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Research Note

Effects of Dry Storage and Resubmersion of Oysters on Total *Vibrio vulnificus* and Total and Pathogenic (*tdh*+/*trh*+) *Vibrio parahaemolyticus* Levels

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ABSTRACT

Vibrio vulnificus (Vv) and *Vibrio parahaemolyticus* (Vp) are the two leading causes of bacterial illnesses associated with raw shellfish consumption. Levels of these pathogens in oysters can increase during routine antifouling aquaculture practices involving dry storage in ambient air conditions. After storage, common practice is to resubmerge these stored oysters to reduce elevated Vv and Vp levels, but evidence proving the effectiveness of this practice is lacking. This study examined the changes in Vv and in total and pathogenic (thermostable direct hemolysin gene and the *tdh*-related hemolysin gene, *tdh*+ and *trh*+) Vp levels in oysters after 5 or 24 h of dry storage (28 to 32°C), followed by resubmersion (27 to 32°C) for 14 days. For each trial, replicate oyster samples were collected at initial harvest, after dry storage, after 7 days, and after 14 days of resubmersion. Oysters not subjected to dry storage were collected and analyzed to determine natural undisturbed vibrio levels (background control). Vibrio levels were measured using a most-probable-number enrichment followed by real-time PCR. After storage, vibrio levels (excluding *tdh*+ and *trh*+ Vp during 5-h storage) increased significantly ($P < 0.001$) from initial levels. After 7 days of resubmersion, Vv and total Vp levels (excluding total Vp in oysters stored for 5 h) were not significantly different ($P > 0.1$) from levels in background oysters. Vv and total and pathogenic Vp levels were not significantly different ($P > 0.1$) from levels in background oysters after 14 days of resubmersion, regardless of dry storage time. These data demonstrate that oyster resubmersion after dry storage at elevated ambient temperatures allows vibrio levels to return to those of background control samples. These results can be used to help minimize the risk of Vv and Vp illnesses and to inform the oyster industry on the effectiveness of routine storing and resubmerging of aquaculture oysters.

Vibrio vulnificus (Vv) and *Vibrio parahaemolyticus* (Vp) are two of the leading seafood-borne pathogens in the world and are most commonly associated with consumption of raw shellfish, including oysters *(7, 12)*. These bacteria occur naturally in marine environments and bioaccumulate in the tissues of filter feeders, such as oysters *(11)*. High water temperatures (>15°C) are correlated with increased frequency of Vv and Vp detection in the environment and illnesses in the summer months *(13, 16, 24, 30, 31)*. Vv infections tend to be the most severe, frequently resulting in septicemia, especially in patients with immune system deficiencies and/or liver disease *(4, 31)*. The Centers for Disease Control and Prevention has reported the annual mortality rate for Vv cases in the United States to be ~35%, making it one of the most deadly foodborne pathogens *(28, 36)*. Infections associated with Vp normally result in gastroenteritis and are the most frequent cause of vibriosis, accounting for almost 40% of all *Vibrio* spp. infections and 53% of all mollusk-related illnesses *(2, 20, 31)*. These two bacteria are estimated to account for 45,000 illnesses, 330 hospitalizations, and 80 deaths annually in the United States *(36)*. The annual frequency of Vv and Vp infections has steadily risen for the past 15 years *(31)*. There have been no definitive virulence markers identified for Vv. However, two genetic markers (thermostable hemolysin direct gene [*tdh*+] and *tdh*-related hemolysin gene [*trh*+]) are generally considered to be linked to higher pathogenicity in Vp containing these genes *(18, 29, 38)*.

Annually, the oyster industry along the Gulf coast is a >$50 million market and is a >$40 million market along the Atlantic coast *(3)*. The nationwide financial cost associated with Vv cases per year was estimated at ~$250 million in 2012, a 20% increase from the U.S. Food and Drug Administration's (FDA) 1995 estimate *(1, 17, 34)*. A much lower cost estimate in 2011 for Vp illnesses was ~$20 million, which is still significant with respect to the market income *(34)*. The safety of the consumer is an important factor in the success of this industry, and several safe harvesting practices have been explored in an attempt to reduce the risk of infections associated with raw oyster

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consumption. Postharvest processing, depuration, wet storage, and other aquaculture practices can be effective in inhibiting growth and, in some cases, reducing the levels of vibrios in shell stock oysters *(19, 27, 30, 32)*.

Antifouling is a common practice that reduces or removes unwanted organisms and deposits from shell stock oysters, which can damage or harm the shellfish or equipment used in aquaculture settings *(14)* and can also reduce the quality of the shellfish product. A specific antifouling practice to prevent the overaccumulation of biofouling organisms involves the dry storage of oysters in ambient air conditions. However, this practice can increase Vv levels by 1 to 2 log CFU/g during 14-h storage at temperatures >15°C and can increase Vp levels 2 to 3 log CFU/g after 10 h when stored at temperatures >20°C *(9, 16, 19, 21)*. This demonstrates the potential for an increased risk involved in consuming oysters that undergo this type of handling *(16)*. In an attempt to mitigate these elevated bacteria levels in ambient air stored oysters, the typical practice is to resubmerge these oysters and harvest them after approximately 7 days *(37)*. Few data exist on the effects of this resubmersion on levels of Vv and Vp that have proliferated during storage. The objective of this study was to determine the changes in total Vv levels, total Vp levels, and pathogenic Vp levels (Vp containing the *tdh* and/or *trh* genes) in oysters stored under ambient air, dry storage conditions, with subsequent resubmersion at the same site after 7 and 14 days.

MATERIALS AND METHODS

Experimental design. All oysters used for this study were collected by dredge from Mobile Bay, AL, relocated to Dauphin Island Bay, AL, and held in wet storage cages for at least 2 weeks before experimentation. For each trial, ~100 oysters were collected from wet storage cages and placed in hanging aquaculture baskets (SEAPA Pty. Ltd., Edwardstown, Australia) in Dauphin Island Bay. Two oyster samples (six live oysters per sample, ~200 g) were collected for initial vibrio level measurements. One basket of ~50 oysters, for oysters with natural undisturbed vibrio levels (background control samples), was immediately resubmerged in the same location by suspending the basket in the water column. The remaining oysters were dry stored in hanging baskets (~50 oysters per basket). Oysters in dry storage were exposed to ambient air (28 to 32°C) in a nonshaded area. For two trials these oysters were stored for 5 h, and for three trials they were stored for 24 h. One oyster per trial was fitted with a Smart Button temperature logger (ACR Systems, Surrey, British Columbia, Canada), recording every 2 min for 5-h storage trials and every 15 min for 24-h storage trials. The Smart Button was sealed inside the oyster using zip ties, and the oyster was placed in the middle of the basket during storage to measure a representative internal oyster temperature during dry storage. After dry storage, two samples were collected from the baskets for vibrio level measurements. The remaining oysters were resubmerged by suspending the basket in the water column in the same location as initial harvest. After 7 and 14 days of resubmersion, four oyster samples were collected from the resubmerged baskets, along with four concurrent oyster samples from the background control basket. On each sampling occasion, water temperature and salinity were measured with a YSI model 85 (YSI, Inc., Yellow Springs, OH).

Sample preparation and enrichment. Each sample was analyzed within 1 h of collection, using a three-tube, most-probable-number (MPN) enrichment as described in the FDA's *Bacteriological Analytical Manual (26),* with slight modifications. Oysters (six live oysters per sample, ~200 g) were cleaned with freshwater and then were scrubbed with a brush, shucked, and homogenized in a blender on high for ~90 s. One gram of oyster homogenate was added to 9 ml of phosphate buffered saline (PBS; 7.65 g of NaCl, 0.724 g of Na_2HPO_4 [anhydrous], 0.21 g of KH_2PO_4 in 1 liter of distilled H_2O, pH 7.4) for a 1:10 dilution, and subsequent 10-fold dilutions were made in PBS. One-milliliter aliquots of each PBS dilution were added to three tubes of 9 ml of alkaline peptone water (APW; 10 g of Bacto Peptone, 10 g of NaCl, 1 liter of dH_2O). All media components for PBS and APW were purchased from Fisher Scientific, Pittsburgh, PA. One gram of oyster homogenate was added to 9 ml of APW in triplicate to complete the three-tube MPN series. All tubes were incubated (37°C) for 18 to 24 h. After incubation, a 1-ml aliquot from each tube displaying visible bacteria growth was transferred to a microcentrifuge tube (Fisher Scientific), heated to 100°C for 10 min, and then immediately frozen. Boiled samples were stored frozen at −20°C in a manual defrost freezer until real-time PCR (Rti-PCR) analysis was performed. Samples were completely thawed and centrifuged at 13,000 × *g* for 2 min immediately preceding Rti-PCR analysis, and 2 μl of the resulting supernatant was used as template.

Rti-PCR analysis. Three Rti-PCR assays were performed separately for total Vv (*vvhA*), total Vp (*tlh*), and pathogenic Vp (*tdh*+/*trh*+) on the Applied Biosystems 7500 Fast Real-Time PCR system (AB 7500, Life Technologies, Waltham, MA). Each reaction contained 2 μl of Internal Amplification Control (IAC) DNA as previously published *(33),* 0.06 μl of ROX reference dye (Invitrogen, Carlsbad, CA), 5 mM final concentration of $MgCl_2$ (Invitrogen), and 300 nM final concentration of each of the deoxynucleoside triphosphates (Roche, Indianapolis, IN). *Taq* polymerase, primer, and probe concentrations for each of the reactions are described below. All primers and probes were purchased from Integrated DNA Technologies (Coralville, IA) or Life Technologies. Oligonucleotide sequences are listed in Table 1. The final volume for each reaction was brought up to 25 μl with PCR-grade water (Ambion, Austin, TX). Default instrument parameters were utilized, with the exceptions that the threshold cycle was set to 0.02 and the background end cycle was set at 10.

The Vv Rti-PCR assay used *vvhA* primers and probe as published *(6)* and cycling parameters and reaction mixture concentrations as described *(15),* with modifications for the AB 7500 platform. The cycling parameters were 95°C for 1 min, 45 cycles of 95°C for 15 s (denature), 57°C for 15 s (anneal), and 72°C for 25 s (extend). Final reagent concentrations were 1.12 U Platinum *Taq* DNA polymerase (Invitrogen), 300 nM each *vvhA* primer, 150 nM each IAC primer, 200 nM *vvhA* nuclease probe, and 150 nM IAC nuclease probe.

The *tlh* Rti-PCR assay protocol was as described *(15),* with modifications to the reaction mixture for the AB 7500 platform. Final concentrations for *Taq* polymerase, primers, and nuclease probes were 1.5 U Platinum *Taq* DNA polymerase (Invitrogen), 200 nM each *tlh* primer, 75 nM each IAC primer, and 150 nM each *tlh* and IAC nuclease probes.

The Rti-PCR assay for *tdh* and *trh* analysis was as previously described *(22),* with modifications to reaction concentrations for the AB 7500 platform. The final reagent concentrations per reaction were 2.25 U Platinum *Taq* DNA polymerase (Invitrogen), 100 nM each *tdh* primer, 300 nM each *trh* primer, 25 nM each IAC primer, 75 nM *tdh* and *trh* nuclease probes, and 150 nM IAC nuclease probe.

TABLE 1. *Oligonucleotide sequences for real-time PCR on the AB 7500 Fast*

	Sequences (5′→3′)	Modifications
tdh 89F	TCCCTTTTCCTGCCCCC	—
tdh 321R	CGCTGCCATTGTATAGTCTTTATC	—
tdh Probe	TGACATCCTACATGACTGTG	5′FAM-3′MGBNFQ[a]
trh 20F	TTGCTTTCAGTTTGCTATTGGCT	—
trh 292R	TGTTTACCGTCATATAGGCGCTT	—
trh Probe	AGAAATACAACAATCAAAACTGA	5′NED-3′MGBNFQ
tlh 884F	ACTCAACACAAGAAGAGATCGACCA	—
tlh 1091R	GATGAGCGGTTGATGTCCAA	—
tlh Probe	CGCTCGCGTTCACGAAACCGT	5′JOE-3′BHQ2[b]
*vvh*F	TGTTTATGGTGAGAACGGTGACA	—
*vvh*R	TTCTTTATCTAGGCCCCAAACTTG	—
vvhA Probe	CCGTTAACCGAACCACCCGCAA	5′Cy5-3′BHQ2
IAC 46F	GACATCGATATGGGTGCCG	—
IAC 186R	CGAGACGATGCAGCCATTC	—
IAC probe	TCTCATGCGTCTCCCTGGTGAATGTG	5′Cy5-3′BHQ2[c]
IAC probe	TCTCATGCGTCTCCCTGGTGAATGTG	5′JOE-3′BHQ2[d]

[a] MGBNFQ, minor groove binding nonfluorescent quencher.
[b] BHQ2, black hole quencher 2.
[c] When used with *V. parahaemolyticus* primers and probes.
[d] When used with *V. vulnificus* primers and probes.

Data analysis. Results from Rti-PCR were used with standard MPN tables to estimate total Vv and total and pathogenic Vp densities in each sample *(5)*. A one-way analysis of variance (ANOVA) was used to estimate and compare mean vibrio densities for each of the two treatments (5-h and 24-h dry storage) and each of the four gene targets. Differences among treatment groups (initial harvest, dry storage, 7 and 14 days resubmerged) and in comparison to control samples were assessed by multiple pairwise

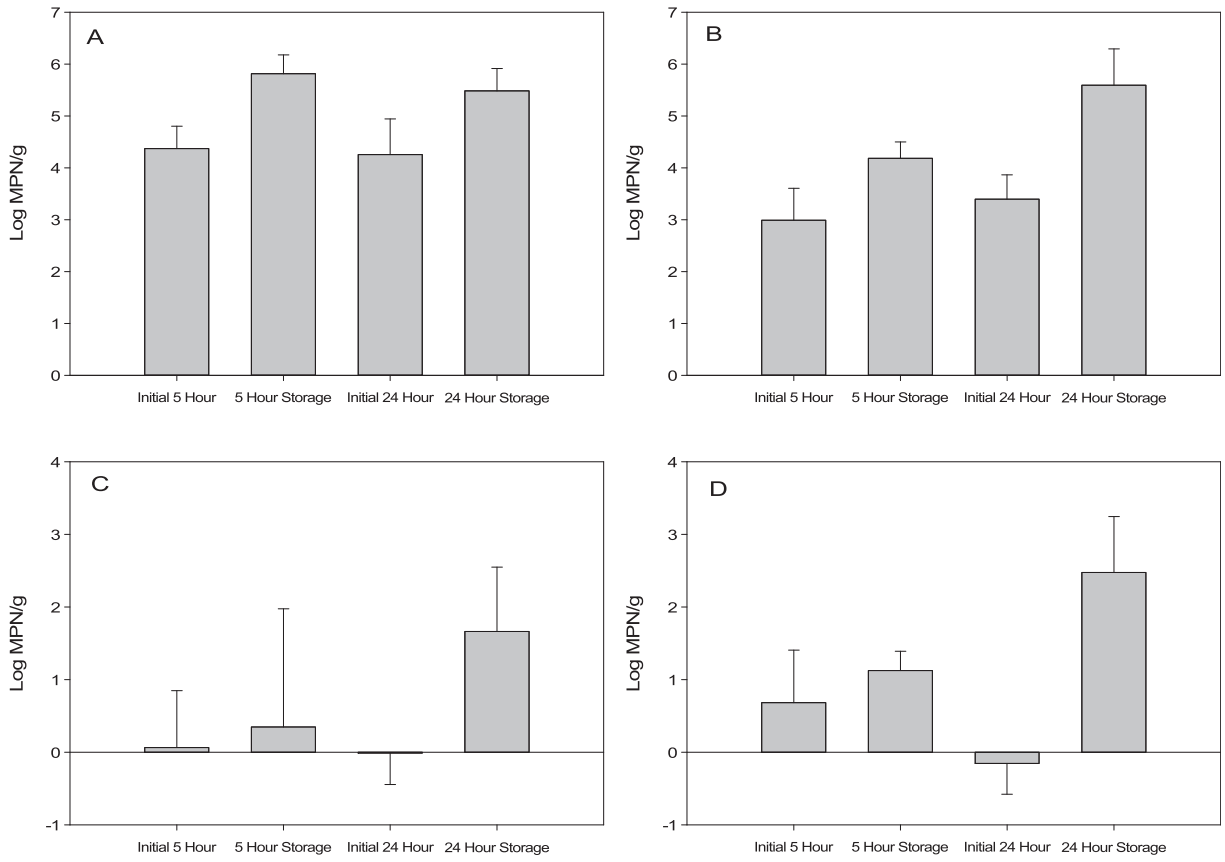

FIGURE 1. *Total* V. vulnificus *(A), total* V. parahaemolyticus *(B),* tdh+ V. parahaemolyticus *(C), and* trh+ V. parahaemolyticus *(D) levels in oysters upon initial harvest, after 5-h dry storage, and after 24-h dry storage. Bars are representative of mean levels, with error bars indicating the standard deviation.*

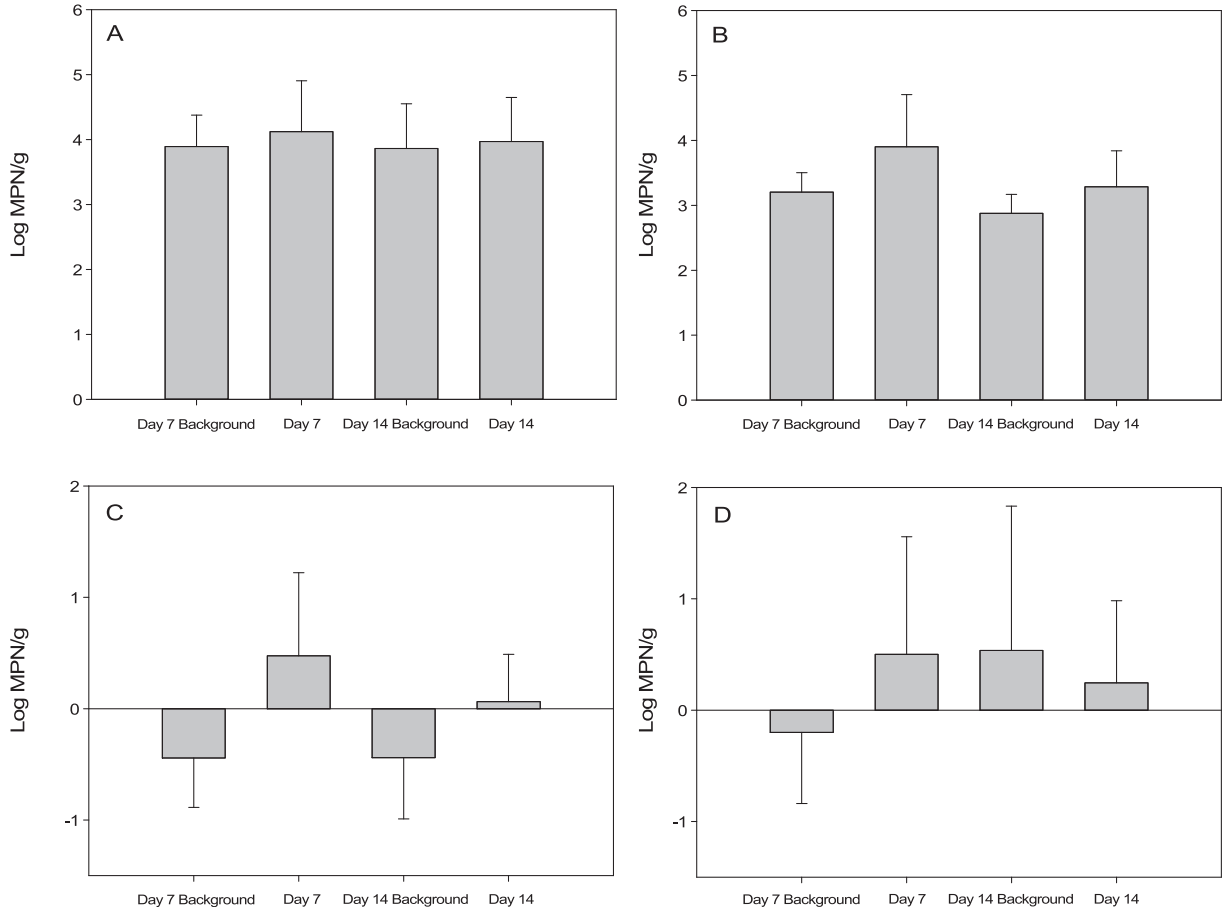

FIGURE 2. *Total* V. vulnificus *(A), total* V. parahaemolyticus *(B),* tdh+ V. parahaemolyticus *(C), and* trh+ V. parahaemolyticus *(D) levels in oysters after 7 and 14 days of resubmersion following 5-h dry storage and levels in concurrent background samples at 7 and 14 days. Bars are representative of mean levels, with error bars indicating the standard deviation.*

comparisons. Estimates of abundance were log-transformed prior to statistical analysis. For instances in which results from Rti-PCR for *tdh* and *trh* indicated levels below the limit of detection, half the limit of detection was substituted. Data from the five individual trials were pooled for the purpose of ANOVA. An alpha level of 0.05 was chosen as the criterion for statistical significance. All statistical calculations were performed using the R programming language *(35).*

RESULTS AND DISCUSSION

During this study, oysters were removed from the water, stored at ambient temperatures for 5 h or 24 h, and then resubmerged in the same location for 2 weeks to observe the effects of dry storage and subsequent resubmersion on total Vv and on total and pathogenic Vp levels in oysters. Average water temperature during the study was 29.0°C (range: 27.1 to 31.7°C) and salinity was 17.6 ppt (range: 12.2 to 21.3 ppt). The average oyster internal temperature was 27.9°C (±1.1°C) during the two 5-h dry storage trials and was 27.7°C (±2.7°C) during the three 24-h dry storage trials.

Vv. Mean Vv levels increased from 4.4 to 5.8 log MPN/g during 5-h storage and from 4.3 to 5.5 log MPN/g during 24-h storage (Fig. 1A). These increases were statistically significant for both storage times ($P < 0.001$) compared with initial harvest levels. After 7 days of resubmersion, statistically significant reductions in mean Vv levels to 4.1 log MPN/g were observed for both 5 and 24 h storage times ($P < 0.001$; Figs. 2A and 3A, respectively). These 7-day resubmersion levels were not statistically different ($P > 0.4$) than mean levels in concurrent background oyster samples (5 h, 3.9 log MPN/g; 24 h, 4.3 log MPN/g) or the mean levels at initial harvest (5 h, $P = 0.52$; 24 h, $P = 0.64$). No additional reduction in Vv levels occurred after 14 days of resubmersion for both storage times (5 h, 4.0 log MPN/g; 24 h, 4.0 log MPN/g).

These results are consistent with previous studies in which Vv proliferated (1 to 3 log MPN/g increase) in oysters during high temperature (15 to 30°C) storage *(8, 10).* In another experiment, oysters were harvested, stored, and resubmerged in a different location with a higher salinity; similar to our results, even under different conditions, Vv levels returned to levels measured at initial harvest after 7 days of resubmersion *(37).*

Total Vp. Mean total Vp increased from 3.0 to 4.2 log MPN/g during 5-h storage and from 3.4 to 5.6 log MPN/g during 24-h storage (Fig. 1B). These increases were statistically significant for both storage times ($P < 0.001$). After subsequent resubmersion for 7 days, mean total Vp levels in 5-h stored oysters (3.9 log MPN/g) were

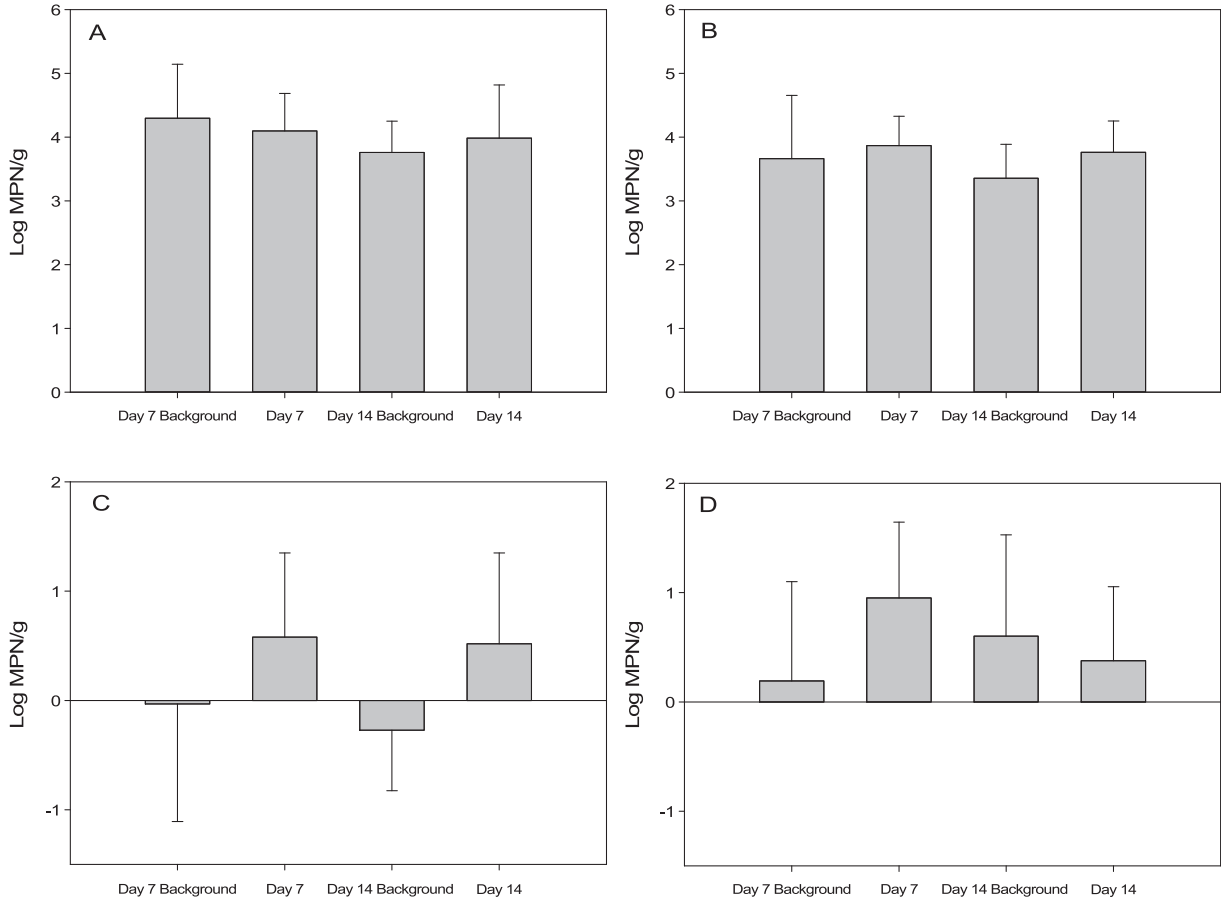

FIGURE 3. *Total* V. vulnificus *(A), total* V. parahaemolyticus *(B),* tdh+ V. parahaemolyticus *(C), and* trh+ V. parahaemolyticus *(D) levels in oysters after 7 and 14 days of resubmersion following 24-h dry storage and levels in concurrent background samples at 7 and 14 days. Bars are representative of mean levels, with error bars indicating the standard deviation.*

significantly higher than mean levels in background oysters (3.2 log MPN/g, $P = 0.004$) and mean levels at initial harvest ($P < 0.002$) (Fig. 2B). However, oysters that were stored for 24 h and then resubmerged for 7 days showed no statistically significant difference ($P > 0.4$) in mean total Vp levels (3.9 log MPN/g) compared with concurrent background levels (3.7 log MPN/g) or initial harvest levels (Fig. 3B). Vp levels in oysters resubmerged for 14 days (5 h, 3.3 log MPN/g; 24 h, 3.8 log MPN/g) were not statistically different from levels in concurrent background samples (5 h, 2.9 log MPN/g; 24 h, 3.4 log MPN/g) or at initial harvest, regardless of dry storage time ($P > 0.1$).

The observation of increasing Vp levels in this study during both storage times is similar to a previous report of a 50-fold increase of Vp in oysters stored for 10 h at 26°C *(16)*. Walton et al. *(37)* observed that Vp levels in stored oysters returned to levels at initial harvest after 14 days of resubmersion. Our finding that 5-h stored oysters required 2 weeks of resubmersion to return to initial harvest levels for Vp is consistent with the Walton study. However, we observed that Vp levels returned to those seen at initial harvest and in concurrent background oysters in only 7 days for 24-h stored oysters. The reduction of elevated Vp levels in 24-h stored oysters, but not 5-h stored oysters, after 7 days of resubmersion could possibly be due to oysters being packed too tightly during 5-h storage and subsequent resubmersion, barring their ability to open. However, this was considered unlikely; results from another oyster aquaculture practice study suggest that tight packing of oysters in a holding container does not hinder the oysters' ability to pump *(23)*.

Because oysters pump water for food, respiration, and release of waste, the oysters that were stored for 24 h could possibly have opened to pump more hastily after resubmersion, as if to gasp, to replenish O_2 and release respiration waste that had built up during storage, effectively flushing out Vp that had multiplied during storage. Conversely, the oysters stored for 5 h may not have resumed pumping as quickly or as intensely after resubmersion and, therefore, would not have flushed out increased bacteria levels as efficiently as seen in 24-h stored oysters.

Pathogenic (*tdh*+ and *trh*+) Vp. No significant increase ($P > 0.4$) from mean *tdh*+ and *trh*+ Vp initial levels (0.1 and 0.7 log MPN/g, respectively) was observed after 5-h dry storage (0.3 and 1.1 log MPN/g, respectively) (Fig. 1C and 1D, respectively). Mean *tdh*+ levels after 7 days of resubmersion (0.5 log MPN/g) showed a significant difference ($P < 0.03$) compared with levels in background oysters (−0.4 log MPN/g) (Fig. 2C). Mean *trh*+ levels were

not significantly different ($P > 0.1$) from background levels (−0.2 log MPN/g) after 7 days of resubmersion (0.5 log MPN/g) (Fig. 2D). Mean *tdh*+ and *trh*+ levels after 14 days of resubmersion (0.1 and 0.2 log MPN/g, respectively) were not significantly different ($P > 0.1$) compared with background levels (−0.4 and 0.5 log MPN/g, respectively) (Fig. 2C and 2D, respectively).

During 24-h dry storage, pathogenic Vp levels showed similar trends as seen for total Vp levels, with significant increases in levels for both *tdh*+ and *trh*+ ($P < 0.001$; from 0.0 to 2.2 and from −0.2 to 2.5 log MPN/g, respectively) (Fig. 1C and 1D). Significant reductions in *tdh*+ and *trh*+ levels in these oysters were seen after 7 days of resubmersion ($P < 0.001$; 0.9 and 1.0 log MPN/g, respectively). However, *tdh*+ and *trh*+ levels in 7-day resubmerged oysters were also significantly different from levels in concurrent background oysters ($P < 0.05$; 0.06 and 0.2 log MPN/g, respectively) and were significantly different from levels at initial harvest ($P < 0.05$). After 14 days of resubmersion, *tdh*+ and *trh*+ levels (0.1 and 0.4 log MPN/g, respectively) were not statistically different from those measured in concurrent background samples ($P > 0.1$; −0.4 and 0.6 log MPN/g, respectively) or oysters at initial harvest ($P > 0.1$) (Fig. 3C and 3D, respectively).

In 2003, Kaufman et al. *(25)* reported a singular ''hot'' oyster with a concentration of *tdh*+ Vp that was 10 times greater than any other oysters collected at the same time and same area, but this occurrence was determined to be rare when using a pooled sample of multiple oysters. They also reported a standard deviation among oysters sampled to be 0.4 to 0.6 log CFU/g *(25)*. In this study, we estimated standard deviations ranging from 0.5 to 0.9 log MPN/g throughout all experiments. The variability of *tdh*+ and *trh*+ Vp levels was greater than those of total Vp, which may explain why some of these results appear incongruent with the total Vp data.

In summary, throughout this study we observed vibrio levels in oysters, using handling practices similar to those in an approved aquaculture setting. Under the conditions of this study, total Vv and Vp levels increased significantly during dry storage, as expected. In most cases, these levels were observed to return to levels similar to those measured at initial harvest and in concurrent background samples after 7 days of resubmersion. In all cases, *Vibrio* spp. levels after 14 days of resubmersion, including pathogenic Vp, were similar to initial harvest and background levels. The levels reported are representative of the simulated aquaculture handling practices in this experiment, but they may not be representative of oyster handling using equipment or techniques not employed in this study. With these understood limitations, these data support the resubmersion of shellstock oysters after ambient air dry storage as a viable method to reduce Vv and total Vp that proliferated during dry storage. However, additional evidence, such as further experimentation in an approved aquaculture setting, may be required for these data to be used in development of best management practices for oyster aquaculture and handling.

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