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Uptake and Retention of *Vibrio cholerae* O1 in the Eastern Oyster, *Crassostrea virginica*†

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***Vibrio cholerae* O1, the causative agent of cholera, is known to persist in estuarine environments as endogenous microflora. The recent introduction of *V. cholerae* O1 into estuaries of the North and South American continents has stimulated the need to determine the effect of controlled purification on reducing this pathogen in edible molluscan shellfish. Experiments defined parameters for the uptake and retention of *V. cholerae* O1 in tissues of *Crassostrea virginica*, and these parameters were compared with those for *Escherichia coli* and *Salmonella tallahassee*, bacteria which are usually eliminated from moderately contaminated shellfish within 48 h. Oysters accumulated greater concentrations of *V. cholerae* O1 than *E. coli* and *S. tallahassee*. When *V. cholerae* O1 was exposed to controlled purification at 15, 19, and 25°C over 48 h, it persisted in oysters at markedly higher levels than *E. coli* and *S. tallahassee*. The concentration of a *V. cholerae* O1-specific agglutinin did not positively correlate with the uptake or retention of *V. cholerae* O1. These data show that state and federally approved controlled purification techniques are not effective at reducing *V. cholerae* O1 in oysters.**

It is estimated that 1 in 2,000 meals of raw molluscan shellfish results in disease (19), making these shellfish one of the most hazardous foods (22, 34). Such risk is related to the high numbers of microorganisms that oysters accumulate from overlying waters (11, 18–20) and the consumption by humans of the entire animal in a raw or undercooked form. Risk is further enhanced by pollution of shellfish growing waters, during product handling from harvest to retail market, and by compromised host defenses of the consumer.

Of the numerous human diseases that can be transmitted by oysters, cholera is a significant cause of morbidity worldwide (4). *Vibrio cholerae* O1, the etiological agent (3, 6, 31), is easily spread over large geographical areas by water, infected travelers, and imported seafoods. Once the bacterium is endemic, both fresh and estuarine waters and plankton can be reservoirs of *V. cholerae* O1 and vectors of seafood disease (29). Such a situation was recently realized during the epidemic of *V. cholerae* O1 in Latin America, where very high levels of *V. cholerae* O1 have been reported in Peruvian environments (31). This intensifies the need to investigate interactions between this pathogen and shellfish and to determine possible methods for reducing its numbers.

A state and federally approved postharvest process termed controlled purification (depuration) can increase the safety of shellfish products (21). It can reduce the number of pathogenic organisms present in shellfish harvested from moderately polluted (restricted) waters to levels acceptable for human consumption (35). However, the effect of depuration on *V. cholerae* O1 is not understood. Research to determine the efficacy of the process and the specific factors involved in the elimination of *V. cholerae* O1 is needed.

A factor that is hypothesized to influence the retention of *V. cholerae* O1 in oysters is a *V. cholerae* O1 agglutinin found in oyster hemolymph and on the surface of oyster tissues (28). This agglutinin reacts with all serotypes and biotypes of *V. cholerae* O1 and has been demonstrated in oysters (i.e., *Crassostrea virginica*) from different environments along the Gulf and Atlantic coasts (28). It remains to be determined whether the presence or absence of this *V. cholerae* O1 agglutinin affects the persistence of *V. cholerae* in oyster tissues.

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MATERIALS AND METHODS

Bacterial strains. Bacteria were suspended in alkaline peptone broth containing 12% glycerol (Sigma, St. Louis, Mo.) and stored in liquid nitrogen. One day prior to experimentation, *V. cholerae* O1 (National Institutes of Health isolates 102 [Inaba-El Tor], 114 [Hikojima-El Tor], and 124 [Ogawa] from Lima, Peru), *Escherichia coli* (ATCC 35270), and *Salmonella tallahassee* (Florida Department of Agriculture and Consumer Services) were plated on tryptic soy agar (Difco, Detroit, Mich.) containing 1% NaCl. A mixture of all three *V. cholerae* O1 strains was used in certain experiments (as described).

Bacterial culture. Bacteria were cultured overnight on tryptic soy agar–1% NaCl at 37°C, and a colony was transferred to 100 ml of tryptic soy broth (Difco) and incubated in a 37°C water bath until logarithmic growth. The culture was centrifuged for 15 min at 3,000 × *g*, the supernatant was removed, and the cell pellet was suspended in 50 ml of phosphate-buffered saline (PBS) (0.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.13 M NaCl [pH 7.4]). The cells were washed three times with PBS, and the final pellet was adjusted to an optical density at 420 nm of 1.0 (ca. 10^{10} CFU/ml). Tenfold serial dilutions of the suspension were made in 10 ml of PBS.

Preparation of oysters. Oysters were collected from approved waters near Cedar Key, Fla., transported at less than 10°C according to standard procedures, and processed within 4 h of collection (35).

Identification and enumeration of bacteria. Except as noted, 10 oysters were scrubbed to remove loose particles, and the meat and liquor were pooled and homogenized as previously described (27). Three 1-ml aliquots from each sample homogenate were separately serially diluted in 10-fold increments in 9 ml of PBS for triplicate most probable number (MPN) measurements.

V. cholerae O1 and *Vibrio vulnificus* were enumerated with alkaline peptone broth by the three-tube MPN enrichment method. After 6 to 8 h of incubation at 37°C, all turbid tubes were streaked on thiosulfate-citrate-bile salts-sucrose agar (Difco) for the isolation of *V. cholerae* O1 (5, 8). The same alkaline peptone enrichment broths were reincubated at 37°C for a total of 12 to 16 h and streaked on modified colistin-polymyxin B-cellobiose agar for the isolation of *V. vulnificus* (30). Thiosulfate-citrate-bile salts-sucrose and modified colistin-polymyxin B-cellobiose plates were incubated at 37°C for 18 to 24 h. For species identification, two suspect colonies from each agar plate were transferred to sterile 96-well tissue culture plates (Costar, Charlotte, N.C.) containing 100 μl of alkaline peptone broth and incubated at 37°C for 4 h, and then 25 μl of each turbid well was transferred to an enzyme immunosorbent assay plate (Costar) containing 25 μl of 0.02% Triton X-100 (Sigma) in PBS. The enzyme immunosorbent assay procedure, which utilizes species-specific monoclonal antibodies for *V. cholerae* O1 and *V. vulnificus* (Global Diagnostics, Alachua, Fla.), has been described

previously (30). Positive enzyme immunosorbent assay reactions were recorded with the corresponding MPN tube, and results were reported as MPN per gram.

E. coli was enumerated by the five-tube MPN procedure (12). Results were reported as MPN per gram.

S. tallahassee was enumerated by the three-tube MPN method (2). Colonies were confirmed with an API 20E test kit (Analytab Products, Plainview, N.Y.). Results were reported as MPN per gram.

Controlled purification. A 30-gal (ca. 110-liter) aquarium was filled with artificial seawater (Instant Ocean; Aquarium Systems, Mentor, Ohio) which was recirculated with a pump (Little Giant, Oklahoma City, Okla.) through a 5-μm-pore/size cartridge filter (Omni Corporation, Hammond, Ind.) at a rate of 30 liters/min and was exposed to a 30-W UV light (Aquanetics Systems, San Diego, Calif.). Experiments were conducted at 15, 19, and 25°C in 25-ppt seawater.

Bactericidal effect of UV light. The disinfecting efficiency of UV light was studied with *V. cholerae* O1, *E. coli*, and *S. tallahassee* added to 25°C seawater exposed to UV light during recirculation. Each species was added to the aquarium separately at final concentrations of 10^7 *V. cholerae* O1, 10^5 *E. coli*, and 10^5 *S. tallahassee* CFU/ml. Samples were removed immediately after inoculation and every 30 min during 7 h of UV treatment. Bacteria were enumerated as described previously.

Uptake of *V. cholerae* O1, *E. coli*, and *S. tallahassee* by oysters. Approximately 100 oysters were placed in an aquarium containing 25°C recirculating seawater that was not exposed to UV light or passed through a filter. *V. cholerae* O1 strains were cultured and washed as previously described and added to the aquarium at 10^4 CFU/ml. Ten oysters were randomly selected and removed from the aquarium at predetermined time intervals over 7 h. In a separate experiment, *V. cholerae* O1, *E. coli*, and *S. tallahassee* were added to 19 and 25°C seawater at a concentration of 10^4 CFU/ml. At selected time intervals, 10 oysters were sampled.

Retention of *V. cholerae* O1, *E. coli*, *S. tallahassee*, and *V. vulnificus* under conditions of controlled purification. Retention of *V. cholerae* O1 was measured for oysters previously exposed to 10^4 *V. cholerae* O1 CFU/ml of seawater for 4 h and then maintained in 25°C seawater recirculated through a filter and exposed to UV light. Ten oysters were sampled before experimentation, at 4 h following the uptake, and at selected time intervals over 48 h of treatment. In a separate experiment, oysters were exposed for 4 h at 19°C to seawater containing 10^2 *V. cholerae* O1, 10^2 *E. coli*, and 10^2 *S. tallahassee* CFU/ml and then were subjected to depuration in a separate aquarium at 19°C. Ten oysters were sampled at selected time intervals over 48 h. Endogenous *V. vulnificus* populations were also measured. In a third experiment, oysters were exposed for 4 h at 25°C to 10^5 *V. cholerae* O1, 10^5 *E. coli*, and 10^5 *S. tallahassee* CFU/ml, divided into equal groups, and then placed in either 15 or 25°C seawater. Ten oysters were sampled from each aquarium over 48 h of controlled purification.

Relationship between oyster hemolymph agglutinin titer and persistence of *V. cholerae* O1. The shells of approximately 150 oysters were notched with a 4-in. (ca. 10-cm) disc grinder, which formed a small V-shaped notch in the shell near the adductor muscle. The oysters were rinsed with distilled water and drained for 30 min to remove excess fluid from the mantle cavity. Next, approximately 1 ml of hemolymph was removed from the adductor muscle sinus with a 22-gauge needle. Hemolymph samples and oyster shells were labelled for later reference, and the oysters were stored at 10°C overnight until the completion of the bacterial agglutination assay. Individual hemolymph samples were centrifuged for 15 s at 15,000 × *g*, and the titers of the samples were determined in 96-well plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.) in duplicate by following the procedure of Tamplin and Fisher (28).

Following the agglutination assay, oysters with low and high agglutinin titers were removed from the refrigerator and placed into an aquarium containing 25°C seawater, and they acclimated for 30 min. Low- and high-titer oysters were then separated and transferred to individual 10-gal (ca. 38-liter) aquaria (25°C) and exposed to 10^2 *V. cholerae* O1 CFU/ml. At selected time intervals, *V. cholerae* O1 was enumerated in pooled oyster meats from five oysters.

In a separate experiment, oysters were segregated into low- and high-titer groups as described above. After a 4-h exposure in an aquarium containing 10^4 *V. cholerae* O1 CFU/ml of seawater (25°C), groups of oysters were transferred to 10-gal aquaria containing filtered 15°C seawater that had been exposed to an 8-W UV light. Pooled meats from five animals were sampled before exposure to *V. cholerae* O1, after 4 h of exposure, and at selected time intervals of controlled purification.

Statistical methods. Significant differences in mean values were determined by Student's two-tailed *t* distribution test ($P \leq 0.05$; degrees of freedom, 4) (7, 25).

RESULTS

Bactericidal effect of UV light on *V. cholerae* O1, *E. coli*, and *S. tallahassee*. UV light was bactericidal for all three species. *V. cholerae* O1, *E. coli*, and *S. tallahassee* were not detected in seawater at 3.5, 1.0, and 2.5 h posttreatment, respectively.

Uptake and retention of *V. cholerae* O1, *E. coli*, and *S. tallahassee*. Oysters contained no detectable *V. cholerae* O1 and *S. tallahassee* and 1.4 *E. coli* organisms per g prior to exposure.

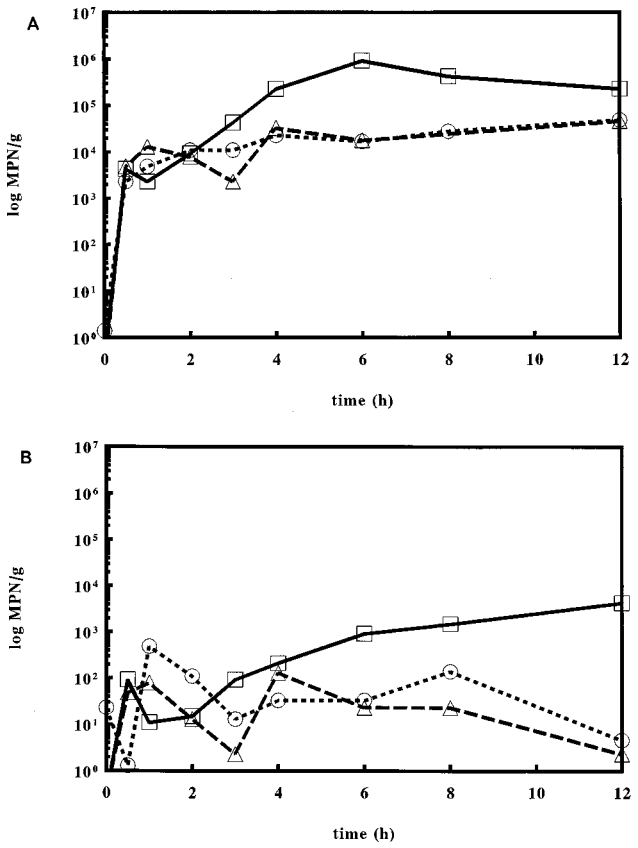

FIG. 1. Uptake of *V. cholerae* O1 (□), *E. coli* (○), and *S. tallahassee* (△) by *C. virginica* at 25°C, 25 ppt, and a seawater inoculum of 10^4 organisms per ml (A) and 19°C, 25 ppt, and a seawater inoculum of 10^4 organisms per ml (B).

Exposure to 10^4 *V. cholerae* O1, 10^4 *E. coli*, and 10^5 *S. tallahassee* CFU/ml in 25°C seawater resulted in the accumulation of all the species in oyster tissues. After 1 h of treatment, all species exceeded 10^3 organisms per g of tissue (Fig. 1A). *E. coli* and *S. tallahassee* concentrations remained relatively stable through 12 h. However, *V. cholerae* O1 continued to increase in oyster tissue through 6 h and then remained at $>10^5$ organisms per g through 12 h. A similar pattern of uptake among the species was observed at 19°C, although the quantity accumulated for each strain was smaller by more than 100-fold (Fig. 1B).

Typical Gulf of Mexico and commercial depuration temperatures were more closely simulated by exposing a batch of oysters to the three bacterial species at 25°C and then splitting them into aquaria containing 15 or 25°C depuration seawater. Prior to experimentation, oysters contained no detectable *V. cholerae* O1 or *S. tallahassee* and only 0.56 *E. coli* organisms per g. After a 4-h exposure to seawater containing 10^5 *V. cholerae* O1, 10^5 *E. coli*, and 10^5 *S. tallahassee* organisms per ml, oysters accumulated approximately 10^2 *V. cholerae* O1, 10^3 *E. coli*, and 10^2 *S. tallahassee* organisms per g (Fig. 2). Following 48 h of treatment, oysters at both 15 and 25°C retained greater levels of *V. cholerae* O1 than *E. coli* or *S. tallahassee*. Specifically, at 15°C, the *V. cholerae* O1 level was approximately 30 times greater than that of *S. tallahassee* and approximately 500 times greater than that of *E. coli*. At 25°C, *V. cholerae* O1 reached levels approximately 100 times greater than those of *S. tallahassee* and approximately 10 times greater than those of *E. coli.*

To compare the retentions of *V. cholerae* O1, *E. coli*, *S. tallahassee*, and *V. vulnificus* at 19°C, oysters containing non-

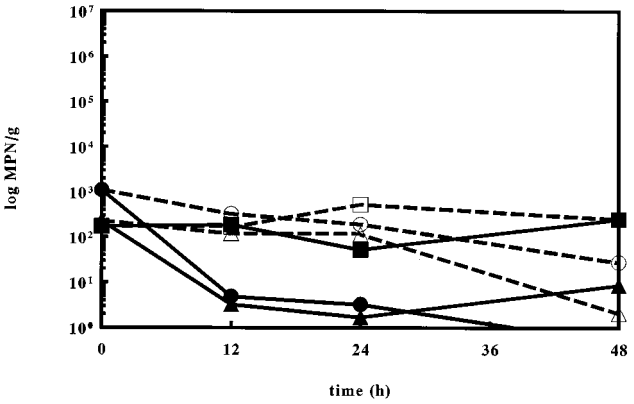

FIG. 2. Retention of *V. cholerae* O1, *E. coli*, and *S. tallahassee* by *C. virginica* under conditions of controlled purification at 15 and 25°C, 25 ppt, a seawater inoculum of 10^5 organisms per ml, and an uptake temperature of 25°C. ■, *V. cholerae* O1 at 15°C; ●, *E. coli* at 15°C; ▲, *S. tallahassee* at 15°C; □, *V. cholerae* O1 at 25°C; ○, *E. coli* at 25°C; △, *S. tallahassee* at 25°C.

detectable levels of *V. cholerae* O1 and *S. tallahassee*, 75 *V. vulnificus* organisms per g, and 0.2 *E. coli* organisms per g were placed in 19°C seawater containing 10^2 *V. cholerae*, 10^2 *E. coli*, and 10^2 *S. tallahassee* CFU/ml for 4 h. Following exposure, oysters contained approximately 10^2 *V. cholerae* O1, 10^2 *E. coli*, 10^2 *S. tallahassee*, and 23 naturally occurring *V. vulnificus* organisms per g (Fig. 3). After 48 h of treatment, oysters retained 4.3 *V. cholerae* O1 and 1.5×10^2 *V. vulnificus* organisms per g; however, *E. coli* and *S. tallahassee* were not detected (Fig. 3).

Relationship between oyster agglutinin titer and uptake and retention of *V. cholerae* O1. Oysters in the low-titer group had no detectable agglutinin (<2). Those selected for the high-titer group had a mean agglutinin titer of 35. When the oysters were exposed to *V. cholerae* O1, there was no significant difference in bacterial levels for low- and high-titer groups at all time intervals tested (Fig. 4A). For example, low-titer oysters contained 1.1×10^4 *V. cholerae* O1 organisms per g and the high-titer group contained 3.6×10^4 organisms per g after an 8-h exposure (Fig. 4A).

In a separate experiment measuring the retention of *V. cholerae* O1 against the agglutinin level, the low-titer group had a mean agglutinin titer of 4 and oysters in the high-titer group had a mean titer of >128. Both groups were exposed to *V. cholerae* O1 for 4 h at 25°C in the same aquaria containing 10^4 *V. cholerae* O1 CFU/ml. After uptake, high-titer oysters contained 460 *V. cholerae* O1 organisms per g and the low-titer group contained 36 organisms per g. These levels were not significantly different. Oysters were then exposed to controlled purification at 15°C and tested for retention of *V. cholerae* O1 over 48 h. There was no significant difference in *V. cholerae* O1 levels for the high- and low-lectin-titer groups over 48 h of treatment (Fig. 4B).

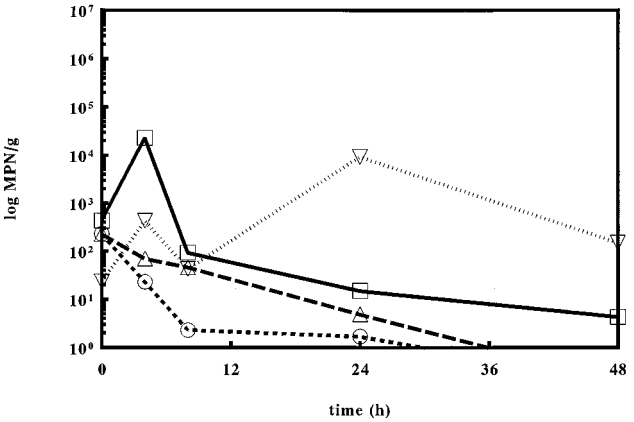

FIG. 3. Retention of *V. cholerae* O1 (□), *E. coli* (○), *S. tallahassee* (△), and naturally occurring *V. vulnificus* (▽) by *C. virginica* under conditions of controlled purification at 19°C, 25 ppt, a seawater inoculum of 10^2 organisms per ml, and an uptake temperature of 19°C.

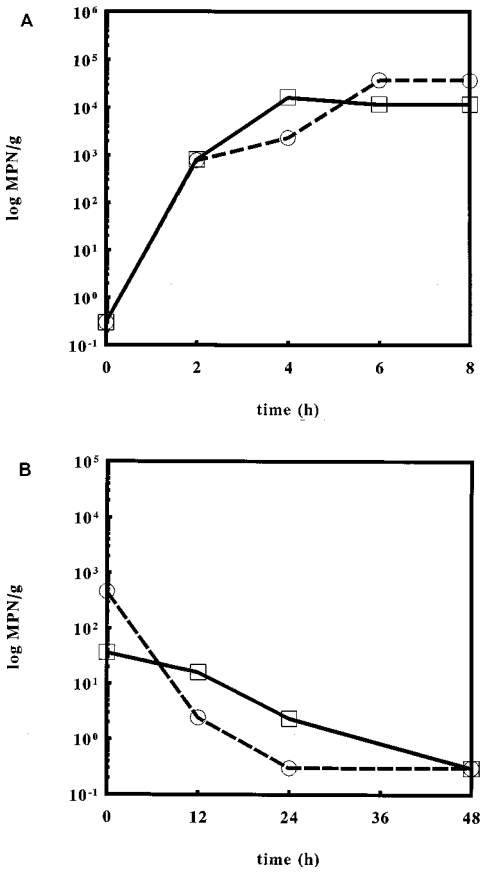

FIG. 4. Effect of hemolymph lectin concentration on uptake of *V. cholerae* O1 by *C. virginica* at 15°C, 25 ppt, and a seawater inoculum of 10^2 organisms per ml (A) and retention at 15°C, 25 ppt, a seawater inoculum of 10^2 organisms per ml, and an uptake temperature of 15°C (B). □, low titer; ○, high titer.

DISCUSSION

Although shellfish have been implicated in the transmission of cholera and the persistence of *V. cholerae* O1 in the environment, reports of the effect of postharvest controlled purification methods on this pathogen are lacking. This information is important to understanding the efficacy of depuration for increasing the safety of shellfish when *V. cholerae* O1 contaminates aquatic environments.

Results showed that oysters exposed to seawater containing *V. cholerae* O1 at levels similar to those in contaminated waters in Peru (31) accumulated high numbers of *V. cholerae* O1 in as few as 4 h of exposure and that levels continued to increase with time. This concentration may have resulted from the multiplication of *V. cholerae* O1 in oyster tissues, as has been suggested for *V. vulnificus* (27). Compared with the accumu-

lation of *E. coli* and *S. tallahassee* in both 19 and 25°C seawater, the accumulation of *V. cholerae* O1 was greater over 12 h of exposure. However, accumulation of all species at 19°C was less than that at 25°C (Fig. 1), indicating that reduced temperature may affect (i) oyster physiology (i.e., pumping), allowing less accumulation of bacteria; (ii) the surface affinity of bacteria and oyster tissue; and/or (iii) the survival of bacteria in oyster tissues.

Regardless of the inoculum level or treatment temperature, experiments showed that *V. cholerae* O1 persisted in oyster tissues through 48 h of controlled purification. In some experiments (Fig. 2), the levels of *V. cholerae* O1 observed at 48 h were greater than those seen at the initial exposure. This may have been due to the multiplication of *V. cholerae* O1 in oyster tissues at elevated temperature (25°C). At lower temperatures, multiplication of *V. cholerae* O1 appeared to be inhibited, possibly allowing UV light to reduce the numbers of *V. cholerae* O1 in recirculating seawater and leading to lower-level accumulation in oysters.

These results are in agreement with other studies showing that *E. coli* and salmonellae are reduced to nondetectable levels by 36 h (13, 16, 21, 26) and that *Vibrio* spp. survive in shellfish longer than *E. coli* and *S. tallahassee* (14, 24). Furthermore, we observed that natural *V. vulnificus* populations increased 10-fold after 48 h of controlled purification (Fig. 3), confirming the conclusions of previous studies (27) that controlled purification does not reduce *V. vulnificus* numbers in shellfish held above 15°C (27).

Several authors have reported that elimination of salmonellae is less rapid than that of *E. coli* (1, 15, 17, 32) and that the difference in elimination may depend on the temperature and salinity of the process water (23). Jones et al. (16) reported that elimination of fecal coliforms is most effective during warm months (July, August, and September) and less effective in December. This information gives some possible explanations relative to this experiment, since oysters which were harvested from colder waters (20°C) retained greater numbers of both *E. coli* and *S. tallahassee.*

Tamplin and Fisher (28) have reported the presence of a *V. cholerae* O1 agglutinin in oyster tissues. Experiments conducted to study the effect of agglutinin concentration on the potential retention of *V. cholerae* O1 showed a marked contrast in *V. cholerae* O1 oyster hemolymph agglutinin titers of warm- and cold-water collections. At the July harvest date, many oysters had an agglutinin titer of <2 ($n = 33$), while no oysters sampled had an agglutinin titer of >128 (Fig. 4A). In contrast, the majority of oysters collected in January had agglutinin titers of >128 ($n = 44$) and few had titers of <2 ($n = 2$) (Fig. 4B). Fisher et al. (10) has reported that *V. cholerae* O1 agglutinin levels are lower in summer and higher in winter months. The low bacterial agglutination titers for the warm-water collections (July) may have resulted from the reproductive state (spawning) of the oysters. Spawning stresses their physiological state and reduces the function of oyster defense mechanisms (9).

There was no significant difference in the accumulation of *V. cholerae* O1 for the low- and high-titer agglutinin groups (Fig. 4B). Similarly, retention of *V. cholerae* O1 under conditions of controlled purification was not significantly different for the low- and high-titer groups (Fig. 4B).

There are several explanations for these results. First, agglutinin may not contribute to the retention of *V. cholerae* O1 in oysters. Second, the bacterial agglutination assay described by Tamplin and Fisher (28) determines the agglutinin titer in oyster hemolymph collected from the sinus of the adductor muscle and may not reflect the amount of agglutinin present in other oyster tissues, which constitute large surface areas exposed to *V. cholerae* O1. Third, the bacterial agglutination assay uses a high concentration of *V. cholerae* O1 per milliliter (i.e., approximately 10^{10} CFU), and results may not predict the fate for lower *V. cholerae* O1 levels used in these experiments (i.e., $\leq 10^5$ CFU). Further studies are needed to determine the amount of agglutinin on specific oyster tissues and to develop better methods for optimizing oyster physiology during experimentation.

Interestingly, in the experiment comparing the retentions of *V. cholerae* O1 by low- and high-titer oysters, *V. cholerae* O1 could not be detected after 48 h of treatment (Fig. 4B), unlike the case with earlier experiments (Fig. 2 and 3). This may have resulted from factors including increased oyster defense mechanisms during winter months and/or a more rapid elimination of *V. cholerae* O1 at a lower exposure dose (10^2 organisms per g) (17). Further experiments are warranted to conclusively demonstrate the effect of *V. cholerae* O1 agglutinin on the uptake and retention of *V. cholerae* O1.

This research demonstrates that controlled purification has a limited effect on *V. cholerae* O1 numbers in oysters compared with its effects on numbers of *E. coli* and *S. tallahassee*, although low seawater temperature appears to enhance reduction. Future experiments could delineate the specific effects of salinity and dose and the relationship between harvest and treatment temperature.

With more waters being closed because of declining bacteriological quality, the shellfish industry will be compelled to rely on postharvest processes, such as controlled purification, to maintain a commercially viable raw product. Although previous surveys of oysters in U.S. waters have shown that only approximately 1% are contaminated with *V. cholerae* O1 and that all are nontoxigenic (33), the recent introduction of virulent *V. cholerae* O1 to the North and South American continents is a reminder of the continued threat of introduced and free-living forms of *V. cholerae* O1 in estuaries (4). In conclusion, current Food and Drug Administration regulations recommending depuration temperatures of 10 to 25°C must be reevaluated to determine the optimal parameters for eliminating such pathogens as vibrios and *Salmonella* spp. Our results also show that elevated seawater temperature may produce a more hazardous product.

ACKNOWLEDGMENTS

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