Test of the effects of geography, gear type, and culture techniques on *Vibrio* risks associated with farm-raised oysters

by

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Abstract

During the off-bottom aquaculture growing process for the eastern oyster, *Crassostrea virginica*, farmers routinely remove the oysters for tumbling and desiccation. These routine handling practices improve oyster quality but may result in an increased public health risk from the elevated levels of *Vibrio vulnificus* and *V. parahaemolyticus* within the oysters. The oysters can be resubmersed in the water, allowing filter feeding to resume and for elevated *Vibrio* spp. levels to return to background, or ambient, levels normally found in oysters. This study investigated how the *Vibrio* spp. recovery times after resubmersion were affected by handling type (desiccation, tumbling, refrigeration), gear type (adjustable longline system, OysterGro® system), geography (Alabama, North Carolina), and time of year (May, July). The results indicate that 7 to 14 days of resubmersion is sufficient for the recovery of elevated *Vibrio* spp. in oysters from both states that were subjected to the same gear and handling types. The recovery times were similar in oysters that were tumbled and oysters that were desiccated, while the recovery times were similar between the gear types when the same handling treatment was applied to the oysters. The tumbled and refrigerated oysters required 14 days or more of resubmersion after handling, so this type of handling would be discouraged as a common industry practice. These data also suggest that the cooler month of May could require a longer resubmersion period than the summer months. Overall, the data can be used to guide public health agencies and inform future studies.

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ALS	Adjustable Longline System
APW	Alkaline Peptone Water
AUSL	Auburn University Shellfish Laboratory
FDA GCSL	Food & Drug Administration Gulf Coast Seafood Laboratory
MPN	Most Probable Number
NSSP	National Shellfish Sanitation Program
NTR	Not Tumbled and Refrigerated
NTNR	Not Tumbled and Not Refrigerated
OG	OysterGro® System
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PSU	Practical Salinity Unit
tdh+	Thermostable Direct Hemolysin
trh+	TDH-Related Hemolysin
TR	Tumbled and Refrigerated
TNR	Tumbled and Not Refrigerated

Chapter I. A Brief Introduction to Oyster Aquaculture and Seafood Safety

Eastern Oyster (*Crassostrea virginica*) Biology and Ecology

The eastern oyster, *Crassostrea virginica* (Gmelin), is an economically important species that belongs to the phylum Mollusca, class Bivalvia, order Ostreoida, and family Ostreidae. Each oyster consists of two asymmetrical valves (the left valve is thicker and more deeply cupped), a large visceral mass, two mantle skirts, a mantle cavity, an adductor muscles, a pair of gills, and a pair of labial palps (*16*, *57*). The valves are opened and closed by the adductor muscle, allowing for survival in suboptimal conditions or outside of the water for extended periods of time (*20*). They are broadcast spawners, releasing gametes into the water column for fertilization. After fertilization and hatching, the larvae are free-swimming and remain in this phase for 2-3 weeks. The larvae then undergo metamorphosis and settlement, where they permanently attach to a solid surface (i.e. oyster shells, rocks, hard surfaces), lose their velum and foot, and become juvenile oyster spat. The preferred surface for attachment is oyster shell, allowing oysters to naturally build large reefs in estuarine waters (*57*).

Eastern oysters are filter feeders, feeding on suspended phytoplankton and detritus (~1-30 µm in size) from the water column (*16*). The filtration rate is regulated by the movement of cilia on the gills, which can increase or decrease the movement of water into the oyster in response to several factors, like the amount and type of food available (*15*). The particles are sorted at the gills and passed to the labial palps, where desired particles (plankton, bacteria, organic matter) are sent to the digestive tract through the mouth, and rejected particles (sediment) are excreted as pseudofeces (*60*, *65*). Overall, their growth rate varies primarily based on the water temperature, salinity, intertidal

exposure, turbidity, and availability of food, with faster growth rates found in the warm, nutrient rich waters of the Gulf of Mexico than along the Atlantic Coast (*16, 57*).

The eastern oyster is native to estuarine waters along the Atlantic and Gulf of Mexico Coasts due to its ability to tolerate wide temperature and salinity ranges (*16, 57*). It can survive in freezing water temperatures and up to 42°C (optimal temperature range for growth: 20-30°C), and salinities ranging from 5-40 ppt (optimal salinity range for growth: 14-28 ppt) (*16, 57*). They can withstand suboptimal salinities because they are osmoconformers, allowing them to adjust their plasma osmolality in response to salinity changes. If suboptimal conditions persist, the oyster can close its valves tightly and switch to anaerobic respiration (*20, 35*). Historically, the natural abundance of oysters supported a thriving commercial fishery that harvested oysters as a valuable food product (*30, 57*).

Decline of Commercial Oyster Fisheries and Overview of Off-Bottom Oyster Aquaculture

In the Gulf of Mexico, oysters were traditionally harvested from natural oyster reefs and beds using tongs and dredges. However, years of overfishing, destructive harvesting techniques, disease, predation, and poor water quality have reduced the productivity of natural oyster reefs and the landings of wild oysters (*30, 33, 53*). To meet the increasing demands for oysters as a food product, oyster aquaculture developed as a new industry, with oysters initially being raised in on-bottom cages to mimic natural oyster reefs. Farmers have since shifted from on-bottom cages to off-bottom cages, allowing the oysters to be grown in areas where the water column can be leased. This

greatly expanded the range of available aquaculture sites, with farmers growing oysters in waters of varying depths, temperatures, salinities, and flow regimes (*63*). Additionally, the development of new gear types, breeding technology, and culture practices has transformed the oyster aquaculture industry into a thriving agricultural industry in the US. In fact, it was estimated that the off-bottom production of oysters in the US in 2015 was approximately 150-200 million oysters (*61*).

In off-bottom aquaculture, hatchery-reared single set oysters (referred to as seed) are raised in floating or suspended culture gear that holds the oysters in the water column. In doing so, the farmer provides protection from predators, eliminates burial in the sediment, and provides greater water flow to bring in food and remove waste products, ultimately increasing the growth and survival of the oysters (*63*). Additionally, culture practices have been developed to produce a more uniform, high quality product. Farmers will remove the oysters from the water for desiccation (air-drying), which prevents biofouling organisms from accumulating on the oysters and gear and eliminates predators such as oyster drills (*1*, *28*). The oysters may also be tumbled through a mechanical grader (Fig. 1.1) that breaks off fragile shell growth to produce a deeper-cupped oyster and sorts the oysters by size (*52*). In the end, the farmer intends to produce a high quality product that is destined for the half-shell market.

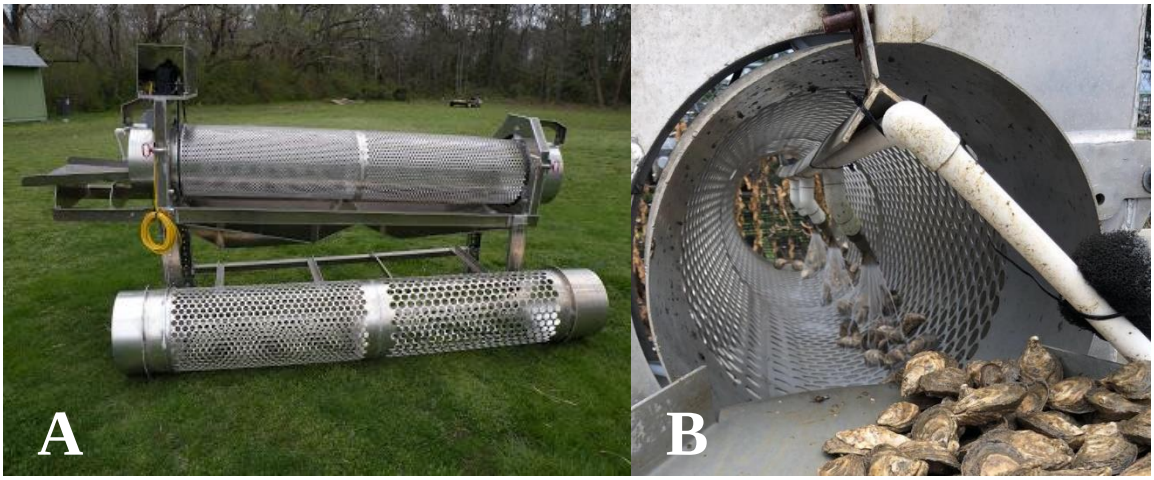

Figure 1.1. A) A mechanical grader (also referred to as a tumbler) with grading tubes. As the oysters are tumbled through the grading tube, they will be sorted by size as they fall through the two sizes of holes, or pass through to the end of the sorter. Photo credit: Chesapeake Bay Oyster Company. B) Inside view of the grading tube during operation. Cultured oysters are tumbled, graded, and washed as they pass through the tumbler.

In the Gulf of Mexico, the two most common gear types used by farmers are the Adjustable Longline System (ALS) and the OysterGro® system. The ALS is a system that originated in Australia, and consists of a longline that is tensioned between two pilings and secured in clips on PVC poles that are distributed along the longline (Fig. 1.2). The baskets are hung on the line that is placed in a middle clip, where the oysters are submersed in the water or in the feeding position. The line can be raised to higher clips for desiccation (Fig. 1.3), or lowered to lower clips for storm protection. Each basket can hold 100-120 market-sized oysters (*21*, *63*).

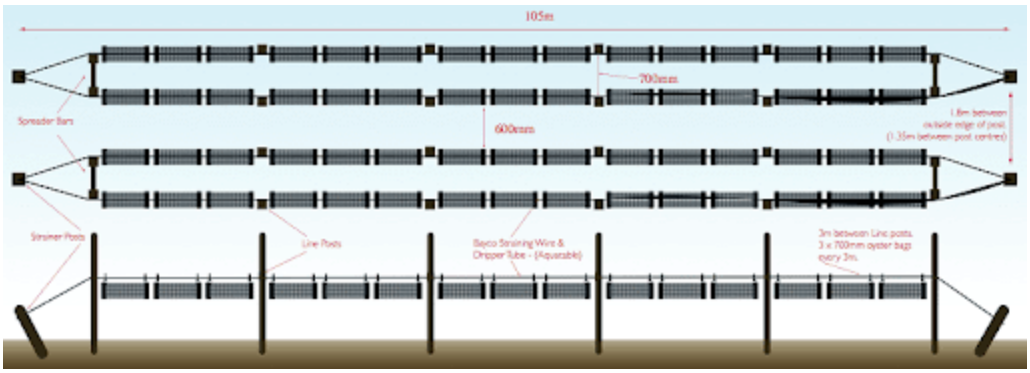

Figure 1.2. The Adjustable Longline System (ALS) used by oyster farmers. Photo Credit: BST Oyster Supplies.

Figure 1.3. The ALS at the Grand Bay Oyster Park Research Site. The baskets of the ALS can be desiccated by moving the longline up and securing it in a higher clip. Photo Credit: US Department of Agriculture.

The OysterGro® system is a floating cage system that consists of metal cage suspended in the water column with plastic air-filled pontoons (Fig. 1.4). Each cage can hold 2 to 6 mesh bags of oysters, with 150-200 market-sized oysters per bag. Normally, the cage is in the feeding position, with the bags of oyster held below the surface of the water. The cages can be flipped 180° into the air-drying or desiccating position, so that the pontoons are in the water and the oysters are exposed to the air for desiccation

(Fig. 1.5). Farmers can sink these cages to the bottom by filling the pontoons with water, in case of severe weather events like hurricanes (*21*, *63*).

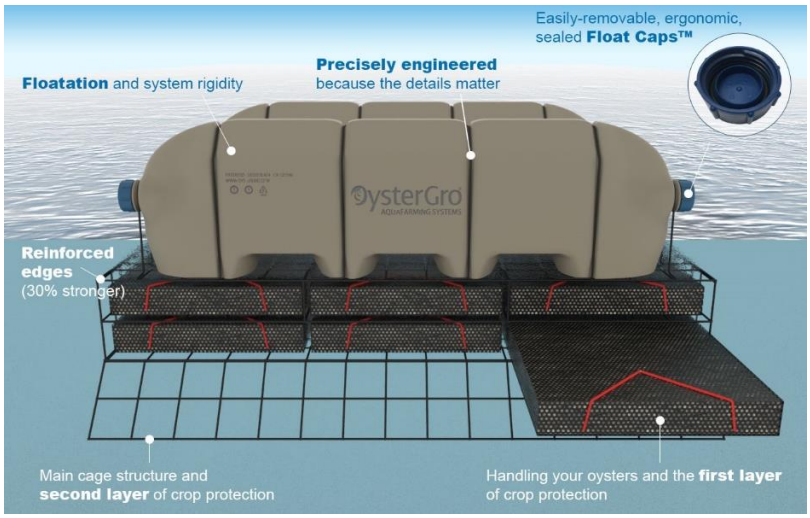

Figure 1.4. The OysterGro® floating cage system used by oyster farmers. Photo Credit: OysterGro®.

Figure 1.5. The OG cage system at the Cedar Island, North Carolina Research Site. The cages in the front are flipped up in the air-drying position, while the cages behind are down in the feeding position.

Oysters and Seafood Safety

While oysters can be prepared and served in many ways, they are commonly consumed raw (on the half shell) or undercooked (e.g., fried oysters), which may pose a public health risk. As filter feeding organisms, oysters entrap and concentrate microbes from the surrounding waters. As a result, the microbial concentration in oysters can be up to 100x greater than the concentration in the surrounding water (*13*). The microbial population in an oyster can consist of both indigenous species that are endemic to the waters (e.g. naturally occurring bacteria from the genus *Vibrio*), and non-indigenous species that are evidence of unnatural contaminants (e.g. fecal contaminants such as *Escherichia coli*) (*8, 32, 62*).

Unlike other foodborne pathogens, *Vibrio* spp. are endemic to estuarine waters and there are no means to prevent the introduction of these pathogens into oysters through water classifications. In fact, the incidence of *Vibrio* spp. infection in the US is increasing, with a 35% increase in infections from 2006 to 2015 (*5*). The two main species that commonly cause infection from raw oyster consumption are *Vibrio vulnificus* and *V. parahaemolyticus*. These species have been well studied in shellfish and estuarine waters, finding that concentrations are highest during the summer months and virtually 100% of oysters can carry one or both species (*40, 67*). This coincides with the highest number of infections occurring during the summer months (*6*). In order to mitigate this risk in the US, oysters that are harvested for raw consumption are under strict harvesting requirements that minimize the transport time from the harvest area to mechanical refrigeration, which prevents growth of *Vibrio* spp. (*9, 17, 41*).

Vibrio vulnificus

Vibrio vulnificus is a gram-negative, halophilic bacterium that was first identified in 1976 by the US Center for Disease Control and Prevention (*47*). It has been found on all coasts of the US, but thrives in warmer water temperatures with intermediate salinities of 5-25 ppt (*40*). The levels of *V. vulnificus* in water are positively correlated with water temperature, and this species is generally undetectable when water temperatures are below 15°C (*50*). When temperatures dip below this threshold, *V. vulnificus* is reported to enter a viable but not culturable (VBNC) state. The cells cannot be cultured on microbiological media but are still viable, and can be resuscitated when the water temperature increases above 15°C (*44*, *45*).

This species has several virulence factors that contribute to its pathogenicity in humans. The first is a polysaccharide capsule that protects it from phagocytosis by the host. The capsule is not present in all strains, but it has been demonstrated that the encapsulated strains cause infection in humans (*55*, *56*). Virulent strains are also known to produce lipopolysaccharide (LPS, or endotoxin), which produces the typical symptoms in humans that are associated with endotoxic shock (*36*). Other factors, such as the production of exoproteins, adhesion to human cell lines, and hydrophobicity have been thought to play a role in this species' pathogenesis (*14*, *38*). In addition to the virulence factors listed above, underlying health conditions (e.g. diabetes, liver disease, cancer) that result in immunodeficiency in the host also contribute to the incidence of *V. vulnificus* infections (*58*).

As a human pathogen, *V. vulnificus* is the number one cause of seafood-related deaths in the US, accounting for 95% of all seafood-related deaths (*46*). This species

causes three types of infections: gastroenteritis, primary septicemia, and wound infections. Both gastroenteritis and primary septicemia are almost always caused by the ingestion of raw or undercooked seafood, while wound infections are contracted from exposing open wounds to seawater (*25*, *46*). The cases of gastroenteritis are typically mild and not reported, while the most significant infections result in primary septicemia. Most of the primary septicemia cases occur in people with one or more underlying disease, and more than 80% of the cases occur in males over the age of 50. Conversely, underlying health conditions are not a risk factor for wound infections, as most cases occur in patients that do not have underlying health conditions. However, underlying health conditions do correlate to the cases where wound infections developed into primary septicemia (*46*).

Vibrio parahaemolyticus

Vibrio parahaemolyticus is a gram negative, halophilic bacterium that can be free-swimming or attached to a variety of surfaces in marine environments (*26*, *27*). It has been isolated from all coasts of the US, and densities in water and shellfish are positively correlated with water temperature (*10*, *13*). *V. parahaemolyticus* thrives in estuarine waters ranging from 15-25 ppt (*3*, *13*). It is thought that *V. parahaemolyticus* survives the winter in the sediment, and is reintroduced to the water column when temperatures rise (*26*). This species can be associated with outbreaks from seafood products, with significant outbreaks in the US having occurred in Maryland (1971), the Pacific Northwest (1997), Galveston Bay, Texas (1998), and the Northeast Atlantic Coast (2012) (*7*, *11*, *34*, *66*).

Not all strains of *V. parahaemolyticus* are truly pathogenic. The pathogenicity of *V. parahaemolyticus* has been correlated to the presence of either the *tdh* gene, that codes for the production of a thermostable direct hemolysin, and/or the *trh* gene, that codes for the production of a *tdh*-related hemolysin (*trh*). Both of these hemolysins have similar hemolytic activity in cells, binding to host cells and forming pores on the surface of the cell membrane (*22, 31, 37, 42, 54*). The *tdh* and *trh* genes are the most commonly used factors to determine pathogenicity, but recent studies have indicated the potential for additional factors contributing to virulence (*24, 49*). Other potential virulence factors include the urease production, adhesiveness adhesion to human cells, and other putative factors (*43, 48, 59*).

Typically, anyone is susceptible to a *V. parahaemolyticus* infection, making the occurrence of infection more common than *V. vulnificus* infections. Infections are most commonly contracted through eating raw or undercooked seafood, resulting in gastroenteritis or septicemia (*12, 39*). Gastroenteritis cases are the most common disease presentation, resulting in diarrhea, vomiting, headache, fever, and abdominal cramps that require hospital treatment in approximately 60% of cases (*51*). Only 5% of cases result in septicemia, which commonly occurs in immunocompromised patients. Similar to *V. vulnificus, V. parahaemolyticus* can also cause wound infections when open wounds are exposed to seawater (*12*).

Aquaculture Practices and *Vibrio* Risk

As mentioned previously, routine handling practices are an important part of off-bottom oyster aquaculture to improve product quality and consistency. Farmers will use

certain practices, like desiccation, weekly or biweekly during the growing process to combat biofouling organisms and predators. Other practices, like tumbling, are performed periodically (e.g., monthly or quarterly) to produce a deeper cupped oyster and to sort the oysters by size (*21*). While these practices are beneficial to the farmer, the oysters are removed from the water for extended periods of time (e.g., 24 hours). This violates any time-temperature harvesting window, exposes the oysters to higher air temperatures, and interrupts their filter feeding, producing the perfect environment for *Vibrio* spp. to proliferate within the oyster while its valves are closed. Previous studies have shown that the *Vibrio* spp. levels can increase by 1-2 log MPN/g within a 24-hour exposure period (*18, 19, 23, 29*).

Despite the increased public health risk that results from routine handling, resubmersion has been shown as an effective strategy to mitigate this imposed risk in cultured oysters. After the handling is complete, the oysters can be placed back into the water, or "resubmersed", to allow the oysters to resume filter feeding. In doing so, the oysters will purge the elevated levels of *Vibrio* spp. back to the ambient levels normally found in oysters. After the appropriate resubmersion period, the farmer can harvest the oysters within an appropriate time-temperature window for raw consumption (*18, 19, 23, 29, 64*).

In Alabama, the Alabama Department of Public Health required handled oysters to be resubmersed for a minimum of 14 days prior to harvest based on the findings from Kinsey *et al.* (*29*). While this study showed that resubmersion was effective, it used longline baskets suspended under a pier, not suspended on the adjustable longline system. Additionally, the longer resubmersion period allows the potential for biofouling

organisms to re-infest the oysters, the occurrence of a large rainfall event that results in harvest area closures due to high bacterial levels, or for the occurrence of a harmful algal bloom that would result in harvest area closures. In response to the concerns from farmers, a follow up study (*18*, *19*) examined how desiccation affects the recovery of elevated *Vibrio* spp., and found that seven days was sufficient for the recovery of elevated *Vibrio* spp. levels. Using these data, the Alabama resubmersion requirement was reduced from 14 to 7 days for oysters that were desiccated and maintained in the ALS system (*2*). However, oyster farmers using other gear or handling types are still required to observe a longer resubmersion period.

Knowledge Gaps

There are several factors that could potentially affect the recovery of elevated *Vibrio* spp. after resubmersion that are not fully understood. The first factor is handling type: the effect of desiccation has been previously studied (*18*, *19*), but other forms of handling, such as tumbling, have not. Tumbling oysters through a mechanical grader subjects the oyster to a much rougher form of handling than simply being raised out of the water to desiccate. Tumbling is a rougher form of handling that may place additional stress on the oysters (*4*), which could delay filter feeding after resubmersion, thus changing the recovery time. Additionally, the idea of adding an overnight refrigeration step while out of the water has been proposed as a way to prevent *Vibrio* spp. growth during handling (*9, 17*). While refrigeration is effective at preventing *Vibrio* spp. growth, the literature regarding the resubmersion of refrigerated oysters is limited and provides conflicting results (*23*, *64*).

Second, the type of culture gear could affect resubmersion periods. The reduction in the resubmersion requirement for Alabama farmers was restricted to those using the ALS system based on Grodeska *et al.* (*18, 19*). It is thought that the two gear types (ALS and OG systems) hold the oysters at different positions in the water column and are subject to different levels of wave action, which could affect the filter feeding activity of the oysters and, thus, the recovery times. Third, the time of year could play a role in determining resubmersion periods. The resubmersion of oysters has been studied between June and September, (*18, 19, 29*), when the *Vibrio* spp. levels are the highest in oysters and the risk of infection is thought to be highest. While these months are known to have the highest *Vibrio* infection rates, the risk is still present during other times of year, especially in the warmer waters of the Gulf of Mexico. Finally, resubmersion research has been geographically limited in scope, with most of the research performed in Alabama (*18, 19, 29, 64*), New Jersey, and Washington (*23*). While several states utilize the same culture gear and handling types, the environmental conditions and the *Vibrio* spp. populations in oysters may differ between the states and create differences in recovery times.

Research Aims and Objectives

As commercial fisheries continue to decline, off-bottom oyster aquaculture is becoming more prevalent across the US to satisfy the demand for oysters. The culture techniques used by the farmers create a high quality product that are intended to earn a higher profit on the half shell market. With most farmed oysters consumed raw, the success of this industry depends on the ability of the farmers to produce a safe product for

consumers. However, the knowledge gaps surrounding the resubmersion of oysters after routine handling prevents public health agencies from making informed regulations. In order to address these knowledge gaps, my research goal was to determine how geography, gear type, handling type, and time of year affect the recovery of elevated *Vibrio* spp. levels after resubmersion.

The first research objective determined the effects of tumbling and refrigeration on the levels of *Vibrio* spp. in oysters over time during resubmersion. Cultured oysters maintained in an ALS system were subjected to a series of tumbling and refrigeration treatments, then resubmersed at the farm site in Portersville Bay, Alabama. The levels of *Vibrio* spp. were measured over time, and the recovery time for each *Vibrio* spp. was determined. The goal of the second research objective was to determine the recovery times for oysters raised in different gear types and at different times of the year. The resubmersion of oysters in two common gear types (ALS and OG systems) was compared during the months of May and July in Grand Bay, Alabama to determine if the recovery of *Vibrio* spp. differed across the factors. Finally, the third research objective investigated the question of geographical variation in resubmersion times. Using similar experimental factors from the second objective, the *Vibrio* spp. recovery times were compared in oysters from Grand Bay, Alabama and Cedar Island, North Carolina. Through this series of resubmersion studies, we provide data that can be used to inform public health officials and guide future research for the industry.

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

Abstract

The routine handling of oysters is a common industry practice for off-bottom oyster aquaculture, which aims to produce a high-quality oyster. These practices may 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. 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 pre-treatment levels, while the *Vibrio* spp. levels in refrigerated oysters were not significantly higher than the 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 levels after 1-7 days of resubmersion, depending on the handling treatment and the *Vibrio* spp. The results of this study 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 (*21*). In Alabama, 22 commercial oyster aquaculture operations reported 1.92 million oysters harvested in 2018 (*11*). 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 (*1*, *27*). 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 (*12*, *20*, *27*).

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 (*9*, *23*). Both *V. vulnificus* and *V. parahaemolyticus* infections are contracted from consuming raw or undercooked seafood or through contact with an open wound, with *V. vulnificus* causing primary septicemia and potentially fatal wound infections, and *V. parahaemolyticus* causing gastroenteritis

and wound infections (*9*, *15*). 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 (*15*). *V. parahaemolyticus* infections are more common than *V. vulnificus,* accounting for 48% of vibriosis (*4*). 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 (*5*, *7*, *8*, *10*, *24*). 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 (*12*, *13*, *14*, *19*). 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 (*12*, *13*, *19*). However, previous studies have only focused on desiccating, or air-drying, oysters for up to 27 hours 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 (*2*).

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 (*6*, *10*), but research on the potential effects of refrigeration on resubmersed oysters is limited. Walton *et al.* (*28*) 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 (*14*). 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 (*19*). 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) were 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 two minutes 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 2.1), 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 removed from the water and transported to the Auburn University Shellfish Laboratory (~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 (0-4ºC) 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 separate 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* (*18*, *22*). 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 ± 2ºC, and then examined for turbidity. Crude DNA extracts were prepared for all tubes positive for bacterial growth by heating a 1 mL aliquot to 95ºC for 10 min, which were cooled on ice, or immediately frozen, and stored in a manual defrost freezer (-20 ± 5ºC) until analysis. Prior to testing by real-time PCR, extracts were thawed

completely and centrifuged at 12,500 x *g* 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 (*19*). Levels of each *Vibrio* spp. were determined using a standard MPN table (*3*).

2.4 Statistical Analysis.

An average daily mean, minimum, and maximum were calculated for the water temperature and salinity data. Similarly, the mean, minimum, and maximum air temperatures during the 24 h treatment period were calculated. 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, and a random effect of trial was included in the 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 (*25*, *26*). Figures were created in SigmaPlot Version 13.0 (Systat Software, San Jose, CA).

3. Results

3.1. Environmental and Control Data.

There were significant differences ($p < 0.05$) among trials for the three environmental parameters measured (Table 2.1). 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 (*29*). 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 salinities were typical for *Vibrio* spp. (*9*). 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*. The control oysters from Trials III and V had significantly lower total *V. parahaemolyticus* levels than the other trials, and control oysters from Trial III had 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 2.2).

Table 2.1. Environmental data collected during the trials[a].

Trials	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.

Table 2.2. *Vibrio* spp. levels in submersed control oysters, by trial[a].

Trials	*V. vulnificus*	Total *V. parahaemolyticus*	Pathogenic *V. parahaemolyticus* (*tdh+*)	Pathogenic *V. parahaemolyticus* (*trh+*)
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.

During the treatment period, the average internal oyster temperature depended on the treatment type. The refrigerated oysters (TR, NTR) had an average internal

temperature of 5.43 (range, 2.38-28.3ºC) and 5.60ºC (range, 2.69-29.1ºC), respectively. The non-refrigerated oysters (TNR, NTNR), which were left exposed to ambient outdoor conditions, experienced an average internal temperature of 25.8 (range, 24.3-29.3ºC) and 25.6ºC (range, 24.2-28.4ºC). 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 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 the post-treatment samples, the *V. vulnificus* levels in treated oysters were affected by refrigeration ($p < 0.001$), but with no significant interaction between tumbling and refrigeration ($p = 0.75$). Tumbling did not have a significant effect on *V. vulnificus* levels compared to pre-treatment levels ($p = 0.97$). 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. 2.1).

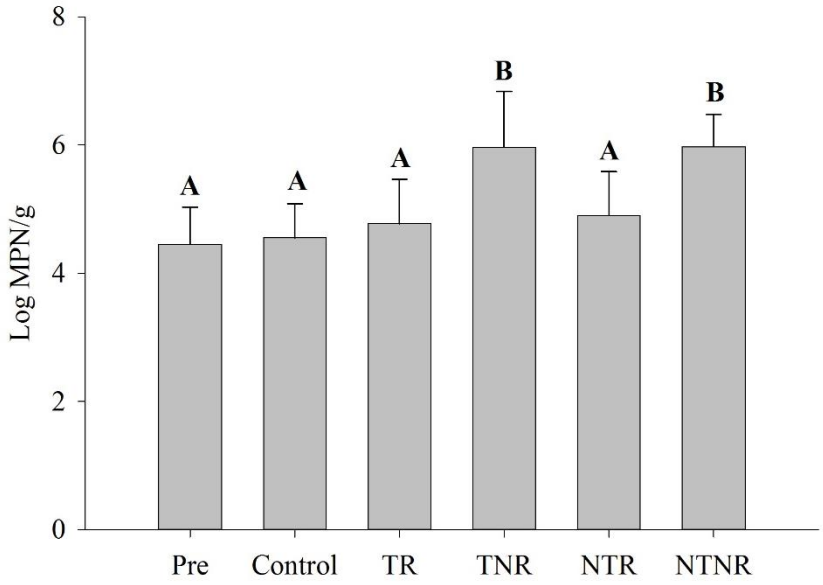

Figure 2.1. Mean log-transformed *V. vulnificus* levels before (Pre) and after the handling treatments were applied: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). Bars represent standard deviation, and letters represent significant differences in *V. vulnificus* levels, as determined by the mixed effects model (n=15).

The individual trial models and the mixed effects model showed significant interactions between treatment and the days since resubmersion (Table 2.3; Fig. 2.2). 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. 2.2). 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, *V. vulnificus* levels were not significantly higher than the control levels ($p \geq 0.05$) in all treated oysters after four days of resubmersion (Table 2.4). 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 2.5).

Table 2.3. Summary statistics from mixed effects models, by *Vibrio* spp.[a]

Vibrio spp.	Source	DF	F-Value	*p*-value
V. vulnificus	Treatment	4	1.41	0.23
	Time	**6**	**51.4**	**<0.0001**
	Treatment*Time	**24**	**4.39**	**<0.0001**
V. parahaemolyticus	**Treatment**	**4**	**9.32**	**<0.0001**
	Time	**6**	**64.8**	**<0.0001**
	Treatment*Time	**24**	**5.35**	**<0.0001**
Pathogenic *V. parahaemolyticus* (*tdh*+)	**Treatment**	**4**	**20.0**	**<0.0001**
	Time	**6**	**55.0**	**<0.0001**
	Treatment*Time	**24**	**5.55**	**<0.0001**
Pathogenic *V. parahaemolyticus* (*trh*+)	**Treatment**	**4**	**25.7**	**<0.0001**
	Time	**6**	**67.1**	**<0.0001**
	Treatment*Time	**24**	**7.71**	**<0.0001**

[a]Lines in bold represent significant effects ($\alpha = 0.05$).

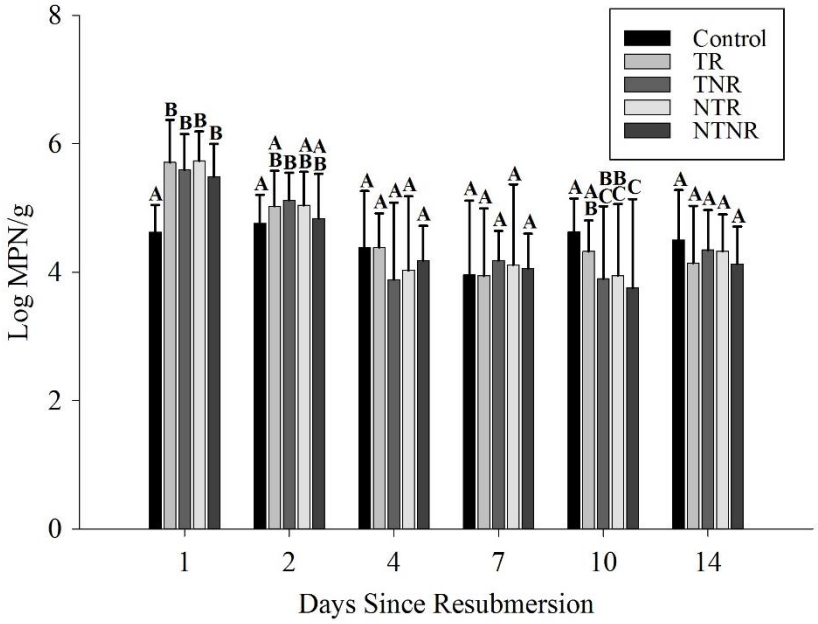

Figure 2.2. Mean log-transformed *V. vulnificus* levels during the resubmersion period for the handling treatments: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days 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).

Table 2.4. *Vibrio* spp. recovery times, as determined by the 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. parahaemolyticus* (*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 2.5. *Vibrio* spp. recovery times by trial, as determined by general linear models.

		Day[a]			
Trial	*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.

3.3. Treatment Effects on Total Vibrio parahaemolyticus.

Similar to the results for *V. vulnificus*, the effect of 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 ($p \geq 0.28$; Fig. 2.3).

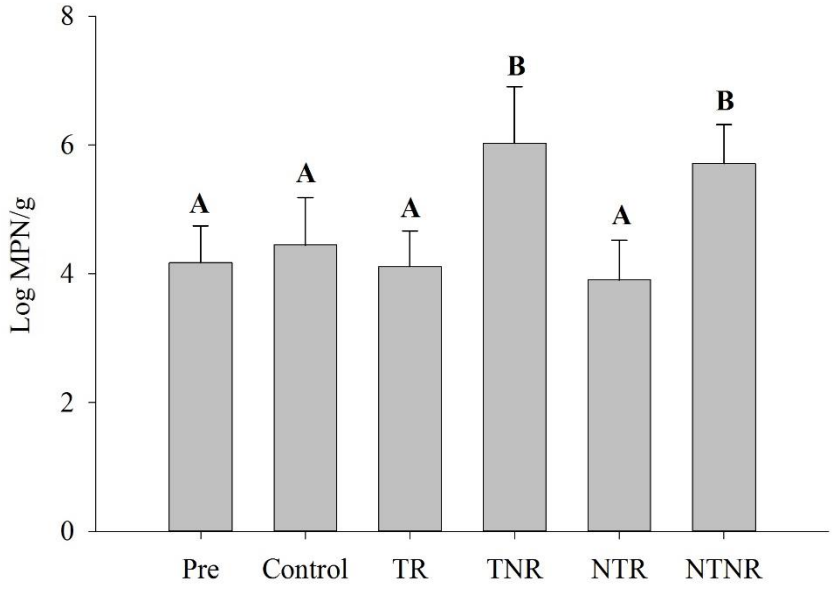

Figure 2.3. Mean log-transformed total *V. parahaemolyticus* levels before (Pre) and after the handling treatments were applied: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). Bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* levels, as determined by the mixed effects model (n=15).

Both sets of models showed a significant interaction between treatment and days since resubmersion, similar to the results from *V. vulnificus* (Table 2.3). Therefore, the results for *V. parahaemolyticus* were analyzed in the same manner as the results for *V. vulnificus*. After one day of resubmersion, the *V. parahaemolyticus* levels in treated oysters were significantly higher than in the submersed control oysters, 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. 2.4) after four days of resubmersion ($p > 0.05$; Table 2.4). 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 2.5).

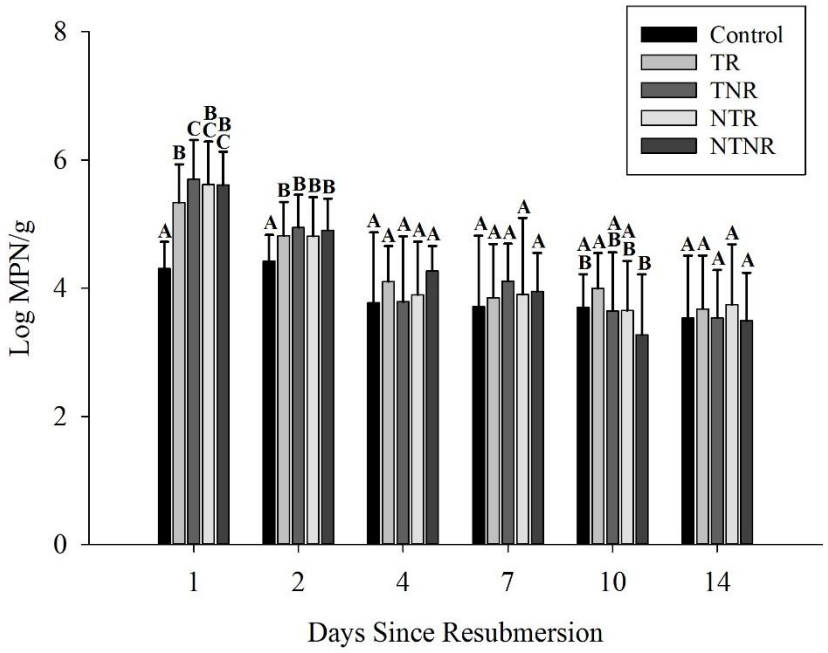

Figure 2.4. Mean log-transformed total *V. parahaemolyticus* levels during the resubmersion period for the handling treatments: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days since resubmersion. Error bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* levels, as determined by the mixed effects model (n=15).

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. 2.5-2.6).

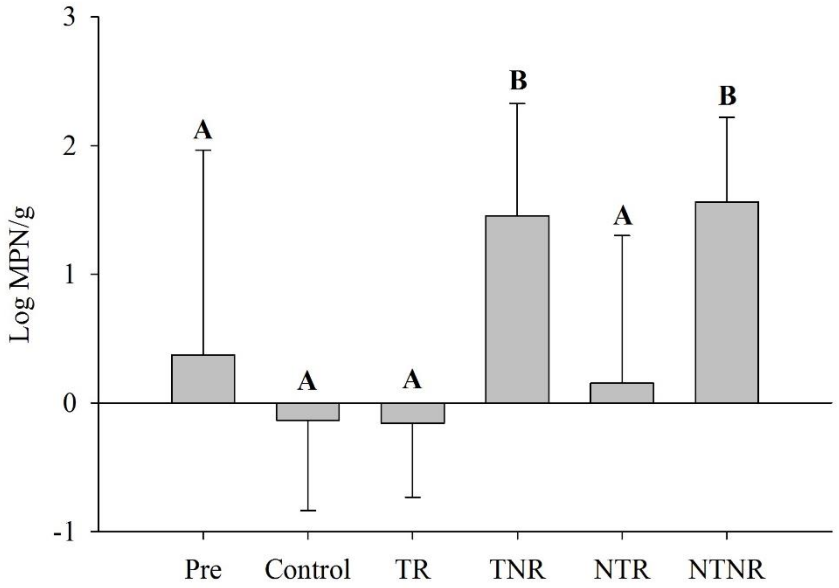

Figure 2.5. Mean log-transformed pathogenic *V. parahaemolyticus* (*tdh*+) levels before (Pre) and after the handling treatments were applied: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). Bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* (*tdh*+) levels, as determined by the mixed effects model (n=15).

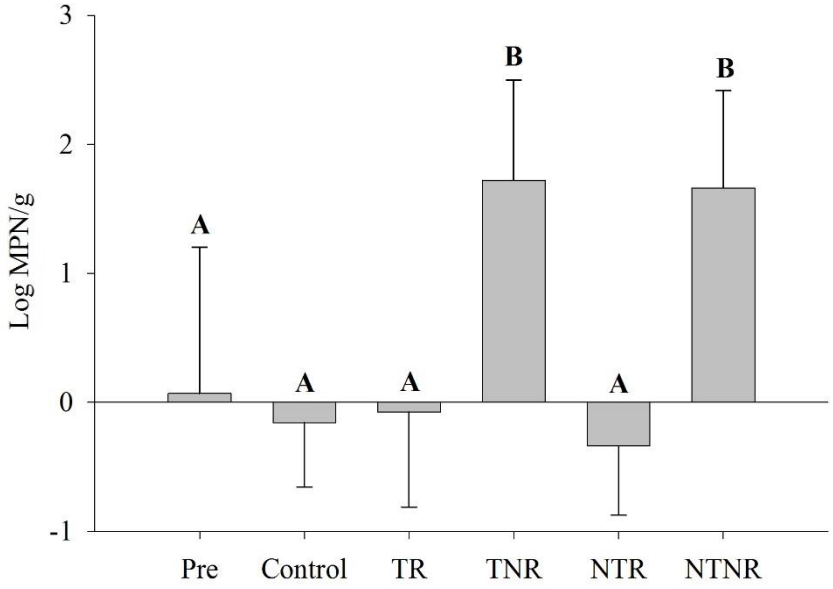

Figure 2.6. Mean log-transformed pathogenic *V. parahaemolyticus* (*trh*+) levels before (Pre) and after the handling treatments were applied: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). Bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* (*trh*+) levels, as determined by the mixed effects model (n=15).

With a similar significant interaction between treatment and days since resubmersion for both *tdh*+ and *trh*+ models (Table 2.3; Fig. 2.7-2.8), the same approach was used. The levels of both *tdh*+ and *trh*+ in the refrigerated oysters increased after one day of resubmersion (Fig. 2.7-2.8). The mixed effects model showed the pathogenic strains in treated oysters required a longer recovery time than *V. vulnificus* and total *V. parahaemolyticus*. When the trials were analyzed together, all treated oysters had *tdh*+ and *trh*+ levels that were not 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 2.4). 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 2.5).

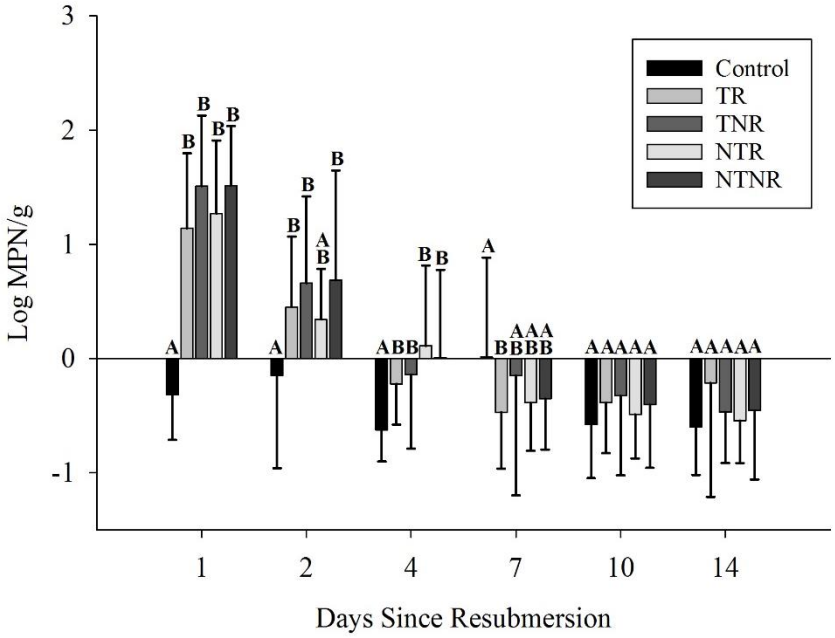

Figure 2.7. Mean log-transformed pathogenic *V. parahaemolyticus* (*tdh*+) levels during the resubmersion period for the handling treatments: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days since resubmersion. Error bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* (*tdh*+) levels, as determined by the mixed effects model (n=15).

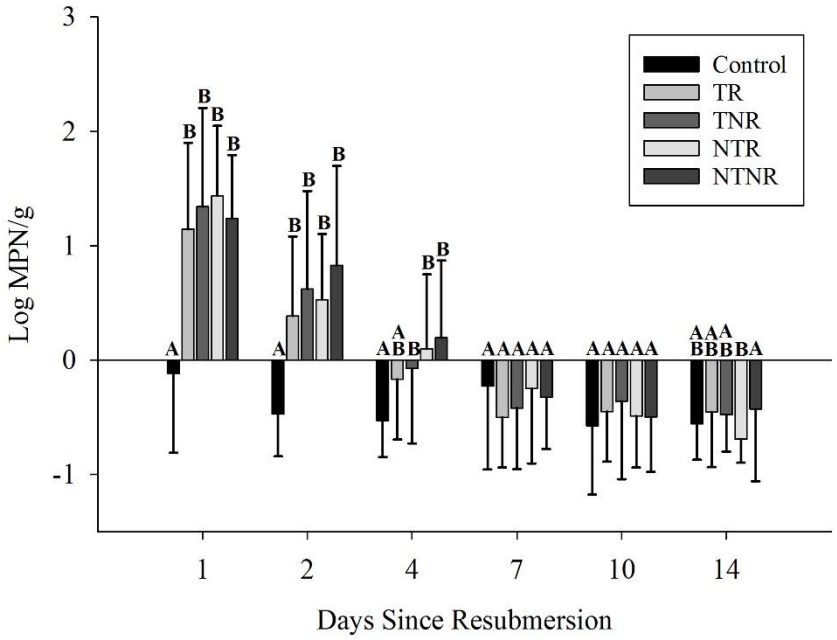

Figure 2.8. Mean log-transformed pathogenic *V. parahaemolyticus* (*trh*+) levels during the resubmersion period for the handling treatments: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, not refrigerated), NTR (not tumbled, refrigerated), NTNR (not tumbled, not refrigerated). The X-axis shows the days since resubmersion. Error bars represent standard deviation, and letters represent significant differences in *V. parahaemolyticus* (*trh*+) levels, as determined by the mixed effects model (n=15).

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*+ and *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 air temperatures during handling were subjected to conditions that were conducive for *Vibrio* spp. growth (*5*, *6*, *7*, *8*, *10*, *16*, *24*). *Vibrio* spp. significantly increased in the non-refrigerated oysters, consistent with previous studies that exposed oysters to ambient conditions during a routine handling practice (*12*, *13*, *19*). 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 control levels, the addition of a refrigeration treatment prior to resubmersion was successful at preventing significant increases in *Vibrio* spp. levels, as seen previously (*5*, *6*, *7*, *8*, *10*, *16*, *24*). *Vibrio* spp. levels increased in refrigerated oysters after they were placed back in the water, similar to previous studies (*14*, *28*). The change between the 0-4ºC cooler to the 27-31ºC 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 (*14*). 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 (*12*, *13*, *14*).

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.* (*12*), examined the trials individually with a linear model to determine the appropriate recovery time. These analyses suggest in 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 2.5) 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 trial, and therefore increasing 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 errors.

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 2.6). The mixed effects models were more conservative than the first statistical approach, as they 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 (*17*, *19*, *29*). 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. That level of difference 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.

Table 2.6. Model comparisons by *Vibrio* spp., with partial likelihood ratio test results[a]

Vibrio spp.	Simple Model	Mixed Effects Model		Partial Likelihood Ratio Test *p*-value
	Residual Standard Error	Error due to Trial	Residual Standard Error	
V. vulnificus	**0.8037**	**0.4191**	**0.7038**	**<0.0001**
Total *V. parahaemolyticus*	**0.7685**	**0.2714**	**0.7262**	**<0.0001**
Pathogenic *V. parahaemolyticus* (*tdh*+)	**0.6809**	**0.1299**	**0.6701**	**0.0025**
Pathogenic *V. parahaemolyticus* (*trh*+)	**0.6269**	**0.0997**	**0.6201**	**0.0191**

[a]Lines in bold represent that the mixed effects model is a significant improvement in fit over the simple linear model ($\alpha = 0.05$).

This study examined the levels of pathogenic *V. parahaemolyticus* (*tdh*+ and *trh*+), as well as *V. vulnificus* and *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 was previously found in Zimmerman *et al.* (*29*) and Kinsey *et al.* (*19*), 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 (*22*).

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.* (*12*) 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.

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Chapter III. Effects of gear type on *Vibrio* spp. levels in farm-raised oysters (*Crassostrea virginica*) after routine handling and resubmersion

Abstract

During routine handling, cultured oysters are removed from the water and exposed to elevated temperatures, causing growth of *Vibrio vulnificus* and *V. parahaemolyticus* within. Farmers can resubmerse oysters in the water, allowing elevated *Vibrio* spp. levels to return to ambient levels within the oysters. Previous resubmersion research is limited to one aquaculture gear type during studies performed from June-September. This study aims to expand existing knowledge on the recovery times needed for elevated *Vibrio* levels in handled oysters from two common gear types (adjustable longline system [ALS] and OysterGro system [OG]) during early and mid-summer periods. Oysters held in both gear types were subjected to being tumbled and refrigerated (TR) or desiccated, then resubmersed into water in May and July of 2018 and 2019. *Vibrio* spp. levels were measured before (pre) and after (post) the treatments, and 3, 7, and 14 days after resubmersion, and compared to levels in submersed oysters. All samples were tested for *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+). Water temperatures in May were significantly lower (~5ºC; $p \leq 0.009$) than in July, corresponding to lower *V. vulnificus* levels (-0.67 log MPN/g) and higher *tdh+/trh+* levels (+0.56-0.63 log MPN/g) in control oysters. The average *Vibrio* spp. levels in control oysters from each trial did not differ between the gear types ($p \geq 0.08$). Elevated *V. vulnificus* levels recovered to ambient levels after seven days in May and three days in July, regardless of gear or handling. For *V. parahaemolyticus*, the desiccated oysters required 14 days to recover in May, and 7 days in July, while the TR oysters required 14 days or more in both months. This study had limited replication in each month, but the data suggest that the resubmersion times differ

between the gear types, treatment types and months. Future studies with more replication are needed to determine if these trends continue.

1. Introduction

Off-bottom oyster aquaculture has expanded to multiple sites in the Gulf of Mexico to provide a higher quality oyster for the half-shell market and increase the value of oyster products. The two most common systems used in this region are the adjustable longline system (ALS), with baskets of oysters suspended from horizontal lines, and the floating cage system, with bags of oysters held in floating cages such as the OysterGro® system (OG) (*33*). Both gear types suspend oysters in the water column, allowing the shellfish greater access to food and protection from predators than on-bottom oysters, while providing farmers ease of access for routine handling (*32, 33*). Farmers routinely remove the oysters from the water to desiccate (air drying), tumble through a mechanical grader, and sort into size classes by hand to produce a deep-cupped oyster free of biofouling organisms (*18*, *26*). While routine handling produces a more consistent, high quality product, it may also create an increased public health risk regarding the *Vibrio* spp. naturally found within the oysters unless properly managed.

During filter feeding, oysters concentrate *Vibrio* spp. within their tissues, presenting a potential public health risk when consumed raw. The most common species associated with foodborne illness are *V. vulnificus* and *V. parahaemolyticus*, which are commonly contracted from consuming raw or undercooked shellfish (*14*, *22*). *V. vulnificus* infections are sporadic and tend to occur in patients with compromised immune systems, causing primary septicemia and mild gastroenteritis. *V. parahaemolyticus*

infections are more common and likely associated with outbreaks, resulting in gastroenteritis (*10*, *20*, *22*). *Vibrio* spp. infections mostly occur in warmer temperatures, with 85% of cases occurring between May and October (*17*, *20*). Routine handling practices are performed year-round, with an increase in handling during the summer months when biofouling organisms are more prevalent (*15*). When the oysters are removed from the water for handling, the filter feeding process is interrupted and the oysters are exposed to higher ambient air temperatures, creating ideal conditions for *Vibrio* spp. to multiply (*7*, *9*, *11*, *17*, *28*). Oysters can then be resubmersed in the water to resume filter feeding, and the elevated levels of *Vibrio* bacteria are purged from the oyster (*18*, *19*, *24*, *30*). After the *Vibrio* spp. levels have recovered to ambient levels found in oysters that were not removed from the water, the oysters can be harvested appropriately. The resubmersion of oysters allows for farmers to continue their best farm management practices, while minimizing the associated public health risks.

Previous resubmersion studies of cultured oysters have focused on the effects of different handling methods on the *Vibrio* spp. levels in oysters, reporting the resubmersion periods required after desiccation, tumbling, and refrigeration (*18*, *30*). Both studies used the ALS in Portersville Bay, Alabama during the high-risk period for *Vibrio* infections (July-September), and found that a seven-day resubmersion period was sufficient for elevated levels of *Vibrio* spp. to return to ambient levels. Data from Grodeska *et al.* (*18*) were used to support a change in the resubmersion requirement from 14 to 7 days for Alabama farmers using the ALS with baskets stocked at 100-120 oysters and desiccating their oysters (*1*). However, this reduction in resubmersion time is not

applicable to farmers using other handling methods or gear types, as their effect on resubmersion times is unknown.

The ALS and OG systems suspend, or float, oysters in the water column at different depths (Fig. 3.1), but it is unclear if this difference affects the *Vibrio* spp. levels during resubmersion. Walton *et al.* (*32*) found no difference between the ambient *V. vulnificus* and *V. parahaemolyticus* levels in oysters raised in these two culture systems, but they did not test the *Vibrio* spp. levels after handling and subsequent resubmersion. Oysters in the ALS system are suspended on a rigid structure made of wooden pilings and PVC poles and can be hung at any height in the water column, while oysters in the OG system are in floating cages at the surface of the water. Therefore, the OG oysters may be subjected to wave action for a longer period of time than the ALS oysters. Increased wave action has been shown to negatively impact the filtration rate of oysters in OG cages (*5*, *25*), which could reduce the oysters' ability to efficiently purge the elevated levels of *Vibrio* spp. and, therefore, require longer resubmersion times.

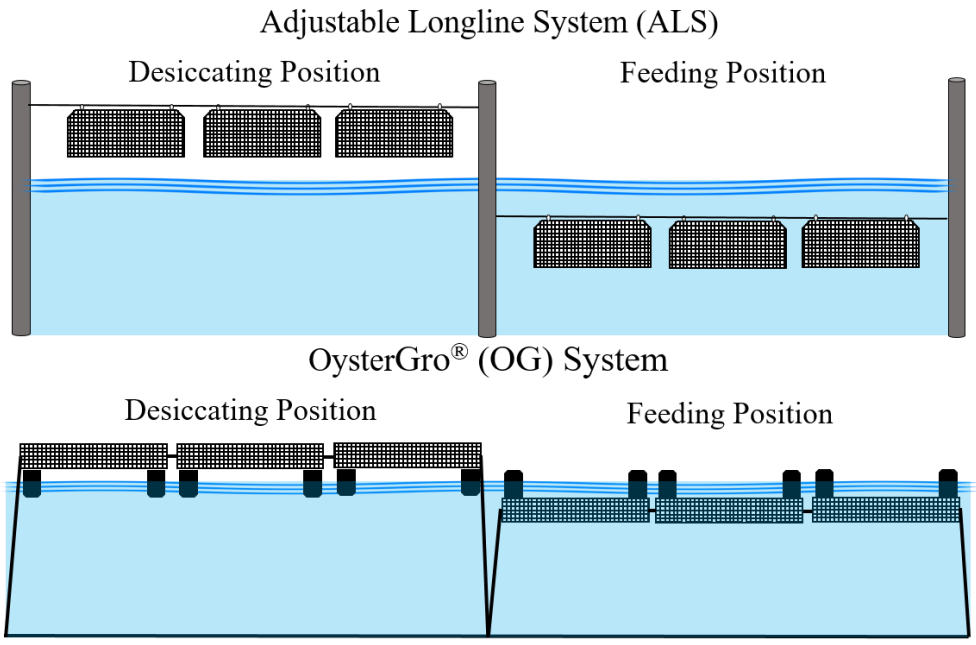

Figure 3.1. Diagram of the Adjustable Longline System (ALS) and OysterGro® (OG) gear types. The ALS system consists of a series of wooden pilings and PVC poles with lines tensioned between, and mesh baskets of oysters hanging from the line. The line can be raised up and down and secured in clips at various heights to allow for desiccation and feeding. The OG system consists of floating cages buoyed by air-filled pontoons at the surface of the water, filled with mesh bags containing oysters. Cages can be flipped up to expose oysters for desiccation, or flipped down in the water for feeding.

Previous resubmersion studies in Alabama were performed between June and September, during the high risk period for *Vibrio* infection (*18*, *24*, *30*); however, no research has been conducted during the beginning of the increased risk period (May) when water temperatures may be cooler. Both Kinsey *et al.* (*24*) and Pruente *et al.* (*30*) found that pathogenic *V. parahaemolyticus* required longer resubmersion periods after routine handling than did *V. vulnificus* and total *V. parahaemolyticus* during June-September. While it is well known that *V. vulnificus* and total *V. parahaemolyticus* levels are highest during that high risk period, pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) levels in oysters can be higher during late April-early May than June-September (*12*, *13*,

21, *34*). While the lower air temperatures during early spring may result in smaller increases in *Vibrio* spp. during routine handling, the ambient pathogenic *V. parahaemolyticus* levels are higher within oysters before they are removed for handling. Combined with the longer recovery time needed for those pathogenic *Vibrio* spp., the recovery time needed to purge in late April-early May could be longer. Cooler water temperatures are known to reduce the filtration rate of oysters and could likely reduce the purging efficiency of all *Vibrio* spp. during this time as well (*5*, *6*, *16*). Collectively, these factors could result in oysters needing a longer resubmersion period for recovery of elevated levels of all *Vibrio* spp., especially pathogenic *V. parahaemolyticus*, at the beginning of the high-risk season.

In order to expand the existing knowledge of handling effects on *Vibrio* spp. in oysters, the study objective was to determine the effects of two gear types on the levels of *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* (*tdh+*/*trh+*) in cultured oysters before and after handling, and over time after resubmersion. Farm-raised oysters maintained in the ALS and OG systems were subjected to a tumbled and refrigerated, or a desiccated treatment, and then returned to the water in May and July. The *Vibrio* spp. levels were measured over time in order to determine when the levels in the treated oysters, elevated during routine handling, returned to ambient levels in submersed oysters that served as a control. The results from this study will further inform public health officials and oyster farmers and aid in making informed decisions on appropriate resubmersion times for handled oysters.

2. Materials and Methods

2.1. Field Site and Environmental Monitoring.

This study was performed at Auburn University's research farm site in the Grand Bay Oyster Park, Alabama (Grand Bay). Single-set diploid oysters (*Crassostrea virginica*) were cultured in two off-bottom gear types: in BST bags suspended ~1 ft below the surface on the adjustable longline system (ALS; BST Oyster Supplies, Cowell, Australia), and in the floating six pack OysterGro® cage system (OG; OysterGro®, New Brunswick, Canada). Oysters were stocked at 100-120 oysters per bag for ALS, and 150-200 oysters per bag for OG; the stocking densities are different between the two gear types as standard stocking densities for the respective gear types were used. The oysters remained submersed at the farm site for a minimum of two weeks before starting each trial. Water temperature and salinity were recorded using a HOBO Saltwater Conductivity Data Logger (Onset Computer Corporation, Bourne, Massachusetts). During the treatment period, when oysters were out of the water, the air temperature was collected from the Dauphin Island weather station at mymobilebay.com. Additionally, Smart Button data loggers (ACR Systems Inc., British Columbia, Canada) were placed inside two oysters subjected to each treatment to monitor the internal oyster temperatures while exposed.

2.2. Treatments and Sample Collection.

A total of four trials were performed during 2018-2019 (Table 3.1): May 2018, July 2018, May 2019, July 2019. During the trials, three treatments were tested for each gear type (ALS, OG), with six replicate bags for each of the six combinations: a

submersed control, a tumbled and refrigerated treatment (TR), and a desiccated treatment. The control oysters remained submersed throughout each trial in each gear type. Bags of oysters from the two handling treatments were removed from the water and transported to the Auburn University Shellfish Laboratory (~1 h), where the handling treatments were applied over a 24 h period. The tumbled and refrigerated oysters were tumbled separately by bag, allowing the oysters from each bag to be passed through the mechanical grader once (Chesapeake Bay Oyster Company, Wake, Virginia), before being returned to their respective bag and placed into a walk-in cooler (0-4^{o}C) for 18 ± 2 h. The desiccated oysters remained in their bags and were exposed to ambient outdoor conditions for 24 h. After 24 ± 2 h, the handled oysters were resubmersed in their respective gear types at the farm site.

Triplicate samples (15 oysters/sample) were collected from the control oyster bags before the treatments were applied (pre-treatment); then, triplicate samples were collected from separate bags from each of the six gear/treatment combinations after handling treatments were applied but prior to resubmersion (post-treatment), and 3, 7, and 14 days after resubmersion. All oyster samples were collected at the farm site, placed into sample bags, packed with gel ice packs into a cooler, and transported to the FDA Gulf Coast Seafood Laboratory for further analysis.

2.3. MPN and Real-Time PCR.

The samples were processed following the National Shellfish Sanitation Program (NSSP) methods and analyzed using a three-tube-most-probable-number (MPN) as described in the FDA's *Bacteriological Analytical Manual* (*4, 23, 27*). In brief, oysters

were cleaned under cold tap water with a sterile brush, aseptically shucked into a sterile blender, and blended for 90 s. Then, 1 g of 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 [anhydrous] in 1 L distilled H_2O, pH 7.4 ± 0.2), and 1 mL of each dilution was inoculated into triplicate tubes of alkaline peptone water (APW; 10 g Bacto Peptone, 10 g NaCl in 1 L of distilled H_2O, pH 8.5 ± 0.2). Three tubes containing 10 mL of APW were inoculated with 1 g of oyster homogenate each to allow for a limit of detection of 0.3 MPN/g. MPN tubes were incubated for 18-24 h at 35°C, then visually examined for turbidity. For each tube that was turbid, a 1 mL aliquot was heated at 95°C for 10 min, resulting in a crude DNA extract that was cooled on ice, or directly stored at -20°C until further analysis. For real-time PCR analysis, extracts were thawed and centrifuged at 12,500 x *g* for 2 min. A 2 µL aliquot of the supernatant was tested for the presence of *Vibrio vulnificus*, total *V. parahaemolyticus* (*tlh*), and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) using the real-time PCR assays as previously described (*24*, *27*). The number of MPN tubes positive for each target was used to determine the levels of each *Vibrio* spp. using a standard MPN table (*4*).

2.4. Statistical Analysis.

The environmental data (water temperature, air temperature, and salinity) were used to calculate average daily means, minimums, and maximums. A general linear model was used to determine any statistical differences in these parameters among the trials. The internal oyster temperature data were averaged across the four trials to report a

mean and range for each treatment type during the 24 h period the oysters were out of the water.

Prior to analysis, all *Vibrio* spp. data (reported as MPN/g of oyster homogenate) were log transformed. In instances where *Vibrio* spp. were not detected (<0.3 MPN/g), half of the limit of detection was substituted prior to log transformation. General linear models were performed to compare the *Vibrio* spp. levels in control oysters between the two gear types for each individual trial. This model was also used to compare the *Vibrio* spp. levels in control oysters among the May and July trials. Based on these results, the May trials were analyzed separately from the July trials to explore the differences between the months. All *Vibrio* spp. data is reported as log MPN/g ± 95% confidence interval.

The data from the May 2018 and 2019 trials (Trials I and III) were pooled and analyzed using a linear mixed effects model to determine the effects of gear/treatment and days since resubmersion, and the interaction between the two fixed effect variables. A random effect of trial was included to account for any between-trial variation. The data from July 2018 and 2019 trials (Trials II and IV) were analyzed with a similar model for each *Vibrio* spp. Initially, pre-treatment *Vibrio* spp. levels were compared to the post-treatment levels in the handled oysters to determine how handling affected the *Vibrio* spp. levels. Then, if a significant interaction between treatment and days since resubmersion was detected, individual models were performed for each time point. Using the models, each *Vibrio* spp. was considered "recovered" when the treatment levels were not significantly higher than the submersed control levels for each gear type ($\alpha = 0.05$). All

data analyses were performed in R Studio using the nlme package (*29, 31*). All figures were created in SigmaPlot Version 13.0 (Systat Software, San Jose, CA).

3. Results

3.1. Environmental Data and Vibrio spp. Variation.

May trials had significantly lower (~5 ºC) air and water temperatures than July trials (Table 3.1; $p \leq 0.01$). Trial IV had a significantly higher mean ambient air temperature at 30.2 ºC than any other trial. Water temperatures did not significantly differ ($p \geq 0.51$) within the May or July trials. There were significant differences between the average daily salinities amongst the trials, with higher salinities during 2018 (Trials I and II) than 2019 (Trials III and IV).

Table 3.1. Environmental data collected during the trials[a]

Trial	Air Temp (ºC)[b]	Water Temp (ºC)[c]	Salinity (PSU)[c,d]
I (Apr 29-May 14, 2018)	21.4 (18.0-24.9)^{A}	25.7 (24.1-27.6)^{A}	16.2 (7.8-18.8)^{A}
II (Jul 8-Jul 23, 2018)	27.0 (23.6-28.2)^{B}	30.7 (28.2-33.5)^{B}	17.0 (6.7-23.4)^{A}
III (Apr 28-May 13, 2019)	21.6 (20.1-23.6)^{A}	25.4 (23.6-26.9)^{A}	6.7 (4.5-7.7)^{B}
IV (Jul 7-Jul 22, 2019)	30.2 (27.3-33.6)^{C}	30.3 (29.3-31.6)^{B}	12.4 (10.6-13.0)^{C}

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

Vibrio spp. levels in control oysters of each gear type did not significantly differ (Table 3.2; $p \geq 0.08$), except for one instance. On average, total *V. vulnificus* levels in the OG control oysters in Trial I were 0.52 ±0.51 log MPN/g higher than the levels in the ALS control oysters ($p = 0.04$). During the treatment period (~24 h), the desiccated

oysters had an average internal temperature of 19.9ºC (range, 14.9-30.8 ºC) in May and 26.8ºC (range, 24.3-31.0ºC) in July. The TR oysters had an average internal temperature of 4.4ºC (range, 2.6-25.9ºC) in May and 4.3ºC (range, 2.4-31.3ºC) in July. The internal temperatures were recorded during the entire treatment period, including transport, handling, and desiccation/refrigeration, resulting in a large temperature range for the TR oysters. On average, the internal temperature of the refrigerated oysters decreased by 22.3°C in May, and by 26.9°C in July.

Table 3.2. *Vibrio* spp. levels in submersed control oysters by trial[a]

Trial	*V. vulnificus*	Total *V. parahaemolyticus*	Pathogenic *V. parahaemolyticus* (*tdh*+)	Pathogenic *V. parahaemolyticus* (*trh*+)
I	3.18 (±0.71)^{A}	3.51 (±0.60)^{A}	0.43 (±0.63)^{A}	0.36 (±0.71)^{A}
II	4.36 (±0.48)^{B}	3.33 (±0.52)^{AB}	-0.42 (±0.41)^{B}	-0.48 (±0.36)^{BC}
III	3.88 (±0.42)^{C}	3.07 (±0.44)^{C}	-0.01 (±0.62)^{C}	-0.24 (±0.53)^{C}
IV	4.04 (±0.44)^{C}	3.20 (±0.38)^{BC}	-0.41 (±0.58)^{B}	-0.52 (±0.34)^{B}

[a]Average *Vibrio* spp. levels (n=15) in submersed control oysters during each trial (±standard deviation), reported as log MPN/g. Means in the same column with different letters are significantly different.

3.2. Treatment and Gear Effects on Vibrio vulnificus.

Prior to treatment in the May trials, the levels of *V. vulnificus* in the control oysters were 3.15 ± 0.62 and 3.55 ± 0.61 log MPN/g for ALS and OG, respectively (Fig. 3.2A). Immediately after treatment, the effects on the *V. vulnificus* levels depended on the handling type. In the ALS TR and OG TR oysters, the *V. vulnificus* levels were 0.32 ± 0.88 and 0.61 ± 0.86 log MPN/g lower than pre-treatment levels but did not differ significantly ($p \geq 0.16$). The *V. vulnificus* levels significantly ($p \leq 0.002$) increased from

pre-treatment levels by 1.96 ± 0.88 and 1.49 ± 0.86 log MPN/g, in the ALS and OG desiccated treatments, respectively.

During the July trials (Fig. 3.3A), the *V. vulnificus* levels in the pre-treatment oysters were 4.14 ± 0.39 and 3.94 ± 0.46 log MPN/g for ALS and OG, respectively. After the treatments were applied and prior to resubmersion, the levels in the TR oysters (regardless of gear type) increased by 0.20 ± 0.56 and 0.06 ± 0.46 log MPN/g from pre-treatment levels, but these increases were not significant ($p \geq 0.47$). The *V. vulnificus* levels in the ALS and OG desiccated oysters significantly ($p < 0.001$) increased by 1.90 ± 0.56 and 2.03 ± 0.46 log MPN/g, respectively. Additionally, the post-treatment *V. vulnificus* levels in the ALS control oysters were significantly ($p = 0.03$) greater than the pre-treatment levels by 0.57 ± 0.50 log MPN/g, but this effect was not observed in the OG control oysters ($p = 0.18$).

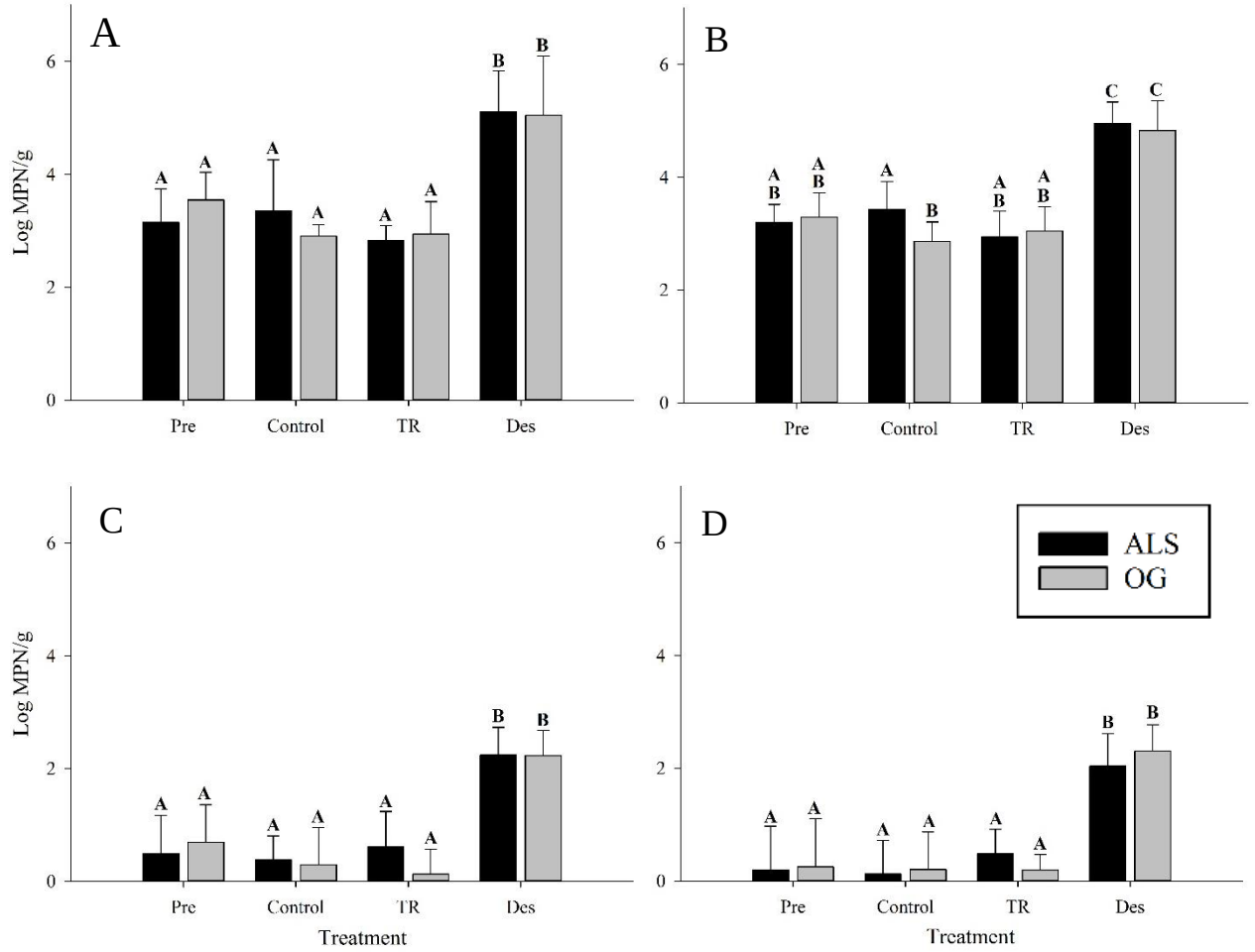

Figure 3.2. Mean log-transformed *Vibrio* levels for A) *V. vulnificus*, B) total *V. parahaemolyticus*, C) pathogenic *V. parahaemolyticus* (*tdh*+), and D) pathogenic *V. parahaemolyticus* (*trh*+) before (Pre) and after the handling treatments were applied (prior to resubmersion) during the May trials: Control (submersed control), TR (tumbled, refrigerated), Des (Desiccated). Bars represent standard deviation, and letters represent significant differences in *Vibrio* spp. levels (n=6).

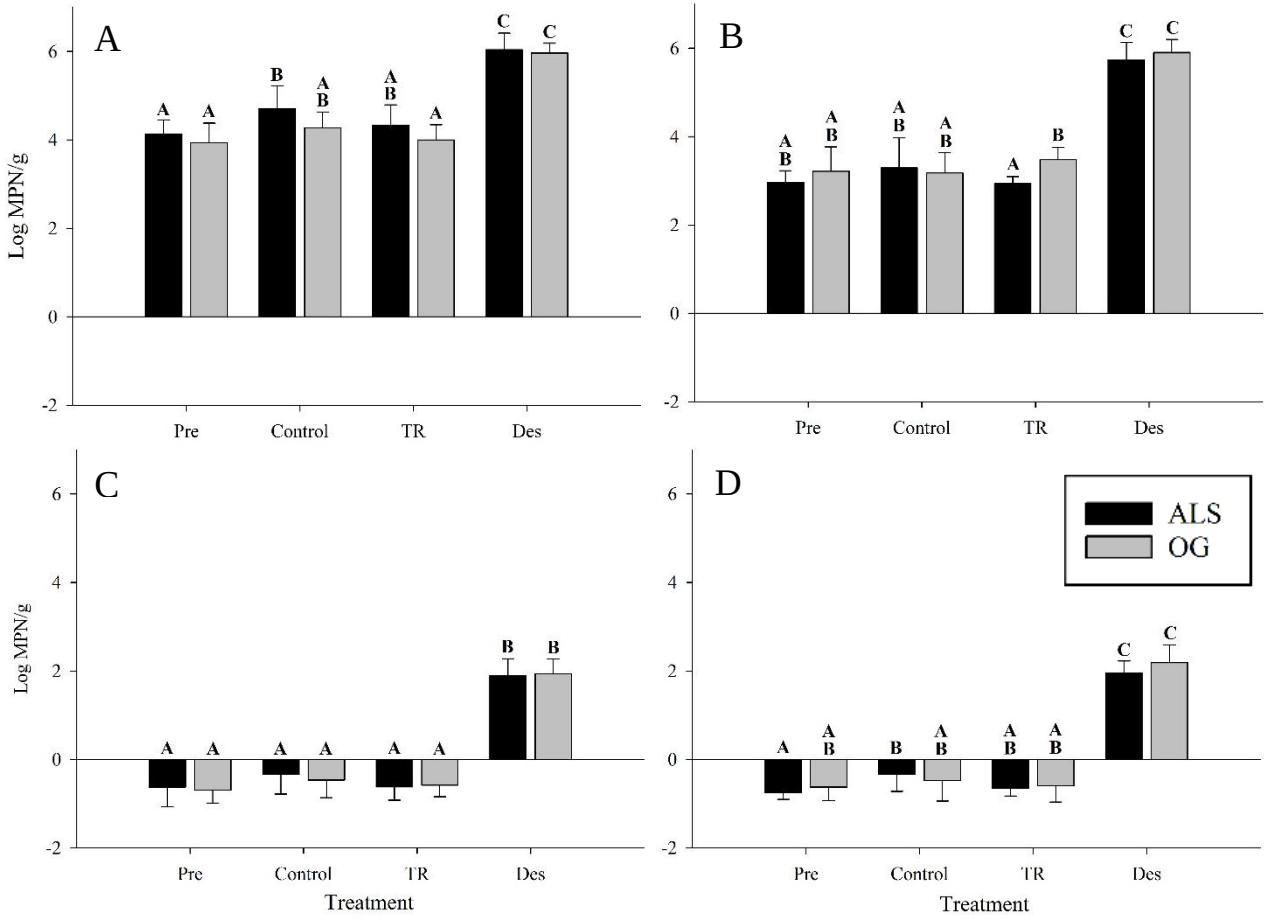

Figure 3.3. Mean log-transformed *Vibrio* levels for A) *V. vulnificus*, B) total *V. parahaemolyticus*, C) pathogenic *V. parahaemolyticus* (*tdh*+), and D) pathogenic *V. parahaemolyticus* (*trh*+) before (Pre) and after the handling treatments were applied (prior to resubmersion) during the July trials: Control (submersed control), TR (tumbled, refrigerated), Des (Desiccated). Bars represent standard deviation, and letters represent significant differences in *Vibrio* spp. levels (n=6).

For both May and July, there were significant interactions between treatment type and time since resubmersion on the levels of *V. vulnificus* (Table 3.3). Therefore, individual analyses were performed at each sampling time point to determine when the *V. vulnificus* levels recovered to control levels by gear type. In May, the *V. vulnificus* levels in the treatment oysters were not significantly higher than the control of each gear type after seven days of resubmersion ($p \geq 0.11$; Fig. 3.4A). In contrast, all treatment levels were not significantly higher than control levels after three days of resubmersion in July ($p \geq 0.05$; Fig. 3.5A). For *V. vulnificus*, the recovery times did not differ between the gear types within the month but tended to be longer in May than in July (Table 3.4).

Table 3.3. Summary statistics from mixed effects models, by *Vibrio* spp.[a]

	Vibrio spp.	Source	DF	F-Value	*p*-value
May Trials Trials I and III	*V. vulnificus*	**Treatment**	**5**	**5.78**	**0.0001**
		Time	3	1.77	0.16
		Treatment*Time	**25**	**7.16**	**<0.0001**
	V. parahaemolyticus	**Treatment**	**5**	**21.7**	**<0.0001**
		Time	**3**	**16.2**	**<0.0001**
		Treatment*Time	**25**	**6.74**	**<0.0001**
	Pathogenic *V. parahaemolyticus* (*tdh*+)	**Treatment**	**5**	**21.0**	**<0.0001**
		Time	**3**	**23.2**	**<0.0001**
		Treatment*Time	**15**	**3.35**	**<0.0001**
	Pathogenic *V. parahaemolyticus* (*trh*+)	**Treatment**	**5**	**20.1**	**<0.0001**
		Time	**3**	**20.2**	**<0.0001**
		Treatment*Time	**15**	**3.00**	**<0.0001**
July Trials Trials II and IV	*V. vulnificus*	**Treatment**	**5**	**8.94**	**<0.0001**
		Time	**3**	**51.2**	**<0.0001**
		Treatment*Time	**15**	**8.59**	**<0.0001**
	V. parahaemolyticus	**Treatment**	**5**	**24.5**	**<0.0001**
		Time	**3**	**41.8**	**<0.0001**
		Treatment*Time	**15**	**26.5**	**<0.0001**
	Pathogenic *V. parahaemolyticus* (*tdh*+)	**Treatment**	**5**	**8.30**	**<0.0001**
		Time	**3**	**14.2**	**<0.0001**
		Treatment*Time	**15**	**9.54**	**<0.0001**
	Pathogenic *V. parahaemolyticus* (*trh*+)	**Treatment**	**5**	**24.4**	**<0.0001**
		Time	**3**	**38.4**	**<0.0001**
		Treatment*Time	**15**	**17.5**	**<0.0001**

[a]Lines in bold represent significant differences ($\alpha = 0.05$).

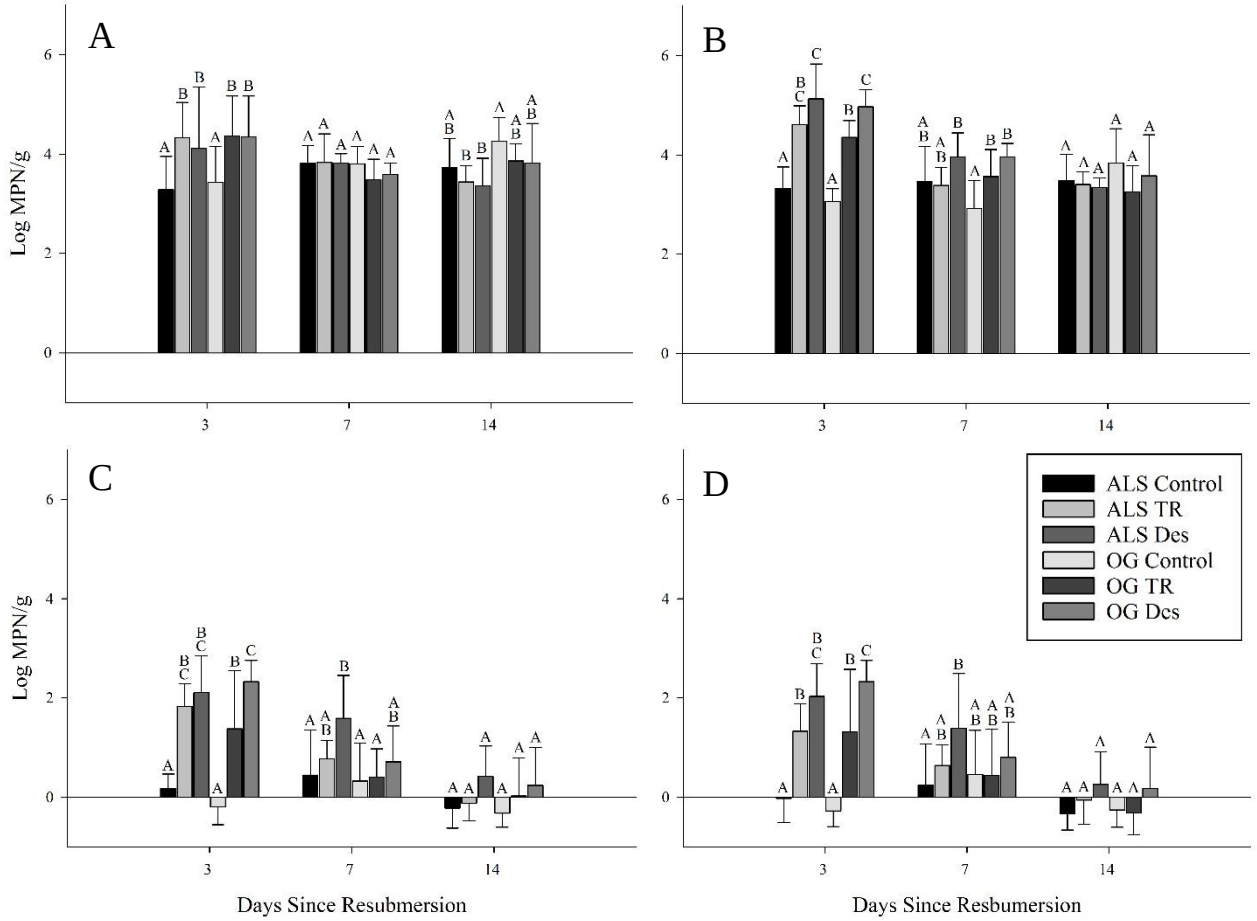

Figure 3.4. Mean log-transformed *Vibrio* levels for A) *V. vulnificus*, B) total *V. parahaemolyticus*, C) *V. parahaemolyticus* (*tdh*+), and D) *V. parahaemolyticus* (*trh*+) during May resubmersion trials: ALS (Adjustable Longline System gear), OG (OysterGro gear), Control (submersed control), TR (tumbled, refrigerated), Des (Desiccated). X-axis shows the days since resubmersion. Bars represent standard deviation, and letters represent significant differences in *Vibrio* spp. levels (n=6).

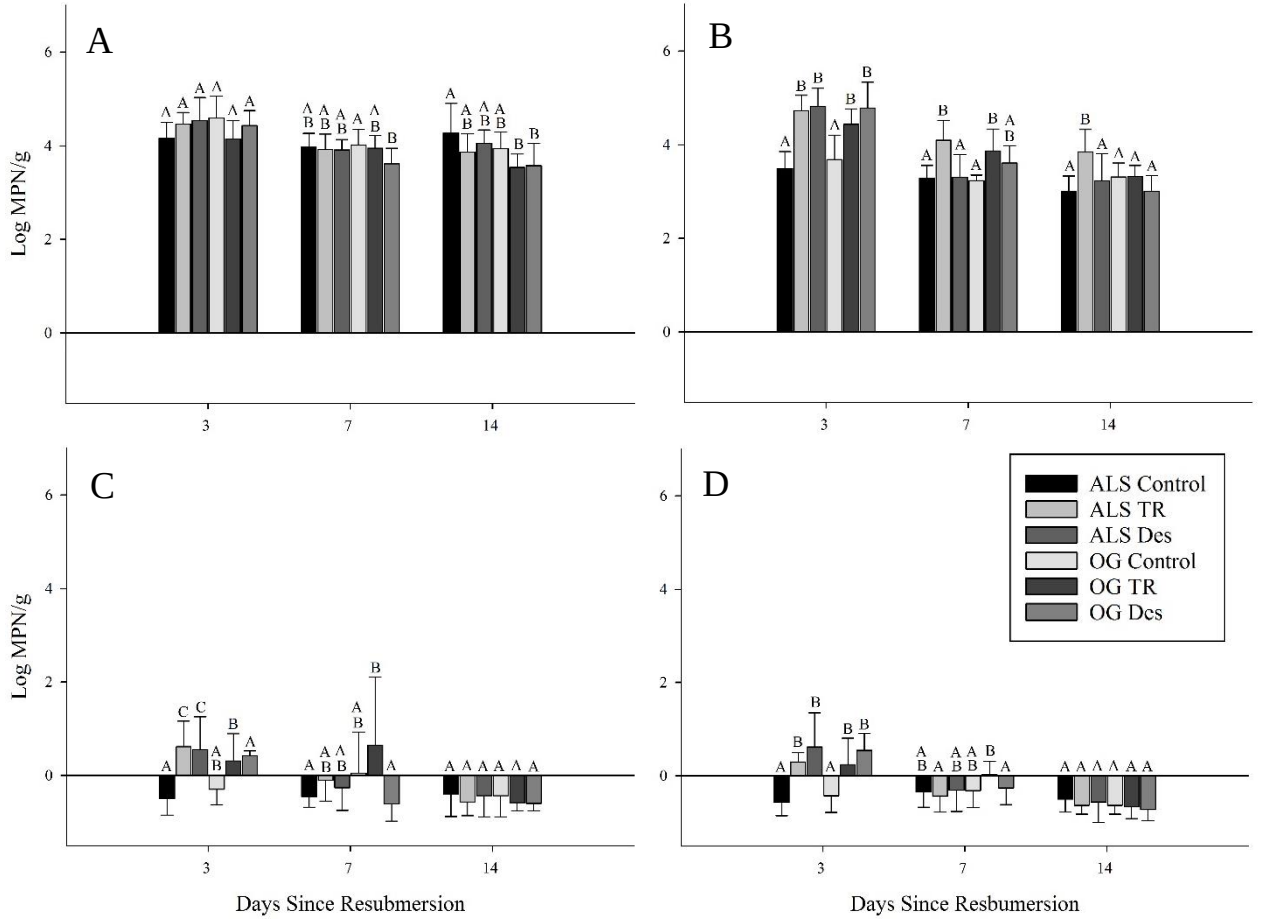

Figure 3.5. Mean log-transformed *Vibrio* levels for A) *V. vulnificus*, B) total *V. parahaemolyticus*, C) *V. parahaemolyticus* (*tdh*+), and D) *V. parahaemolyticus* (*trh*+) during July resubmersion trials: ALS (Adjustable Longline System gear), OG (OysterGro gear), Control (submersed control), TR (tumbled, refrigerated), Des (Desiccated). X-axis shows the days since resubmersion. Bars represent standard deviation, and letters represent significant differences in *Vibrio* spp. levels (n=6).

Table 3.4. *Vibrio* spp. recovery times, by trial[a]

		Days[a]			
	Vibrio spp.	ALS TR[b]	ALS Desiccated[c]	OG TR[d]	OG Desiccated[e]
May Trials (2018-19)	*V. vulnificus*	7	7	7	7
	Total *V. parahaemolyticus*	7	7	14	14
	Pathogenic *V. parahaemolyticus* (*tdh*+)	7	14[f]	7	7
	Pathogenic *V. parahaemolyticus* (*trh*+)	7	14[f]	7	7
July Trials (2018-19)	*V. vulnificus*	3	3	3	3
	Total *V. parahaemolyticus*	>14	7	14	7
	Pathogenic *V. parahaemolyticus* (*tdh*+)	7	7	7[f]	7
	Pathogenic *V. parahaemolyticus* (*trh*+)	7	7	7	7

[a]Number of days after re-submersion when *Vibrio* spp. levels in treatment oysters were not significantly higher than control oysters ($p>0.05$), as determined by the mixed effects model.
[b]Adjustable longline system, tumbled and refrigerated treatment.
[c]Adjustable longline system, desiccated treatment.
[d]OysterGro® system, tumbled and refrigerated treatment.
[e]OysterGro® system, desiccated treatment.
[f]Cases where statistical significance does not agree with biological relevance (i.e. *Vibrio* spp. levels in the treatment oysters were still greater than 0.5 log MPN/g higher than levels in control oysters).

3.3. Treatment and Gear Effects on Total Vibrio parahaemolyticus.

In May, the pre-treatment levels of *V. parahaemolyticus* in the ALS and OG oysters were 3.20 ± 0.39 and 3.29 ± 0.41 log MPN/g (Fig 3.2B). After tumbling and refrigeration, the *V. parahaemolyticus* levels in the ALS and OG TR oysters were 0.25 ± 0.56 and 0.24 ± 0.58 log MPN/g lower than pre-treatment levels; however, these decreases were insignificant ($p \geq 0.35$). Conversely, the levels in ALS and OG desiccated oysters significantly ($p < 0.001$) increased from pre-treatment levels by 1.76 ± 0.56 and 1.53 ± 0.58 log MPN/g.

In July, the levels of *V. parahaemolyticus* in the ALS and OG pre-treatment oysters were 2.96 ± 0.39 and 3.22 ± 0.38 log MPN/g, respectively (Fig. 3.3B). After tumbling and refrigeration, the *V. parahaemolyticus* levels decreased from pre-treatment levels by 0.02 ± 0.56 log MPN/g in the ALS TR oysters and increased by 0.26 ± 0.54 log MPN/g in the OG TR oysters. Neither of these changes in *V. parahaemolyticus* levels in the TR oysters were significant ($p \geq 0.34$). The *V. parahaemolyticus* levels significantly ($p < 0.001$) increased from pre-treatment levels by 2.78 ± 0.56 and 2.68 ± 0.54 log MPN/g for the ALS and OG desiccated oysters, respectively.

There were significant interactions between treatment and time since resubmersion for total *V. parahaemolyticus* levels (Table 3.3), so individual analyses were used at each sampling time point. In May, the elevated *V. parahaemolyticus* levels in the ALS oysters (regardless of handling) were not significantly higher than control levels after 7 days ($p \geq 0.08$), while the levels in the OG oysters (regardless of handling) were not significantly higher after 14 days ($p \geq 0.06$; Fig. 3.4B). In July, the levels in the desiccated oysters of both gear types were not significantly higher than control levels after seven days of resubmersion ($p \geq 0.12$; Fig 3.5B). Oysters in the OG TR treatment required 14 days to reach levels similar to the control ($p = 0.98$). The total *V. parahaemolyticus* levels in the ALS TR oysters were 0.84 ± 0.50 log MPN/g higher than in the control treatment ($p = 0.002$) after 14 days of resubmersion, and did not return to ambient *V. parahaemolyticus* levels during the July study (Table 3.4).

3.4. Treatment and Gear Effects on Pathogenic Vibrio parahaemolyticus (tdh+/trh+).

In May, the *tdh*+ levels in ALS and OG pre-treatment oysters were 0.49 ± 0.52 and 0.69 ± 0.53 log MPN/g, and *trh*+ levels were 0.20 ± 0.56 and 0.25 ± 0.57 log MPN/g (Fig. 3.2C-3.2D). After refrigeration, the *tdh*+ and *trh*+ levels in ALS TR oysters were 0.12 ± 0.80 and 0.28 ± 0.80 log MPN/g higher than pre-treatment levels, while the levels in OG TR oysters were 0.56 ± 0.75 and 0.06 ± 0.80 log MPN/g lower than pre-treatment levels. These changes were not significant ($p \geq 0.13$). After desiccation, the *tdh*+ and *trh*+ levels significantly ($p < 0.001$) increased from pre-treatment levels by 1.74 ± 0.74 and 1.84 ± 0.80 log MPN/g in the ALS desiccated oysters, and by 1.54 ± 0.75 and 2.05 ± 0.80 log MPN/g in the OG desiccated oysters.

Prior to treatment in July, the *tdh*+ levels in ALS and OG oysters were -0.63 ± 0.37 and -0.69 ± 0.30 log MPN/g, and the *trh*+ levels were -0.76 ± 0.24 and -0.63 ± 0.36 log MPN/g (Fig. 3.3C-3.3D). Following refrigeration, the *tdh*+ levels in ALS TR oysters decreased by 0.003 ± 0.52 log MPN/g from pre-treatment levels, while the *trh*+ levels increased by 0.10 ± 0.34 log MPN/g. The *tdh*+ and *trh*+ levels in OG TR oysters increased by 0.11 ± 0.43 and 0.04 ± 0.51 log MPN/g. However, these changes from pre-treatment *tdh*+ and *trh*+ levels were insignificant ($p \geq 0.55$). In contrast, *tdh*+ and *trh*+ levels in the desiccated oysters significantly ($p < 0.001$) increased from pre-treatment levels by 2.52 ± 0.52 and 2.72 ± 0.34 log MPN/g for ALS, and by 2.63 ± 0.43 and 2.82 ± 0.51 log MPN/g for OG.

A significant interaction was found between the treatment and time since resubmersion for the pathogenic *V. parahaemolyticus* levels (Table 3.3), so the same approach as above was used. In May, the *tdh*+ and *trh*+ levels in oysters from OG,

regardless of treatment, were not significantly higher than control levels after seven days of resubmersion ($p \geq 0.39$; Fig. 3.4C-3.4D). The levels in the ALS oysters were not significantly higher than the control levels after 7 days for the TR oysters ($p = 0.44$), and 14 days for the desiccated oysters ($p = 0.06$). During the July trials, the pathogenic *V. parahaemolyticus* levels in oysters of both gear types were not significantly higher than control levels after 7 days of resubmersion ($p \geq 0.14$), with one exception: the OG TR oysters required 14 days for the elevated *tdh*+ levels to return to ambient levels ($p = 0.51$; Table 3.4).

4. Discussion

Farm-raised oysters were placed in two common gear types (adjustable longline system and the OysterGro® system) and subjected to two routine handling treatments that resulted in elevated levels of *Vibrio* spp. within the oysters. These routine handling practices were followed by a two-week resubmersion period in order to allow the oysters to purge elevated levels of *Vibrio* spp. back to ambient levels. Data from the four trials, performed under varying environmental conditions typical of those observed in the region, were used to determine the recovery times for *Vibrio* spp. in oysters of four handling-gear type combinations using a mixed effects model.

Although water temperatures were lower during the May trials than the July trials, the *Vibrio* spp. levels in the submersed control oysters were not always lower during that time. The effect was species-specific, as oysters had lower *V. vulnificus* levels and higher pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) levels in May than in July, similar to previous findings (*12*, *13*, *21*, *34*). There was only one instance in which *Vibrio* spp.

levels significantly differed between the control oysters in each gear (*V. vulnificus* levels in Trial I). This was similar to the findings from Walton *et al.* (*32*) where no differences in *Vibrio* spp. levels were found among the gear types. The higher *V. vulnificus* levels in the OG oysters during Trial I could have been a result of the oysters experiencing higher surface water temperatures or greater wave action than the ALS oysters. The mean air temperatures were lower in May, corresponding to lower increases of *Vibrio* spp. in oysters during desiccation in May than in July (Fig. 3.2-3.3). Regardless of these differences, the air temperatures in both months created optimal conditions for *Vibrio* spp. growth (*7*, *9*, *11*, *17*, *28*) and resulted in significant increases in *Vibrio* levels during oyster exposure. The tumbled and refrigerated oysters had insignificant initial increases in *Vibrio* spp. levels, and in some cases the levels decreased, as previously described for refrigeration (*7*, *8*, *11*, *17*, *28*).

The recovery times required for *Vibrio* levels in oysters to return to ambient levels in this study varied among the *Vibrio* spp. (Table 3.4). For example, the *V. vulnificus* levels in oysters of all gear and treatment combinations were not significantly higher than control levels after seven days in May and three days in July. For *V. parahaemolyticus*, however, there were differences in recovery times based on month, gear type, and handling treatment. When looking at the handling effects in July, the TR oysters (regardless of gear type) required 14 days or more of resubmersion for elevated total *V. parahaemolyticus* levels to recover, while the desiccated oysters only required 7 days. This was in contrast to previous findings from Portersville Bay (*30*), where recovery times were the same between the TR and desiccated treatments. The TR oysters in this study experienced a delay in filter feeding after resubmersion, possibly due to the effect

of different environmental conditions experienced in Grand Bay, indicating the potential of spatial and temporal variability in recovery times. There was no difference in recovery time (seven days) between desiccated oysters in either gear type in July for *V. parahaemolyticus*. This same trend did not hold true in May, as the desiccated oysters of both gear types required 14 days of resubmersion to allow all *Vibrio* spp. to recover. The variation in recovery times between May and July indicate that the cooler month of May requires a longer resubmersion period of 14 days than June-September for *V. parahaemolyticus*. This could be due to the variability in total and pathogenic *V. parahaemolyticus* levels found during early May in previous studies, combined with the reduced filtration rate of oysters when the water temperatures are cooler (*6*, *12*, *13*, *16*, *21*, *34*).

The data from this study were analyzed using a mixed effects model as previously described (*30*); however, the question of biological relevance versus statistical significance was raised. It can be inferred from the Quantitative Risk Assessments that a 0.5 log MPN/g increase in levels increases the risk of infection by 3-6 fold for *V. vulnificus* and 3-fold for *V. parahaemolyticus* (*2*, *3*). Additionally, this threshold of 0.5 log MPN/g takes into account an average combined method error and sample-to-sample variability of 0.5 log MPN/g (*18*, *19*, *24*, *30*). Therefore, observed differences in means above this threshold could be assumed as "real" (not an artifact of sample or method variability) and raise concerns about risk of illness. This biological relevance "threshold" and the mixed effects model were in agreement on determination of recovery times, except for 2 of the 32 conditions examined in the pooled analyses: in May, the *tdh*+ and *trh*+ levels in the ALS desiccated oysters were 0.63 (±0.73) and 0.60 (±0.63) log MPN/g

higher than the control levels on day 14, and the model showed these differences as insignificant ($p \geq 0.06$). Using this biological threshold appears to be a more conservative approach for public health, suggesting longer recovery times are needed for *Vibrio* spp. levels in oysters to return to ambient than are determined by the model. In both cases of discrepancy between the model and the biological threshold, the model is less conservative, with no significant difference at day 14 when, biologically, the risk appears to persist to day 14. While the use of a more conservative alpha with the models is one option, the use of a biologically relevant difference in means of 0.50 MPN/g could be used as an alternative metric for decision-making.

This study subjected cultured oysters to routine handling practices that elevated *Vibrio* spp. levels within the oysters, and determined the time required for the elevated levels to return to ambient levels after resubmersion. As a result of the lower level of replication, the data from this study are limited in statistical power and may not be well suited for use in making regulatory decisions about resubmersion periods. Despite these limitations, this study has revealed valuable trends that have started to fill in existing knowledge gaps, and ultimately can be used to inform future studies to further investigate resubmersion. Several factors from similar studies were tested (i.e. ALS gear type, summer months, handling treatments) (*18*, *19*, *24*, *30*) along with several new factors, including resubmersion in a different water body (Grand Bay, Alabama), a cooler shoulder month (May), and an additional gear type (OG). The addition of new factors revealed that geographical location and time of year may have an effect on the resubmersion time required for cultured oysters to purge *Vibrio* spp. levels after routine handling.

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Effects of tumbling, refrigeration, and resubmersion on *Vibrio* spp. levels in North Carolina cultured oysters (*Crassostrea virginica*)

Abstract

Routine handling of cultured oysters is necessary to produce a high quality product, but removing the oysters from the water can increase the *Vibrio* spp. levels within the oyster. To mitigate this public health risk, oysters are resubmersed after handling to allow elevated *Vibrio* spp. levels to "recover" to ambient levels. The majority of previous resubmersion research has been conducted in Alabama, leaving open the question of potential geographic variability in recovery times. This study aims to expand existing knowledge by employing an experimental design previously used in Alabama in Cedar Island, North Carolina. Four handling treatments (tumbled and refrigerated [TR], tumbled and not refrigerated [TNR], not tumbled and refrigerated [NTR], and not tumbled and not refrigerated [NTNR]) were applied to farmed oysters, followed by resubmersion. The levels of *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) were measured before and after handling treatment, and 1, 3, 7, 10, and 14 days after resubmersion. The levels in treated oysters were compared to levels in untreated submersed control oysters to determine the recovery times. After handling, the refrigerated oysters did not have significant increases in *Vibrio* spp. levels (-0.08-0.46 log MPN/g), while the non-refrigerated oysters had significant increases (1.06-2.06 log MPN/g). The refrigerated oysters, however, had a significant spike (0.60-1.03 log MPN/g) in *V. parahaemolyticus* levels after one to three days of resubmersion, but this effect was not seen for *V. vulnificus*. Elevated *V. vulnificus* levels recovered to ambient levels after one day of resubmersion in all treatments. Total and pathogenic *V. parahaemolyticus* recovered in 1 to 7 days in all treatments, except for the TR treatment, which recovered after 14 days. The recovery times in North Carolina were similar to

previous findings from Alabama, with the levels of all *Vibrio* spp. in NTNR oysters recovering after 7 days and after 14 days for TR oysters. The data indicate that geography does not affect the recovery times of cultured oysters under the conditions tested.

1. Introduction

The decline in wild oyster landings over the last century has led to the development of oyster aquaculture in North Carolina. Historically, oyster aquaculture consisted of bottom leases that were planted with a substrate on which wild oyster larvae would settle (*10*). Within the last decade, the number of water column leases has substantially increased, resulting in the adoption of off-bottom culture techniques that utilize hatchery-reared oyster seed (*10*). Single-set oysters are raised in floating cages to give the oysters greater access to food and increased protection from predators than oysters on bottom leases, while allowing farmers access to their oysters at any time (*2, 32*). Farmers will remove the oysters from the water for periodic desiccation (air-drying) to reduce biofouling, and to tumble the oysters through a mechanical grader for improved shell shape and for sorting into different size grades (*18, 32*).

While the oysters are removed from the water for handling, the combination of higher ambient air temperatures and the interruption of filter feeding causes *Vibrio* spp. levels to increase within the oysters, resulting in potential increased public health risk (*12, 13, 17, 27, 28*). *Vibrio vulnificus* and *V. parahaemolyticus* are naturally occurring bacteria that are concentrated in oysters during filter feeding, with the majority of illnesses in humans caused by consuming raw or undercooked shellfish (*22, 30*). *V. parahaemolyticus* infections can occur in anyone, resulting in gastroenteritis and, rarely,

septicemia (*7*), while *V. vulnificus* infections are more common in immunocompromised individuals and result in mild gastroenteritis and primary septicemia (*15*, *22*, *23*).

Although routine handling may increase the public health risk, the oysters can be resubmersed after ambient exposure to mitigate this risk prior to harvest for consumption (*12*, *13*, *17*, *27*, *28*). Farmers may resubmerse the oysters in the water and as filter feeding resumes, the elevated levels of *Vibrio* spp. are purged back to ambient levels normally present in unexposed oysters. The resubmersion of cultured oysters has been well studied in Alabama, finding that 7 to 14 days of resubmersion is sufficient for elevated *Vibrio* spp. levels to return to ambient levels in oysters raised in different off-bottom culture gears and subjected to several handling types (*12*, *13*, *17*, *27*, *28*). As these studies were geographically limited, it is uncertain if the recovery times would be similar in other regions or states due to differences in the regional ecology of *Vibrio* spp., environmental conditions (water temperature, salinity), and farming techniques.

In areas where resubmersion research has not been conducted, like North Carolina, a resubmersion requirement has been added into the state's Vibrio Control Plan. More specifically, oyster farmers performing routine handling practices in North Carolina have a 14 day resubmersion requirement for oysters that are removed from the water for more than 5 hours between May 1 and October 14 (S. Jenkins, North Carolina Department of Environmental Quality, pers. comm., Jan. 10, 2020). Using the results from previous studies, however, the Vibrio Control Plan from Alabama was modified to allow as little as seven days of resubmersion for specific gear and handling types that are also used by farmers in North Carolina (*3*). Despite the similar gear and handling, the

environmental conditions and levels of *Vibrio* spp. present in oysters may differ between the states, raising the question of whether recovery times are similar between the regions.

In order to begin addressing this question of geographic variability in recovery times, a resubmersion study was performed at a farm site in Cedar Island, North Carolina. Four combinations of tumbling and refrigeration handling treatments were applied to cultured oysters, and levels of *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) were measured after treatment and resubmersion. The recovery time required for the elevated *Vibrio* spp. levels in the treated oysters to return to ambient levels in submersed control oysters was determined for each treatment type. By using a similar experimental design to previous resubmersion studies (i.e. time of year, handling treatments, gear type) (*28*), the goal of this study is to evaluate the resubmersion of cultured oysters in a previously unstudied area, North Carolina.

2. Materials and Methods

2.1. Field Site and Environmental Monitoring.

An existing commercial oyster lease in Cedar Island Bay, North Carolina was used as the field site for this study. Market sized triploid oysters (*Crassostrea virginica*) were stocked into mesh bags (150-200 oysters per bag) and placed into six-pack OysterGro® cages (OysterGro®, New Brunswick, Canada) for a minimum of two weeks prior to each experimental trial. The water temperature and salinity were recorded hourly with a HOBO Saltwater Conductivity Data Logger (Onset Computer Corporation, Bourne, Massachusetts), while the air temperature during the handling period was collected from the North Carolina State Climate Office (NCDI Cedar Island Station).

Additionally, Smart Button data loggers (ACR Systems Inc., British Columbia, Canada) were inserted into two oysters from each treatment to monitor the internal oyster temperature every two minutes during the handling period.

2.2. Treatments and Sample Collection.

Four trials were performed during 2018-2019 (Table 4.1), and 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. An OysterGro® cage was randomly assigned to each treatment (six replicate bags per treatment) in order to streamline the application of treatments and sample collection. The submersed control oysters remained submerged during each trial, and the oysters assigned to the handling treatments were removed from the water and transported back to land (~10 min) to apply the treatments. Tumbled oysters were passed through the mechanical grader once (~10 min), then returned to the original bag; the not tumbled oysters remained in their bags. After tumbling, the refrigerated oysters were placed inside a pre-chilled portable refrigeration unit (≤7.2ºC) for 18 ± 2 h (range), while the non-refrigerated oysters were exposed to ambient outdoor conditions overnight, generating four handling treatments: TR, TNR, NTR and NTNR. After handling, all treated oysters were transported back to the farm and resubmersed in the cages within 24 ± 2 h of removal.

Before oysters were removed from the water for treatment, triplicate samples (15 oysters/sample) were collected from 3 separate submersed control bags (pre-treatment). Then, triplicate samples (15 oysters/sample, collected from separate treatment bags) were

taken from each of the five treatments after the handling treatments were applied but prior to resubmersion (post-treatment), and 1, 3, 7, 10, and 14 days after resubmersion. All samples were collected from the farm, placed in a cooler with gel ice packs, and transported to the University of North Carolina's Institute of Marine Sciences for processing.

2.3. MPN and Real-Time PCR.

Upon arrival at the lab, oyster samples were processed using the standard three-tube-most-probable-number (MPN) method (*5, 16, 20*). Oysters were cleaned under cold tap water with a sterile brush, aseptically shucked into a sterile blender, and homogenized for 90 s. Then, oyster homogenate was serially diluted 10-fold to 1:100,000 in phosphate-buffered saline (PBS), and 1 mL of each dilution was inoculated into triplicate tubes of alkaline peptone water (APW; *5, 16*). A set of three APW tubes were each inoculated with 1 g oyster homogenate to complete the dilution series for a limit of detection of 0.3 MPN/g. The MPN tubes were incubated overnight (18-24 h) at 35 ± 2ºC and then examined for turbidity. DNA extracts were prepared from each turbid tube by heating a 1 mL aliquot at 95ºC for 10 min, then directly stored in a freezer (-80ºC). All DNA extracts were shipped on dry ice to the FDA Gulf Coast Seafood Laboratory (Dauphin Island, AL) for further analysis. Prior to PCR analysis, DNA extracts were thawed and centrifuged at 12,500 x *g* for 2 min. Then, 2 µL of the resulting supernatant was tested for the presence of *Vibrio vulnificus,* total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) using the real-time PCR assays as previously described

(*17*, *20*). The number of positive tubes was used to determine the levels of each *Vibrio* spp. target using a standard MPN table (*5*).

2.4. Statistical Analysis.

The average daily mean, minimum, and maximum values were calculated for the air temperature, water temperature, and salinity. A general linear model was used to determine any statistical differences in average daily means among the trials. The internal oyster temperatures from the handling period (24 h) were averaged across the four trials to report a mean and range for the treatments. When *Vibrio* spp. levels were below the limit of detection, half of the limit was substituted prior to log transformation. General linear models were used to compare the average *Vibrio* spp. levels in the control oysters during each trial, and to compare the *Vibrio* spp. levels in the pre-treatment oysters to the post-treatment control and treated oysters (all trial data pooled).

To determine the recovery time required for elevated *Vibrio* spp. levels to return to ambient levels, the data from the four trials were pooled and a mixed effects model was used (*27*, *28*). The model tested the effects of treatment type and time since resubmersion (fixed effects), the interaction between treatment and time, and a random effect of trial to account for between-trial variation. If a significant interaction was detected, individual models for each time point were used to compare the *Vibrio* spp. levels in the treated oysters to the control oysters. *Vibrio* spp. levels were considered recovered when the levels in the treated oysters were not significantly higher than levels in control oysters ($\alpha = 0.05$). All *Vibrio* spp. data are reported as log MPN/g ± 95% confidence interval. Data analyses were performed in R Studio using the nlme package

(*26, 31*), and figures were created using SigmaPlot version 13.0 (Systat Software, San Jose, California).

3. Results

3.1. Environmental Data.

The water temperatures were similar across the trials except for Trial III, which had significantly lower (~2ºC) water temperatures (Table 4.1). The average daily salinity was significantly different across all trials ($p < 0.001$), while the air temperatures during the treatment period were similar across all trials ($p \geq 0.39$). The environmental conditions were conducive for the presence of *Vibrio* spp. in oysters, as all four gene targets were detected in submersed control oysters during each trial (Table 4.2). The levels of all *Vibrio* spp. were significantly higher ($p \leq 0.03$) during Trials I and II than Trials III and IV. During the handling period (~24 h), the refrigerated oysters had an average internal oyster temperature of 8.1ºC (range, 4.7-27.9ºC), while the non-refrigerated oysters had an average internal oyster temperature of 24.9ºC (range, 23.8-27.9ºC). Internal temperatures were monitored during the entire treatment period, including transport, handling, and desiccation/refrigeration, resulting in the large temperature ranges for the refrigerated oysters. The internal temperatures of the refrigerated oysters decreased by 23.2°C during refrigeration.

Table 4.1. Environmental data collected during trials[a]

Trials	Air Temp (ºC)[b]	Water Temp (ºC)[c]	Salinity (PSU)[c,d]
I (Jun 10-25, 2018)	26.2 (23.3-30.0)[A]	28.5 (26.9-30.4)[A]	23.5 (17.3-24.5)[A]
II (Aug 5-20, 2018)	26.5 (23.3-30.6)[A]	29.4 (27.6-31.2)[AC]	26.8 (19.7-29.0)[B]
III (Jun 9-24, 2019)	26.7 (24.4-29.4)[A]	26.9 (25.3-28.7)[B]	20.5 (19.8-21.2)[C]
IV (Aug 4-19, 2019)	26.3 (22.8-30.6)[A]	29.6 (28.2-31.2)[C]	17.7 (17.0-18.2)[D]

[a] Means in the same column with different letters are significantly different ($p < 0.05$).
[b]Average air temperature, with range in parentheses, during the treatment period, collected from the North Carolina State Climate Office, Cedar Island (NCDI) station.
[c]Average daily means, with daily ranges in parentheses.
[d]PSU, practical salinity units.

Table 4.2. *Vibrio* spp. levels in submersed control oysters, by trial[a]

Trials	*V. vulnificus*	Total *V. parahaemolyticus*	Pathogenic *V. parahaemolyticus* (*tdh*+)	Pathogenic *V. parahaemolyticus* (*trh*+)
I	2.38 (±0.59)[A]	2.77 (±0.44)[AB]	-0.03 (±0.63)[A]	0.37 (±0.41)[A]
II	3.03 (±0.54)[B]	2.87 (±0.40)[A]	-0.11 (±0.36)[A]	0.20 (±0.34)[A]
III	0.93 (±0.50)[C]	2.49 (±0.18)[B]	-0.34 (±0.37)[AB]	-0.14 (±0.29)[B]
IV	0.17 (±0.59)[D]	1.99 (±0.39)[C]	-0.51 (±0.30)[B]	-0.66 (±0.16)[C]

[a]Average *Vibrio* spp. levels, reported as mean log MPN/g (± standard deviation). Means in the same column with different letters are significantly different ($p \leq 0.01$).

3.2. Treatment Effects on Vibrio spp.

After the treatments were applied, but prior to resubmersion, the effects of tumbling and refrigeration depended on the treatment type (Fig. 4.1). The levels in refrigerated oysters slightly ($p \geq 0.05$) increased from pre-treatment levels by as little as 0.07 ± 0.49 log MPN/g for total *V. parahaemolyticus* (NTR; Fig. 4.1B), up to an increase of 0.46 ± 0.46 log MPN/g for *V. parahaemolyticus* (*trh*+) (TR; Fig. 4.1D). The overnight refrigeration decreased the *Vibrio* spp. levels in one case, where the total *V. parahaemolyticus* levels in the NTR oysters were 0.08 ± 0.39 log MPN/g lower than the pre-treatment levels ($p = 0.68$). Conversely, the *Vibrio* spp. in non-refrigerated oysters significantly increased ($p < 0.001$) from pre-treatment levels, ranging from an increase of 1.06 ± 0.42 log MPN/g for *V. vulnificus* (NTNR; Fig. 4.1A) to an increase of 2.06 ± 0.39 log MPN/g for total *V. parahaemolyticus* (TNR; Fig. 4.1B).

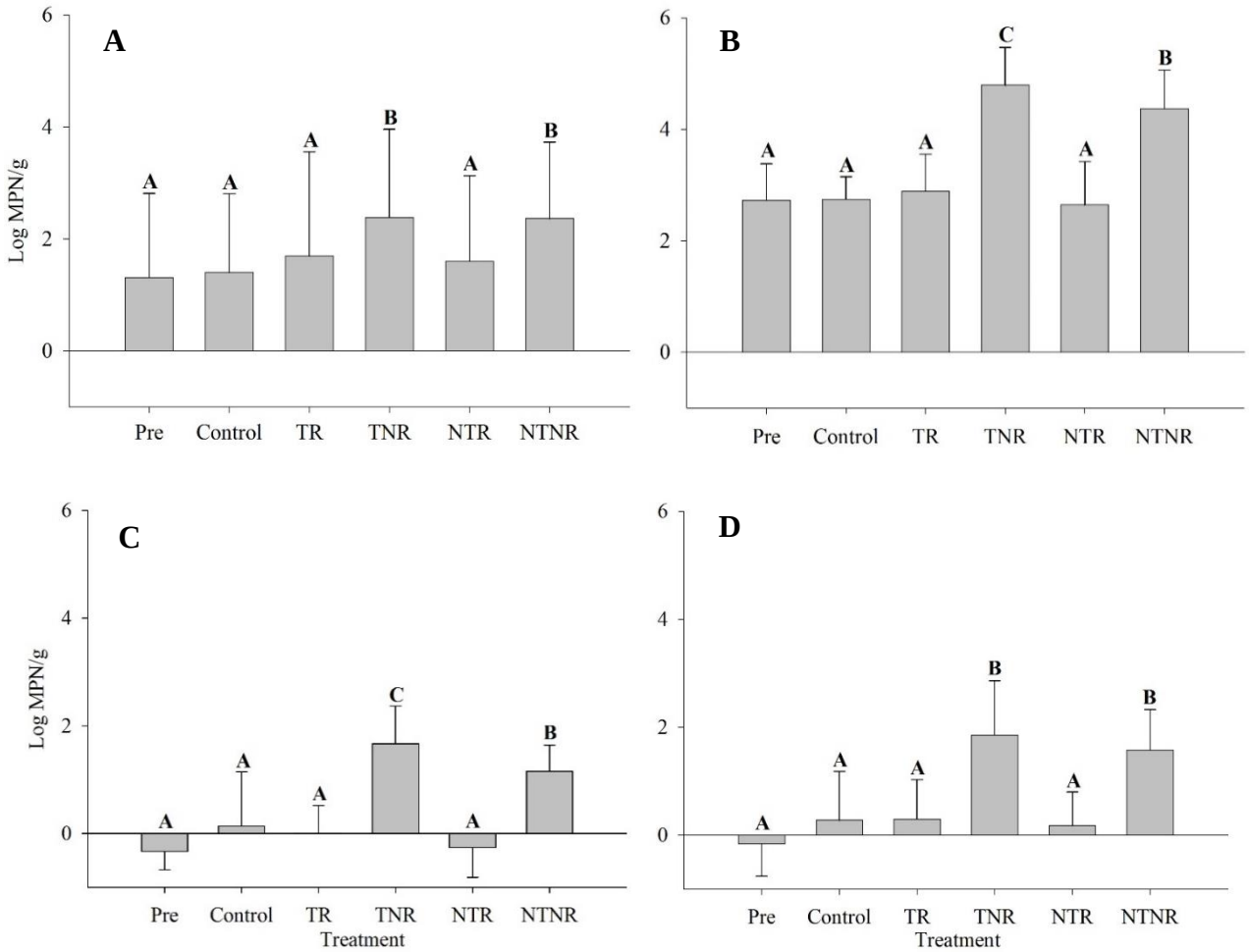

Figure 4.1. Mean log-transformed *Vibrio* levels for A) *V. vulnificus*, B) total *V. parahaemolyticus*, C) *V. parahaemolyticus* (*tdh*+), D) and *V. parahaemolyticus* (*trh*+) before (Pre) and after the handling treatments were applied: Control (submersed control), TR (tumbled, refrigerated), TNR (tumbled, 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=12).

3.3. Recovery of Elevated Vibrio spp. After Resubmersion.

After one day of resubmersion, some of the refrigerated oysters experienced a spike in *Vibrio* spp. levels, while others did not (Fig. 4.2). For example, the TR and NTR oysters did not experience a spike in *V. vulnificus* levels after resubmersion, with levels remaining 0.18 ± 0.58 and 0.30 ± 0.58 log MPN/g lower than the control levels and never significantly elevating (Fig 4.2A; $p \geq 0.30$). Meanwhile, the total *V. parahaemolyticus* levels in the TR and NTR oysters spiked significantly (0.98 ± 0.45 log MPN/g higher than in the control oysters) after one day of resubmersion (Fig. 4.2B; $p < 0.001$). For pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+), the changes in *Vibrio* spp. levels after one day of resubmersion depended on if the oysters were tumbled or not in combination with refrigeration. The *V. parahaemolyticus* (*tdh*+) levels in the TR oysters were 0.99 ± 0.57 log MPN/g higher than control oysters ($p = 0.001$), while the levels in the NTR oysters were 0.25 ± 0.57 log MPN/g higher than control oysters (Fig. 4.2C; $p = 0.38$), with a similar effect observed for *V. parahaemolyticus* (*trh*+; Fig. 4.2D). Meanwhile, the *Vibrio* spp. levels in the non-refrigerated oysters remained significantly higher than the levels in control oysters, except for the levels of *V. vulnificus*, which were not significantly higher than control levels after one day of resubmersion (Fig. 4.2A; $p \geq 0.10$).

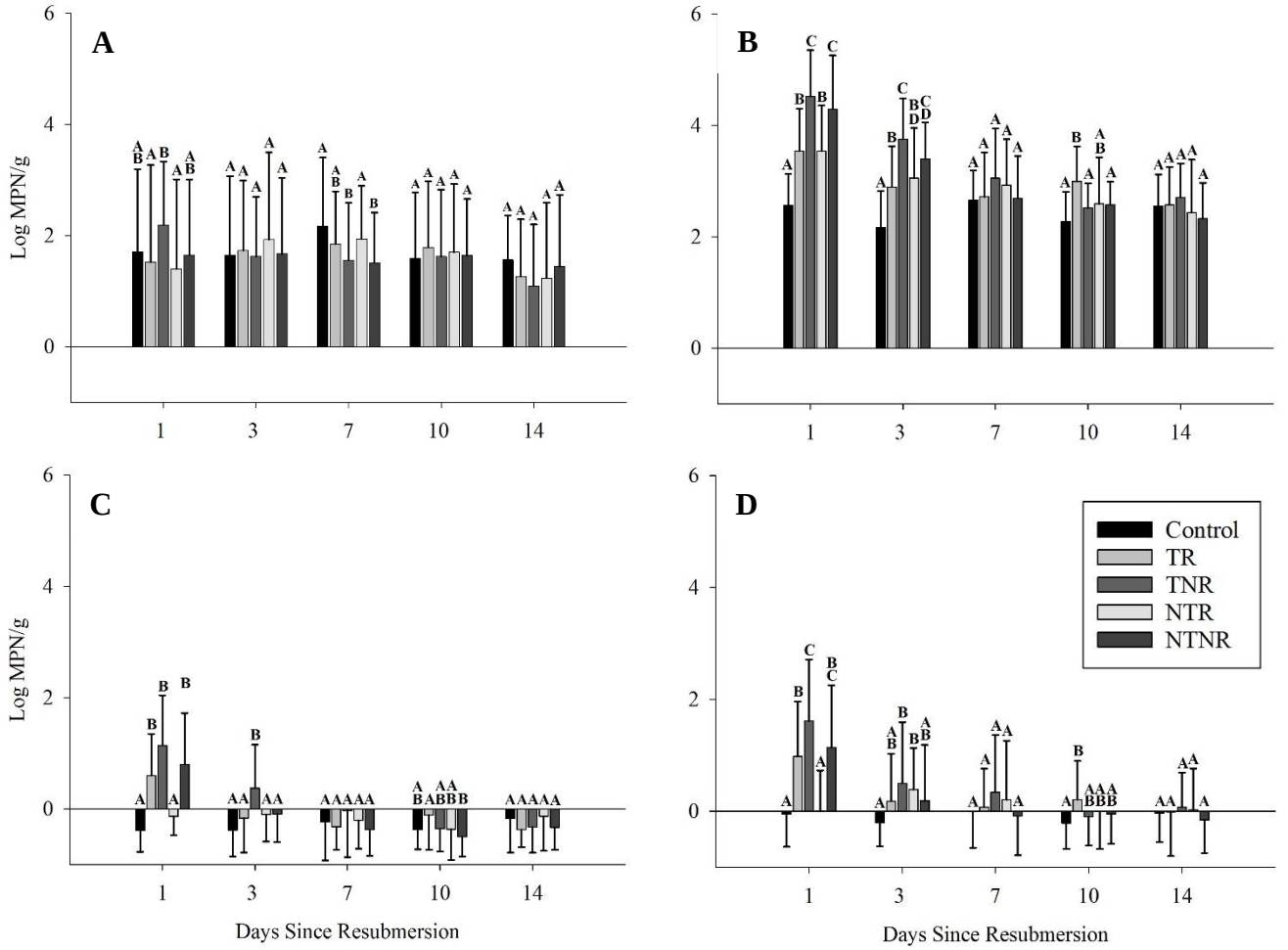

Figure 4.2. Mean log-transformed levels of A) *V. vulnificus*, B) total *V. parahaemolyticus*, C) pathogenic *V. parahaemolyticus* (*tdh*+), D) pathogenic *V. parahaemolyticus* (*trh*+) in oysters during the resubmersion period for the handling treatments: Control (submersed control, TR (tumbled, refrigerated), 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 *Vibrio* spp. levels, as determined by the mixed effects model (n=12).

There were significant interactions ($p \leq 0.01$; Table 4.3) between treatment and days since resubmersion for all *Vibrio* spp., so individual models were performed at each time point to compare the levels in control and treated oysters. Regardless of handling type, the levels of *V. vulnificus* in treated oysters were not significantly higher than the levels in control oysters after one day of resubmersion ($p \geq 0.10$; Table 4.4; Fig. 4.2A). After seven days of resubmersion, the levels of total *V. parahaemolyticus* in all treated oysters were not significantly higher than control levels ($p \geq 0.13$), except for the TR oysters (Fig. 2B). Despite having total *V. parahaemolyticus* levels similar to control levels on day 7 ($p = 0.80$), the levels in the TR oysters were significantly higher on day 10 ($p = 0.001$) before returning to levels similar to control levels on day 14 ($p = 0.92$).

Table 4.3. Summary statistics from mixed effects models, by *Vibrio* spp.[a]

Vibrio spp.	Source	DF	F-Value	*p*-value
V. vulnificus	Treatment	4	0.31	0.87
	Time	**5**	**4.32**	**0.001**
	Treatment*Time	**20**	**1.88**	**0.01**
Total *V. parahaemolyticus*	**Treatment**	**4**	**34.8**	**<0.001**
	Time	**5**	**40.2**	**<0.001**
	Treatment*Time	**20**	**7.65**	**<0.001**
Pathogenic *V. parahaemolyticus* (*tdh*+)	**Treatment**	**4**	**14.5**	**<0.001**
	Time	**5**	**24.2**	**<0.001**
	Treatment*Time	**20**	**5.53**	**<0.001**
Pathogenic *V. parahaemolyticus* (*trh*+)	**Treatment**	**4**	**14.0**	**<0.001**
	Time	**5**	**20.7**	**<0.001**
	Treatment*Time	**20**	**4.66**	**<0.001**

[a]Lines in bold represent significant effects ($\alpha = 0.05$).

The recovery times for elevated pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) varied among the treatment types. For *V. parahaemolyticus* (*tdh*+), the levels in tumbled oysters (TR, TNR) were not significantly higher than control levels after three to seven days of resubmersion, while the not tumbled oysters (NTR, NTNR) required one to three days of resubmersion ($p \geq 0.17$; Table 4.4). Regardless of tumbling, the refrigerated oysters had shorter recovery times (1-3 days) than their matching non-refrigerated treatment (3-7 days). Unlike the recovery times for *V. parahaemolyticus* (*tdh*+), the recovery times for elevated *V. parahaemolyticus* (*trh*+) varied among the treatments with no distinct trend, and the levels in treated oysters were not significantly higher than control levels after three to seven days of resubmersion ($p \geq 0.12$; Table 4.4).

Table 4.4. *Vibrio* spp. recovery times, by treatment.

	Day[a]			
Vibrio spp.	TR[b]	TNR[c]	NTR[d]	NTNR[e]
V. vulnificus	1	1	1	1
Total *V. parahaemolyticus*	14	7	7	7
Pathogenic *V. parahaemolyticus* (*tdh*+)	3	7	1	3
Pathogenic *V. parahaemolyticus* (*trh*+)	3	7	7	3

[a]Number of days after resubmersion when Vibrio spp. levels were not significantly different from control levels ($p \geq 0.05$), determined by the mixed effects model.
[b]Tumbled and refrigerated treatment.
[c]Tumbled and not refrigerated treatment.
[d]Not tumbled and refrigerated treatment.
[e]Not tumbled and not refrigerated treatment.

4. Discussion

Oysters were farm-raised in a floating cage system in Cedar Island, North Carolina and subjected to four routine handling treatments, similar to current industry practices (tumbled and refrigerated, tumbled and not refrigerated, not tumbled and

refrigerated, not tumbled and not refrigerated). The levels of *Vibrio* spp. were monitored after the oysters were resubmersed at the farm site, and the recovery time for elevated *Vibrio* spp. levels to return to levels not significantly higher than control levels was determined for each handling treatment. With a similar experimental design to a previous study (*28*), the goal of this study was to expand the existing knowledge about resubmersion of farmed oysters to a new geographical region.

The trials for this study were performed during the warmest months when *Vibrio* spp. levels in oysters are known to be highest in this region, and therefore risk of infection is assumed to be the highest (*4, 19, 25*). While variation was found in the water temperature and salinity among the trials, the observed conditions were within the optimal ranges for *Vibrio* spp. (*4, 9, 19*) and all four targets were detected in the submersed control oysters. While out of the water, the refrigerated oysters had an average internal oyster temperature below 10ºC and therefore did not experience significant increases in *Vibrio* spp. as previously shown (*6, 8, 11, 24, 27, 28*). The non-refrigerated, or desiccated, oysters experienced significant increases of all *Vibrio* spp., which was expected based on the length of exposure, observed air temperatures, and previous findings (*12, 13, 14, 17, 21, 27, 28*).

After resubmersion, the effects of tumbling and refrigeration were variable depending on the *Vibrio* spp. The refrigerated oysters experienced a spike in total and pathogenic *V. parahaemolyticus* after one to three days of resubmersion, but the delayed increase (~1 log MPN/g) was lower than the initial increase in *V. parahaemolyticus* levels in the non-refrigerated oysters (~1.5-2 log MPN/g). This effect was not seen for *V. vulnificus* in refrigerated oysters, in contrast to previous findings (*27, 28*). The difference

in temperature (~17ºC) between the refrigeration unit and the farm could have placed additional stress on the oysters and delayed the resumption of filter feeding, resulting in the proliferation of *V. parahaemolyticus*. Additionally, *V. parahaemolyticus* has a faster growth rate than *V. vulnificus* (*8*, *24*) and possesses several characteristics that allow it to colonize the oyster and avoid hemocyte phagocytosis (*1*, *29*, *30*). Therefore, *V. parahaemolyticus* could have had a growth advantage over *V. vulnificus*, allowing it to proliferate faster and outcompete *V. vulnificus*. Tumbling had an effect on the recovery times for elevated pathogenic *V. parahaemolyticus* (*tdh*+) levels, with the tumbled oysters requiring longer recovery times than the not tumbled oysters (Table 4.4). While this could be a result of the additional stress from rough handling affecting filter feeding, the same effect of tumbling on recovery times was not observed for the other *Vibrio* spp.

Overall, *V. vulnificus* had the lowest increases in levels after the treatment period and the shortest recovery time for all treatments. Both total and pathogenic *V. parahaemolyticus* required one to seven days of resubmersion to recover to control levels, except for the TR oysters, which required 14 days for total *V. parahaemolyticus* levels to recover. These recovery times were determined using the same statistical model from past resubmersion studies (*27*, *28*), and the issue of statistical significance and biological relevance in relation to the difference in mean *Vibrio* spp. levels in treatment and control oysters was also considered here. In the analysis, the statistical significance groupings agreed with the more conservative approach of a biologically relevant difference in means. Therefore, when the model showed that the *Vibrio* spp. levels in the treated oysters were not significantly different from the control oysters, the difference in means between the two groups was less than 0.5 log MPN/g, and vice versa.

Comparing these recovery times for the common treatments from a recent study in Alabama (*28*), similar trends were observed. Of the targets measured, *V. vulnificus* had the shortest recovery time in Alabama (three days) and North Carolina (one day) and did not differ between the TR and NTNR (desiccated) treatments. Total *V. parahaemolyticus* had the longest recovery time (up to 14 days in both areas) and differed between the handling treatments, with TR oysters requiring 14 days of resubmersion and the NTNR (desiccated) oysters requiring 7 days. Pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+) had a shorter recovery time (3 days) in North Carolina than in Alabama (7-14 days depending on treatment). Despite the differences in recovery times among the individual *Vibrio* spp., the overall recovery times were essentially the same between the states: all *Vibrio* spp. levels in TR oysters recovered to control levels after 14 days of resubmersion, and the levels in NTNR oysters recovered after 7 days.

Cultured oysters were subjected to routine handling practices that resulted in elevated *Vibrio* spp. levels within the oysters, which were purged after the oysters were resubmersed at the farm and resumed filter feeding. The study was performed in a previously unstudied region (Cedar Island, North Carolina), and designed to allow for comparison to a similar study on cultured oysters in Grand Bay, Alabama (*28*). The results from this study support the previous finding that tumbling and refrigerating should not be considered as a best farm management practice, as recovery times for elevated *Vibrio* spp. were longer for those oysters. Excluding this tumbling and refrigeration (TR) treatment, a seven day resubmersion period was sufficient for the recovery of elevated *Vibrio* spp. levels in the other treatments tested, similar to previous findings in Alabama (*12, 13, 27, 28*). While the handling practices used in this study were representative of

those used by farmers in the Southeastern United States, and the resultant data might not be applicable to other handling practices, gear types, or geographical regions not examined in this study, the results provide further evidence to support a seven day resubmersion period for oysters that are desiccated (under refrigeration or ambient conditions) or tumbled followed by ambient desiccation, but not those tumbled and then refrigerated overnight.

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Chapter V. Summary for the Industry

Background

In off-bottom oyster aquaculture, farmers will routinely use different culture practices to improve the quality and consistency of their oysters. When farmers perform these routine handling practices, they must be aware that they may increase the public health risk due to the potential increases in vibrio bacteria within the oysters as a result of this handling. Vibrio are naturally occurring bacteria in the marine environment and are concentrated within oysters during filter feeding. Certain species are known to cause illness in humans, and most vibrio infections occur from eating raw or undercooked seafood. During routine handling, farmers will pull the oysters out of the water for prolonged periods of time, 'breaking the time-temperature window' by exceeding the maximum allowed time of ambient exposure prior to refrigeration for harvest. This also interrupts the filter feeding activity of the oysters. This interruption, combined with warmer temperatures outside of the water, allows the vibrio bacteria to multiply within the oyster. Therefore, after handling, the oysters have higher levels of vibrio bacteria, possibly resulting in an increased public health risk, and cannot be sold for raw consumption.

Resubmersion of Oysters After Handling

Farmers can easily mitigate the increased public health risk by putting the oysters back in the water after handling. This process, referred to as resubmersion, allows the oysters to resume filtering in the water, and the higher levels of vibrio bacteria are purged from the oyster. After an appropriate resubmersion period, the levels of vibrio bacteria in the oysters have returned to the levels before the routine handling occurred. Then, the

farmers may harvest the oysters within an appropriate time-temperature window to be sold for raw consumption (as prescribed by the local permitting authority). Resubmersion is advantageous for farmers because it allows them to remove oysters from the water to improve product quality and consistency, resubmerse the oysters to mitigate the increased health risk, then sell the oysters for the half-shell market to earn a higher profit.

Each state has different resubmersion requirements for farmers; for example, Alabama farmers have either a 7 or 14 day resubmersion requirement based on the culture gear type the oysters are raised in, and the handling type used on the oysters. In order for resubmersion to be successful, the oysters should be actively filtering in order to purge the higher vibrio levels. Anything that affects an oyster's ability to effectively filter feed, such as crowded or overstocked bags, can affect the oyster's ability to purge. While studies have shown that resubmersion is effective following desiccation, there are several other factors that could potentially affect the length of a resubmersion requirement, including handling type, culture gear type, time of year, and geography. We designed a series of studies to investigate these factors, and we present the following summary of the findings for the industry.

Effects of Handling Type

- The recovery times for oysters subjected to four different handling types were determined: 1) desiccation only, 2) tumbling followed by overnight desiccation, 3) tumbling followed by overnight refrigeration, 4) overnight refrigeration only.
- The vibrio recovery time after tumbling (a rougher form of handling) was similar to the recovery time after desiccation only.

- Refrigerating oysters overnight prevented vibrios from increasing while the oysters are removed from the water, but the vibrio levels increased after one day of resubmersion, before decreasing to background levels. Refrigeration did not reduce the overall recovery time for vibrio, so refrigerating oysters overnight (instead of desiccating) would not be recommended as a beneficial practice.

- A combined treatment of tumbling oysters followed by refrigerating overnight required the longest recovery time out of the four handling types tested. Therefore, a combined treatment of tumbling and refrigerating oysters is discouraged as a common industry practice in the Gulf of Mexico.

- Overall, oysters that were maintained in an adjustable longline system (stocked at 100-120 oysters per bag) and desiccated for 24 hours, or tumbled and then desiccated for 24 hours, required 7 days of resubmersion for elevated vibrio levels to recover.

Effects of Gear Type

- The recovery times for oysters maintained in two common gear types (adjustable longline system, OysterGro® system) were compared.

- The recovery times were similar for oysters in both the gear types when the oysters were subjected to the same handling types.

- Oysters that were desiccated for 24 hours, then resubmersed in the adjustable longline system (100-120 oysters per basket) or the OysterGro® system (150-200 oysters per bag) required 7 days of resubmersion for vibrio levels to recover.

- Oysters that were tumbled, refrigerated overnight, then resubmersed in the adjustable longline system (100-120 oysters per basket) or the OysterGro® system (150-200 oysters per bag) required 14 days or more of resubmersion.

- A low number of replicate trials was performed in this study in comparison to previous resubmersion studies, so additional research is needed with more replication to further investigate the effects of culture gear type.

Effects of Time of Year

- Previous research has focused on the vibrio recovery times during the hottest months of the year (June-September), when the vibrio infection risk is thought to be the highest. However, no research has been conducted during the beginning of the increased risk period (May), when water temperatures can be cooler. This study compared the vibrio recovery times between early May and July.

- The recovery times for desiccated oysters were longer in May than in July, suggesting that May could require a longer resubmersion period than July.

- In May, oysters that were desiccated for 24 hours and resubmersed in the adjustable longline system or the OysterGro® system required 14 days or more of resubmersion for vibrios to recover.

- In July, oysters that were desiccated for 24 hours, and resubmersed in the adjustable longline system or the OysterGro® system required 7 days of resubmersion for vibrios to recover.

- In both May and July, oysters that were tumbled and refrigerated overnight, and resubmersed in the adjustable longline system or the OysterGro® system required 14 days or more of resubmersion for higher levels of *Vibrio* to recover.
- More research is needed with a higher level of replication to further investigate the observed seasonal trend.

Effects of Geography

- This study determined the recovery times for oysters that were maintained in the OysterGro® system and either desiccated or tumbled and refrigerated in Grand Bay, Alabama and Cedar Island, North Carolina.
- The data suggest that geography did not have an effect on the recovery times for vibrio under the conditions tested.
- In both states, the desiccated oysters required 7 days of resubmersion for higher vibrio levels to recover, while the tumbled and refrigerated oysters required 14 days or more of resubmersion.
- However, these sites should not be considered representative for each state, and future studies are needed to further investigate the potential effects of geography.

In summary, routine handling practices utilized by farmers potentially exceed the allowable amount of time out of water for harvest and may increase the vibrio levels within oysters, creating a public health risk. Tumbling and refrigerating oysters overnight produced the longest vibrio recovery times, so adding a refrigeration step while the

oysters are removed from the water for handling is discouraged as a common industry practice for Gulf of Mexico farmers. Based on this series of studies, oysters should be resubmersed for at least 7 days, and in some cases 14 days or more, depending on the handling type, gear type, time of year, and location. Due to time and sampling constraints, the studies that tested the effects of gear type, time of year, and geography had low levels of replication. Therefore, future studies should focus on these factors with higher levels of replication to confirm the trends discovered here.