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Ice Immersion as a Postharvest Treatment of Oysters for the Reduction of *Vibrio vulnificus*†

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ABSTRACT

Vibrio vulnificus produces serious illnesses that are commonly associated with shellfish consumption, particularly raw oysters. Ingestion can result in fatal septicemia in susceptible individuals with hepatitis, cirrhosis, immune dysfunction, diabetes, or hemochromatosis (metabolic iron overload). Therefore, postharvest treatments to reduce vibrio levels in oysters have been recommended. In this study, rapid chilling by immersion of unwashed whole oysters in ice for 3 h was assessed as a postharvest treatment for reduction of *V. vulnificus.* Treated oysters were subsequently refrigerated at 45°F (7.2°C), whereas control oysters were not iced but were maintained at 45°F throughout the study. Homogenized meats were monitored for total heterotrophic aerobic bacteria, *V. vulnificus,* and fecal coliform content before and after treatment over a 2-week period. *V. vulnificus* was enumerated by DNA probe hybridization of colonies from standard plate counts on nonselective medium, and recovery was compared for several media. Loss of plating efficiency was observed on standard selective and differential media compared with nonselective agars. Numbers of *V. vulnificus* generally declined in treated samples compared with controls; however, increases in total heterotrophic bacteria and fecal coliforms were also observed in treated samples at some time points. This study does not support the use of ice immersion as a postharvest method because of the relatively small declines in *V. vulnificus* numbers and the possibility of concomitant increases in fecal coliform and total bacterial contamination.

Vibrio vulnificus produces fatal septicemia, which is associated with the consumption of raw or undercooked oysters *(4, 14, 30, 31).* Handling of seafood products or exposure of lesions to seawater can also result in serious wound infections. Unlike *Vibrio parahemolyticus, V. vulnificus* infections do not produce large outbreaks of disease, and relatively few sporadic cases (generally <50) are reported annually. However, high mortality rates (>50%) resulting from *V. vulnificus* disease have eroded consumer confidence in seafood safety, creating tremendous burdens for the industry. Life-threatening systemic disease is limited to persons with underlying conditions that increase susceptibility to infection, such as immune system dysfunction, insulin-dependent diabetes, hepatic disease, or metabolic iron imbalance (hemochromatosis). Attempts to reduce exposure of populations at risk to disease through consumer education have not resulted in decreased disease incidence *(13).*

V. vulnificus is indigenous to coastal waters and shellfish, and increased prevalence of this organism is observed in warmer summer months and corresponds to increased incidence of disease *(7, 8, 18, 27, 29, 31, 32, 37).* These epidemiological observations indicated that infections are dose dependent. Thus, effective postharvest treatments for the reduction of this bacterium in oysters have been instigated and include high pressure *(12, 19),* low-temperature pasteurization *(1),* irradiation *(9),* or ultra–low temperature freezing *(2, 15, 21).* These methods can effectively lower bacterial levels but will also kill the oyster. Therefore, they do not have application to the commercially important raw ''half-shell'' market. Most efforts to reduce vibrio contamination in live oysters have not been successful, but reduction of *V. vulnificus* in live oysters was reported with prolonged immersion of shellstock in ice immediately following harvest *(28).* Ice immersion offers several advantages as a postharvest treatment: the procedure does not require specialized technology, processing can be done during or immediately following harvest, the method is cost-effective and does not kill the oyster in the process. Therefore, we evaluated rapid chilling by ice immersion as a postharvest treatment method by monitoring *V. vulnificus,* total heterotrophic bacteria, and fecal coliform content in oysters before and after treatment.

Evaluation of postharvest treatments requires quantitative assessment, and numerous methodologies have been used for the enumeration of vibrios in oysters (for a recent review, see Harwood et al. *(11)*); however, application of molecular probe technology has provided more rapid detection of vibrios with increased specificity and sensitivity *(6, 10, 20, 22, 23, 25, 36–38)* compared with standard methodology *(17, 24, 34).* Enzyme immunoassays have also been described *(33),* but antibody is not currently available. The study described herein used a previously described DNA probe *(38)* derived from a sequence of the *vvhA* hemolysin gene to hybridize with *V. vulnificus* colonies on filters for enumeration by standard plate counts. Nonselective media are generally used for colony detection by DNA probe, whereas selective media are used for enumeration from enrichment assays; therefore, we also com-

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pared plating efficiency of several types of media for recovery from oyster homogenates with endogenous and artificially inoculated *V. vulnificus.*

MATERIALS AND METHODS

Sample collection and ice immersion treatment. Oysters were harvested from Apalachicola Bay, Fla., and transported immediately to the Florida Department of Agriculture and Consumer Services Aquaculture Laboratory in Apalachicola for processing. Ice immersion treatment consisted of covering oysters with ice in open coolers that were stored at 40 to 45°F (4.4 to 7.2°C) for 3 h. Control oysters were maintained in separate coolers in the same refrigerator but without ice. Following treatment, oysters were removed from ice, and all samples were refrigerated for the remainder of the study. Surface water temperatures at harvest ranged from 81 to 85°F (27.2 to 29.4°C). The internal oyster and external ambient air temperatures were monitored (Ellab, Tracksense II recorder, Arvada, Colo.) throughout the study. Internal temperature of iced oyster reached 32°F after about 1 h of treatment, whereas control oyster temperature declined to 45°F after 4 h of refrigeration. To determine the effectiveness of ice immersion as a postharvest treatment specifically for reduction of *V. vulnificus* in oysters, samples were collected in summer months when the prevalence of vibrios is greatest. Samples were collected for three independent trials conducted 14 August 2000 (study 1), 18 June 2001 (study 2), and 26 September 2000 (study 3). Data were analyzed by Student's *t* test paired analysis with two-tailed distribution.

Fecal coliform and total heterotrophic bacterial assessment of oyster homogenates. Oysters were analyzed for total bacteria and fecal coliforms at pretreatment (0 h); immediately posttreatment (3 h); and at 1, 7, and 14 days posttreatment as previously described *(23).* Oysters were scrubbed in tap water with sterile brushes, shucked, and weighed in sterile equipment to avoid cross-contamination. Samples ($n = 3$) of oyster meats (200 g each) were diluted 1:2 (wt/wt) in phosphate-buffered saline and homogenized for 90 s in a Waring blender. Homogenates were assayed for fecal coliform most probable number (MPN) according to standard protocol by the Florida Department of Aquaculture Laboratory in Apalachicola, Fla. Total numbers of heterotrophic, aerobic bacteria were determined from spread plates of serial dilutions of homogenates on T1N1 agar (1% NaCl, 1% tryptone, 1.5% agar) incubated overnight at 35 to 37°C. All media were purchased from Difco (Sparks, Md.), and unless otherwise stated, all other reagents were from Sigma Aldrich (St. Louis, Mo.).

***V. vulnificus* enumeration of oyster homogenates.** *V. vulnificus* numbers were determined by hybridization of the *V. vulnificus* alkaline phosphatase–labeled DNA probe to colonies that were transferred to filters from the standard plate counts as previously described *(38).*

Briefly, colonies were transferred from T1N1 spread plates by overlay with filter papers (85 mm Whatman #541). Filters with transferred colonies were heated to dryness in a microwave oven (Panasonic Inverter, Secaucus, N.J.) for approximately 2 to 5 min on the highest setting in lysis solution (0.5 M NaOH, 1.5 M NaCl), followed by neutralization in ammonium acetate (Fisher Scientific, Pittsburgh, Pa.) buffer and rinses in standard saline citrate buffer. Filters were treated with proteinase K (20 μg/ml) to remove background enzymatic activity and hybridized with alkaline phosphatase–labeled oligonucleotide probes (DNA Technologies, Aarhus, Denmark) derived from the species-specific *V. vulnificus vvhA* gene under stringent conditions (56°C) for 1 h. Filters were rinsed in standard saline citrate buffer with 1% sodium dodecyl sulfate at hybridization temperature, followed by additional rinses with standard saline citrate buffer at room temperature. NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidinium) substrate (Roche Diagnostics, Indianapolis, Ind.) was used for detection of alkaline phosphatase label and filters developed in the dark at room temperature. Appropriate control filters with colonies of *V. vulnificus, V. parahemolyticus, V. cholerae,* and *Escherichia coli* colonies were developed concurrently for all time points. A detailed protocol can be accessed at the U.S. Food and Drug Administration (FDA) Web site *(35).*

Evaluation of different media for recovery of *V. vulnificus* from oyster homogenates. Fresh market oysters were obtained in winter months to ensure reduced *V. vulnificus* concentrations and were homogenized as described previously. Aliquots (10 ml) of the homogenates were seeded with dilutions of overnight cultures of *V. vulnificus* MO6-24/O to achieve inocula ranging from 0 to 10^7 CFU/g. The concentrations of inocula were determined by optical density at 600 nm (A_{600}) and plate counts. Indigenous *V. vulnificus* in unseeded homogenate was $<10^2$ CFU/g. Plating media included Luria broth agar (LA; 1% tryptone, 0.5% yeast extract, 1.5% agar, 1% NaCl), artificial seawater agar (ASW; LA with Instant Ocean [Mentor, Ohio] at 16 ppt substituted for NaCl), LA prepared with 3% agar (3% LA), T1N1 prepared as described previously, and T1N3 (1% tryptone, 3% NaCl, 1.5% agar). Plates were incubated overnight at 35 to 37°C, and colonies were enumerated by DNA probe colony hybridization, as described previously. *V. vulnificus* colonies were also enumerated from selective media by transferring all typical single colonies from thiocitrate bile salts sucrose agar (TCBS; Difco) or modified cellobiose polymyxin colistin (MCPC) agar *(32)* to nonselective LA for subsequent DNA probe colony hybridization identification as described previously. Indigenous *V. vulnificus* in oysters from uninoculated Apalachicola oysters obtained in the summer were also enumerated as described previously.

RESULTS AND DISCUSSION

Effect of ice immersion on total bacterial content in oysters. Total aerobic, heterotrophic bacteria were enumerated in oyster meats by standard plate count from growth on nonselective media (T1N1). As shown in Table 1, bacterial numbers generally increased over time in both studies for either ice immersion–treated oysters or for control samples, approaching 10^5 CFU/g at some time points. In study 2, day 7, bacterial content was significantly higher ($P < 0.05$) in iced samples compared with control oysters. These data suggested that rapid chilling by ice immersion was not effective in reducing the total bacterial load in oysters and might in fact increase bacterial content.

Effect of ice immersion on survival of *V. vulnificus* in oysters. Pretreatment numbers of *V. vulnificus* in oysters were about 10^3 CFU/g. Our results concurred with a previous study that demonstrated rapid chilling on ice reduced the numbers of *V. vulnificus* in oysters *(28).* As shown in Table 2, numbers of *V. vulnificus* were lower in iced oysters compared with refrigerated controls in most samples (six of eight). These data represented the mean of triplicate samples of oyster homogenates, and similar results were obtained for the two independent experiments. Significant decreases ($P < 0.05$) were observed for treated compared with control samples on days 14 and 7 in studies 1 and 2, respectively.

TABLE 1. *Effect of ice immersion on total heterotrophic bacteria in oysters*

	Total heterotrophic bacteria (mean log CFU/g ± SD)[b]				
	Day 0				
Treatment[a]	Pretreatment	Posttreatment	Day 1	Day 7	Day 14
Study 1					
Control	4.6 ± 0.08	4.9 ± 0.09	4.7 ± 0.07	4.5 ± 0.30	5.3 ± 0.23
Iced		4.7 ± 0.16	4.3 ± 0.44	3.9 ± 0.27	5.5 ± 0.34
Study 2					
Control	3.6 ± 0.13	4.1 ± 0.46	3.7 ± 0.14	3.3 ± 0.28	NA[c]
Iced		4.1 ± 0.22	3.8 ± 0.09	5.0 ± 0.27[d]	NA

[a] Control samples were maintained at 45°F. Iced samples were immersed in ice for 3 h and then stored at 45°F as described in ''Materials and Methods.''

[b] Enumeration of bacteria was based on the mean log CFU/g ± standard deviation of triplicate oyster homogenates for each time point as described in the text.

[c] NA, data not available.

[d] Significantly different from control sample by Student's two-tailed *t* test ($P < 0.05$).

In study 1, numbers of *V. vulnificus* decreased over time for treated oysters; however, in study 2, numbers of *V. vulnificus* in treated samples exceeded those of controls after 14 days of incubation. The discrepancy between these studies could not be attributed to differences in initial concentrations of *V. vulnificus* before treatment because they were similar for both studies. Other variables, as yet unknown, might influence vibrio survival. For example, differences in oyster health and condition can affect bacterial levels *(17).* Although these factors were not specifically investigated, oyster mortality was not observed in either study. Changes in the physiological state of *V. vulnificus* could also influence survival in oysters; low temperature ''acclimatization'' induces cold shock proteins, which could enhance survival during stressful conditions *(16).* Alternatively, decreased bacterial competition could account for increased *V. vulnificus* concentrations during storage because initial total bacterial content was about 10-fold less in study 1 compared with study 2 (Table 1).

The FDA currently recommends that postharvest treatments should reduce *V. vulnificus* to levels of 30 MPN/g *(13).* Contamination in oysters following ice immersion generally exceeded this recommended level for validation of postharvest treatments; in fact, reductions in *V. vulnificus* numbers were not observed for all time points. Unfortunately, in this study, ice immersion treatment generally produced <10-fold decreases in *V. vulnificus* concentrations compared with noniced control samples. Reductions at this level would still leave large numbers of *V. vulnificus* remaining in oysters, particularly because *V. vulnificus* concentrations have been reported to be as high as 10^5 CFU/g, representing as much as 50% of the total culturable bacterial population *(37).* Thus, decreases in *V. vulnificus* concentrations resulting from ice immersion treatment would not be sufficient to fulfill postharvest mandates by the FDA and probably would not affect public health through disease reduction.

Ice immersion of oysters and fecal coliform content. For studies 1 and 2, rapid immersion of oysters in ice resulted in subsequent increased fecal coliforms compared with refrigerated controls for several samples (Table 3). A significant difference ($P < 0.01$) between treated and con-

TABLE 2. *Effect of ice immersion on* V. vulnificus *survival in oysters*

	No. of *V. vulnificus* in oysters (mean log CFU/g ± SD)[b]				
	Day 0				
Treatment[a]	Pretreatment	Posttreatment	Day 1	Day 7	Day 14
Study 1					
Control	2.9 ± 0.15	3.4 ± 0.39	3.1 ± 0.11	2.6 ± 0.03	3.0 ± 0.08
Iced		3.0 ± 0.09	2.8 ± 0.46	1.6 ± 0.77	1.2 ± 1.08[c]
Study 2					
Control	2.9 ± 0.10	3.0 ± 0.39	2.5 ± 0.46	3.5 ± 0.06	3.5 ± 0.31
Iced		3.5 ± 0.33	2.2 ± 0.13	2.2 ± 0.82[c]	4.0 ± 0.78

[a] Control samples were maintained at 45°F. Iced samples were immersed in ice for 3 h and then stored at 45°F as described in ''Materials and Methods.''

[b] Enumeration was based on the mean log CFU/g ± standard deviation of triplicate oyster homogenates for each time point as determined by colony hybridization with *V. vulnificus* alkaline phosphatase–labeled DNA probe as described in the text.

[c] Significantly different from control sample by Student's two-tailed *t* test ($P < 0.05$).

TABLE 3. *Effect of ice immersion on fecal coliform content in oysters*

	Fecal coliform content (mean MPN/100 g)[b]				
	Day 0				
Treatment[a]	Pretreatment	Posttreatment	Day 1	Day 7	Day 14
Study 1					
Control	<1.0	1.2 ± 1.06	<1.0	1.4 ± 0.09	1.4 ± 0.81
Iced		2.4 ± 0.39	2.8 ± 0.38[c]	1.0 ± 0.62	1.6 ± 0.11
Study 2					
Control	1.1 ± 0.17	1.1 ± 0.17	<1.0	2.2 ± 0.34	1.5 ± 1.3
Iced		1.1 ± 0.17	<1.0	1.9 ± 0.15	2.3 ± 0.18
Study 3					
Control	1.3 ± 0	1.3 ± 0.35	2.8 ± 0.30	2.3 ± 0.55	4.8 ± 0.39
Iced		1.5 ± 0.47	3.2 ± 0.44	2.0 ± 0.20	4.8 ± 0.38

[a] Control samples were maintained at 45°F and treated samples were immersed in ice for 3 h and then stored at 45°F as described in ''Materials and Methods.''

[b] Enumeration of fecal coliforms was based on mean log MPN per 100 g ± standard deviation from triplicate oyster homogenates at each time point as assayed by the standard protocol described in ''Materials and Methods.''

[c] Significantly different for treated versus control samples by Student's two-tailed *t* test ($P < 0.01$). For statistical purposes, values <10 CFU/100 g were calculated as 1.0 log CFU/100 g.

trol oysters was observed for only one time point, but these numbers exceeded the fecal coliform limits (>230 MPN/100 g, or 2.48 log MPN/100 g) established by the state of Florida, rendering this product unsafe for human consumption. Fecal coliform analysis for study 3 produced similar results. In summary, ice immersion did not consistently reduce fecal coliform content and might actually serve to increase numbers under some conditions. Hence, ice immersion treatment could further augment health risks posed by non-*Vibrio* contamination through growth of psychrophilic bacteria or increased stability of viral pathogens. Increases in fecal coliforms following ice immersion might be a consequence of using unwashed oysters in this study because sediments can harbor significant numbers of bacteria. Thorough rinsing of oysters prior to treatment might eliminate this problem.

Fecal coliform analysis is generally used to assess safety of shellfish-harvesting waters; however, it should be noted that this assessment is not effective for monitoring vibrio contamination. Several studies have shown that prevalence of both *V. vulnificus* and *V. parahemolyticus* are independent of fecal indicators *(8, 18),* probably because vibrios are indigenous to estuarine environments, whereas fecal contamination is generally a result of groundwater runoff. Our investigations also suggested that vibrios and coliforms respond differently to postharvest treatments because time points with significant reduction in *V. vulnificus* did not correspond to a reduction in fecal coliform MPN and visa versa. Thus, both populations need to be assessed for safety evaluation of postharvest treatments.

Plating efficiency for recovery of *V. vulnificus* from oyster homogenates. Colony hybridization with DNA probe has been used to enumerate *V. vulnificus* in oysters *(21, 37, 38),* and similar molecular detection assays are available for *V. cholerae (23, 36)* and *V. parahemolyticus (10, 25).* Compared with standard microbiological methods, these techniques provide more rapid, accurate evaluation of postharvest treatments of oysters *(11).* Colonies for DNA probe hybridization are derived from growth on standard plate counts with the use of nonselective media because dyes in typical selective and differential media interfere with DNA probe detection. On the other hand, selective agar is generally used for vibrio isolation and enumeration following enrichment in standard MPN protocols. Therefore, we evaluated different media for recovery of *V. vulnificus* with DNA probe enumeration.

Comparison of recovery on different nonselective media for *V. vulnificus* in artificially inoculated oyster homogenates did not reveal greatly increased plating efficiency for any particular medium (Table 4). However, as expected, recovery was generally lower on standard selective and differential media (TCBS and MCPC) compared with nonselective agars, and *V. vulnificus* was frequently not detectable on selective media at lower dilutions. Recovery of indigenous *V. vulnificus* in uninoculated oysters was also comparable for most of the media examined (Table 5) but was reduced about 10-fold on both T1N3 and the selective and differential agars. The NaCl concentration for T1N3 is threefold higher than the other media and was probably responsible for the reduced recovery because the optimum salinity for *V. vulnificus* is about 1% NaCl *(18).* Recovery of indigenous bacteria from TCBS or MCPC, as confirmed by DNA probe, was 10- to 100-fold lower than that of nonselective media, indicating that enumeration on these agars might not provide accurate assessment.

The sensitivity of DNA probe colony hybridization assays for detection of vibrios in oyster homogenates approaches 10 CFU/g *(36).* However, application of this technology might be restricted by overgrowth of background colonies, especially at lower concentrations of target organisms. Also, spreading colonies can completely cover plates and prevent transfer of target colonies to filters. We ob-

TABLE 4. *Plating efficiency of selective and nonselective media for* V. vulnificus *from inoculated oyster homogenates*

Medium	No. of *V. vulnificus* recovered (CFU/g) for each inoculum (CFU/g)[a]:						
	10^7	10^6	10^5	10^4	10^3	10^2	10^1
LA	9.2×10^6	1.2×10^6	9.7×10^3	9.7×10^3	1.7×10^3	3.0×10^2	1.7×10^1
T1N1	9.7×10^6	1.7×10^6	8.4×10^3	1.2×10^4	2.6×10^3	2.3×10^1	1.7×10^1
T1N3	1.5×10^7	3.3×10^6	1.1×10^4	1.5×10^4	4.8×10^3	2.0×10^2	2.3×10^1
3% LA	1.1×10^7	1.5×10^6	1.1×10^4	1.5×10^4	1.2×10^3	1.0×10^2	4.7×10^1
ASW	1.0×10^7	1.9×10^6	1.2×10^4	1.1×10^4	4.4×10^3	9.7×10^1	7.7×10^1
TCBS	6.8×10^5	ND	1.3×10^2	2.5×10^4	ND	ND	ND
MCPC	5.8×10^5	3.8×10^5	ND	2.0×10^3	1.6×10^3	ND	ND

[a] Numbers of *V. vulnificus* were enumerated from oyster homogenates on nondifferential media as determined by colony blot hybridization with the *V. vulnificus* alkaline phosphatase–labeled probe method, as described in ''Materials and Methods.'' Media included Luria broth agar (LA; 1.5% agar, 1% NaCl), LA with artificial seawater agar at 16 ppt substituted for NaCl (ASW), LA with 3% agar (3% LA), and T1N1 and T1N3 (1% tryptone agar with either 1% or 3% NaCl, respectively). Enumeration on selective and differential media (TCBS and MCPC) was determined by transferring all typical colonies to LA for subsequent identification by colony blot hybridization, as described in the text. ND, none detected.

served that the use of very dry plates incubated at 37 to 40°C instead of 30 to 35°C reduced the spreading of background bacteria (data not shown). Spreading or swarming on solid medium results from the expression of lateral flagella, which are expressed by vibrios and aeromonads, but not by *V. vulnificus.* For *V. parahemolyticus,* differential expression of lateral flagella was temperature regulated, whereby optimum expression was obtained at 25° and declined at 37°C *(3).* These data are consistent with our observations of natural populations of swarming bacteria on solid media.

This study does not support the use of ice immersion as an effective postharvest treatment for the reduction of vibrios in oyster stock. Although our data and a previous study *(28)* demonstrated reduction of indigenous vibrios by this method, the numbers of surviving *V. vulnificus* always exceeded recommended limits. Furthermore, treatment generally did not reduce fecal coliform or total bacterial content and occasionally produced significant increases for treated samples over controls. Another consideration that was not addressed in this study was the induction of nonculturability in response to temperature downshift, which could render *V. vulnificus* nondetectable but still virulent *(26)* or could induce adaptive responses that enhance survival *(5).* Currently available approved methods for compliance with FDA-mandated implementation of postharvest treatment of oysters do not provide a live ''half-shell'' product. Ice immersion potentially offers a nonlethal alternative that could be applied onboard harvesting vessels to further limit exposure of oysters to elevated temperatures that have been shown to increase vibrio growth *(7, 18).* Ice immersion is also less expensive than available methodologies, which require purchase of expensive processing equipment. Further examination of relevant parameters related to ice immersion treatment is needed to develop rapid chilling as a postharvest alternative. For example, extending treatment time beyond 3 h with iced seawater or prewashing oysters before treatment might improve results. Rapid chilling methods could also be combined with other nonlethal treatments, such as depuration, to adequately reduce vibrios while maintaining a live product. Ice immersion offers potential benefits to the oyster industry in terms of cost savings and increased food safety that merit further research in this area.

TABLE 5. *Plating efficiency of different media for indigenous* V. vulnificus *in oyster homogenates*

Medium	No. of *V. vulnificus* recovered (CFU/g)[a]	
	Study 1	Study 2
L-agar	4.2×10^3	ND
T1N1	5.3×10^3	5.0×10^1
T1N3	7.0×10^2	ND
3% LA	1.6×10^3	7.5×10^1
ASW	Sp[b]	8.5×10^1
TCBS[b]	2.0×10^2	ND
MCPC[b]	1.0×10^2	ND

[a] Numbers of *V. vulnificus* were enumerated from oyster homogenates on nondifferential media as determined by colony blot hybridization with *V. vulnificus* alkaline phosphatase–labeled probe method, as described in ''Materials and Methods.'' Media included Luria broth agar (LA; 1.5% agar, 1% NaCl), LA with artificial seawater agar at 16 ppt substituted for NaCl (ASW), LA with 3% agar (3% LA), and T1N1 and T1N3 (1% tryptone agar with either 1% or 3% NaCl, respectively). Enumeration on selective and differential media (TCBS and MCPC) was determined by transferring all typical colonies to LA for subsequent identification by colony blot hybridization as described in the text. ND, none detected.

[b] Individual colonies were not enumerated because of overgrowth of spreading colonies.

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