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Isolation and Characterization of *Vibrio vulnificus* from Two Florida Estuaries

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Vibrio vulnificus was enumerated in seawater and shellfish from two Florida estuaries at selected seasonal intervals. There were significant fluctuations in the presence and numbers of *V. vulnificus*. Relatively high seawater temperature and salinity favored the presence of *V. vulnificus* in both seawater and shellfish samples.

Vibrio vulnificus is a lactose-fermenting, halophilic, gram-negative, and potentially pathogenic marine vibrio. Reports of its occurrence in seawater and association in human infections are increasing (2, 3, 5).

It has been suggested that some *Vibrio* spp. may be indigenous to estuarine microflora (4). Kaneko and Colwell (4) have reported high levels of *Vibrio parahaemolyticus* in seawater during the warm months and low levels in cold months, appearing mostly in sediments.

This study reports the seasonal occurrence of *V. vulnificus* in both seawater and shellfish from two Florida estuaries which are closely associated with both shellfish harvesting and recreational activities.

Seawater, oyster, and clam samples were collected from three Tampa Bay and eight Apalachicola Bay stations (Fig. 1–3). Tampa Bay samples were collected in May and October 1980 and in January, April, and July 1981. Apalachicola Bay samples were obtained in April, July, and November 1980 and in February, May, and August 1981. Stations were classified as approved, unapproved, or conditionally approved for shellfish harvesting by the Florida State Department of Natural Resources (Fig. 1 and 3). Seawater was obtained 2 ft (ca. 61 cm) or less below the surface on an outgoing tide. Shellfish were collected with the associated seawater samples, transferred to the laboratory, and prepared for bacteriological analysis by established techniques (1, 6).

Specifically, oysters and clams were cleaned, shucked, weighed, and homogenized in a twofold dilution (wt/vol) of peptone water. Seawater and homogenized shellfish samples were serially diluted in 10-fold increments of 100 ml of 0.5% peptone water (pH 7.2). Serially diluted sample (1 ml) was transferred immediately into triplicate tubes containing 9 ml of alkaline peptone broth (pH 8.4). After 12 h of incubation at 37°C, these inocula were streaked from alkaline peptone broth onto thiosulfate-citrate-bile salts-sucrose agar (Difco Laboratories). Plates were incubated for 24 h at 37°C. All typical green and gray colonies were inoculated into API (Analytab Products) biochemical strips for species identification. A most-probable-number (MPN) estimate of *V. vulnificus* numbers was then determined from standard MPN tables for a three-tube series.

Seawater temperature and salinity were measured in situ with a Beckman RS5-3 thermister-salinometer. Fecal coliform MPN was determined by standard methods (6).

The levels of *V. vulnificus* and selected environmental data at Apalachicola Bay are shown in Table 1. *V. vulnificus* was isolated more often from both seawater and oyster samples during the months of July and November than at the other sampling times. *V. vulnificus* was not detected during April and February in either seawater or oyster samples. In May, relatively high levels were detected in some seawater samples but not in oysters. In August, *V. vulnificus* was again primarily detected in seawater; however, at one site, oysters contained relatively high counts. Examination of environmental data shows that *V. vulnificus* was isolated more often when the water temperature was greater than 17°C and in a greater proportion of samples over 29°C. In addition, *V. vulnificus* was isolated more frequently in waters with a salinity greater than 17.0‰ and in a higher proportion of samples greater than 23.0‰. Interestingly, *V. vulnificus* was found more often in waters with a fecal coliform MPN of less than 3 per 100 ml of seawater.

The numbers of *V. vulnificus* detected at

TABLE 1. Levels of *V. vulnificus* and environmental data[a] at selected seasonal intervals at Apalachicola Bay

Date and station no.	Temp (°C)	Salinity (‰)	Fecal coliform MPN	*V. vulnificus* MPN	
				Oyster	Seawater
April 1980					
29	20.5	0.0	15	<0.3	<0.3
36	18.0	12.0	<0.3	<0.3	<0.3
37CB	23.4	12.1	<0.3	<0.3	<0.3
8C	22.4	1.2	23	<0.3	<0.3
34	23.3	5.4	<0.3	<0.3	<0.3
19	21.2	0.0	7	<0.3	<0.3
16	22.5	0.9	11	<0.3	<0.3
15B	21.9	0.9	15	<0.3	<0.3
July 1980					
29	29.4	28.0	<0.3	<0.3	430
36	29.0	10.9	<0.3	2,300	2,300
37CB	29.0	14.5	<0.3	43	930
8C	29.5	31.0	<0.3	93	<0.3
34	28.9	8.2	<0.3	43	43
19	29.9	30.3	<0.3	4.3	930
16	29.4	31.2	<0.3	<0.3	430
15B	29.4	30.4	<0.3	93	430
November 1980					
29	18.6	21.9	93	9.3	23
36	18.9	23.8	4	46	240
37CB	18.9	23.5	23	110	280
8C	19.3	25.3	9	24	150
34	19.2	22.5	<0.3	110	93
19	19.2	20.7	43	15	93
16	19.2	32.2	<0.3	15	43
15B	19.1	25.3	93	9.3	43
February 1981					
29	10.7	0.8	240	<0.3	<0.3
36	7.7	22.3	4	<0.3	<0.3
37CB	8.5	19.5	<0.3	<0.3	<0.3
8C	9.2	12.7	9	<0.3	<0.3
34	3.1	18.5	23	<0.3	<0.3
19	9.4	10.3	9	<0.3	<0.3
16	9.5	21.0	9	<0.3	<0.3
15B	9.5	13.3	15	<0.3	<0.3
May 1981					
29	24.3	17.3	13	<0.3	<0.3
36	24.0	20.8	7	<0.3	46,000
37CB	24.3	17.3	<0.3	<0.3	<0.3
8C	24.5	25.8	7	1.1	<0.3
34	24.3	17.1	13	<0.3	24,000
19	25.1	15.3	13	<0.3	<0.3
16	23.8	25.2	<0.3	110	46,000
15B	24.4	24.7	<0.3	<0.3	<0.3
August 1981					
29	30.4	20.2	43	<0.3	150
36	29.3	19.8	<0.3	<0.3	240
37CB	29.6	19.8	<0.3	3,000	<0.3
8C	29.7	25.1	<0.3	<0.3	<0.3
34	29.5	19.7	<0.3	<0.3	93
19	30.3	16.4	<0.3	<0.3	<0.3
16	29.4	25.7	<0.3	<0.3	43
15B	29.5	22.7	7	<0.3	<0.3

[a] Values for fecal coliform MPN are per 100 ml of seawater. Oyster-associated *V. vulnificus* MPN calculated per gram of oyster (wet weight); seawater-associated *V. vulnificus* MPN calculated per 100 ml of seawater.

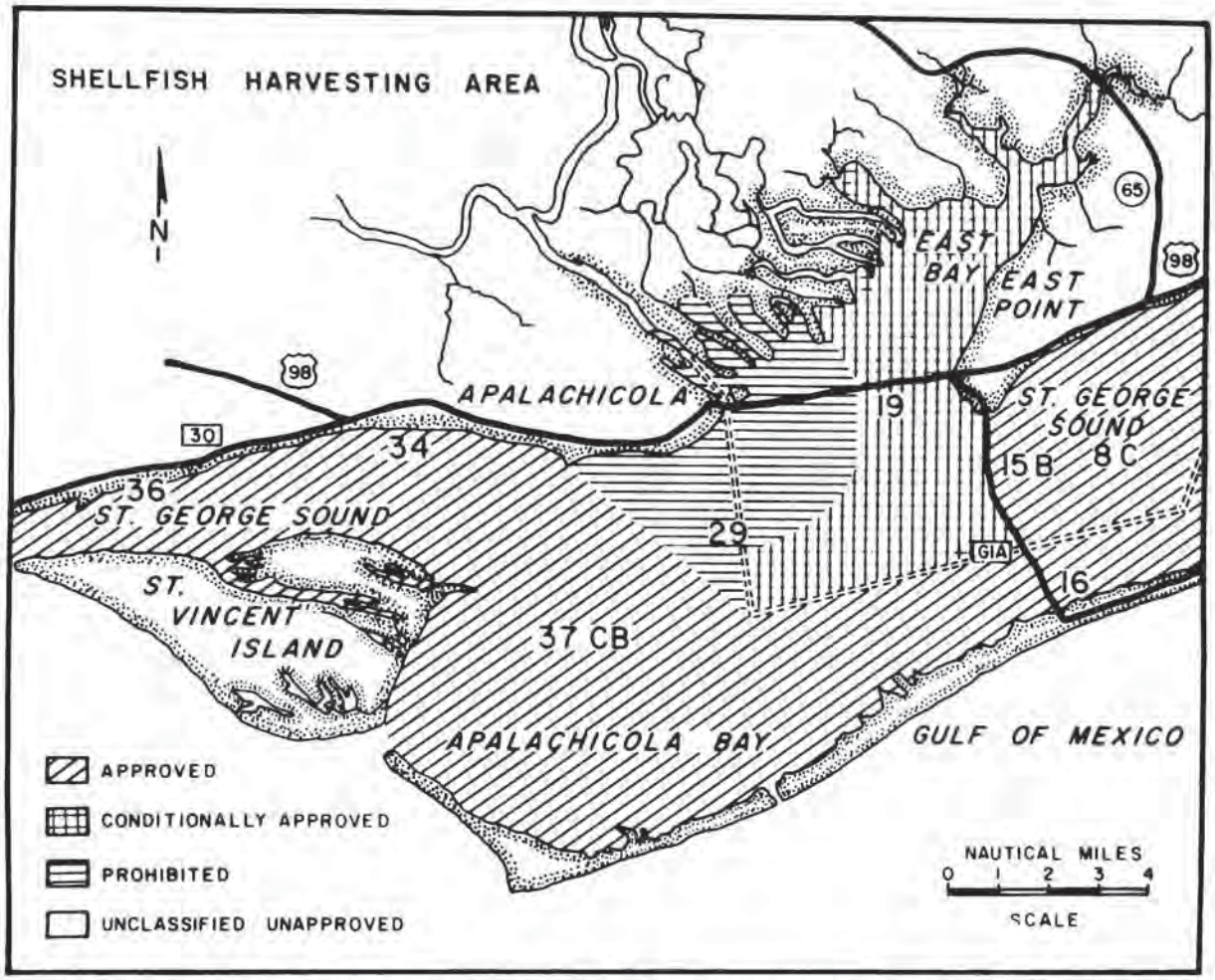

FIG. 1. Eight collection stations in Apalachicola Bay, Fla. (stations no. 29, 36, 37CB, 8C, 34, 19, 16, and 15B).

TABLE 2. Levels of *V. vulnificus* and environmental data[a] at selected seasonal intervals at Tampa Bay

Date and station no.	Temp (°C)	Salinity (‰)	Fecal coliform MPN	*V. vulnificus* MPN		
				Oyster	Clam	Seawater
May 1980						
1	28.0	25.6	240	<0.3	<0.3	<0.3
2	28.0	31.5	43	—	<0.3	<0.3
3	28.2	32.4	9	<0.3	<0.3	<0.3
October 1980						
1	30.8	20.7	4,600	930	—	15,000
2	31.6	27.1	23	—	9.3	12,000
3	36.2	31.4	<0.3	<0.3	<0.3	110
January 1981						
1	8.5	18.2	110,000	<0.3	—	<0.3
2	9.6	28.2	460	—	<0.3	<0.3
3	13.2	28.1	<0.3	<0.3	<0.3	<0.3
April 1981						
1	24.7	29.0	39	<0.3	—	<0.3
2	25.1	32.7	4	—	<0.3	<0.3
3	27.6	33.0	11	<0.3	<0.3	<0.3
July 1981						
1	31.0	30.5	39,000	<0.3	—	700,000
2	31.0	34.0	<0.3	—	<0.3	<0.3
3	31.0	35.0	23	<0.3	<0.3	<0.3

[a] Values for fecal coliform MPN are per 100 ml of seawater. MPNs for oyster- and clam-associated *V. vulnificus* are per gram of shellfish (wet weight); seawater-associated organism counts are per 100 ml of seawater. —, Not available for testing at the site.

Tampa Bay are shown in Table 2. *V. vulnificus* was not detected in the January or April samplings. However, a single seawater sample taken in July contained relatively high numbers of *V. vulnificus*. In addition, high numbers and frequent isolations were observed during the October sampling in both shellfish and seawater samples from unapproved sites. A comparison of environmental data and *V. vulnificus* numbers at Tampa Bay differed slightly from that of Apalachicola Bay. Specifically, *V. vulnificus* was isolated only in waters with a temperature greater than 30°C. In addition, *V. vulnificus* was found only in waters with a salinity greater than 17.0°/$_{oo}$. *V. vulnificus* was also isolated from waters with either a high or low fecal coliform MPN.

The two estuaries which we examined contained significant numbers of *V. vulnificus* that fluctuated greatly between samplings. In both estuaries, there were times when seawater, oysters, and clams, all showed no detectable levels of *V. vulnificus*. Preliminary data suggest that seawater temperature and salinity may play important roles in this variance in numbers from sampling to sampling. *V. vulnificus* was detected frequently when the water temperature was greater than 17°C, but never below 17°C. In addition, salinities greater than 17.0°/$_{oo}$ appear to be favorable for isolation of *V. vulnificus*. There appeared to be higher numbers and more frequent isolation of *V. vulnificus* from samples taken during the summer and fall months. Waste comtamination and land runoff may contribute to the presence of this bacterium, either directly by supplying needed nutrients or indirectly by enriching for some other physical or biological factor. The ecology of *V. vulnificus* at this time is unclear. Obviously, more ecological investigations are needed to understand the relationship of this organism within the marine environment. It must be emphasized that our studies have dealt with one-point-in-time analyses at different stations over seasonal intervals. Fluctuations in biological or physical factors may occur over shorter time intervals, necessitating intensive samplings for a truly statistical analysis of the presence and numbers of *V. vulnificus* in the marine environment.

It is interesting to note that reported human vibrio infections in Florida occurred primarily

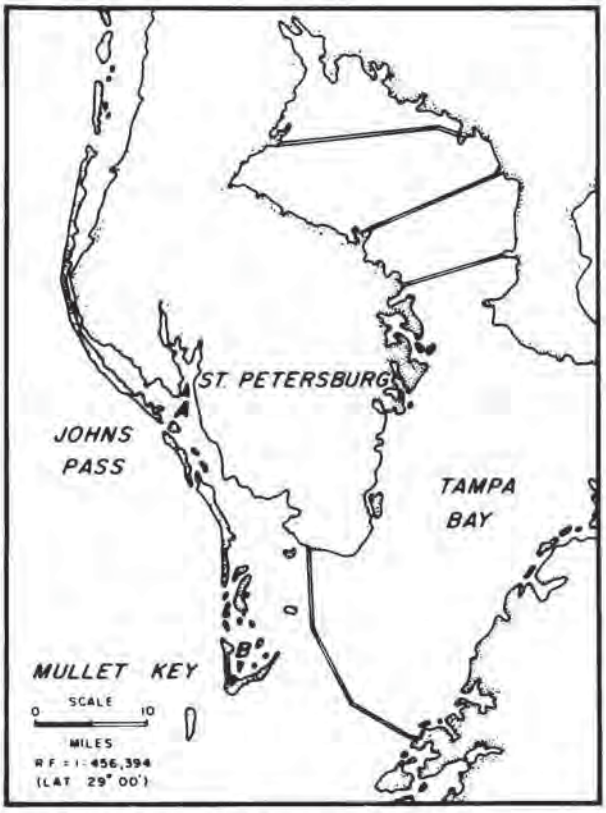

FIG. 2. Collection sites (A and B) in Tampa Bay, Fla.

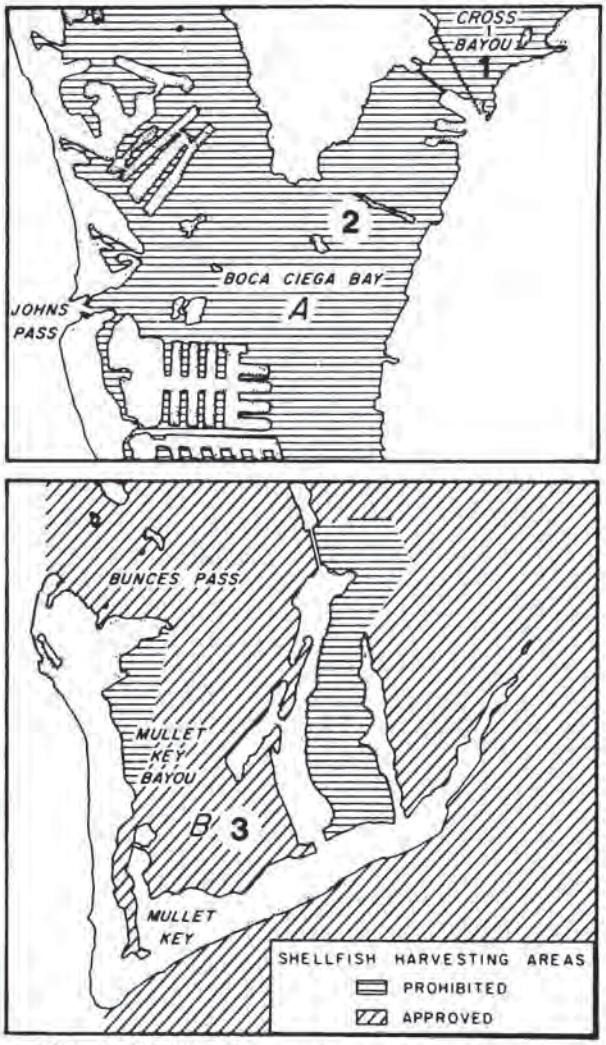

FIG. 3. Shellfish harvesting stations (A, 1 and 2 and B, 3) at the Tampa Bay collection sites.

during the fall months, correlating with our isolation of vibrios in shellfish and seawater for that period.

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Offshore Suspension Relaying To Reduce Levels of *Vibrio vulnificus* in Oysters (*Crassostrea virginica*)

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Oysters naturally contaminated with 10^3 to 10^4 most probable numbers (MPN) of *Vibrio vulnificus* per g were relayed to offshore waters (salinity, 30 to 34 ppt), where they were suspended in racks at a depth of 7.6 m. *V. vulnificus* counts in oysters were reduced to <10 MPN/g within 7 to 17 days in five of the six studies. At the end of the studies (17 to 49 days), *V. vulnificus* levels were reduced further and ranged from a mean of 0.23 to 2.6 MPN/g. Oyster mortalities during relaying were <6%. The reduction of *V. vulnificus* in relayed oysters is associated with exposure to high-salinity environments essentially devoid of *V. vulnificus*. Offshore suspension relaying may be a method that industry can employ to reduce *V. vulnificus* levels in raw Gulf Coast oysters.

The human pathogen *Vibrio vulnificus*, part of the normal microflora of estuarine waters and molluscan shellfish, continues to be a significant concern to public health agencies and the shellfish industry. Consumption of raw Gulf Coast oysters contaminated with *V. vulnificus* can cause primary septicemia in individuals with certain underlying diseases (1, 11). Infections associated with *V. vulnificus* are usually swift, aggressive, and often fatal. This pathogen is responsible for 95% of all seafood-related deaths in this country (11). There were 31 cases and 14 fatalities associated with shellfish and *V. vulnificus* reported in 1995 (2).

Experimental methods to reduce *V. vulnificus* in live oysters after harvest include irradiation and depuration. Gamma irradiation, although effective in reducing *V. vulnificus* in oysters at doses of 1.0 to 1.5 kGy, also increases oyster mortalities during storage (9). This process is currently not approved by the U.S. Food and Drug Administration. Conventional depuration of oysters with an indigenous microflora of *V. vulnificus* has been unsuccessful (6, 14) and, if conducted at temperatures higher than 21°C, may actually increase the number of *V. vulnificus* in oysters (14).

Relaying, a Food and Drug Administration-approved process, is normally employed to free contaminated shellfish of human enteric pathogens and involves the transfer of shellfish from restricted to open areas for natural biological cleansing. Relaying has not been used as a means of removing *V. vulnificus* from Gulf Coast oysters because inshore Gulf Coast waters contain large numbers of *V. vulnificus*. This study evaluates suspension relaying of Gulf Coast oysters to offshore waters that are normally free of *V. vulnificus.*

Oysters were harvested from conditionally approved growing areas at either Cedar Point or Buoy Reef in Mobile Bay, Ala., with oyster tongs or dredge, respectively. After culling and washing, 100 oysters were placed in baskets and suspended from a pier in Dauphin Island Bay (DIB) for 1 to 7 days for acclimation. DIB is close to the mouth of Mobile Bay and usually has higher salinities than the harvest sites. Oysters were removed from DIB after the acclimation period and transported to the offshore relay site (a natural gas production platform) located ca. 6.5 km south of the Alabama coast in 15.2 m of water. The 7 July 1995 study was conducted at an alternate gas production platform located ca. 37 km offshore in 30.4 m of water. At each relay site, oysters were divided into two groups, placed in plastic trays, and suspended at a depth of ca. 7.6 m.

Oysters for the 0-h *V. vulnificus* counts were collected after acclimation at DIB and just prior to relaying. Periodically, the remaining oysters were examined for mortalities and 10 to 12 oysters were removed from each tray and held separately for analysis. Seawater samples (500 ml) were collected at the depth of the suspended oysters with a submersible pump. All samples were held on ice and analyzed within 4 to 6 h of collection.

V. vulnificus was enumerated by a three-tube, most probable number (MPN) technique as recommended in the Food and Drug Administration Bacteriological Analytical Manual (4). Values less than the lower limit of sensitivity (0.30) were assigned a value of 0.15 to distinguish them from values equal to 0.30 and for calculation of geometric means. Seawater was similarly examined except that 10- and 100-ml portions were inoculated 10:1 into 10× strength alkaline peptone water. One-milliliter portions of seawater were inoculated into single-strength alkaline peptone water tubes. Isolates were confirmed by an enzyme immunoassay using a monoclonal antibody specific to *V. vulnificus* (15).

Temperature and salinity were determined at the harvest, acclimation, and relay sites with a bimetallic dial thermometer and a refractometer (American Optical). Measurements at the relay sites were made at the surface and on water pumped from the depth of the suspended oysters prior to each sampling. All other readings were surface measurements.

Ambient surface water temperatures at the harvest sites ranged from 22.7 to 31.1°C, and salinities ranged from 15 to 24 ppt (Table 1). Surface water temperatures at the acclimation and relay sites were similar to those recorded at harvest. Average water temperatures at the depth of the suspended oysters were normally lower than the average surface water temperatures at the relay sites; average salinities were consistently higher and ranged from 32.0 to 35.3 ppt.

Studies were conducted between July and October when the mean numbers ($n = 2$) of *V. vulnificus* in oysters after acclimation and immediately prior to relaying ranged from 1,991 to 14,019 MPN/g. *V. vulnificus* in oysters was reduced to <10

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TABLE 1. Environmental parameters at harvest, acclimation, and relay sites[a]

Date of harvest (mo/day/yr)	Harvest (surface value)		Acclimation (surface value[b])		Date of relay (mo/day/yr)	Relay			
						Surface[c]		Suspension depth[c]	
	Temp (°C)	Sal (ppt)	Temp (°C)	Sal (ppt)		Temp (°C)	Sal (ppt)	Temp (°C)	Sal (ppt)
08/23/94	29.6	25.0	30.4	28.0	08/24/94	29.8 ± 1.3	27.0 ± 1.4	28.8 ± 0.3	32.0 ± 1.6
09/12/94	29.2	15.0	28.1	23.0	09/19/94	26.7 ± 1.6	29.5 ± 3.0	25.4 ± 1.7	32.5 ± 1.0
10/17/94	22.7	24.0	24.6	25.0	10/19/94	22.4 ± 2.5	29.8 ± 1.7	22.2 ± 2.1	32.8 ± 0.5
06/30/95	28.6	24.0	28.7	25.0	07/07/95	29.3 ± 0.5	32.3 ± 1.5	27.6 ± 1.6	34.8 ± 1.5
06/30/95	28.6	24.0	28.7	25.0	07/07/95[d]	30.0 ± 1.6	31.7 ± 1.5	29.1 ± 0.8	32.7 ± 1.2
08/21/95	31.1	18.0	30.6	18.0	08/28/95	30.7 ± 1.1	31.3 ± 6.4	29.7 ± 0.3	35.3 ± 0.6

[a] Temp, temperature; Sal, salinity.
[b] Temperature and salinity recorded on the day that the oysters were relayed.
[c] Mean of observed values ± standard deviation.
[d] Alternate relay site located ca. 37 km south of Dauphin Island in 30.4 m of water. Oysters were suspended at 7.6 m.

MPN/g after 7 to 17 days of relaying in five of the six studies (Fig. 1). By the end of each study (17 to 49 days), *V. vulnificus* levels were reduced further and ranged from a mean of 0.23 to 2.6 MPN/g. These counts are similar to those seen in Gulf Coast oysters harvested between January and March (10) when *V. vulnificus* infections are minimal.

Twenty-one seawater samples collected at the depth of the suspended oysters were analyzed for *V. vulnificus* (data not shown). Seven of the samples had *V. vulnificus* concentrations between 0.3 and 10 MPN/liter; concentrations in the remaining seawater samples were <0.3 MPN/liter. Concentrations in other molluscs (barnacles and small oysters) examined from the relay site were <0.3 MPN/g. Apparently, offshore high-salinity environments are not a significant source of *V. vulnificus.*

There is a definite seasonal influence on the concentration of *V. vulnificus* in oysters along the Gulf Coast; at temperatures of <20°C, counts in oysters are greatly reduced (8, 10, 13). A similar ecological relationship with salinity is not as obvious. Tamplin et al. (13) reported that *V. vulnificus* favors relatively high-salinity environments; Kelly (8) and Vanoy et al. (17) suggested that *V. vulnificus* favors low-salinity environments. Recent work by Kaspar and Tamplin (7) in seawater microcosms supports this observation. They found that after 21 days at 25-ppt salinity the survival of *V. vulnificus* in pure culture was adversely affected.

In a 14-month survey, *V. vulnificus* was rarely recovered from

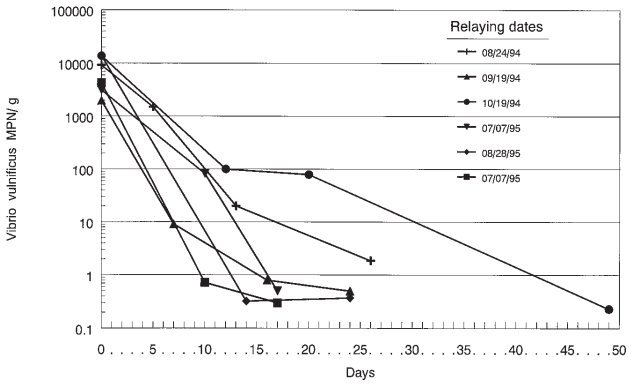

FIG. 1. Change in numbers of *V. vulnificus* in oysters following relaying to offshore high-salinity environments. Each point represents the mean ($n = 2$) of the MPN of *V. vulnificus* per gram of oyster meat.

Atlantic Coast oysters harvested from waters with average salinities of >30 ppt (10). In a study conducted in southern Maine, Jones (5) was able to eliminate *V. vulnificus* from oysters relayed to relatively high-salinity waters (25 ppt) free of *V. vulnificus*. In the present study, the oysters were relayed into waters with average salinities greater than 32 ppt (Table 1).

We conclude that the reduction in numbers of *V. vulnificus* in relayed oysters was primarily brought about by the adverse effect of the high-salinity waters on the ability of the organism to survive. Suspension relaying, in which shellfish were held off the bottom, offered an additional advantage in that shellfish were not exposed to the microflora associated with the sediments, a significant source of *V. vulnificus* in estuarine environments (3, 12, 17). Since the offshore waters were essentially free of *V. vulnificus*, the result was a progressive reduction to lower numbers.

The average salinities at the relay sites were 33% higher than those at the harvest sites. Consequently, we were concerned that this salinity change might affect the survival of the oysters. National Shellfish Sanitation Program guidelines (16) recommend that salinities in process waters at a facility for the controlled purification of oysters not vary more than 20% from the harvest water salinities. Average oyster mortalities during the study were <6%. Apparently, the oysters acclimated sufficiently in the intermediate-salinity waters of DIB prior to relaying to prevent significant mortalities.

Relaying of oysters into high-salinity offshore waters during the warm months reduces *V. vulnificus* to levels typically observed in oysters harvested during January and February, two months in which food-borne *V. vulnificus* illness has never been reported. Thus, offshore relaying is a relatively simple process that the shellfish industry may employ to reduce *V. vulnificus* levels in raw Gulf Coast oysters.

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