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Persistence of *Vibrio vulnificus* in Tissues of Gulf Coast Oysters, *Crassostrea virginica*, Exposed to Seawater Disinfected with UV Light

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***Vibrio vulnificus* is an estuarine bacterium which can cause opportunistic infections in humans consuming raw Gulf Coast oysters, *Crassostrea virginica*. Although *V. vulnificus* is known as a ubiquitous organism in the Gulf of Mexico, its ecological relationship with *C. virginica* has not been adequately defined. The objective of the present study was to test the hypothesis that *V. vulnificus* is a persistent microbial flora of oysters and unamenable to traditional methods of controlled purification, such as UV light depuration. Experimental depuration systems consisted of aquaria containing temperature-controlled seawater treated with UV light and 0.2-μm-pore-size filtration. *V. vulnificus* was enumerated in seawater, oyster shell biofilms, homogenates of whole oyster meats, and tissues including the hemolymph, digestive region, gills, mantle, and adductor muscle. Results showed that depuration systems conducted at temperatures greater than 23°C caused *V. vulnificus* counts to increase in oysters, especially in the hemolymph, adductor muscle, and mantle. Throughout the process, depuration water contained high concentrations of *V. vulnificus*, indicating that the disinfection properties of UV radiation and 0.2-μm-pore-size filtration were less than the rate at which *V. vulnificus* was released into seawater. Approximately 10^5 to 10^6 *V. vulnificus* organisms were released from each oyster per hour, with 0.05 to 35% originating from shell surfaces. These surfaces contained greater than 10^3 *V. vulnificus* organisms per cm^2. In contrast, when depuration seawater was maintained at 15°C, *V. vulnificus* was not detected in seawater and multiplication in oyster tissues was inhibited.**

Vibrio vulnificus is a common bacterium in estuarine waters in temperate and tropical climates. It is distributed widely in the Gulf of Mexico and other waters where it is found in seawater and sediment and with various marine life forms (7, 9, 14, 21, 22), particularly when seawater temperatures are elevated (9, 21). Occasionally, *V. vulnificus* causes invasive disease and life-threatening infections in humans who suffer from preexisting illnesses and consume raw Gulf Coast oysters, *Crassostrea virginica* (3, 11).

Although some information of the physicochemical growth requirements of *V. vulnificus* exists (9), very little is known of detailed interactions between it and other marine biota, such as oysters. Such information may determine natural and postharvesting processes that could reduce *V. vulnificus* counts in oyster meats, thereby decreasing the risk of human infections.

The literature contains numerous reports which describe shellfish purification methods (e.g., depuration) in which physicochemical parameters of seawater are controlled to reduce bacterial pollutants in shellfish meats (2, 5, 15). In the majority of these studies, UV radiation was used to disinfect seawater and lower the number of fecal bacteria. Since *V. vulnificus* and other pathogenic *Vibrio* spp. have been recognized as significant seafood pathogens, depuration has been proposed. Although some studies indicate that laboratory-grown vibrios can be depurated from oysters (10), there is little published information which validates this process for shellfish contaminated with natural vibrios.

We hypothesize that the autochthonous form of *V. vulnificus* in estuarine waters has evolved commensal or symbiotic associations with oysters which make it unamenable to depuration using UV light-disinfected seawater. In the present study, we examined whether natural populations of *V. vulnificus* persisted in Gulf of Mexico oysters, *C. virginica*, after exposure to UV light-disinfected seawater.

MATERIALS AND METHODS

Specimen collection and preparation for bacterial analyses. Oysters were collected with tongs from the Mississippi Sound, southeast of Mobile Bay, Alabama, placed in insulated coolers, and transferred to the laboratory within 1 h.

Before being shucked, oyster shells were scrubbed with a brush under deionized water. For bacterial analyses of tissues, a masonry sawblade was used to make a perpendicular cut through the lateral edge of the shell adjacent to the adductor muscle, avoiding contact with tissues. A sterile 22-gauge needle, fitted to a sterile syringe, was inserted through the notch into the adductor muscle sinus, and 1 to 3 ml of hemolymph was aspirated. Earlier experiments determined that insignificant numbers of *V. vulnificus* were introduced into the hemolymph from adductor muscle aspirations. Shells were opened with a sterile oyster knife, the adductor muscle was cut from one valve, and the entire unseparated contents were washed with approximately 100 ml of sterile phosphate-buffered saline (PBS; 0.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.13 M NaCl [pH 7.4]). By following anatomical descriptions by Galtsoff (4), the digestive tract, gills, mantle, and adductor muscle were removed, sequentially, by using sterile scissors and forceps. Tissues were rinsed with sterile PBS and transferred to preweighed

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sterile plastic tubes. Before homogenization, all tissues were diluted 10-fold in 10 ml of PBS (wt/wt), except for the digestive tract which was diluted 1:1 (1). After homogenization, tissues were diluted in 10-fold serial increments from 10^{-1} to 10^{-6} in 10 ml of PBS. Tissues were homogenized with a Kinematica blender (Kriens-lu, Switzerland) at setting 4 for 1 min. Previous experiments showed that homogenization did not reduce bacterial viability. The blender was sterilized with 70% ethyl alcohol and rinsed with sterile PBS between each tissue homogenization.

For tests of whole undissected oysters, specimens were shucked, weighed, mixed 1:1 (wt/wt) with PBS, homogenized with a blender (Waring Products Division, New Hartford, Conn.) for 90 s at high speed, and diluted 10^{-1} to 10^{-6} in PBS.

Isolation and enumeration of *V. vulnificus* organisms. For depuration experiments, *V. vulnificus* was enumerated in dissected tissues (i.e., hemolymph, gills, mantle, adductor muscle, and digestive tract) of three oysters or in pooled tissue homogenates of 10 undissected specimens. Three sample replicates were tested per time interval. The basic procedures and formulations for media and reagents were described previously (8, 20). A three-tube most-probable-number enumeration technique using alkaline peptone water was employed. After inoculation with a 1.0-ml sample, alkaline peptone water was incubated for 12 to 16 h at 35°C. Turbid alkaline peptone water was streaked on modified CPC agar (12, 20), and the agar was incubated for 18 to 24 h at 39 to 40°C. *V. vulnificus*-like colonies were selected and identified by an enzyme immunoassay described by Tamplin et al. (20).

Depuration systems. Before depuration experiments were performed, the bactericidal effect of UV light systems was determined. Environmental strains *V. vulnificus* 4600, 4965, and 4832 were cultured on tryptic soy agar (Difco Laboratories, Detroit, Mich.) for 18 h at 35°C, suspended in equal numbers in PBS, washed with PBS by centrifugation at 5,000 × *g*, and added to 500 ml of sterile seawater (salinity, 15‰) at 10^5 CFU/ml. After 2 days of incubation at approximately 24°C, the bacterial suspensions were added to two aquaria filled with 50 liters of seawater (salinity, 15‰; 15 or 23°C) at 10^4 CFU/ml. Seawater was recirculated by a pump (2-MD; Little Giant Pump Co., Oklahoma City, Okla.) through 60 W of UV light (Aquanetics Systems, San Diego, Calif.) at 1,000 gal/h (ca. 3,800 liters/h); the control aquarium did not have UV lights. The UV light chambers were standard commercial water-tight polyvinylchloride hollow chambers (4 by 41 in. [ca. 10 by 100 cm]), each containing a 30-W bulb. Earlier tests utilizing water-soluble dye confirmed that the pump tube positions allowed thorough mixing of seawater. At predetermined time intervals, *V. vulnificus* was enumerated in seawater.

One depuration system consisted of 80-liter aquaria containing 40 oysters and filled with 50 liters of autoclaved natural seawater (salinity, 16‰) which was recirculated at 1,000 gal/h with pumps through 60-W UV light chambers. Suspended particles were filtered from seawater by using a pump (Magnum 330; Marineland Aquarium Products, Simi Valley, Calif.) and a diatomaceous earth filter. Water temperature was maintained at 24°C by using a manifold filled with polyethylene glycol and connected to a recirculating, refrigerated bath (LKB Instruments, Bromma, Sweden). Sedimented particles (e.g., oyster feces) were removed daily from the bottom of the aquaria by manually vacuuming the glass surface with the inlet port of a pump connected to a diatomaceous earth filter (Magnum 330; Marineland Aquarium Products). For control aquaria, seawater was recirculated with a pump and filtered but not treated with UV light. Whole oyster meats, individual tissues, and seawater were tested at selected time intervals.

The second depuration system was identical to that described above, with the following modifications: (i) seawater (salinity, 15‰; 21°C) was also filtered at 6 liters/h through a 0.22-μm-pore-size membrane filter (Gelman Science Inc., Ann Arbor, Mich.) which was changed daily, and (ii) pooled whole oyster meats, not individual tissues, were tested.

A separate experiment measured the effect of temperature on depuration of *V. vulnificus* by using aquaria containing 50 oysters and equipped with 60-W UV light bulbs, diatomaceous earth filters, and recirculating pumps. Refrigerated manifolds were placed in depuration tanks to maintain seawater temperature at 15 or 23°C. Seawater salinity was adjusted to 16‰. Pooled meats of 10 oysters and 10 ml of seawater were sampled at each time interval.

Measurement of *V. vulnificus* released from oysters. The quantity of *V. vulnificus* organisms and their rate of release from oysters (mean shell surface area, 123 cm^2) were determined by using four cylindrical chambers (16 cm long by 8 cm wide; internal volume, ≈600 ml) containing three specimens each. UV light-disinfected artificial seawater (Instant Ocean; Aquarium Systems, Mentor, Ohio; salinity, 11‰; 24°C) was pumped in parallel (one-pass disinfected seawater) through each chamber at 9.1 liters/h by using a four-head peristaltic pump (Cole-Palmer Instrument Co., Chicago, Ill.). At each time interval, *V. vulnificus* organisms were enumerated in the effluent seawater of all chambers and in representative samples of oyster meats before and after experimental protocol.

In a separate experiment, the quantities of *V. vulnificus* released from filter-feeding and non-filter-feeding oysters were determined. One chamber contained three unbound and filter-feeding oysters. A second flowthrough chamber contained three secured oysters (closed with rubber bands) to measure the quantity of *V. vulnificus* released from shell surfaces. *V. vulnificus* organisms were enumerated in effluent seawater from each chamber at selected time intervals.

Enumeration of *V. vulnificus* organisms on oyster shell surfaces. Ten oyster shells were scrubbed with a sterile brush in 4 liters of sterile PBS. A 10-ml subsample of scrub water was homogenized at 15,000 rpm (Omni International, Waterbury, Conn.) for 30 s, and *V. vulnificus* organisms were enumerated as described above. Shell surface area was measured by using aluminum foil molds (13).

RESULTS

Retention of *V. vulnificus* in oyster shellstock. At 15 and 23°C, UV radiation was bactericidal for pure cultures of *V. vulnificus* suspended in aquarium seawater in the absence of oysters (Fig. 1). *V. vulnificus* counts were reduced approximately 10,000-fold to undetectable levels after 2 h of UV light treatment at both temperatures; no significant reduction in *V. vulnificus* counts was observed in untreated water. In contrast, when oysters containing natural populations of *V. vulnificus* were present in depuration aquaria, *V. vulnificus* counts remained elevated in seawater for 3 days, i.e., 10^3 to 10^5 organisms per ml (Fig. 2). At days 1 and 3, *V. vulnificus* levels were approximately 10-fold greater in control seawater than those in UV light-treated seawater (Fig. 2); *V. vulnificus* counts were similar in oysters exposed to UV light and those exposed to untreated seawater. For both test and control specimens, *V. vulnificus* counts increased more than

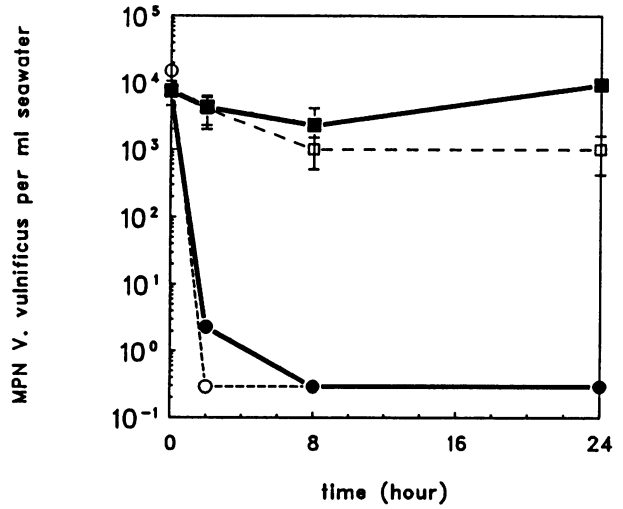

FIG. 1. Effect of UV light on survival of *V. vulnificus* in depuration systems at 15 and 23°C. Symbols: ●, with UV light at 15°C; ■, control at 23°C; ○, with UV light at 23°C; □, control at 15°C. MPN, most probable number. Bars represent standard deviations.

100-fold in oyster meats within 24 h and remained elevated for 3 days of treatment (Fig. 2).

In a separate experiment, 0.2-μm-pore-size membrane filters were added to depuration systems to reduce residual *V. vulnificus* organisms in seawater, limit feedback to oysters, and potentially increase depuration efficiency. This procedure reduced *V. vulnificus* counts in seawater compared to those in depuration systems without filtration, but *V. vulnificus* counts in oysters meats and seawater remained elevated throughout 7 days of treatment.

The effect of UV light-assisted depuration on *V. vulnificus* counts in selected tissues was also examined. In the environment, the largest concentrations of *V. vulnificus* were found in the digestive tract, gills, and adductor muscle, followed by the mantle and hemolymph (Fig. 3a). After 3 days of depuration treatment, *V. vulnificus* counts increased progressively in the mantle, hemolymph, and digestive tissues (Fig. 3a).

The tissue distribution of *V. vulnificus* also changed during treatment. In the environment, the digestive tract and adductor muscle contained approximately 55 and 35% total *V. vulnificus* counts, respectively (Fig. 3b). Less than 10% was found in the mantle, gills, and hemolymph. After 1 day of treatment, the percent *V. vulnificus* in adductor muscle and gill tissue increased to approximately 36 and 30%, respectively. At 3 days, the digestive tract harbored approximately 70% of the *V. vulnificus* organisms found in oyster meats.

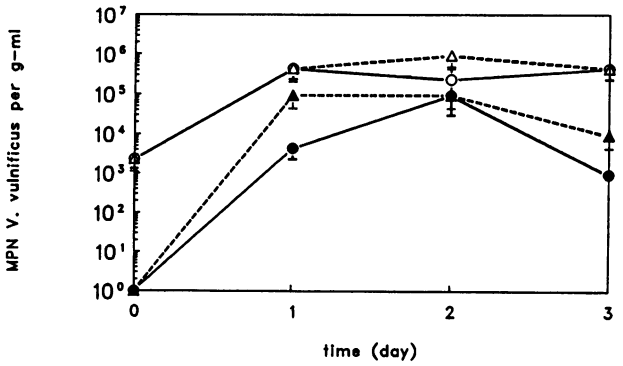

FIG. 2. Survival of *V. vulnificus* in seawater and oyster meats under depuration conditions. Symbols: ●, seawater with UV light; ○, oyster with UV; ▲, seawater control; △, oyster control. MPN *V. vulnificus* per g-ml, most probable number per milliliter of seawater or gram of oyster tissue.

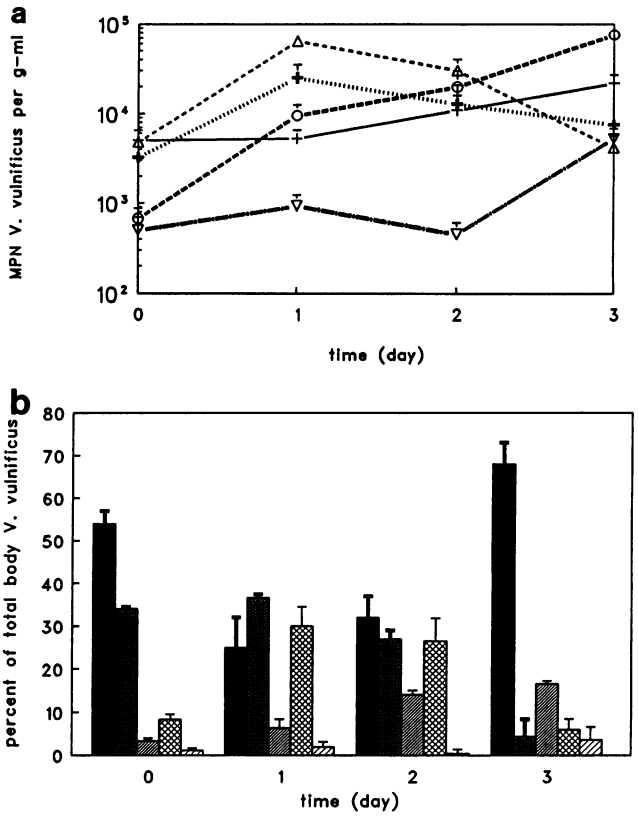

FIG. 3. Concentration and distribution of *V. vulnificus* in specific oyster tissues (n = 3) exposed to UV light-treated seawater. (a) Most-probable-number (MPN) *V. vulnificus* per gram of oyster tissue or milliliter of hemolymph. Symbols: +, digestive tissue; △, adductor muscle; ○, mantle; +, gills; ▽, hemolymph. (b) Percent *V. vulnificus* per tissue. Symbols: ■, digestive tissue; ▦, adductor muscle; □, mantle; ▩, gills; ▨, hemolymph.

Effect of temperature on depuration of *V. vulnificus*. The temperature of depuration seawater markedly affected the growth of *V. vulnificus* in oysters and seawater (Fig. 4). At 15°C, *V. vulnificus* was not detected in seawater (i.e., <0.3 *V. vulnificus* organisms per ml), and the counts in oyster tissues remained low after 5 days of treatment, similar to pretreatment counts. In contrast, oysters maintained in 23°C seawater contained 4.3×10^4 to 2.3×10^6 *V. vulnificus* organisms per g, concentrations 10^5 greater than pretreatment concentrations. Likewise, *V. vulnificus* concentrations in 23°C seawater were markedly elevated (i.e., $>10^6$/ml).

Measurement of *V. vulnificus* released from oysters. Large counts of *V. vulnificus* organisms ($>10^6$/h) were released from oysters into surrounding seawater within 8 h (Fig. 5). The rate of release declined over 5 days to approximately 10^5 *V. vulnificus* organisms per oyster per h. After 7 days of exposure to one-pass disinfected seawater (see Materials and Methods), a total of more than 10^8 *V. vulnificus* organisms were released per oyster; however, during the same

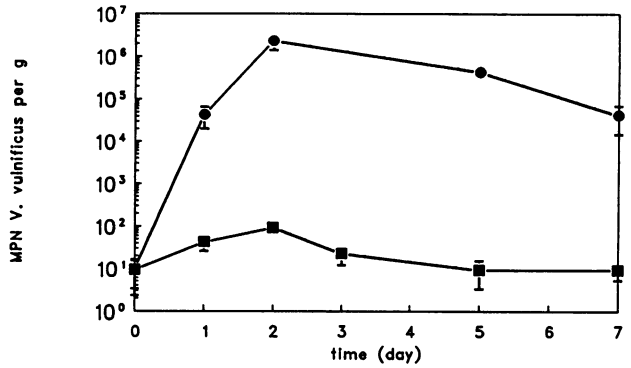

FIG. 4. Effect of seawater temperatures of 15°C (■) and 23°C (●) on survival of *V. vulnificus* in oyster meats. MPN, most probable number.

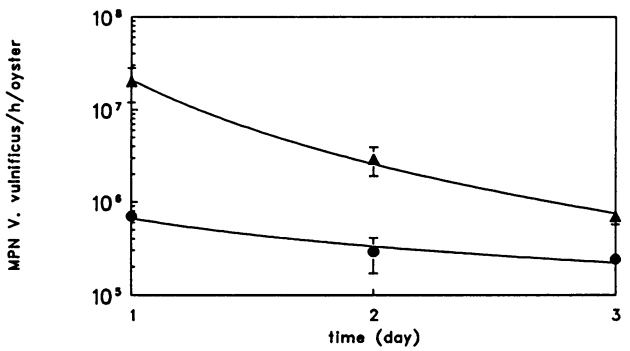

FIG. 6. Rate of *V. vulnificus* released from internal and external oyster surfaces in flowthrough chambers. The line plot represents logarithmic regression analysis of datum points. MPN, most probable number. ▲, unclamped oysters; ●, clamped oysters.

time interval, *V. vulnificus* counts in oyster meats were reduced by only 10^5.

The quantities of *V. vulnificus* organisms released from internal and external oyster surfaces were also determined (Fig. 6). Similar to the previous experiment (Fig. 5), from 1 to 7 days, *V. vulnificus* counts released from filter-feeding specimens declined from $\approx 1 \times 10^7$ to 8×10^5/h. *V. vulnificus* organisms released from oyster shell surfaces decreased from approximately 7×10^5 to 2×10^5/h. In separate tests, oyster shell surfaces were found to contain more than 2.4×10^3 *V. vulnificus* organisms per cm^2. Data were recorded through 3 days only, since oyster mortality increased in clamped specimens at 4 days. We speculate that internal anaerobic conditions of clamped oysters reduced specimen viability.

DISCUSSION

These findings demonstrate that *V. vulnificus* does not depurate from oyster shellstock when UV light and/or 0.2-μm-pore-size filtration treatments of seawater were used. Importantly, at elevated seawater temperatures (e.g., >21°C), *V. vulnificus* multiplies markedly in oyster tissues and large numbers are released into surrounding seawater at rates exceeding the bactericidal activities of UV light. Depuration treatment also changes the distribution of *V. vulnificus* organisms in tissues, especially in the digestive tract, gills, mantle, and adductor muscle. In such closed systems, *V. vulnificus* replication may be enhanced by a combination of nutrients released from oysters and elevated temperature. Furthermore, seawater containing *V. vulnificus* can recontaminate oyster tissues.

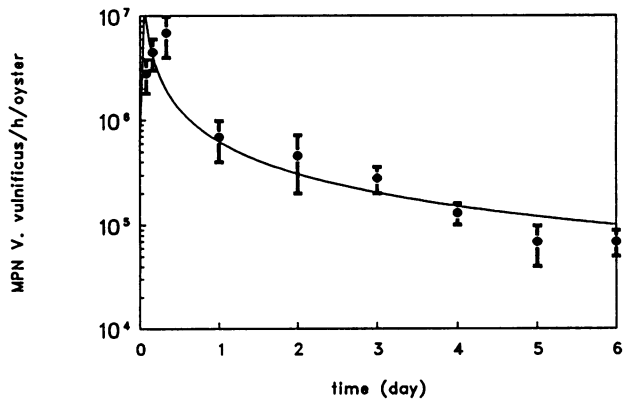

FIG. 5. Release of *V. vulnificus* from filter-feeding oysters into seawater in one-pass flowthrough chambers. The line plot represents a logarithmic regression analysis of datum points. MPN, most probable number.

The close association of *V. vulnificus* with oyster tissues is further shown by its systemic distribution in freshly harvested and treated specimens. This is particularly illustrated by isolation of *V. vulnificus* from the hemolymph. In estuarine environments, this close association may influence the ecology of *V. vulnificus*. Undoubtedly, other marine biota, such as finfish, crustaceans, mollusks, and plankton, may also provide environments where *V. vulnificus* can reproduce.

As has been shown in other environmental and laboratory studies (9, 22), low seawater temperature markedly inhibits *V. vulnificus* growth. Under depuration conditions at 15°C, *V. vulnificus* is not detected in seawater and its counts in oyster meats remain low, similar to pretreatment concentrations (Fig. 4). In contrast, at 23°C, *V. vulnificus* counts in oyster meats can increase 100,000-fold. We have regularly observed marked multiplication of *V. vulnificus* organisms in oysters when they are harvested from cold (<13°C) environmental waters and then maintained at temperatures greater than 20°C (17–19). During the coldest months (e.g., January and February), *V. vulnificus* is not isolated from freshly harvested oysters but is detected in large numbers when shellfish are incubated at 25°C for 24 h (17–19). This observation can be explained by the presence of low levels of *V. vulnificus* which is not detected by the relatively nonselective properties of alkaline peptone water enrichment broth and/or cold-stressed cells. Further experiments are needed to identify the specific low temperatures which initiate and inhibit *V. vulnificus* growth.

These results concur with investigations which report the persistence of other *Vibrio* species in shellfish exposed to standard depuration protocols. Eyles and Davey (2) showed that UV light-treated seawater had no significant effect on levels of naturally occurring *Vibrio parahaemolyticus*, yet depuration significantly reduced the aerobic plate count and counts of coliforms and *Escherichia coli*. Likewise, Greenberg et al. (5) showed that *V. parahaemolyticus* and *Vibrio harveyi* counts remained elevated in clams exposed to UV light-treated seawater, while *E. coli* counts were reduced 100-fold in 24 h.

However, these reports are in contrast to the findings of Kelly and DiNuzzo (10) who, by using laboratory-contaminated oysters and no bactericidal treatments of seawater, found that *V. vulnificus* depurated to low concentrations after 7 days. These conflicting results may be related to very low inoculum levels, differences in bacteriological isolation and identification procedures, and/or use of laboratory versus environmentally-contaminated specimens.

In this regard, Richards (15) suggests that natural populations of vibrios may be more resistant to the effects of depuration than vibrios grown in the laboratory. Similarly, we have observed that laboratory and natural strains of *V. vulnificus* show different survival patterns under depuration and dry-storage conditions. Unlike environmental strains, laboratory strain counts are easily reduced, apparently by rapidly growing endogenous bacterial flora, including natural populations of *V. vulnificus* (6). Consequently, we recommend that oysters containing natural *V. vulnificus* populations should be tested when the effects of postharvesting processes are assessed. We speculate that close associations have evolved between endogenous *V. vulnificus* and shellfish in estuarine environments, and that long time intervals may be necessary to induce *V. vulnificus* to associate with oyster tissues by processes which are not expressed by laboratory strains.

The present studies also indicate that depuration and wet-storage operating conditions described in the *National Shellfish Sanitation Program Manual of Operations* (23) may need new evaluation. The manual specifies that approved facilities can depurate oysters at seawater temperatures of 10 to 25°C (23). However, our results indicate that seawater temperatures higher than 21°C, and possibly lower, can support rapid multiplication of *V. vulnificus* and may increase health risks.

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