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Effects of tumbling, refrigeration and subsequent resubmersion on the abundance of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in cultured oysters (*Crassostrea virginica*)

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ABSTRACT

Routine handling of oysters is a common industry practice for off-bottom oyster aquaculture, which aims to produce a high-quality oyster. These practices expose oysters to elevated temperatures and interrupt filter feeding, which can increase *Vibrio vulnificus* and *V. parahaemolyticus* levels within the oyster. The resubmersion of oysters after exposure to conditions where the time-temperature controls are exceeded is as an effective mitigation strategy to allow elevated levels of *Vibrio* spp. to "recover", or return to ambient levels, prior to harvest. Previous work examined the effect of desiccation on recovery times; the objective of this study was to evaluate the effect of additional handling treatments [tumbled and refrigerated (TR), tumbled and not refrigerated (TNR), not tumbled and refrigerated (NTR), and not tumbled and not refrigerated (NTNR)] on the time needed for *V. vulnificus,* total *V. parahaemolyticus,* and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) to recover in oysters. A set of non-treated (control) oysters remained submerged throughout the study to determine the ambient *Vibrio* spp. (inclusive of genotypes) levels within oysters. *Vibrio* spp. levels were measured immediately before (pre) and after (post) the treatments, and 1, 2, 4, 7, 10, and 14 days after resubmersion using a three-tube MPN real-time PCR method. The non-refrigerated oysters (TNR, NTNR) had *Vibrio* spp. levels 1.54 to 2.10 log MPN/g higher than the pre-treatment levels, while the *Vibrio* spp. levels in refrigerated oysters were not significantly higher than pre-treatment levels. After resubmersion, *Vibrio* spp. levels increased by 0.84 to 1.78 log MPN/g in the refrigerated oysters (TR, NTR). *Vibrio* spp. levels in oysters returned to ambient after 1–7 days of resubmersion, depending on the handling treatment and the *Vibrio* spp. These results provide data on handling treatments not previously reported and further support the seven-day resubmersion requirement for farmers in Alabama using the adjustable longline system.

1. Introduction

Off-bottom oyster aquaculture has increased steadily over the past 10–12 years in the Gulf of Mexico (National Marine Fisheries Service, 2018). In Alabama, 22 commercial oyster aquaculture operations reported 1.92 million oysters harvested in 2018 (Grice and Walton, 2018). In off-bottom aquaculture, oysters are maintained in floating cages or suspended baskets, which protects oysters from predators and provides greater access to food, allowing for faster growth. The gear allows farmers to improve the quality of their oyster through various culture techniques, which aim to produce a deep-cupped oyster free of biofouling (Adams et al., 2011; Walton et al., 2013a). Common culture techniques involve the routine handling of oysters to produce a consistent product, including periodic desiccation (air drying) of oysters to reduce biofouling, tumbling through a mechanical grader to improve shell shape, and grading and sorting of oysters by hand (Grodeska et al., 2017; Mizuta and Wikfors, 2019; Walton et al., 2013a).

While routine handling of oysters is beneficial for farmers, there is concern about how routine handling prior to harvest could affect *Vibrio* spp. levels within the oysters and associated risks to consumers. *Vibrio vulnificus* and *V. parahaemolyticus* are human pathogenic bacteria that are ubiquitous in estuarine waters and can be concentrated within the oyster during the filter feeding process (Drake et al., 2007; Oliver, 2013). Both *V. vulnificus* and *V. parahaemolyticus* infections are contracted from consuming raw or undercooked seafood or through contact

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with an open wound, with *V. vulnificus* causing primary septicemia and potentially fatal wound infections, and *V. parahaemolyticus* causing gastroenteritis and wound infections (Drake et al., 2007; Jones and Oliver, 2009). While *V. vulnificus* cases are relatively infrequent and mainly occur in patients who are immunocompromised, they have the highest case fatality rate of any foodborne pathogen and are responsible for 95% of all seafood related deaths (Jones and Oliver, 2009). *V. parahaemolyticus* infections are more common than *V. vulnificus,* accounting for 48% of vibriosis (Centers for Disease Control and Prevention, 2016). During routine handling practices described above, oysters are removed from the water for extended periods of time and exposed to higher ambient air temperatures, creating conditions that are conducive for the growth of *Vibrio* spp. within the oysters (Cook, 1994; Cook and Ruple, 1989; DaSilva et al., 2012; Gooch et al., 2002; Parveen et al., 2013). Farmers can resubmerse oysters after handling, allowing the oysters to resume filter feeding and purge the elevated levels of *Vibrio* bacteria, thus returning to the ambient *Vibrio* spp. levels in non-handled oysters (Grodeska et al., 2017, 2019; Jones et al., 2016; Kinsey et al., 2015). In the end, the practice of resubmersion allows oyster farmers to produce a high-value product for the half-shell market, while minimizing public health risks introduced through routine handling.

The resubmersion of temperature-abused oysters is an effective mitigation strategy for recovery from elevated *Vibrio* spp. levels after the desiccation of oysters (Grodeska et al., 2017, 2019; Kinsey et al., 2015). However, previous studies have only focused on desiccating, or air-drying, oysters for up to 27 h and determining the time needed for elevated *Vibrio* spp. levels to "recover", or return to ambient levels. These previous studies resulted in the reduction of regulatory resubmersion times from 14 days to 7 days for some aquaculture operations in Alabama (i.e., adjustable long-line systems with 100–120 oysters per basket), but farmers who use routine handling practices other than desiccation or freshwater rinsing followed by desiccation still require 14 days of resubmersion (Alabama Seafood Marketing Commission, 2020). What remains unclear is how additional handling, such as tumbling through a mechanical grader, may affect oysters resuming filter feeding once they are returned to the water. Tumbling oysters through a rotating mechanical grader (similar to a rock tumbler) allows for improved shell shape but subjects the oyster to rough handling while out of the water, potentially causing additional stress that could affect the purging of elevated *Vibrio* spp. after resubmersion. Additionally, refrigerating oysters overnight following handling has been suggested to reduce the recovery time. Refrigeration can be used to prevent the growth of *V. vulnificus* and *V. parahaemolyticus* in post-harvest oysters (Cook, 1997; Gooch et al., 2002), but research on the potential effects of refrigeration on resubmersed oysters is limited. Walton et al. (2013b) demonstrated that temperature-abused oysters that were shipped and refrigerated prior to being transplanted to a different growing area experienced an initial spike in *Vibrio* spp. levels after being resubmersed, before recovering after 14 days of resubmersion. Similarly, a study in New Jersey showed that containerized oysters that were refrigerated overnight and then resubmersed in the water prior to harvest experienced increases in *Vibrio* spp. levels after one day of resubmersion, but the *Vibrio* levels recovered after two days of resubmersion (Jones et al., 2016). Therefore, additional research is needed to determine if refrigerating oysters during routine handling to prevent significant increases in *Vibrio* spp. levels could reduce the recovery time after resubmersion.

The goal of this research was to determine the effects of four different tumbling and refrigeration combinations (tumbled and refrigerated, tumbled and not refrigerated, not tumbled and refrigerated, and not tumbled and not refrigerated) on the levels of *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) after treatment and over time following resubmersion. By monitoring the levels of *Vibrio* spp. over time relative to a non-treated control sample, the resubmersion time required for elevated *Vibrio* spp. levels to return to ambient levels within oysters was determined for each treatment type. Results from this study will contribute to the existing knowledge about routine handling and resubmersion practices and provide public health officials data to make informed regulatory decisions.

2. Materials and methods

2.1. Field site and environmental monitoring

The field work for this study was performed at Auburn University's research farm site in Portersville Bay, Alabama (Mississippi Sound). Hatchery-spawned diploid oysters (*Crassostrea virginica*) were cultured on an adjustable longline system in BST bags (BST Oyster Supplies, Cowell, Australia). Prior to each trial, 100–120 oysters were stocked in BST bags and submersed at the farm for a minimum of two weeks (Kinsey et al., 2015). Water temperature and salinity were recorded hourly using an Aqua TROLL 600 multiparameter sonde (In-Situ, Fort Collins, Colorado). The hourly air temperatures during treatment (the time period when the oysters were out of the water) was collected from the Dauphin Island weather station at mymobilebay.com. Smart Button data loggers (ACR Systems Inc., British Columbia, Canada) were placed inside two oysters in each treatment to monitor the internal oyster temperatures every 2 min during the treatment period.

2.2. Treatments and sample collection

A total of five resubmersion trials were performed in 2016–2017 during the summer months (June–September; Table 1), when the risk of *Vibrio* infection is assumed to be the highest. Multiple trials were performed to increase the number of replicates while capturing variations in environmental conditions. In each trial, five treatments were tested: tumbled and refrigerated (TR), tumbled and not refrigerated (TNR), not tumbled and refrigerated (NTR), not tumbled and not refrigerated (NTNR), and a submersed control. Six replicate BST bags were randomly assigned to each treatment type per trial. The oysters in the submersed control treatment remained submerged at the farm site throughout each trial. The oysters from the handling treatments were

Table 1
Environmental data collected during the trials[a].

Trial	Air temp (°C)[b]	Water temp (°C)[c]	Salinity (PSU)[c,d]
I (Jul 10–25, 2016)	29.2 (26.4–30.8)^{A}	31.2 (30.2–32.4)^{A}	20.2 (15.9–21.2)^{A}
II (Aug 14–29, 2016)	28.0 (25.0–29.5)^{B}	31.3 (30.1–32.6)^{A}	18.7 (16.5–21.2)^{A,B}
III (Jun 18-Jul 3, 2017)	28.0 (26.2–30.0)^{C}	28.0 (26.4–29.4)^{B}	8.4 (5.7–10.3)^{C}
IV (Aug 13–28, 2017)	29.3 (27.0–31.7)^{D}	30.5 (28.9–31.9)^{A}	13.6 (9.0–15.4)^{D}
V (Sep 24-Oct 9, 2017)	25.7 (23.3–27.7)^{E}	27.1 (25.9–28.2)^{C}	17.3 (15.1–18.5)^{B}

[a] Means in the same column with different letters are significantly different ($p < 0.05$).
[b] Average air temperature during the treatment period, collected from mymobilebay.com from the Dauphin Island station.
[c] Average daily means, with ranges in parentheses.
[d] PSU, practical salinity units.

removed from the water and transported to the Auburn University Shellfish Laboratory (less than 1 h) for handling. For the tumbling treatment, oysters were removed from the bags, allowed to pass through the rotating mechanical grader once (~10 min), and then returned to the bags. The oysters that were not tumbled as part of their treatment remained in bags out of the water, exposed to ambient outdoor conditions. After the tumbling treatments were applied, the refrigerated oysters were placed in a walk-in cooler (0–4 °C) for 18 ± 2 h. The non-refrigerated oysters remained in their bags for 18 ± 2 h and exposed to ambient outdoor conditions, equivalent to an overnight desiccation. Following the refrigeration period, the bags from all four handling treatments were returned to the farm site and resubmersed in the water within 24 ± 2 h of removal.

To examine the levels of *Vibrio* spp. over time, triplicate oyster samples (15 animals/sample) were collected from the replicate bags of each treatment type at multiple time points. Initially, three samples were taken from the submersed control oysters prior to any treatment (pre-treatment). Then, three samples were taken from each of the four treatment types and submersed control after the handling treatments were applied but immediately prior to resubmersion (post-treatment), and 1, 2, 4, 7, 10, and 14 days after resubmersion. Oysters were gathered from the respective bags at the farm, placed into a cooler with ice packs, and transported to the Food and Drug Administration's Gulf Coast Seafood Laboratory for analysis.

2.3. MPN and real-time PCR

Oyster samples were processed according to the three-tube most-probable-number (MPN) method adopted by the National Shellfish Sanitation Program and in the FDA's *Bacteriological Analytical Manual* (Kaysner et al., 2004; National Shellfish Sanitation Program, 2017). Oysters were rinsed under cold tap water with a sterile brush, aseptically shucked into a sterile blender, and blended for 90 s. The oyster homogenate was serially diluted 10-fold to 1:100,000 in phosphate-buffered saline (PBS; 7.65 g NaCl, 0.724 g Na_2HPO_4 [anhydrous], 0.21 g KH_2PO_4 in 1 L distilled H_2O, pH 7.4), and inoculated in triplicate into alkaline peptone water (APW; 10 g Bacto Peptone, 10 g NaCl, 1 L distilled H_2O, pH 8.5 ± 0.2). The MPN tubes were incubated for 18–24 h at 35 ± 2 °C, and then examined for turbidity. Crude DNA extracts were prepared for all tubes positive for bacterial growth by heating a 1 mL aliquot to 95 °C for 10 min, which were cooled on ice, or immediately frozen, and stored in a manual defrost freezer (−20 ± 5 °C) until analysis. Prior to testing by real-time PCR, extracts were thawed completely and centrifuged at 12,500 xg for 2 min. The resultant supernatants were tested for the presence of *V. vulnificus*, total *V. parahaemolyticus* (*tlh*), and pathogenic *V. parahaemolyticus* (*tdh/trh*) using the real-time PCR assays previously described (Kinsey et al., 2015). Levels of each *Vibrio* spp. were determined using a standard MPN table (Blodgett, 2010).

2.4. Statistical analysis

The water temperature and salinity data that were collected for the duration of each trial were used to calculate the average daily mean, minimum, and maximum. Similarly, the air temperatures during the 24 h treatment period were used to calculate the mean, minimum, and maximum air temperature. A general linear model was used to determine any statistical differences in average daily means among the trials. Similarly, for each *Vibrio* spp., a general linear model was used to compare *Vibrio* spp. levels in the submersed control oysters among trials. The internal oyster temperature data was averaged across the five trials to report a mean and range for each treatment type.

The *Vibrio* spp. levels, reported as MPN/g of oyster homogenate, were log transformed to normalize the data. In cases where *tdh*+ and *trh*+ levels were below the limit of detection (0.3 MPN/g), half of the limit of detection value was substituted prior to the log transformation. General linear models were used to compare *Vibrio* spp. levels between the pre-treatment and post-treatment time points to determine if the treatments elevated *Vibrio* spp. levels. Additionally, general linear models were used to determine the effects of tumbling and refrigeration on *Vibrio* spp. levels for the treatments only (i.e., pre-treatment levels were left out), to test for interactions among those variables. For these analyses, the data from the five trials were pooled. All *Vibrio* spp. data is reported as log MPN/g ± 95% confidence interval.

The resubmersion times required for the elevated *Vibrio* spp. levels to return to ambient levels were determined in two ways. First, the five trials were analyzed separately using general linear models to determine the effects of treatment and days since resubmersion, as well as the interaction between the two variables, on *Vibrio* spp. levels. Then, the data from the five trials were pooled and a similar linear mixed effects model was performed, but a random effect of trial was included in this mixed effects model to account for any between-trial variation. For both analyses, if a significant interaction between treatment and days since resubmersion was detected, individual models were performed for each time point to determine the minimum recovery time for each *Vibrio* spp. *Vibrio* spp. levels within the treated oysters were considered “recovered” when the treatment levels were not significantly higher than the submersed control levels ($\alpha = 0.05$). All data analyses were performed in R Studio using the nlme package (Pinheiro et al., 2018; R Core Team, 2018).

3. Results

3.1. Environmental and control data

There were significant differences ($p < 0.05$) among trials for the three environmental parameters measured (Table 1). Trials III and V had significantly lower water temperatures than the other trials, but the water temperatures in all the trials were typical for this region in the summer months (Zimmerman et al., 2007). The average daily salinity showed greater variation, ranging from 8.4 PSU in Trial III to 20.2 PSU in Trial I; regardless, the observed salinity ranges were typical for *Vibrio* spp. (Drake et al., 2007). Similar to the variation in environmental conditions, there were significant differences in levels among the submersed control oysters between trials for *V. vulnificus* and total *V. parahaemolyticus*, with control oysters from Trials III and V having

Table 2
Vibrio spp. levels in submersed control oysters, by trial.

Trial	*V. vulnificus*[a]	Total *V. parahaemolyticus*[a]	Pathogenic V. parahaemolyticus (*tdh*+)[a]	Pathogenic V. parahaemolyticus (*trh*+)[a]
I	4.64 (± 0.60)^{AB}	4.44 (± 0.61)^{A}	−0.22 (± 0.64)^{A}	−0.03 (± 0.64)^{A}
II	4.35 (± 0.50)^{A}	4.02 (± 0.45)^{AB}	−0.42 (± 0.46)^{A}	−0.35 (± 0.49)^{A}
III	4.88 (± 0.49)^{B}	3.66 (± 0.96)^{B}	0.13 (± 1.41)^{A}	−0.26 (± 0.99)^{A}
IV	4.75 (± 0.42)^{AB}	4.27 (± 0.68)^{AB}	−0.31 (± 0.77)^{A}	−0.52 (± 0.43)^{A}
V	3.81 (± 1.00)^{C}	3.65 (± 1.08)^{B}	−0.43 (± 0.55)^{A}	−0.45 (± 0.57)^{A}

[a] Average *Vibrio* spp. levels, reported as mean log MPN/g (± standard deviation). Means in the same column with different letters are significantly different.

significantly lower total *V. parahaemolyticus* levels than the other trials, and control oysters from Trial III having higher *V. vulnificus* levels ($p \leq 0.03$). Levels of pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) were not significantly different in control oysters among trials ($p \geq 0.05$; Table 2).

During the treatment period, the average internal oyster temperature depended on the treatment type. The oysters in the TR and NTR treatments had an average internal temperature of 5.43 (range, 2.38–28.3 °C) and 5.60 °C (range, 2.69–29.1 °C), respectively. The oysters in the non-refrigerated treatments (TNR, NTNR), which were left exposed to ambient outdoor conditions, experienced an average internal temperature of 25.8 (range, 24.3–29.3 °C) and 25.6 °C (range, 24.2–28.4 °C). The temperatures were recorded for the entire treatment period (24 h), including the transport and handling time as well as the refrigeration or desiccation period, resulting in a larger temperature range for the refrigerated oysters. Despite the large temperature range, the internal temperatures of the refrigerated oysters decreased by 26.2 °C on average during refrigeration, as reflected by the lower average internal temperature.

3.2. Treatment effects on Vibrio vulnificus

In samples taken immediately after the treatments were applied, *V. vulnificus* levels in treated oysters were affected by tumbling and refrigeration, but with no significant interaction between these treatments ($p = 0.75$). Tumbling did not have a significant effect on *V. vulnificus* levels compared to pre-treatment levels ($p = 0.97$), while refrigeration did ($p < 0.01$). Prior to treatment, *V. vulnificus* levels in the submersed control oysters were 4.45 ± 0.36 log MPN/g. The *V. vulnificus* levels in non-refrigerated oysters increased by 1.53 ± 0.51 and 1.52 ± 0.51 log MPN/g from the pre-treatment levels for NTNR and TNR, respectively ($p < 0.01$). Conversely, the *V. vulnificus* levels in the refrigerated oysters increased by 0.45 ± 0.51 and 0.32 ± 0.51 log MPN/g for NTR and TR, respectively ($p \geq 0.08$), and did not statistically differ from the pre-treatment levels (Fig. 1A).

The individual trial models and the mixed effects model showed significant interactions between treatment and the days since resubmersion (Table S1; Fig. 2). Therefore, for both sets of analyses, individual models were performed at each time point to determine when the *V. vulnificus* levels recovered. Although the refrigeration treatments prevented significant increases prior to resubmersion, after one day of resubmersion *V. vulnificus* levels increased significantly (Fig. 2). The levels in the NTR and TR treatment oysters were 1.11 ± 0.37 and 1.09 ± 0.37 log MPN/g higher than the levels in the submersed control ($p < 0.01$). According to the mixed effects model, after four days of resubmersion, *V. vulnificus* levels were not significantly higher than the control levels ($p \geq 0.05$) in oysters from any treatment (Table 3). When the trials were analyzed separately, the recovery times for *V. vulnificus* varied from one to four days, depending on trial and treatment type (Table 4).

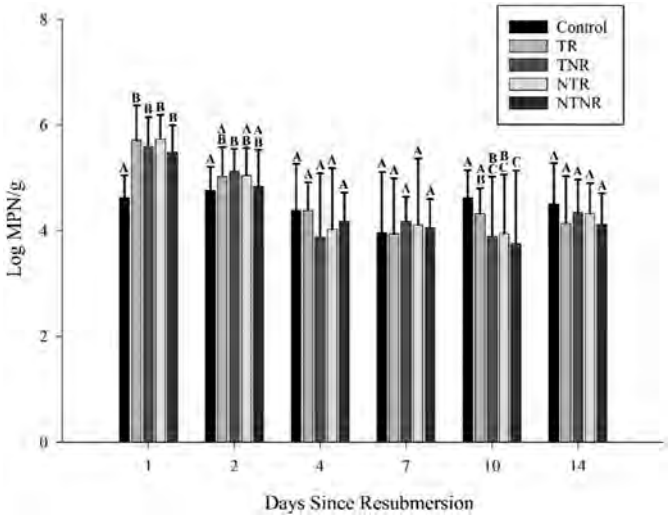

Fig. 2. Mean log-transformed *V. vulnificus* levels during the resubmersion period for the handling treatments: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days since resubmersion. Error bars represent standard deviation, and letters represent significant differences in *V. vulnificus* levels, as determined by the mixed effects model (n = 15).

3.3. Treatment effects on total Vibrio parahaemolyticus

Similar to the results for *V. vulnificus*, the effect of treatment

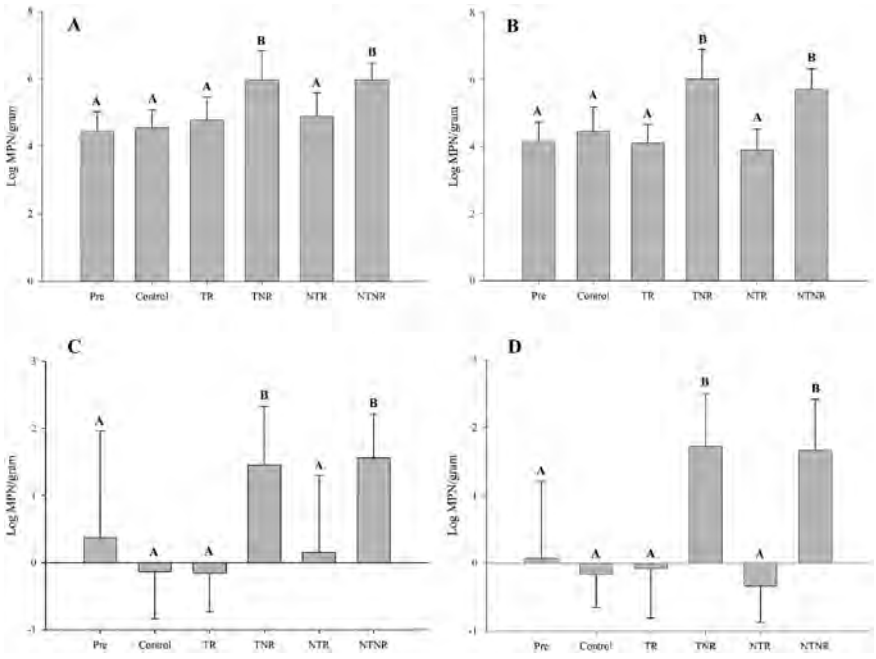

Fig. 1. Mean log-transformed *Vibrio* levels for *V. vulnificus* (A), total *V. parahaemolyticus* (B), pathogenic *V. parahaemolyticus* (*tdh*+) (C), and pathogenic *V. parahaemolyticus* (*trh*+) (D) before (Pre) and after the handling treatments were applied: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). Bars represent standard deviation, and letters represent significant differences in *Vibrio* levels, as determined by the mixed effects model ($n = 15$).

Table 3
Number of days for *Vibrio* spp. levels to return to control levels in oysters of each treatment, determined by mixed effects models.

Days[a]				
Vibrio spp.	TR[b]	TNR[c]	NTR[d]	NTNR[e]
V. vulnificus	2	4	2	2
Total V. parahaemolyticus	4	4	4	4
Pathogenic V. parahaemolyticus (tdh+)	7	7	7	7
Pathogenic V. parahaemolytics (trh+)	4	7	7	7

[a] Number of days after resubmersion when *Vibrio* spp. levels were not significantly higher than control levels ($p > 0.05$).
[b] Tumbled and refrigerated treatment.
[c] Tumbled and not refrigerated treatment.
[d] Not tumbled and refrigerated treatment.
[e] Not tumbled and not refrigerated treatment.

Table 4
Number of days for *Vibrio* spp. levels to return to control levels in oysters of each treatment determined by general linear models.

Trial	Day[a]				
	Vibrio spp.	TR[b]	TNR[c]	NTR[d]	NTNR[e]
I	V. vulnificus	1	2	2	2
	Total V. parahaemolyticus	1	2	2	2
	Pathogenic V. parahaemolyticus (tdh+)	1	2	1	1
	Pathogenic V. parahaemolyticus (trh+)	4	7	4	7
II	V. vulnificus	1	4	4	1
	Total V. parahaemolyticus	2	2	2	2
	Pathogenic V. parahaemolyticus (tdh+)	2	2	2	4
	Pathogenic V. parahaemolyticus (trh+)	2	1	4	4
III	V. vulnificus	2	2	2	4
	Total V. parahaemolyticus	2	7	7	7
	Pathogenic V. parahaemolyticus (tdh+)	1	4	7	4
	Pathogenic V. parahaemolyticus (trh+)	2	4	4	4
IV	V. vulnificus	2	2	2	2
	Total V. parahaemolyticus	2	2	2	2
	Pathogenic V. parahaemolyticus (tdh+)	2	2	2	7
	Pathogenic V. parahaemolyticus (trh+)	4	1	4	4
V	V. vulnificus	2	1	2	1
	Total V. parahaemolyticus	2	4	2	2
	Pathogenic V. parahaemolyticus (tdh+)	2	2	2	2
	Pathogenic V. parahaemolyticus (trh+)	2	2	2	2

[a] Number of days after resubmersion when *Vibrio* spp. levels were not significantly higher than control levels ($p > 0.05$), as determined by the individual models for each trial.
[b] Tumbled and refrigerated treatment.
[c] Tumbled and not refrigerated treatment.
[d] Not tumbled and refrigerated treatment.
[e] Not tumbled and not refrigerated treatment.

depended on the treatment type, and no interactions between tumbling and refrigeration were observed ($p = 0.78$). Tumbling did not have a significant effect on *V. parahaemolyticus* levels in oysters ($p = 0.23$), but refrigeration did ($p < 0.01$). Before treatments were applied, the mean *V. parahaemolyticus* level in the submersed control oysters was 4.17 ± 0.35 log MPN/g. The *V. parahaemolyticus* levels in the non-refrigerated oysters increased from the pre-treatment levels by 1.54 ± 0.49 and 1.85 ± 0.49 log MPN/g for NTNR and TNR, respectively ($p < 0.01$). On the other hand, the refrigeration treatments resulted in slightly decreased *V. parahaemolyticus* levels from the pre-treatment levels, with insignificant decreases of 0.27 ± 0.49 and 0.06 ± 0.49 log MPN/g for NTR and TR, respectively ($p \geq 0.27$; Fig. 1B).

Both sets of models showed a significant interaction between treatment and days since resubmersion, similar to the results from *V. vulnificus* (Table S1). Therefore, the results for *V. parahaemolyticus* were analyzed in the same manner as the results for *V. vulnificus*. After one

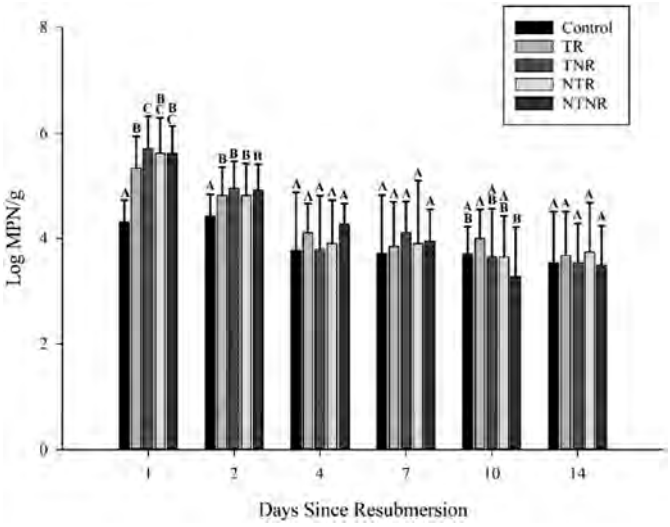

Fig. 3. Mean log-transformed total *V. parahaemolyticus* levels during the resubmersion period for the handling treatments: Control (submersed control, TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days since resubmersion. Bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* levels, as determined by the mixed effects model (n = 15).

day of resubmersion, the treated oysters had *V. parahaemolyticus* levels significantly higher than in the submersed controls, with levels from 1.03 ± 0.32 log MPN/g for TR to 1.40 ± 0.32 log MPN/g for TNR higher than in the control ($p < 0.01$). Similar to *V. vulnificus*, the mixed effects models showed that the levels of *V. parahaemolyticus* in treated oysters were not significantly higher than the levels in submersed control oysters (Fig. 3) after four days of resubmersion ($p > 0.05$; Table 3). When the trials were analyzed separately, the recovery times for *V. parahaemolyticus* ranged from one to seven days, dependent on the trial and treatment type (Table 4).

3.4. Treatment effects on pathogenic Vibrio parahaemolyticus (tdh+/trh+)

While tumbling did not have a significant effect on the levels of *tdh+* or *trh+* in the treated oysters ($p \geq 0.74$), and the interaction between tumbling and refrigeration was not significant ($p \geq 0.59$), refrigeration did have a significant effect on levels ($p < 0.01$). Before treatment, the mean pathogenic *V. parahaemolyticus* levels in the submersed control oysters were 0.37 ± 0.55 and 0.07 ± 0.43 log MPN/g for *tdh+* and *trh+*, respectively. The *tdh+* levels in the NTNR and TNR oysters increased from pre-treatment levels by 1.19 ± 0.78 and 1.09 ± 0.78 log MPN/g, respectively ($p < 0.01$). The *trh+* levels in NTNR and TNR oysters increased by 1.60 ± 0.61 and 1.65 ± 0.61 log MPN/g, respectively ($p < 0.01$). Conversely, the *tdh+* and *trh+* levels in refrigerated oysters did not significantly increase from pre-treatment levels. The *tdh+* levels in NTR and TR decreased by 0.22 ± 0.78 and 0.53 ± 0.78 log MPN/g ($p \geq 0.16$), while *trh+* levels decreased by 0.41 ± 0.61 log MPN/g in NTR and 0.14 ± 0.61 log MPN/g in TR ($p \geq 0.16$; Fig. 1C-D).

With a similar significant interaction between treatment and days since resubmersion for both *tdh+* and *trh+* models (Table S1; Figs. 4–5), the same approach was used as with the other two *Vibrio* targets. The levels of both *tdh+* and *trh+* in the refrigerated oysters increased after one day of resubmersion (Fig. 4-5). The mixed effects model showed the pathogenic strains in treated oysters required a longer period of time to recover to submersed control levels than total *V. vulnificus* and *V. parahaemolyticus*. When the trials were analyzed together, all treated oysters had *tdh+* and *trh+* levels that were not

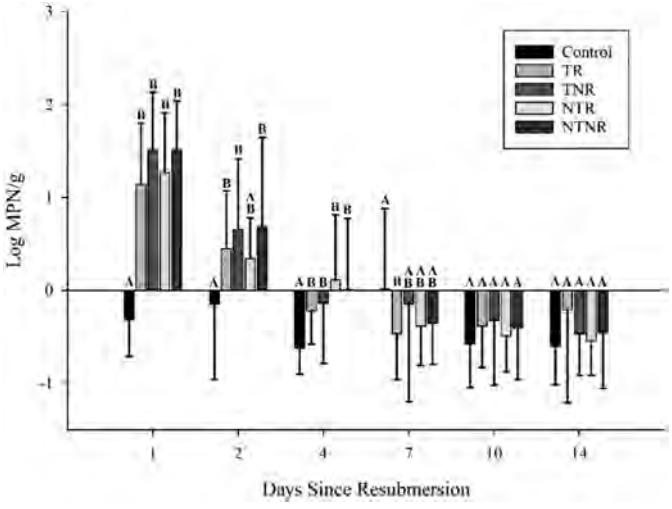

Fig. 4. Mean log-transformed pathogenic *V. parahaemolyticus* (*tdh*+) levels during the resubmersion period for the handling treatments: Control (submersed control, TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days since resubmersion. Bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* (*tdh*+) levels, as determined by the mixed effects model (n = 15).

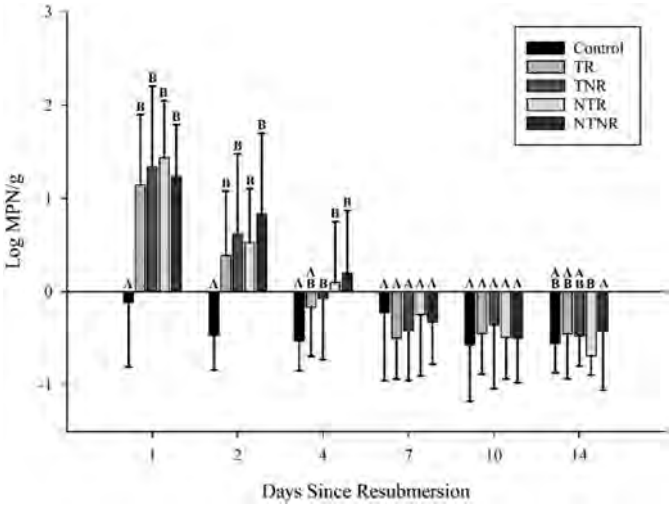

Fig. 5. Mean log-transformed pathogenic *V. parahaemolyticus* (*trh*+) levels during the resubmersion period for the handling treatments: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days since resubmersion. Bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* (*trh*+) levels, as determined by the mixed effects model (n = 15).

significantly higher than the control levels after seven days of resubmersion, with the exception of TR oysters, which were not significantly higher after four days of resubmersion (Table 3). In contrast, the individual trial analyses revealed that the treatment levels were not significantly higher than the control levels after one to seven days of resubmersion (Table 4).

4. Discussion

Farm-raised oysters were subjected to four different routine handling treatments, consisting of common farming techniques (tumbling, desiccation) and a technique not currently in routine use (refrigeration). These handling treatments resulted in elevated *Vibrio* spp. levels within the oysters either immediately post-treatment (non-refrigerated) or one day post-resubmersion (refrigerated). *V. vulnificus,* total *V. parahaemolyticus*, and pathogenic (*tdh*+/*trh*+) *V. parahaemolyticus* levels were monitored over a two-week period to determine the minimum recovery time needed for elevated *Vibrio* spp. levels to return to ambient levels for each handling treatment.

When cultured oysters are removed from the water for routine handling, the storage temperature can affect how the *Vibrio* spp. levels change during that time. In this study, the non-refrigerated oysters stored at ambient outdoor temperatures during handling were subjected to conditions that were conducive for *Vibrio* spp. growth, as demonstrated by the significant increases in *Vibrio* spp. levels in the non-refrigerated oysters (Cook, 1994, 1997; Cook and Ruple, 1989; DaSilva et al., 2012; Gooch et al., 2002; Kaspar and Tamplin, 1993; Parveen et al., 2013). *Vibrio* spp. significantly increased in the non-refrigerated oysters, consistent with previous studies that exposed oysters to ambient conditions for 24 h during a routine handling practice (Grodeska et al., 2017, 2019; Kinsey et al., 2015). These significant increases in *Vibrio* spp. levels confirm that an increased public health risk is inherently imposed on the oysters when they are removed from the water for routine handling.

Prior to resubmersion, the refrigerated oysters experienced less than a 0.50 log MPN/g increase for *V. vulnificus*, while the total and pathogenic *V. parahaemolyticus* levels decreased up to 0.52 log MPN/g. Although the decrease in bacterial levels was non-significant compared to the controls, the addition of a refrigeration treatment prior to resubmersion was successful at preventing increases in *Vibrio* spp. levels, as seen previously (Cook, 1994, 1997; Cook and Ruple, 1989; DaSilva et al., 2012; Gooch et al., 2002; Kaspar and Tamplin, 1993; Parveen et al., 2013). *Vibrio* spp. levels increased in refrigerated oysters after they were placed back in the water, similar to previous studies (Jones et al., 2016; Walton et al., 2013b). The change between the 0–4 °C cooler to the 27–31 °C water at the farm could have placed additional stress on the oysters, affecting how quickly the oysters resumed filtration once back in the water. It is hypothesized that the chilled oysters remained closed, allowing for the *Vibrio* spp. to increase in numbers while resubmersed in the warm water temperatures (Jones et al., 2016). Alternatively, the refrigerated oysters could have immediately resumed filter feeding upon resubmersion, but the increase in temperature could have caused the *Vibrio* spp. population to grow faster than it could be purged by the oyster. Regardless, refrigeration did not affect the overall recovery time, as all *Vibrio* spp. recovered between two and seven days in the refrigerated oysters, similar to the non-refrigerated oysters in this study, and as described previously (Grodeska et al., 2017, 2019; Jones et al., 2016).

Unlike refrigeration, rough handling in the form of tumbling did not have a significant effect on the *Vibrio* spp. levels after resubmersion. It was hypothesized that rough handling, in comparison to simply raising oysters out of the water for desiccation, could increase the stress on the oysters and negatively affect how quickly the oysters resumed filtration upon resubmersion. However, the results show that tumbling did not have any adverse effects, as the oysters from all treatment types recovered to ambient *Vibrio* spp. levels within seven days of resubmersion, with decreases in levels observed as early as one day.

To determine the minimum recovery times required for elevated levels to return to ambient levels, the data for the five trials were analyzed with two sets of models. The first statistical approach, like the approach used in Grodeska et al. (2017), examined the trials individually with a linear model to determine the appropriate recovery time. These analyses suggest shorter recovery times than the second statistical approach, with most of the treatments returning to ambient *Vibrio* spp. levels in two to four days, and some in as little as one day of resubmersion. The recovery times required in each trial (Table 4) were determined based on statistical significance (i.e. when the treatment levels were not significantly higher than the submersed control levels). The simple linear models, however, produced interesting results when

considering biological relevance, most notedly in the pathogenic *V. parahaemolyticus* results. For example, in Trial V, the statistical models show that the *trh*+ levels in TNR and NTNR oysters were not significantly different from the levels in the control oysters after two days of resubmersion. However, the *trh*+ levels in those oysters were ~1.4 log MPN/g higher than the *trh*+ levels in the control oysters. While the difference in *trh*+ levels was not statistically significant, this difference could be considered biologically relevant in terms of an increased public health risk (assuming an increase in levels corresponds to an increased likelihood of illness). In the absence of the feasibility of increasing replication within a study, and therefore increase the statistical power in the individual trial analyses, a more relaxed alpha (0.10–0.15) could be used to better identify these biologically relevant differences and reduce the likelihood of type II error.

The second statistical approach analyzed all five trials together in a mixed effects model with a random effect of trial to account for the between trial variation, possibly due to environmental differences among trials. When compared to the simpler models, the models with the random effect reduced the residual standard error for all *Vibrio* spp., explaining some of the variation as between-trial variation. A partial likelihood ratio test was used to compare the models, which produced significant results for all *Vibrio* spp., indicating that the mixed effects models are a better fit to the data (Table S2). The mixed effects models were more conservative than the first statistical approach, as the replication was higher (had more power and were better at detecting significant differences between *Vibrio* spp. levels in the treatment and control oysters that would also be considered biologically relevant. As a result, the recovery times for elevated *Vibrio* spp. levels to return to ambient levels were longer using the more conservative analysis but remained at seven days or fewer. Where differences in *trh*+ levels of ~1.4 log MPN/g were not significantly different in the first approach, they were significantly different in the second approach. However, the models detected significant differences that might not be considered biologically relevant, but more of a result of variability due to normal variability of *Vibrio* spp. within oysters and/or variation from the test method used (Kaufman et al., 2003; Kinsey et al., 2015; Zimmerman et al., 2007). On day 4, for example, the model showed that the total *V. parahaemolyticus* levels in the TR oysters were significantly higher (0.40 log MPN/g) than levels in the control oysters, but this difference may be explained by natural *Vibrio* spp. variability and/or methodological error. Therefore, we suggest that establishing a level of biological relevance for these types of studies may be appropriate. The biological relevance can be incorporated into study design along with additional factors (e.g., *Vibrio* spp., natural variability of *Vibrio* spp. in oysters, and methodological error) to identify the appropriate replication needed for adequate statistical power and confidence in the results.

This study examined the levels of pathogenic *V. parahaemolyticus* (*tdh*+ and *trh*+), as well as *V. vulnificus* and total *V. parahaemolyticus*. By examining all four *Vibrio* spp. targets concurrently, we note the differences in recovery times required for total *V. parahaemolyticus* in comparison to pathogenic *V. parahaemolyticus*. For all treatment types, total *V. parahaemolyticus* only required four days to return to ambient levels, while *tdh*+ and *trh*+ required up to seven days. The trend of higher variability in pathogenic *V. parahaemolyticus* levels and longer recovery times is similar to previous findings (Kinsey et al., 2015; Zimmerman et al., 2007), and could have resulted from the variations in environmental conditions among the trials. While the *tdh* and *trh* genes do not fully account for pathogenicity, they are the pathogenic markers used to make regulatory decisions and should, therefore, be taken into consideration for recovery times (National Shellfish Sanitation Program, 2017).

5. Conclusions

Regardless of the differences in resubmersion times observed across the *Vibrio* spp. and statistical analyses, a seven-day resubmersion period was sufficient for the recovery from elevated *Vibrio* spp. levels in oysters cultured on the adjustable longline system and subjected to the treatments under the given study conditions. The seven-day resubmersion period previously suggested by Grodeska et al. (2017) was limited to desiccation. In this study, a wider applicability of the seven-day resubmersion time to oysters (cultured on the adjustable long-line system) roughly handled and/or refrigerated prior to being resubmersed was demonstrated. The handling practices used in this study were representative of those that may be, or are currently, utilized by oyster farmers in the Gulf of Mexico; the resultant data may not be applicable to other routine handling practices, gear types, geographical regions, or environmental conditions. These results provide further evidence to support a seven day resubmersion period as a best management practice, as currently described for cultured oysters in Alabama.

CRediT authorship contribution statement

Victoria L. **Pruente**: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Original Draft, Visualization **Jessica** L. **Jones**: Conceptualization, Methodology, Resources, Writing – Review & Editing **Todd D. Steury**: Formal analysis, Writing – Review & Editing **William C. Walton**: Conceptualization, Methodology, Resources, Writing – Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108858.

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