a stiff brush, and aseptically shucked with a sterilized oyster knife. The meat and liquor from 8 to 16 oysters (approximately 100 to 150 g [pooled wet weight]) were homogenized for 90 s in a Waring blender that contained an equal volume of sterile phosphate-buffered peptone water (12).

Water samples were obtained at the same time that oyster collection was performed at low tide by completely immers-

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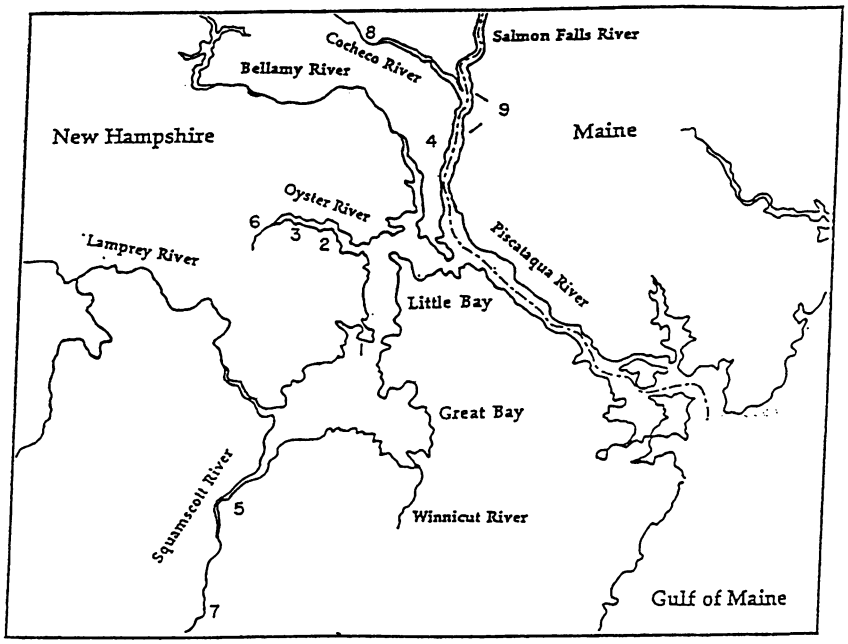

FIG. 1. Sampling sites (1 to 9) in the Great Bay estuary.

ing a covered, sterile 1-liter polycarbonate bottle into the water to a depth of about 10 cm, facing it into the current, and removing the lid. The bottle was capped while still submerged and removed from the water (12). Temperature was measured in situ by using a mercury thermometer. Salinity was determined at the laboratory by use of a refractometer (American Optical, Buffalo, N.Y.) or salinometer (YSI Inc., Yellow Springs, Ohio). Salinity was measured most often at low tide to reflect the greatest influence of inflowing fresh water, thus giving a minimal salinity reading for a given tidal cycle. Samples were collected at precise locations to minimize spatial variability.

Isolation and identification procedures. Water and homogenized oyster samples were inoculated into a three-tube most-probable-number (MPN) dilution series containing alkaline peptone water (pH 8.6, 1% NaCl) for selective enrichment of vibrios. After incubation of MPN tubes at 35°C for 18 h, all tubes showing turbidity were streaked onto thiosulfate-citrate-bile salts-sucrose (Oxoid U.S.A., Columbia, Md.) and colistin-polymyxin B-cellobiose (15) agar plates. Both media were incubated at 35°C for 18 to 24 h. Representative blue-green (sucrose-negative) or yellow (sucrose-positive) colonies on thiosulfate-citrate-bile salts-sucrose medium and yellow colonies on colistin-polymyxin B-cellobiose medium were streaked onto nutrient agar plates to determine purity and restreaked if necessary, and nutrient agar isolates were then inoculated into a series of salt tolerance and differential media to differentiate *V. vulnificus* from other pathogenic and nonpathogenic vibrios. Putative isolates were also Gram stained and tested for the ability to produce oxidase. Oxidase-positive, gram-negative, rod-shaped bacteria which did not grow in peptone water with 0% NaCl were designated halophilic vibrios. Isolates which grew only in 3 and 6% peptone salt broths, were negative for arginine dehydrogenase, were positive for lysine decarboxylase, and fermented cellobiose and salicin were considered presumptive *V. vulnificus* isolates. All putative *V. vulnificus* isolates were verified by using the API 20E identification system (Analytab Products, Inc., Plainview, N.Y.) modified for vibrio identification by using 2.5% saline as an inoculum diluent (14) and confirmed with latex-bound, anti-H (flagellar) monoclonal immunoglobulin G antibody (provided by R. Siebeling, Louisiana State University, Baton Rouge). The order of the isolation steps was such that *Vibrio fluvialis* isolates would have been excluded by biochemical tests from the final verification steps, thus minimizing the possibility of false positives from cross-reaction of *V. fluvialis* with the anti-H antibody, as reported by Oliver et al. (19).

For fecal coliform analysis, water and homogenized oyster samples were inoculated into a five-tube MPN dilution series containing lauryl tryptose broth (Difco Laboratories, Detroit, Mich.) and incubated at 35°C for 24 to 48 h. Dilutions positive for gas were inoculated into Eijkman Coliform broths (Difco), and Eijkman Coliform tubes which produced gas were considered positive for fecal coliforms.

MPN tables were used to calculate the number and confidence intervals of *V. vulnificus* cells and fecal coliforms per 100 g or 100 ml. Other statistical tests were conducted on a Macintosh SE computer by using Statworks (Cricket Software, Philadelphia, Pa.).

RESULTS

V. vulnificus was first isolated from the Great Bay estuary in July of 1989 and 1990 and persisted at three sites (sites 2, 4, and 5) into October. Table 1 shows the incidence of *V. vulnificus* in either water or oyster samples at each site from July through October. Isolation of *V. vulnificus* was most consistent in the Piscataqua (sites 4 and 9) and Squamscott (site 5) rivers, with greater than 50% of either oyster and water (sites 4 and 9) or water (site 5) samples obtained being positive for *V. vulnificus*. The Oyster River (sites 2 and 3) had a lower recovery rate (ca. 30%), and only 2 of 37 samples taken from the Great Bay (site 1) during this period were positive for *V. vulnificus*. *V. vulnificus* was never detected in

TABLE 1. Distribution and frequency of *V. vulnificus* at different sites in the Great Bay estuary from July to October in 1989 and 1990

Site	Site no.	No. of samples with detectable *V. vulnificus*/ total no. of samples	
		Oyster	Water
Piscataqua River	4, 9	18/30	7/12
Great Bay	1	1/19	1/18
Oyster River	2, 3	6/17	8/23
Squamscott River	5	—[a]	7/14
Freshwater sites[b]	6, 7, 8	—	0/18

[a] —, Areas where no oysters were present.
[b] Sites include nontidal Oyster, Squamscott, and Cocheco rivers.

water samples from the freshwater sites (sites 6 to 8) that feed into the estuarine sampling areas.

Levels of *V. vulnificus* increased dramatically from initially low concentrations in July after temperature and salinity levels in the water at low tide had increased to >20°C and >10.0‰, respectively. These values are well within the optimum ranges reported for this species (10). The temperature and salinity of water during low tide at the time of sample collection in areas where *V. vulnificus* was detected ranged from 11.1 to 29.5°C and 5.0 to 27.0‰, respectively. Mean monthly values for temperature and salinity for areas where *V. vulnificus* was detected are shown in Fig. 2. Salinity readings taken at high tide were consistently higher

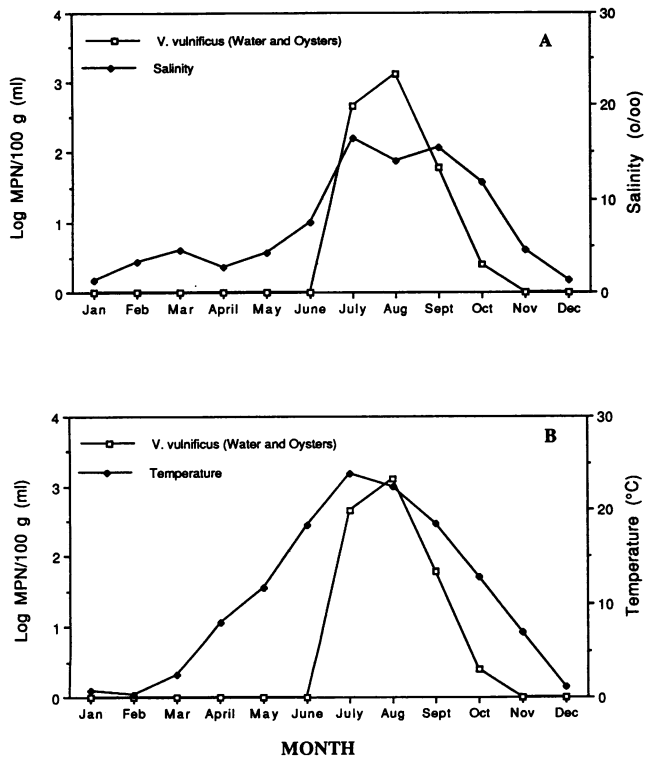

FIG. 2. Effects of salinity and temperature on *V. vulnificus* concentrations in oysters (per 100 g) and low-tide water (per 100 ml) from the Piscataqua, Salmon Falls, Oyster, and Squamscott rivers (July 1989 to December 1990). Values plotted are the mean monthly values for each area.

than those at low tide (data not shown). Thus, the low-tide salinity readings represent a minimum salinity for the sites. The variability in salinity between low tide and high tide was greatest at the Squamscott River and lowest in the Great Bay. Salinity and temperature varied spatially in the estuarine tributaries, depending on the proximity to the source of fresh water and its flow. Little spatial variability for temperature and salinity was observed between Adams Point and other sampling sites in the main portion of Great Bay (data not shown).

The seasonal relationship between the incidence of *V. vulnificus* and temperature and salinity levels in the Great Bay estuary is illustrated in Fig. 2. Values plotted are mean values for each month for sites 2, 3, 4, 5, and 9. There was a gradual decrease in *V. vulnificus* concentrations as both salinity and temperature decreased in the fall. *V. vulnificus* was not detected after October, when temperatures and low-tide salinities were <10.0°C and <5‰, respectively. Seasonal relationships between salinity, temperature, and *V. vulnificus* levels from the Oyster (sites 2 and 3), Piscataqua (sites 4 and 9), and Squamscott (site 5) rivers were investigated by using the statistical means of these parameters for each month. Analysis of variance revealed significant relationships between temperature only and *V. vulnificus* concentrations ($r = 0.79$), salinity only and *V. vulnificus* concentrations ($r = 0.84$), and temperature and salinity together and *V. vulnificus* concentrations ($r = 0.85$). No correlation existed between the levels of fecal coliforms and the levels of *V. vulnificus*.

The mean monthly temperatures of each sampling area where *V. vulnificus* was detected were very similar (Fig. 3). Values for specific sites in the Piscataqua and Oyster rivers were combined and presented as averages for the two rivers. However, salinity varied greatly between these sites, especially from November through May. The salinity of the Squamscott and Piscataqua rivers was less than 5‰ during these months. The salinity of the Oyster River was consistently higher than that in the other rivers; sampling sites in the Oyster River were inaccessible owing to ice formation during December and January, and no data were obtained for these months. Salinity values from Great Bay were the highest of those of all sites, remained greater than 10.0‰ throughout the year for all discrete samples, and in the winter averaged greater than 15‰.

In synoptic oyster and water samples, *V. vulnificus* was found more frequently and at higher concentrations in oysters than in water, except for two paired samples taken from the Oyster River (Table 2). The geometric mean for *V. vulnificus* organisms in oyster meats (11,500 ± 45 per 100 g) was approximately 60 times greater than that in corresponding water samples (190 ± 38 per 100 ml). When *V. vulnificus* was detected in both oysters and water samples from the Piscataqua and Oyster rivers, the levels in oysters were always equal to or greater than the levels in water. The geometric mean for fecal coliform levels in oysters (920 ± 3 per 100 g) was also significantly greater than that for water samples (190 ± 14 per 100 ml). Fecal coliform levels in water samples were consistent with the designated shellfish harvesting classifications of these areas: approved in Great Bay, restricted in the Salmon Falls and Piscataqua rivers, and prohibited in the Oyster and Squamscott rivers.

DISCUSSION

In the Piscataqua, Salmon Falls, Squamscott, and Oyster rivers, *V. vulnificus* was isolated at relatively high levels

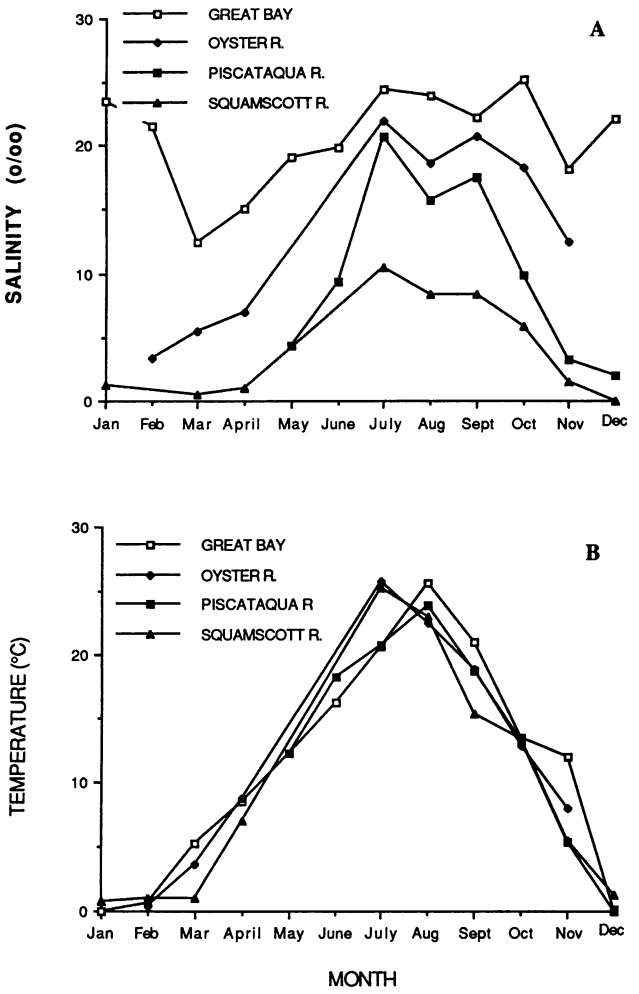

FIG. 3. Average monthly salinity (A) and temperature (B) of water at low tide in the estuarine sampling areas (1989 to 1990).

from oysters and water in early July when temperatures were >20°C. Thereafter, levels peaked in August and slowly decreased, disappearing from oysters in early October (temperature, <15°C) and from water by the middle of October. The latest date for detection of *V. vulnificus* was 11 October 1989, at a temperature of 11.1°C in water from the Squamscott River where no oysters were present. This trend is consistent with the detection of *V. vulnificus* in other parts of the country, although *V. vulnificus* had previously been isolated only from water with temperatures of >13°C (9–11, 18, 20, 23, 26, 27–29). Thus, the present study shows that *V. vulnificus* is detectable in the water column at temperatures lower than those previously thought.

In previous ecological studies in the South, *V. vulnificus* was found in oysters and sediment when the water temperature was <15°C but not in the water column (26, 27). Also, in a study of individual plankton species, *V. vulnificus* was most often associated with benthic species (27). Kaysner et al. (9) isolated *V. vulnificus* from only one sediment sample, out of a total of 112 oyster, water, and sediment samples from Willipa Bay in Washington, and theorized that the lower water temperatures in Washington State were a factor. They detected *V. vulnificus* more frequently in water than in

TABLE 2. Incidence of *V. vulnificus* and fecal coliforms in paired oyster and water samples

Month	Site	Concentration[a] of:			
		V. vulnificus in:		Fecal coliforms in:	
		Oyster	Water	Oyster	Water
July	Oyster River	110,000	430	240	170
		<2	240	800	90
August	Oyster River	150,000	12,000	220	220
		110,000	<2	130	5,500
		3,000	3,000	1,700	40
		110,000	930	1,100	220
	Piscataqua River	110,000	4,300	1,300	170
		46,000	4,600	280	4
		24,000	230	2,400	30
		460,000	9,300	1,700	110
	Great Bay	24,000	<2	1,100	11
September	Oyster River	2,400	<2	700	220
	Piscataqua River	150,000	7,500	1,600	50
		46,000	<2	16,000	220
		15,000	30	3,000	30
		4,300	4	3,000	30
October	Oyster River	<2	4,300	1,100	240

[a] Concentrations were measured as the number of *V. vulnificus* organisms or fecal coliforms per 100 g of oyster or per 100 ml of water.

either shellfish or sediment samples collected from 24 West Coast estuaries. *V. vulnificus* was detected in 11 of 17 synoptic water and oyster samples from sites in the Great Bay estuary, with slightly better recovery in oysters (15 of 17) than in water (13 of 17). The lack of detection of *V. vulnificus* in a given sample type was probably a function of relative numbers present, the amount and types of other bacteria present, or other limitations of the detection methods. Sediment samples were not examined during this study, and additional research may provide information on the factors involved in the survival of *V. vulnificus* at low temperatures.

The isolation of *V. vulnificus* from the Oyster River was less consistent than isolation from either the Squamscott or Piscataqua River, even during July and August. Kelly (10) suggested that the presence of *V. vulnificus* in some estuaries may be due to its growth in localized environments which have optimal conditions, with subsequent seeding via tidal or current action to less-ideal environments. The Oyster River is relatively short and shallow and receives water that may include water from the Piscataqua River on an incoming tide. The salinity of the Oyster River, like that of Great Bay, remains higher in the winter than that of either the Squamscott or the Piscataqua River (Fig. 3). Thus, although *V. vulnificus* has been found in shellfish and water from Oyster River, it may be a transient inhabitant, being seeded into this system during the summer and not maintaining itself through the winter months because of the compounding factors of low temperature and relatively higher salinity.

The apparent positive correlation of incidence of *V. vulnificus* with salinity may not be a true correlation but may be a function of the diminishing influence of fresh water on salinity levels in the tributaries as flow decreases in the

spring and summer while water temperatures increase. The optimum temperature for growth of *V. vulnificus* is >20°C, and low temperatures are known to be a major inducer of the viable, nonculturable form of *V. vulnificus* (3), which probably has a significant influence on the seasonal detection of *V. vulnificus* in the Great Bay estuary.

Water temperatures in all sampling areas were quite similar during any given month throughout the year. Even though the mean temperature (23.7 ± 2.7°C) and salinity (23.5 ± 2.7‰) in Great Bay during the summer were within the optimum ranges for *V. vulnificus*, salinity levels during winter may be a factor in explaining the rarity of *V. vulnificus* in this body of water. Unlike the *V. vulnificus*-positive areas of the Great Bay estuary, the salinity in the Great Bay itself is less temporally and spatially variable and generally remains greater than 15‰ in the winter. Laboratory investigations have shown decreased survival of *V. vulnificus* at low temperatures (14°C) and high salinities (30 to 38‰) (8). When and if *V. vulnificus* becomes seeded into the Great Bay, it may be unable to establish itself as a regular inhabitant when temperatures decrease in the fall because it is more susceptible to the higher salinity of the Bay.

Water from the Piscataqua and Oyster rivers flows into Great Bay on incoming tides, and water from the Squamscott River empties into Great Bay on outgoing tides. These rivers have relatively high levels of total and fecal coliforms and, in New Hampshire, are closed to shellfishing. *V. vulnificus* is detectable from July through October in these rivers, also at relatively high levels. Theoretically, then, these rivers should contribute coliforms and *V. vulnificus* to the Great Bay, and concentrations in the bay might be expected to increase with time to levels similar to levels found in the rivers. The microbial characteristics of Great Bay, however, are quite different from those of these surrounding rivers. Unlike the rest of the estuary, Great Bay has lower levels of fecal coliforms than in all other areas, and it is the only area within the estuary that is open to recreational shellfishing. In spite of intensive sampling throughout the summer months, *V. vulnificus* was not isolated from Great Bay during 1989 and was isolated only twice, once from water and once from oysters, during 1990. Thus, like the fecal indicator bacteria, the incidence and concentration of *V. vulnificus* were lower in Great Bay than in the surrounding waters.

Significant correlations have been seen between the presence of *Vibrio* spp. and densities of fecal coliforms in both the United States and Puerto Rico (20, 23). Conversely, Tamplin et al. (26) found *V. vulnificus* most often in waters with a fecal coliform MPN of less than 3/100 ml. Oliver et al. (20) detected *V. vulnificus* in water with fecal coliform levels ranging from <1 to 800/100 ml. In the Great Bay estuary, fecal coliform levels are highest during autumn and winter when *V. vulnificus* is not detected (7). However, fecal coliform levels are higher where *V. vulnificus* is detected most often, i.e., in the Squamscott, Piscataqua, and Oyster rivers, than in Great Bay, where it is rarely detected.

The reason for the lower levels of fecal indicator bacteria and *V. vulnificus* in Great Bay is not known. It has been suggested that nutrient levels, particularly from sewage discharge and surface runoff, may affect the levels of both fecal coliforms and *V. vulnificus* in estuaries (10, 29). Comparative nutrient analyses of the Great Bay and Squamscott River waters have shown higher levels of ammonium, nitrate, and phosphate to be present in the Squamscott River (13, 24). However, additional work needs to be done to determine what effect these and other environmental parameters (suspended solids, plankton, carbon, and iron levels, etc.) may have on the survival and growth of *V. vulnificus*. Natural processes associated with the eelgrass and the oyster beds may also play some role in controlling concentrations of suspended microorganisms. It is also possible that the reduced current velocity in the shallow areas of Great Bay might allow the indicator bacteria and *V. vulnificus* to settle into the sediment. Finally, the time that water masses take to travel between the riverine areas that contain the coliforms and *V. vulnificus* and Great Bay is such that the bacteria could be in a viable but nonculturable state by the time they enter the apparently less-favorable environment of Great Bay. Earlier work by Grimes and Colwell (4) demonstrated that it required less than 12 h for *Escherichia coli* to become nonculturable in natural seawater, a time frame that is consistent with water movement from the three rivers into Great Bay.

V. vulnificus is not considered to be a public health concern in northern New England because, until recently, it was never detected in the colder-temperature waters north of Boston Harbor (20, 21) and there have been no documented cases of *V. vulnificus* disease in the area (22). However, the warm temperatures and low salinities in the tidal rivers of the Great Bay estuary provide a suitable habitat for this bacterium, and its presence in these waters and shellfish, often in high numbers, is reason for concern. More significantly, a better understanding of the impact of various environmental parameters on the ecology of this species, especially factors that account for the absence of *V. vulnificus* in the Great Bay itself, will facilitate further research aimed at controlling the spread of diseases associated with infection with this organism.

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