Effects of culture methods in the Pacific Northwest on the levels of *Vibrio* spp. in farm-raised oysters (*Crassostrea gigas*)

by

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Abstract

Oyster aquaculture utilizes both on-bottom and off-bottom culture techniques to harvest raw oysters for the half-shell market. In the Pacific Northwest, the farming practices for growing the Pacific oyster, *Crassostrea gigas*, in the intertidal zone expose them to elevated air temperatures for up to several hours each day. These periods of tidal desiccation can improve oyster quality by reducing biofouling but increasing air temperatures in summer months may result in growth of the naturally occurring human pathogens *Vibrio parahaemolyticus* and *V. vulnificus*. Following tidal desiccation, oysters are resubmersed by the incoming tide and can begin filter feeding again, allowing for elevated *Vibrio* spp. levels to return to levels seen prior to air exposure. This study compared the effects of two types of intertidal culture methods used in Samish Bay, Washington on the levels of *Vibrio* spp. in farmed oysters following tidal desiccation and after resubmersion. The two culture methods, beach culture (bottom culture) and flip bag (off-bottom), were compared to determine whether culture method affects the recovery of *Vibrio* spp. levels. Following maximum exposure to air temperatures, *Vibrio* spp. levels generally increased within oysters from both culture methods, although high variation among trials was observed. Subsequent resubmersion from the incoming tide resulted in a decrease in *Vibrio* spp. levels, although variability made general conclusions on the recovery of *Vibrio* spp. difficult. Additionally, while *Vibrio* spp. levels often were not affected by culture method, when they were, levels in flip bag raised oysters were higher than those in beach culture oysters in all cases but one. This study is intended to help oyster growers and public health officials in the region ensure that their culture methods and regulations are not increasing consumer risk from these bacteria.

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List of Abbreviations

APW	Alkaline Peptone Water
CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
MPN	Most Probable Number
NOAA	National Oceanic and Atmospheric Administration
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNW	Pacific Northwest
PSU	Practical Salinity Unit
tdh+	Thermostable Direct Hemolysin
tlh	Thermolabile Direct Hemolysin
trh+	TDH-Related Hemolysin
USD	United States Dollar
Vp	*Vibrio parahaemolyticus*
Vv	*Vibrio vulnificus*
YSI	Yellow Springs Instruments

Chapter I. Introduction to oyster aquaculture and the effects on *Vibrio* spp. and public health

Oyster Aquaculture

The practice of oyster farming, or aquaculture, has existed for over 2,000 years and continues to be a thriving industry in the world of aquaculture (*5, 38*). Oysters are the leading molluscan aquaculture taxon by quantity produced in the world with approximately 6 million tons produced globally (dominated by production in China), with the United States accounting for over 15,000 tons in 2016 (*5, 18*). Oyster aquaculture continues to increase as commercial fisheries harvest has leveled off and the increasing global population look for healthier alternatives to sources of protein such as pork and beef (*4, 5, 34*). Oysters are enjoyed around the world as a source of protein, zinc, copper, vitamin B12, and many other healthy fats, vitamins, and minerals (*32, 62*). The Pacific oyster (*Crassostrea gigas*) is one of the most common species used for aquaculture and, by 2003, was the most produced species by weight of all mollusk, crustacean, and fish species in the world and had a global production value of USD 3.69 billion (*17*).

Globally, oyster aquaculture is generally practiced in shallow coastal waters between intertidal to subtidal depths of about 0.5m to 3m but can extend beyond 30m (*19*). To cultivate oysters, a variety of methods are used, relying either on natural recruitment of oysters to culture grounds or hatchery production of oysters (*35*). In all cases, cultivated oysters, which filter feed phytoplankton, rely on the successful settlement of larvae onto a specific substrate within a hatchery or wild setting, and the management or privately owned or leased land (*44*). Oysters prefer to set on other oyster shell but can also set on a number of other substrates including ceramic tiles and polyester film (*44, 63*). Two of the most common types of oyster aquaculture are on-bottom and off-bottom culture.

In on-bottom culture, oysters are allowed to grow on the natural sea bottom, most similar to a natural reef. In some cases, the natural bottom is made more favorable for oyster recruitment by distributing hard substrate, such as oyster shell (cultching). In others, hatchery-spawned larvae are allowed to attach to shell or other substrate to create 'spat on shell' which are distributed to the on-bottom areas. On-bottom culture generally allows for high levels of production, but production levels are cyclical due to variables such as predation, salinity changes, and years of poor recruitment (*55*). In addition, the oysters grown on-bottom form irregular shapes and grow in clusters which are not desirable traits for the raw-half shell market and therefore tend to obtain lower prices through the shucked meat market (*64*).

Off-bottom methods typically utilize single set hatchery-reared oysters raised above the seafloor in culture gear such as mesh baskets, floating cages, or clipped on lines (*65*). With off-bottom methods, oyster growers are able to control variables such as stocking densities, position in the water column, predation, improving shell shape and appearance, and controlling fouling issues which allows for creating a market ready product faster (*10, 50, 60, 65*). However, off-bottom techniques tend to be more labor intensive and are usually more costly than on-bottom techniques; growers attempt to off-set these costs by consistently producing higher quality oysters (*19*).

Regardless of production method, oyster growers attempt to produce the highest-quality deep cupped oysters to appeal to the high value half-shell market, where oysters are consumed raw. Minimizing any risks that come with raw oyster consumption is not only of vital importance to public health, but a key to success in this industry. Oyster growers have a high interest in the risks surrounding raw consumption and how they can minimize them to produce the safest possible product.

As this project was conducted in Washington state, it is important to highlight the state's extensive history in shellfish aquaculture, dating back to the mid-nineteenth century. Since then, Washington has excelled in the field and as of 2013, is the leading U.S. producer of farmed bivalves producing over 23 million pounds of shellfish through aquaculture, 37.5% of which is the Pacific oyster (*67*). The state's shellfish industry generated over 2,700 jobs and contributed USD 184 million to Washington's economy in 2010 (*67*). If the production of oysters were to be compromised in any way, it could result in drastic economic and health impacts for the region.

Vibrio Bacteria

Oysters are bivalve mollusks which feed by filtering the waters surrounding them, which includes any microbes in the immediate area (*20*). During filter feeding, oysters can accumulate naturally occurring pathogenic microbes such as bacteria from the genus *Vibrio* (*9, 16, 24*). When periods of air exposure occur, and oysters keep their shells closed to conserve moisture, levels of *Vibrio* spp. can rapidly increase within oysters, creating a potential public health concern (*11, 30*). The two main species of *Vibrio* spp. that are commonly associated with illnesses related to raw oyster consumption are *Vibrio parahaemolyticus* and *V. vulnificus* (*13*).

Vibrio parahaemolyticus is a gram-negative, halophilic bacterium that naturally inhabits estuarine environments and coastal areas (*13, 29*). Infections can result from open wounds or eating undercooked or raw seafood, resulting in gastroenteritis or septicemia (*13*). The species is estimated to cause 45,000 illnesses each year in the United States (*7*). Abundances of *V. parahaemolyticus* are known to increase within oysters and other shellfish that are subjected to warm ambient air temperatures that create conditions favorable for growth (*11, 21, 49*). In detection of *V. parahaemolyticus*, a known indicator of the species is the thermolabile direct

hemolysin (*tlh*) gene (*45*). Although not all strains of *V. parahaemolyticus* are truly pathogenic, the pathogenicity may be determined by the presence of the *tdh* gene that codes for the production of a thermostable direct hemolysin and/or the *trh* gene, that codes for the production of *tdh*-related hemolysin. The hemolysins produced have similar activity in cells where they will target host cells through adsorption and rupture the cell membrane causing disintegration (*31, 43, 54*). Although these genes are the most common factors to differentiate pathogenicity in *V. parahaemolyticus*, there is evidence that they do not fully explain its pathogenicity and additional factors contribute to virulence (*26, 47, 58*). Nonetheless, both the *tdh* and *trh* genes are proven virulence factors (*31, 43, 56*).

Vibrio vulnificus is also a gram-negative, halophilic bacterium found on all coasts of the United States (*41*). The species has a variety of virulence factors that can contribute to its pathogenicity (*14, 22, 25, 33*). Aside from virulence factors, hosts that are immune-compromised due to underlying health conditions are at greater risk for *V. vulnificus* infection than healthy individuals (*57*) making all strains a public health concern. Once a host is infected, this species can cause gastroenteritis and primary septicemia, as well as, major wound infections (*28, 46, 61*). The resulting symptoms include nausea, abdominal pain, and secondary lesions (*28*). When oysters are held in air temperatures between 18-34°C, *V. vulnificus* abundances can increase from when initially harvested, creating an increased public health concern (*11*). *V. vulnificus* is responsible for 95% of all seafood-borne deaths (*46*).

During the summer months, concentrations of *Vibrio* spp. in oysters are found to be at their highest compared to the rest of the year and nearly 100% of oysters can carry *V. parahaemolyticus* and/or *V. vulnificus* (*15, 41, 69*). Additionally, Mote and Salathé Jr. (*40*), predict that within the next 20 years, we may see a temperature increase in the Pacific Northwest,

the selected study area of this project, of up to 2.9°C (5.2°F). This projected warming may also be the greatest in the summer months. When oysters are exposed to air temperatures during summer months, *Vibrio* spp. concentrations could rise within their shells as oysters will keep them closed to conserve moisture. Any knowledge on how *Vibrio* spp. levels fluctuate within harvest-ready oysters is of high value to public health.

Pre-Harvest Aquaculture Practices and *Vibrio* spp. in Oyster Aquaculture

As consumption of raw oysters can create a health risk in consumers, managing the entire process from farm through sale on the half-shell market is of great importance to control *Vibrio* spp. infection. This has been regulated through strict harvesting requirements and laws that are made to have rigorous control of the cold-chain after harvest in order to get market ready oysters to mechanical refrigeration in a minimal amount of time. Getting oysters to refrigeration and minimizing their time out of the water prior to refrigeration can prevent growth of, and decrease consumer exposure to, *Vibrio* spp. (*12, 21*).

With the advent of oyster aquaculture, and particularly off-bottom oyster farming, farmers have the opportunity prior to harvest to handle their oysters in a variety of ways to improve yield and/or quality. These manipulations potentially affect the risk level in oysters before they are harvested. One pre-harvest handling technique applied on most oyster farms is desiccation, when oysters are removed from the water to air-dry which can prevent biofouling organisms, such as barnacles and mud worms, from establishing on the oysters' shells and the equipment used to raise them (*1*). Desiccation (either tidal or manually imposed) and other common routine handling practices can expose oysters to elevated air temperatures, often for long periods of time, and put them at risk for an increase in *Vibrio* spp. levels (*11, 21, 30, 52*).

After the period of desiccation is complete, oysters may be resubmersed in water and can begin filter feeding once again. As a result, *Vibrio* spp. levels may be able to return to background levels as oysters begin to filter feed and flush out the bacteria (*3, 50, 52*).

Desiccation practices vary for different locations and regions depending on culture techniques, tidal levels, gear type, and a variety of other factors. This process is controlled by the grower in places, such as the Gulf Coast, where tidal ranges are between 2 and 4 feet. In these areas, growers utilize gear that can expose oysters to air-drying with the low tidal shifts, or they manually remove them from the water and eventually resubmerse them (*65*, Walton, personal communication). In Alabama, following desiccation events, farmers are required to keep oysters submersed for 7 or 14 days prior to harvesting, depending on which gear type is used (*2, 23, 30*). Desiccation can also occur with tidal exposure, such as in the Northeast on the Atlantic Coast and in the Pacific Northwest, where large tidal ranges may expose tidal flats for several hours each day. This subjects all oysters to desiccation, including those on bottom, and is referred to as tidal desiccation.

Several studies have explored how the effect of desiccation might interact with other culture practices, such as gear type used for culturing. On the Gulf Coast in Alabama, previous research has found that handled oysters should be resubmersed for a minimum of 14 days prior to harvesting (*30*). In response to grower concerns about longer resubmersion times affecting factors such as biofouling organisms re-establishing and harvest closures due to increased rainfall occurring during the 14-day period, another study (*23*) was conducted examining the effects of desiccation on levels of *Vibrio* spp. and found that 7 days of resubmersion was sufficient in allowing elevated levels of *Vibrio* spp. to return back to levels prior to air exposure when using a specific off-bottom gear type (adjustable long-line system). In regards to gear type

and how it could affect *Vibrio* spp. recovery times, Walton *et al.* (*66*) found that there was no significant difference in ambient *Vibrio* spp. levels between oysters raised in suspended and floating culture methods after 7 days of resubmersion. Pruente et al. (*52*) examined how gear type and handling affected *Vibrio* spp. levels after resubmersion over time and observed little to no effect of gear type on *Vibrio* spp. levels.

Other than desiccation, a variety of culture practices and routine handling techniques are implemented to help improve the product quality and aid in the farming process that may remove oysters from the water. Tumbling is the practice of using a device such as a mechanical grader to aid in size sorting and to improve shell appearance (*53*). Oysters are removed from the water for extended periods of time, thus violating the regulatory time limits that oysters are allowed to be held out of the water prior to harvesting and exposing them to elevated air temperatures, creating an ideal environment for *Vibrio* spp. growth (*11, 27*). Refrigerating oysters overnight following these handling practices has been hypothesized to reduce the amount of resubmersion time needed for *Vibrio* spp. levels to recover. Pruente *et al.* (*52*) examined the effects of tumbling and refrigeration on *Vibrio* spp. in harvest ready oysters. Their results found that while *Vibrio* spp. levels were not significantly higher following tumbling and refrigeration, 7 to 14 days of resubmersion was needed to observe a recovery to background levels, while all other combinations in the study, such as tumbling and no refrigeration prior to resubmersion, required a maximum of 7 days of resubmersion to recover from elevated levels.

Much of the research done on the effects of pre-harvest handling practices on *Vibrio* spp. has been done in the coastal waters of the northern Gulf of Mexico. To determine the applicability of these results to other regions, Pruente *et al.* (*Submitted*) compared different handling treatments in Alabama and North Carolina, including manual desiccation, and their

effect on recovery times of *Vibrio* spp. in *C. virginica* following resubmersion. They found that recovery times varied between 7 and 14 days dependent upon the treatment in North Carolina, similar to what was viewed in Alabama, and thus it was concluded that geographic location did not affect *Vibrio* spp. level recovery times in cultured oysters using these methods across the tested areas. In addition, McGough *et al.* (*In prep*) examined the effects of geographic variability on resubmersion times at multiple sites along the Gulf Coast (Florida and Alabama). In contrast, these results showed resubmersion periods differed based on *Vibrio* spp. and suggested that there may be variability in the elevation (during desiccation) and recovery (during resubmersion) of *Vibrio* spp. levels at different geographic locations, ultimately stressing the importance of investigating resubmersion research across geographic regions and comparing areas within these regions.

Tidal Desiccation and *Vibrio* spp. in Oyster Aquaculture

Most resubmersion studies have demonstrated the need to understand how farming practices prior to harvest affect *Vibrio* spp. levels, but these studies have been limited to *C. virginica* subjected to manual desiccation, primarily on the Gulf Coast. As such, there is a lack of data on how *Vibrio* spp. levels are affected by different culture practices, including tidal desiccation, and other oyster species.

Intertidal harvest practices are common in places such as the Northeast and Pacific Northwest where oysters are subjected to tidal desiccation on a daily basis. With an increase in outbreaks from areas such as these (*42*, *59*), understanding how *Vibrio* spp. levels may fluctuate during a given tidal cycle is important in mitigating the risk that consuming shellfish raw may pose. In New Jersey, the effects of air exposure were studied in oysters taken from intertidal

flats, refrigerated, and resubmersed by the tidal cycle (*27*). While levels of *Vibrio* spp. did not increase during the refrigeration period, levels did increase following one day of resubmersion. *Vibrio* spp. levels decreased, similar to background levels, following a second day of resubmersion. However, this showed that once oysters were exposed to increased temperatures following refrigeration and desiccation, *Vibrio* spp. levels rose, showing the increased risk associated with oysters exposed to warm temperatures despite being refrigerated and resubmersed by the tidal cycle in an area subject to tidal desiccation. Further confounding effects on risk, *V. parahaemolyticus* outbreaks in the Northeast have been associated with strains from shellfish in the Pacific Northwest (*42*), posing the question of whether these "Pacific Northwest strains" put consumers at an increased risk while also possibly affecting shellfish, and subsequently consumers, in different regions.

In the Pacific Northwest, outbreaks have been prevalent and documented for the past few decades (*6, 8, 59*). Gaining knowledge on what could be the causation of these outbreaks is of vital importance to public health officials. In Washington state, there are many different types of methods used to culture Pacific oysters on tidal flats (*48*). Of the different culture methods used, two of the most common are bottom culture (beach culture) and tumble bag/flip bag culturing (off-bottom) (*48*). Beach culture (Figure 1.1) has been the traditional and predominant means of oyster production in the region where oysters are grown-out directly on the firm bottom of a natural bed. The sandy/muddy substrate gives the oyster a desired long, ruffled shape and lets the oyster look and grow as natural as possible. Flip bag farming (Figure 1.2) is relatively new to the region. With this culture method, a rope runs horizontal to the bottom, two feet off the ground, with mesh bags hanging from it. Attached to the bottom of the mesh bag is a floatation device. The oysters are kept inside of the mesh bag, causing them to rise and fall with the tide levels.

The movement caused from the tides allows for new shell growth to break off, which increases shell quality, and the constant tumbling results in the oyster having to work harder as it grows thereby creating a firmer meat (*48*). The main differences between the oysters in these techniques is that they may be 1) feeding at different levels in the water column, 2) subjected to different levels of disturbance which could stop feeding and respiring at different times in the tidal cycle and 3) are exposed to direct sunlight in different ways. In both methods, growers will prepare for intertidal harvest methods by gathering their oysters during low-tide, when the flats and farms easier to gather oysters on, to be eventually picked up by vessel in large containers and shipped to a processing plant.

Growers in the Pacific Northwest, farming in the intertidal zone, face the task of working with the tidal patterns of the region, which can subject their oysters to the daily process of tidal desiccation. This process, in general, has its own challenges as the levels of *Vibrio* spp. can fluctuate within oysters during a single tidal cycle when they are forced to close due to exposure (*27, 45*). In 2003, Nordstrom *et al*. (*45*) conducted a study along Hood Canal, WA examining the effects of harvest practices on levels of *V. parahaemolyticus* in oysters following an FDA risk assessment stating that the number of illnesses predicted to be associated with oysters from the Pacific Northwest was substantially underestimated (FDA, 2001, as cited in Nordstrom *et al.* 2004). Their findings indicated that densities of *V. parahaemolyticus* increased significantly during exposure to ambient air temperatures caused by tidal desiccation. Those elevated *V. parahaemolyticus* densities returned to background levels after being submerged for a single tidal cycle. A similar study examined *Vibrio* spp. levels in oysters from Washington state subjected to intertidal harvest practices and found that levels did significantly rise following maximum exposure and then returned to background levels following resubmersion for one

whole day (*27*), although, levels of total *V. parahaemolyticus* were dependent on container type used.

Currently in Washington state, before harvesters remove oysters from the tide flats after placing them in a container during intertidal harvest, they must allow a minimum of 4-hours or resubmersion before harvesting (*68*). To help further control *Vibrio* spp. illnesses, from July 1st to August 31st, when water temperatures exceed a certain level (dependent upon location), harvesting cannot occur (*68*). Harvesting regulations are also in place when air temperatures exceed specific amounts, which then affects time of cooling (*68*). These regulations are in place due to the daily tidal desiccation which can expose oysters to direct sunlight, interrupt filter feeding, and effectively produce the perfect environment for *Vibrio* spp. to rapidly proliferate (*11, 21, 49*). Expanding the knowledge in this region can help public health officials refine a *Vibrio* spp. control plan and mitigate the potential risks from raw consumption of oysters.

Objectives of the Study

In recent years, growers in the Pacific Northwest have experienced closures due to an abundance of illnesses caused by *Vibrio* spp. infection associated with consumption of shellfish from the region (*8, 37, 59*). With recent outbreaks, and the potential for temperatures to continue to rise due to global warming (*40*), the chances of future outbreaks exists (*36*). In addition, with the adoption of a new culture method in the region (flip bag farming), it becomes imperative to determine if the risk differs between culture methods and how this affects *Vibrio* spp. levels following tidal resubmersion.

The two-fold objective of this research was to analyze: 1) the effects of tidal desiccation and resubmersion on the levels of total and pathogenic *Vibrio parahaemolyticus* and *V. vulnificus* in cultured Pacific oysters; 2) compare these effects across two culture methods. To determine

the effects of tidal desiccation on *Vibrio* spp. levels, oysters grown in Samish Bay, Washington using traditional beach culturing and flip bag farming were collected at different time points within the tidal cycle. These collection points allowed for the determination of *Vibrio* spp. levels throughout a single tidal cycle and how they fluctuated as the oysters were exposed to air and resubmersed by water over time. The second goal of the research objective was to compare the effects of the two culture practices used in the area on *Vibrio* spp. in the farmed oysters. To determine whether culture method affects *Vibrio* spp. levels in oysters over the course of the tidal cycle, the two techniques were compared. Oysters from each culture method were tested for *Vibrio* spp. levels over time as the oysters were exposed to air and resubmersed by the incoming high tide in an attempt to determine if one culture method gives oysters the chance to recover more quickly from elevated *Vibrio* spp. levels than the other. The data collected in the study will provide additional knowledge for public health officials to make better informed decisions in developing a *Vibrio* spp. control plan for farmed oysters harvested during the summer months in Washington.

Figure 1.1. “Beach culture” oysters grown out using an on-bottom culture method.

Figures 1.2 A) Flip bags during high tide. Attached buoys allow the bags to rise and fall with the tides. The mesh bags allow for water to flow freely so that the oysters may feed. B) Flip bags during low tide.

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Chapter II: Effects of intertidal culture practices on the levels of *Vibrio* spp. in farmed oysters (*Crassostrea gigas*) from the Pacific Northwest

1. Introduction

The culturing of Pacific oysters, *Crassostrea gigas*, along the Pacific Northwest Coast has been prevalent for over 100 years and is the dominant oyster cultivated on the U.S. Pacific coast (*28*). In Washington, two of the most common types of oyster culturing methods used by growers are bottom culture and tumble bag (or flip bag) culture (*28*). Bottom culture, also referred to as beach culturing, is an oyster aquaculture technique where oysters are raised directly on the intertidal grow-out beds providing farmers with a low-cost production method (*28, 35*). Flip bag culturing is a more recently implemented method of off-bottom oyster aquaculture where oysters are cultivated in mesh bags with attached floats hanging on lines held two-to-four feet off the bottom. The floats will cause the mesh bags to rise and fall with the tides, tumbling the oysters inside and improving shell quality while also allowing for protection from predators (*1, 28, 36*). In both culture methods, harvesters commonly prepare the oysters for harvest during low tide by gathering the oysters into large containers and leaving them until the rising tide allows for larger vessels to retrieve the submersed containers and transport them to processing plants. During the intertidal preparation for harvest, and low tidal periods in general, oysters are exposed to direct sunlight and ambient air temperatures of over 30 °C during the summer months in Washington state. This natural exposure can be referred to as tidal desiccation.

During air exposure levels of *Vibrio* spp. within the oysters can rapidly increase (*5, 10, 19, 31*). *Vibrio parahaemolyticus* and *V. vulnificus* are commonly found in estuarine waters where oysters are harvested, are pathogenic to humans, and are of a high public health concern (*6, 8*). These bacteria can be contracted through consuming raw or undercooked shellfish, with 80% of documented cases occurring between the warmer months of May and October and some

of the most well-known outbreaks in the past 25 years coming from the Pacific Northwest region (*3, 4, 9, 12, 15*). The warmer water and air temperatures during these warmer months are correlated with increased densities of *Vibrio* spp. (*3, 8, 16, 33*).

On the incoming high tide, oysters become resubmersed and can resume filter feeding. Resubmersion allows for potentially elevated *Vibrio* spp. levels to recover over time, even when oysters are housed within different gear types (*10, 31*). A previous study in the Puget Sound region (*26*) documented the effects of tidal desiccation on *V. parahaemolyticus* levels in oysters. In this study, oysters were collected both as they emerged from the receding tide and just prior to resubmersion, at maximum exposure. Mean *V. parahaemolyticus* densities increased four to eight times in oysters during exposure to the ambient air temperatures and densities returned to pre-emergent levels over the course of a full tidal cycle (~24 hours). Nordstrom et al. (*26*) also describe the sporadic distribution of *V. parahaemolyticus* in harvest ready oysters from the Pacific Northwest showing the importance of testing in specific harvesting locations. The location in this study (Samish Bay, WA) is a major source of oyster production in the state.

Previous research on air exposure (and associated exposure to increased temperatures) in relation to the effects of different culture methods on *Vibrio* spp. has found that following periods of air-drying, resubmersion times required to see a recovery from elevated levels of *Vibrio* spp. differed between two different off-bottom gear types (*31*). This research shows the importance of monitoring *Vibrio* spp. levels in different grow-out methods, even when in close proximity to one another. To the best of our knowledge, there has been no such research of the effect of different culture methods on *Vibrio* spp. recovery time in the Pacific Northwest, leaving open the question of whether flip bags and traditional bottom culture methods differ. The two culture methods hold oysters at different depths in the water column, exposing them to different

levels of disturbance which could interrupt filter feeding at different times in the tidal cycle. The two methods also expose oysters to direct sunlight in different ways, but it is unclear if this affects *Vibrio* spp. levels between them. Oysters lying directly on the substrate spend the maximum amount of time submersed before the entire tidal flat is exposed to air conditions while oysters held in flip bags are hung off the bottom exposing them to air conditions before waters completely recede during low tide. The mesh bags also float as high as 5 feet off the bottom during high tide, allowing for strong currents and other influences (such as marine mammals and recreational activities) to mimic wave action which has shown to negatively impact the filtration and growth rate of oysters in floating bag gear types (*21*, *28*). These factors could reduce the oyster's ability to effectively recover from elevated *Vibrio* spp. levels over time, thereby leaving a knowledge gap in the industry to allow harvest to be guided by the best available data about different grow-out methods.

During the summer months, warmer temperatures are correlated with increased concentrations of *V. parahaemolyticus* and *V. vulnificus* putting filter feeding shellfish species at risk for accumulation of high densities within them (*7, 23, 29*) . This, coupled with the predicted temperature increases in the Pacific Northwest (up to 2.9°C over the next 20 years), make the summer months of June-August in the region a time of great risk to public health (*22*). These months were chosen as the study time to ensure that *Vibrio* spp. risk was increased to its highest point and to stay within Washington state's "*Vibrio parahaemolyticus* control plan" months (*33*). This study also focused on levels within oysters as Nilsson et al. (*25*) documented the importance of testing *Vibrio* spp. density inside of oysters, rather than testing the density of them in the water column near the farmed oysters, as a better risk assessment tool for the Pacific Northwest.

Previous studies on the effect of intertidal practices on levels of *Vibrio* spp. had also chosen the summer months between July and August to sample (*14*).

This study examined the effects of air exposure during low tidal shifts on the levels of total *V. parahaemolyticus*, pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+), and *V. vulnificus* in oysters from two oyster culture methods over time, following maximum air exposure and then subsequent resubmersion by the incoming tide, in the summer months. Although *V. vulnificus* cases are extremely low in the state of Washington historically (*37*), the species was included in this study as, to the best of our knowledge, the literature concerning their densities in oysters from Washington state and the greater Pacific Northwest is scarce. In order to help fill this knowledge gap, monitoring of their levels throughout a single tidal cycle would provide needed data.

Currently, according to Washington's *"Vibrio parahaemolyticus* control plan", harvesters and shellfish dealers that place oysters in a container or conveyance during intertidal harvesting preparation must wait until the oysters are covered by the incoming tide for a minimum of 4 hours until harvesting and transporting to a processing facility (*38*). However, data on the rates of how levels of *Vibrio* spp. fluctuate within oysters prior to and during intertidal harvest are limited, which restricts the accuracy of risk assessments. The objective of this study was to help fill these data gaps by determining the levels of total and pathogenic *V. parahaemolyticus* and *V. vulnificus* in oysters where intertidal harvest preparation practices are common, using the current industry practices. The data generated provide insight into the responses of *Vibrio* spp. to relevant practices of the industry and public health, which can be incorporated into risk management decisions.

Farm-raised oysters raised using bottom culture (beach culture) and flip bags were sampled at different time points during the course of the tidal cycle: pre-exposure to ambient air conditions, post-exposure to these conditions, 2-hours following resubmersion from the incoming tide, 4-hours of resubmersion, and 24-hours following the original "pre-exposure" time point. With 4-hours of resubmersion following air exposure being the industry standard to be observed before harvesting (*38*), we observed the levels of *Vibrio* spp. before, during, and after this time to evaluate how levels changed during air exposure and tidal desiccation and how these levels fluctuated following resubmersion within the 4-hour time given before harvesting is allowed. Results found from this study will help ensure that oystering farmers, harvesters, and officials in the region are taking the necessary steps to keep *Vibrio* spp. risk at its lowest point during harvest times as well as further guide public health officials to make more informed decisions on resubmersion times for harvest ready oysters.

2. Materials and Methods

2.1. Field Site and Environmental Data

The field work for this study was performed on a commercial shellfish harvest site in Samish Bay, Washington (Puget Sound). The site had a hard mud-bottom with a tidal range of approximately -0.5m to 2.5m. Hatchery spawned Pacific oysters (*Crassostrea gigas*) that were cultured directly on the bottom (beach culture) were utilized for sampling. About 700m off the beach into the bay, mesh bags (Vexar-style 1" mesh, approximately 3' x 1.5' x 4") stocked with hatchery spawned Pacific oysters were hung on lines that were free to rise and fall with the tides (commonly referred to locally as Lentz bags or flip bags) were utilized for sampling. A total of 5 trials were performed in July of 2019 and in July/August of 2020. Water temperature and salinity

were recorded during the collection of samples using a YSI model 55 dissolved oxygen instrument (Table 2.1).

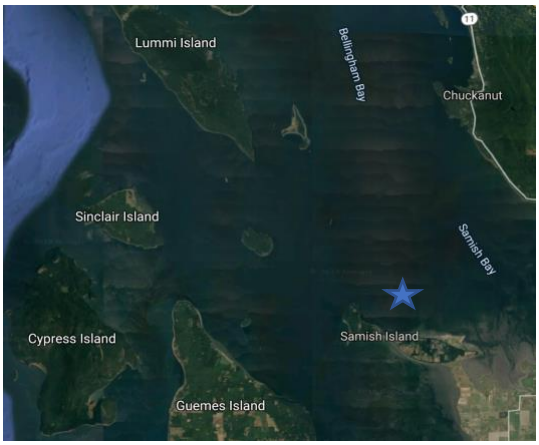

Figure 2.1 Location of sampling site within Samish Bay, Washington.

2.2. Treatment Design and Sample Collection

This study was designed to monitor *Vibrio* spp. levels under two different culture methods located on the same commercial harvest site at a similar tidal height. Flip bag cultured oysters were stocked into mesh bags at 16-32 oysters per bag and attached on the same long lines as bags used for commercial production (Figure 2.2). For beach culture, oysters were sampled among those used for commercial harvest directly on the tidal flats where 15-16 oysters were gathered from the same 1-2m^2 area for each sample. Those samples that were collected during a period of high tide were put into harvest bags with buoys attached, to aid in locating and retrieving them at resubmersed sampling points a later time (Figure 2.3).

For each trial, oysters were collected at five time points: before exposure to air (T_{pre}), at maximum air exposure/before tidal levels resubmersed the oysters (T_{post}), 2-hours after resubmersion (T_2), 4-hours after resubmersion (T_4), and 24-hours from the first collection time

point to represent nearly a full tidal cycle (T_f). At each collection time point, three samples were collected from the two culture methods, with a sample representing a either bag (flip bag culture method) or a 1-2m^2 area (beach culture method). For each sample, 15 oysters were collected. Thus, we collected a total of 6 samples at each time point and 30 samples per trial. For the flip bag culture, at time of sample collection, bags were randomly selected to pull oysters from, with each sample drawn from a separate mesh bag. For the beach culture method, samples collected from T_{pre} and T_{post} time points were gathered directly from the bottom substrate. The remaining beach culture samples were placed in individual harvest bags and collected at the appropriate time points. After collection, oysters were immediately placed in an ice slurry for 10-20 minutes to prevent further bacterial growth. Samples were transported to the University of Washington in a cooler and insulated from ice packs to help maintain samples ≤10°C prior to processing (*17*). Processing of all samples was initiated within 13-hours of collection.

Figure 2.2. A) Sampled flip bags with attached white buoys allowing them to rise and fall with the tides. B) Sampled flip bags at high tide, distinguished from those used by the farmers with attached white buoys.

Figure 2.3. “Beach culture” oysters used for on-bottom culture methods. Harvest bags and white buoys are utilized to aid with collection during high tide.

2.3. MPN and Real-time PCR

Samples were processed according to the standard procedures for the three-tube most probable number (MPN) method in the Food and Drug Administration’s (FDA) *Bacteriological Analytical Manual* (*2, 17, 24)*. Between 10 and 12 oysters from each sample were rinsed with cold tap water, scrubbed with a sterile brush, shucked into a sterile blender, and blended for 60-120 seconds. The homogenate was serially diluted 10-fold to at least 1:100,000 into phosphate buffered saline (PBS; 7.65g NaCl, 0.724g Na_2HPO_4, 0.21g KH_2PO_4, 1L distilled H_2O, pH 7.4 ± 0.2), and 1 mL of each dilution was then inoculated in triplicate into alkaline peptone water (APW; 10g Bacto Peptone, 10g NaCl, 1L H_2O, pH 8.5 ± 0.2). Three tubes containing 10 mL of APW were inoculated with 1 g of oyster homogenate each. The MPN tubes were then incubated at 35±2 ºC for 18-24 hours. Following incubation, all tubes were examined for bacterial growth (turbidity). Those that were positive were then prepared for crude DNA extract by heating 1mL quantities to 95 ºC for 10 min. DNA extracts were then immediately frozen at -20 °C and

prepared for shipment to the FDA's Gulf Coast Seafood Laboratory on Dauphin Island, Alabama.

In preparation for real-time PCR analysis, after completely thawing, samples were centrifuged at 12,500 rcf for 2 min. The resulting supernatants (2 μL) were then tested by real-time PCR (AB7500) for the presence of total *V. parahaemolyticus* (*tlh*), pathogenic *V. parahaemolyticus* (*tdh+/trh+*), and *V. vulnificus* (*19*). Utilizing a standard MPN table/calculator from Appendix 2 of the *Bacteriological Analytical Manual* (*2*) the positive MPN tubes were used to calculate the levels of each target within each sample.

2.4. Statistical Analysis

Water temperature and salinity data that were collected during each time point were used to calculate the average mean, minimum, and maximum for each trial. A general linear model was used to determine any statistical differences in average means among the trials. All *Vibrio* spp. data, reported as MPN/g of oyster homogenate, were log transformed prior to analysis to normalize the data. In cases where *Vibrio* spp. were below the limit of detection (0.3 MPN/g), half of the limit of detection value was substituted before log transformation. General linear models were used to compare *Vibrio* spp. levels within each trial. Given the public health consequences of a Type II error, an alpha of 0.10 was used to determine statistical significance. All *Vibrio* spp. data are reported as log MPN per gram ± 95% confidence interval.

When the data from all trials were combined and analyzed, there was a significant effect of trial on *Vibrio* spp. levels. For consistency, each trial was analyzed separately to better understand what factors were affecting levels throughout the tidal cycle and thus were analyzed separately to determine the effects of culture method, sampling time, and the interaction between

these two variables on *Vibrio* spp. levels. If a significant interaction between culture method and sampling time was detected, a Fisher's Least Significant Difference test was performed to compare all time points for both culture methods within the trial. To assess whether maximum air exposure significantly raised *Vibrio* spp. levels, general linear models were used to compare T_{pre} and T_{post} time points. Subsequently, to determine the effects of resubmersion on *Vibrio* spp. levels, general linear models were used to compare time points T_2, T_4, and T_f to levels viewed following air exposure (T_{post}). The *V. Vulnificus* levels were frequently below the limit of detection, so no statistical analyses of those data were performed. All data analyses were performed in R studio using the nlme, multcomp, and emmenas packages (*11, 20, 30, 32*). Figures were created in SigmaPlot Version 13.0 (Systat Software, San Jose, CA).

3. Results

3.1. Environmental Data

There were significant differences ($p \leq 0.05$) among trials for the two environmental parameters monitored (Table 2.1). Trial C had a significantly higher mean water temperature than the other trials, but water temperatures in all trials were typical for *Vibrio* spp. (*6*). The mean salinities were significantly different in Trials B (higher) and C (lower) compared to other trials. Although, the observed salinities were typical for *Vibrio* spp. (*13*).

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

Trials	Water temp (°C)[b]	Salinity (PSU)[b,c]
A (Jul 16-17, 2019)	20.3 (17.0-24.8)[A]	25.6 (21.1-29.3)[A]
B (Jul 19-20, 2019)	19.4 (15.8-25.8)[A]	28.1 (27.2-29.8)[B]
C (Jul 21-22, 2020)	23.5 (21.1-26.0)[B]	23.1 (21.8-23.7)[C]
D (Jul 31-Aug 1, 2020)	19.7 (15.8-24.7)[A]	25.3 (22.0-27.0)[A]
E (Aug 4-5, 2020)	21.0 (18.1-27.7)[A]	25.5 (21.9-27.4)[A]

[a] Different letters indicate significant differences between means within the same column.
[b]Averages for the trials with ranges in parentheses.
[c]PSU, practical salinity units.

3.2. Total Vibrio parahaemolyticus

For total *V. parahaemolyticus* (*Vp*) in oysters from beach culture samples, levels increased following tidal desiccation (T_{post}) from levels observed at pre-exposure (T_{pre}) in Trial A (1.69 ± 0.51 log MPN/g), Trial B (0.28 ± 0.37 log MPN/g), Trial C (1.10 ± 0.79 log MPN/g), and Trial E (1.65 ± 0.91 log MPN/g) (Figure 2.4). These increases were statistically significant for Trial A and E ($p \leq 0.10$). In Trial D, a decrease in levels was observed (1.12 ± 0.79 log MPN/g). For levels in oysters from flip bag samples, increases following tidal desiccation were seen in Trial A (1.28 ± 0.44 log MPN/g), Trial B (0.82 ± 0.37 log MPN/g), Trial C (1.51 ± 0.54 log MPN/g), and Trial E (0.71 ± 0.55 log MPN/g), with a smaller increase seen in Trial D (0.20 ± 0.33 log MPN/g). These increases were statistically significant for Trials A, B, and C ($p \leq 0.05$).

Following 2-hours of resubmersion (T_2) by the incoming high tide, levels of total *Vp* in oysters from beach culture samples decreased from the post-exposure (T_{post}) samples in Trial A (1.06 ± 0.51 log MPN/g), Trial B (0.35 ± 0.37 log MPN/g), Trial C (0.69 ± 0.79 log MPN/g), Trial D (0.16 ± 0.79 log MPN/g), and Trial E (0.83 ± 0.91 log MPN/g) (Figure 2.4). These decreases were statistically significant for only Trial A ($p \leq 0.06$). For oysters from flip bag

samples, levels from T_2 decreased from T_{post} levels in Trial A (1.01 ± 0.44 log MPN/g), Trial B (1.00 ± 0.37 log MPN/g), Trial C (0.33 ± 0.54 log MPN/g), and Trial D (0.81 ± 0.33 log MPN/g). These observed decrease in levels were statistically significant for Trials A, B, and D ($p \leq 0.04$). Trial E saw an increase in levels following T_2 (0.17 ± 0.55 log MPN/g).

Following 4-hours of resubmersion (T_4) by the incoming high tide, levels of total *Vp* in beach culture oyster decreased from the post-exposure (T_{post}) samples in Trial A (1.69 ± 0.51 log MPN/g), Trial B (0.50 ± 0.37 log MPN/g), Trial D (0.45 ± 0.79 log MPN/g), and Trial E (0.78 ± 91 log MPN/g) (Figure 2.4). These decreases were statistically significant for Trial A ($p \leq 0.01$). Only in Trial C was an increase in levels (0.55 ± 0.79 log MPN/g) observed. For oysters from flip bag samples, levels following T_4 decreased from T_{post} levels in Trial A (2.03 ± 0.44 log MPN/g), Trial B (0.79 ± 0.37 log MPN/g), Trial C (0.28 ± 0.54 log MPN/g), and Trial D (0.38 ± 0.33 log MPN/g). These decreases were statistically significant for Trial A and B ($p \leq 0.07$). Only Trial E saw an increase in levels in T_4 samples (0.49 ± 0.55 log MPN/g).

Approximately 24-hours following the collection of pre-exposure samples (T_f), levels of total *Vp* in oysters from beach culture samples decreased from post-exposure (T_{post}) samples in Trial A (1.00 ± 0.51 log MPN/g), Trial B (0.76 ± 0.37 log MPN/g), Trial C (0.24 ± 0.79 log MPN/g), Trial D (0.46 ± 0.79 log MPN/g), and Trial E (2.04 ± 0.91 log MPN/g) (Figure 2.4). These decreases were statistically significant for Trials A, B, and E ($p \leq 0.07$). For oysters from flip bag samples, levels at T_f decreased from T_{post} in Trial A (0.38 ± 0.44 log MPN/g) and Trial D (0.27 ± 0.33 log MPN/g). Unexpectedly, increases were observed in Trials B, C, and E from T_{post} levels to T_f levels (0.29 ± 0.37 log MPN/g, 0.22 ± 0.54 log MPN/g, and 0.86 ± 0.55 log MPN/g, respectively). There was no statistical difference in any of these changes ($p \geq 0.15$).

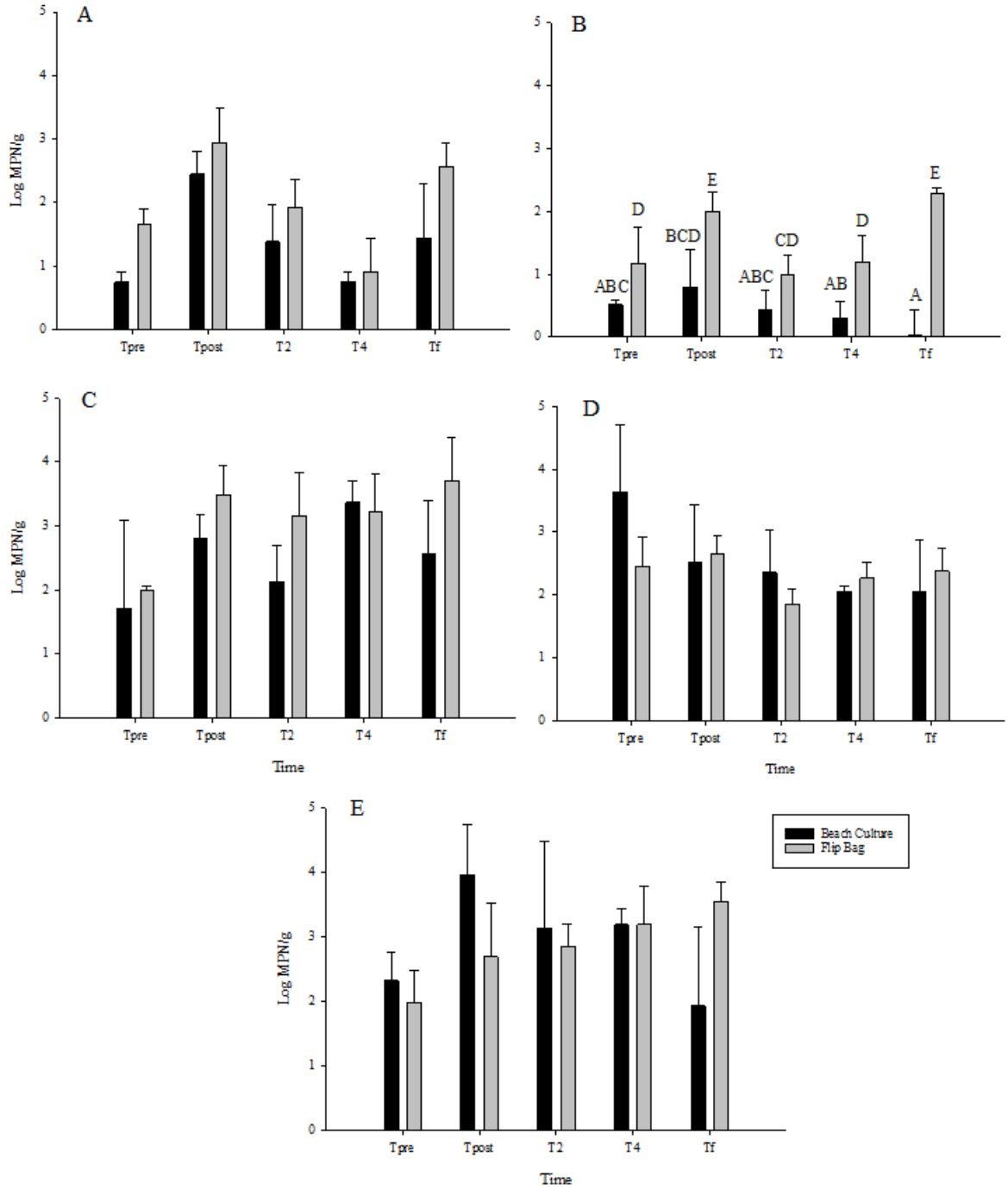

Figure 2.4. Effect of culture method and sampling time on mean log-transformed total *V. parahaemolyticus* levels in oysters from Trial A (A; July 16-17, 2019), Trial B (B; July 19-20, 2019), Trial C (C; July 21-22, 2020), Trial D (D; July 31-August 1, 2020), and Trial E (E; August 4-5, 2020) prior to tidal desiccation (T_{pre}), following maximum air exposure (T_{post}), 2-h following resubmersion from the incoming tide (T_2), 4-h following resubmersion (T_4), and 24-h

following T_{pre} collection (T_f). Bars represent standard error. Letters represent significant differences in *V. parahaemolyticus* levels, as determined by the post hoc comparison test.

3.3. trh+ V. parahaemolyticus

For *trh+ V. parahaemolyticus* in oysters from beach culture samples, levels increased following tidal desiccation (T_{post}) from levels observed pre-exposure (T_{pre}) in Trial A (1.19 ± 0.64 log MPN/g), Trial C (1.97 ± 0.78 log MPN/g), Trial D (0.81 ± 0.50 log MPN/g) and Trial E (0.82 ± 0.61 log MPN/g) (Figure 2.5). These increases were statistically significant for Trial A and C ($p \leq 0.09$). In Trial B levels decreased following intertidal exposure (0.35 ± 0.37 log MPN/g). For levels in oysters from flip bag samples, there were increases following tidal desiccation in Trial A (0.49 ± 0.29 log MPN/g), Trial B (0.39 ± 0.36 log MPN/g), Trial C (1.39 ± 0.47 log MPN/g), and Trial D (0.88 ± 0.40 log MPN/g), and were statistically significant in Trials C and D ($p \leq 0.05$). In Trial E a decrease in levels following intertidal exposure was observed (0.91 ± 0.52 log MPN/g).

Following T_2, levels of *trh+ V. parahaemolyticus* in oysters from beach culture samples decreased from T_{post} samples in Trial A (1.27 ± 0.64 log MPN/g), Trial B (0.12 ± 0.37 log MPN/g), Trial C (0.98 ± 0.78 log MPN/g), and Trial E (0.87 ± 0.61 log MPN/g) (Figure 2.5). These decreases were statistically significant for Trial A ($p \leq 0.07$) only. In Trial D, an increase in levels at T_2 was observed (0.21 ± 0.50 log MPN/g). For oysters from flip bag samples, levels from T_2 decreased from T_{post} in Trial A (0.66 ± 0.29 log MPN/g), Trial B (0.94 ± 0.36 log MPN/g), and Trial C (0.40 ± 0.47 log MPN/g). These observed decrease in levels were statistically significant for Trial A and B ($p \leq 0.04$). In Trials D and E, an increase in levels in

oysters following T_2 (0.14 ± 0.40 log MPN/g and 1.96 ± 0.52 log MPN/g, respectively) was observed.

Following T_4, levels of *trh+ V. parahaemolyticus* in beach culture oysters decreased from T_{post} samples in only Trial A (0.95 ± 0.64 log MPN/g) and were not significant ($p \geq 0.17$). (Figure 2.5). Trial B saw no change in levels and, unexpectedly, minimal increases at T_4 were observed for Trial C (0.12 ± 0.78 log MPN/g), Trial D (0.12 ± 0.50 log MPN/g), and Trial E (0.13 ± 0.61 log MPN/g). For oysters from flip bag samples, levels following T_4 decreased from T_{post} levels in Trial A (1.91 ± 0.29 log MPN/g), Trial B (0.54 ± 0.36 log MPN/g), and Trial C (0.44 ± 0.47 log MPN/g). These decreases were statistically significant for Trial A ($p \leq 0.01$). In Trials D and E, an increase in levels in T_4 samples (0.26 ± 0.40 log MPN/g and 1.86 ± 0.52 log MPN/g, respectively) was observed.

Approximately 24-hours following the collection of pre-exposure samples (T_f), levels of *trh+ Vp* in oysters from beach culture samples decreased from T_{post} samples in Trial A (0.34 ± 0.64 log MPN/g), Trial B (0.38 ± 0.37 log MPN/g), Trial C (0.58 ± 0.78 log MPN/g), and Trial E (0.83 ± 0.61 log MPN/g) and were not significantly different in any trial ($p \geq 0.20$). For levels in oysters from flip bag samples, levels at T_f decreased from T_{post} levels in Trial A (0.47 ± 0.29 log MPN/g) and Trial C (0.15 ± 0.47 log MPN/g) and were not significant ($p \geq 0.14$). Unexpectedly, in Trials B, D, and E, an increase from T_{post} levels to T_f levels (0.25 ± 0.36 log MPN/g, 0.24 ± 0.40 log MPN/g, and 1.56 ± 0.52 log MPN/g, respectively) was observed.

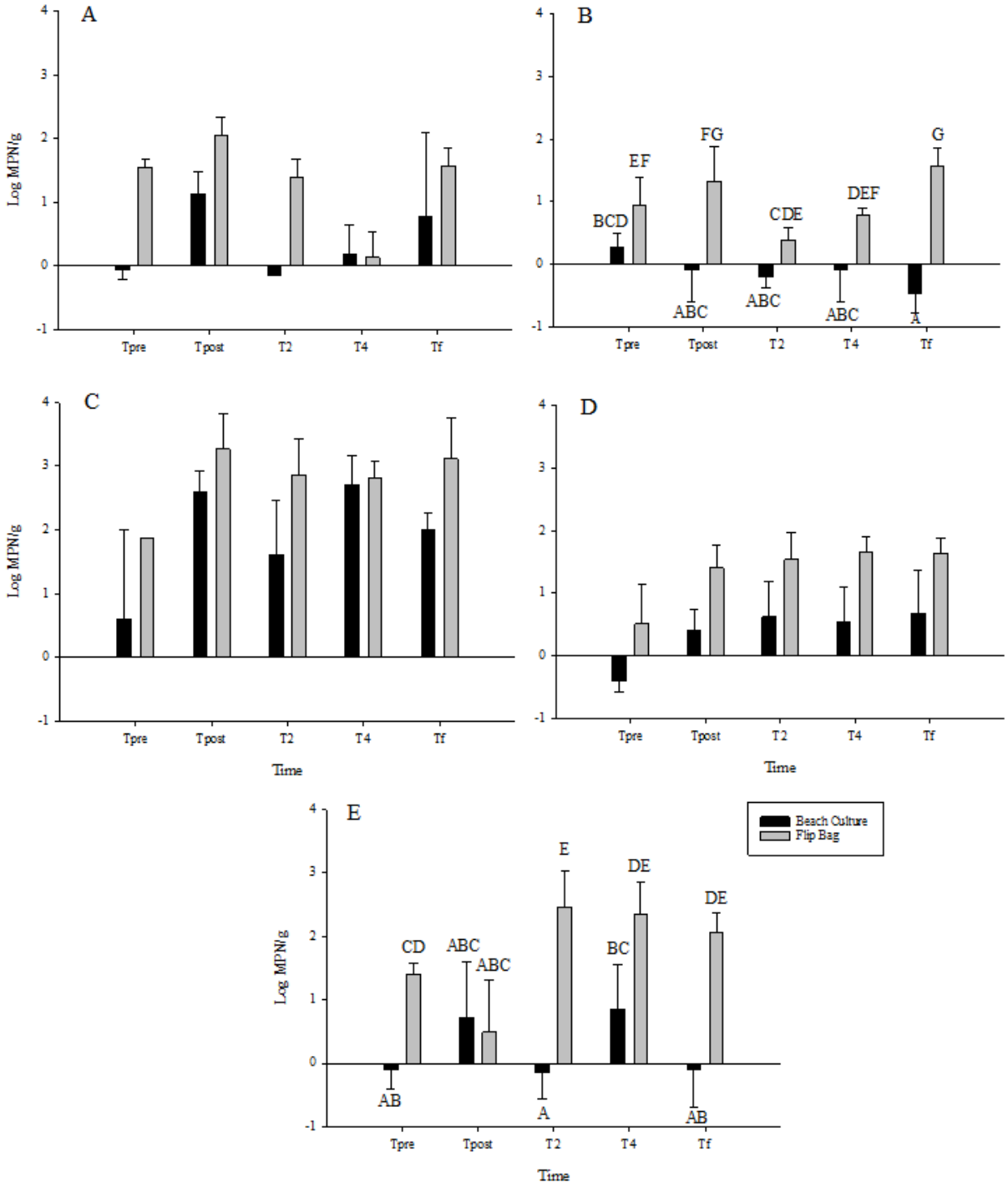

Figure 2.5. Effect of culture method and sampling time on mean log-transformed total *trh*+ *V. parahaemolyticus* levels in oysters from Trial A (A; July 16-17, 2019), Trial B (B; July 19-20, 2019), Trial C (C; July 21-22, 2020), Trial D (D; July 31-August 1, 2020), and Trial E (E; August 4-5, 2020) prior to tidal desiccation (T_{pre}), following maximum air exposure (T_{post}), 2-h following resubmersion from the incoming tide (T_2), 4-h following resubmersion (T_4), and 24-h

following T_{pre} collection (T_f). Bars represent standard error. Letters represent significant differences in *trh+ V. parahaemolyticus* levels, as determined by the post hoc comparison test.

3.4. tdh+ V. parahaemolyticus

For *tdh+ V. parahaemolyticus* (*tdh+ Vp*) in oysters from beach culture samples, levels increased following tidal desiccation (T_{post}) from T_{pre} levels in Trial A (0.45 ± 0.14 log MPN/g), Trial B (0.13 ± 0.18 log MPN/g), Trial C (2.13 ± 0.51 log MPN/g), Trial D (0.46 ± 0.41 log MPN/g), and Trial E (0.92 ± 0.50 log MPN/g) (Figure 2.6). These increases were statistically significant for Trial A, C, and E ($p \leq 0.09$). For levels in oysters from flip bag samples, increases following tidal desiccation in Trial A (0.15 ± 0.51 log MPN/g), Trial B (0.46 ± 0.12 log MPN/g), Trial C (1.39 ± 0.31 log MPN/g), Trial D (0.29 ± 0.24 log MPN/g), and a minimal increase in Trial E (0.06 ± 0.76 log MPN/g) were observed. These increases were statistically significant for Trial B and C ($p \leq 0.01$).

Following T_2, levels of *tdh+ Vp* in oysters from beach culture samples decreased from T_{post} samples in Trial A (0.45 ± 0.14 log MPN/g), Trial C (1.31 ± 0.51 log MPN/g), Trial D (0.59 ± 0.41 log MPN/g), and Trial E (0.59 ± 0.50 log MPN/g) (Figure 2.6). These decreases were statistically significant for Trials A and B ($p \leq 0.01$). In Trial B, a minimal increase in levels at T_2 was observed (0.10 ± 0.18 log MPN/g). For oysters from flip bag samples, levels from T_2 decreased from T_{post} levels in Trial B (0.59 ± 0.12 log MPN/g), Trial C (0.94 ± 0.31 log MPN/g), and Trial D (0.14 ± 0.24 log MPN/g). These observed decrease in levels were statistically significant for Trials B and C ($p \leq 0.01$). In Trials A and E, increases in levels following T_2 (0.67 ± 0.51 log MPN/g and 2.19 ± 0.76 log MPN/g, respectively) were observed.

Following T_4, levels of *tdh+ Vp* levels in beach culture oyster decreased from T_{post} samples in Trial C (0.99 ± 0.51 log MPN/g), Trial D (0.59 ± 0.41 log MPN/g), and Trial E (0.92 ± 0.50 log MPN/g) and were significant for Trials C and E ($p \leq 0.09$). (Figure 2.6). Unexpectedly, minimal increases at T_4 were observed for Trial A (0.06 ± 0.14 log MPN/g) and Trial B (0.10 ± 0.18 log MPN/g). For oysters from flip bag samples, levels following T_4 decreased from T_{post} levels in Trial A (0.13 ± 0.51 log MPN/g), Trial B (0.59 ± 0.12 log MPN/g), and Trial C (1.10 ± 0.31 log MPN/g). These decreases were statistically significant for Trials B and C ($p \leq 0.01$). In Trials D & E, increases in levels at T_4 samples (0.20 ± 0.24 log MPN/g and 0.62 ± 0.76 log MPN/g, respectively) were observed.

Approximately 24-hours following the collection of pre-exposure samples (T_f), levels of *tdh+ Vp* in oysters from beach culture samples decreased from T_{post} samples in Trial A (0.33 ± 0.14 log MPN/g), Trial B (0.13 ± 0.18 log MPN/g), Trial C (1.30 ± 0.51 log MPN/g), and Trial E (0.66 ± 0.76 log MPN/g) and were significantly different in Trials A and C ($p \leq 0.04$). An increase in levels was observed for Trial D (0.24 ± 0.41 log MPN/g). For levels in oysters from flip bag samples, levels at T_f decreased from T_{post} levels in Trial A (0.03 ± 0.51 log MPN/g), Trial B (0.59 ± 0.12 log MPN/g), and Trial C (1.53 ± 0.31 log MPN/g) and were significant in Trials B and C ($p \leq 0.01$). In Trials D and E, increases from T_{post} levels to T_f levels (0.20 ± 0.24 log MPN/g and 1.17 ± 0.76 log MPN/g, respectively) were observed.

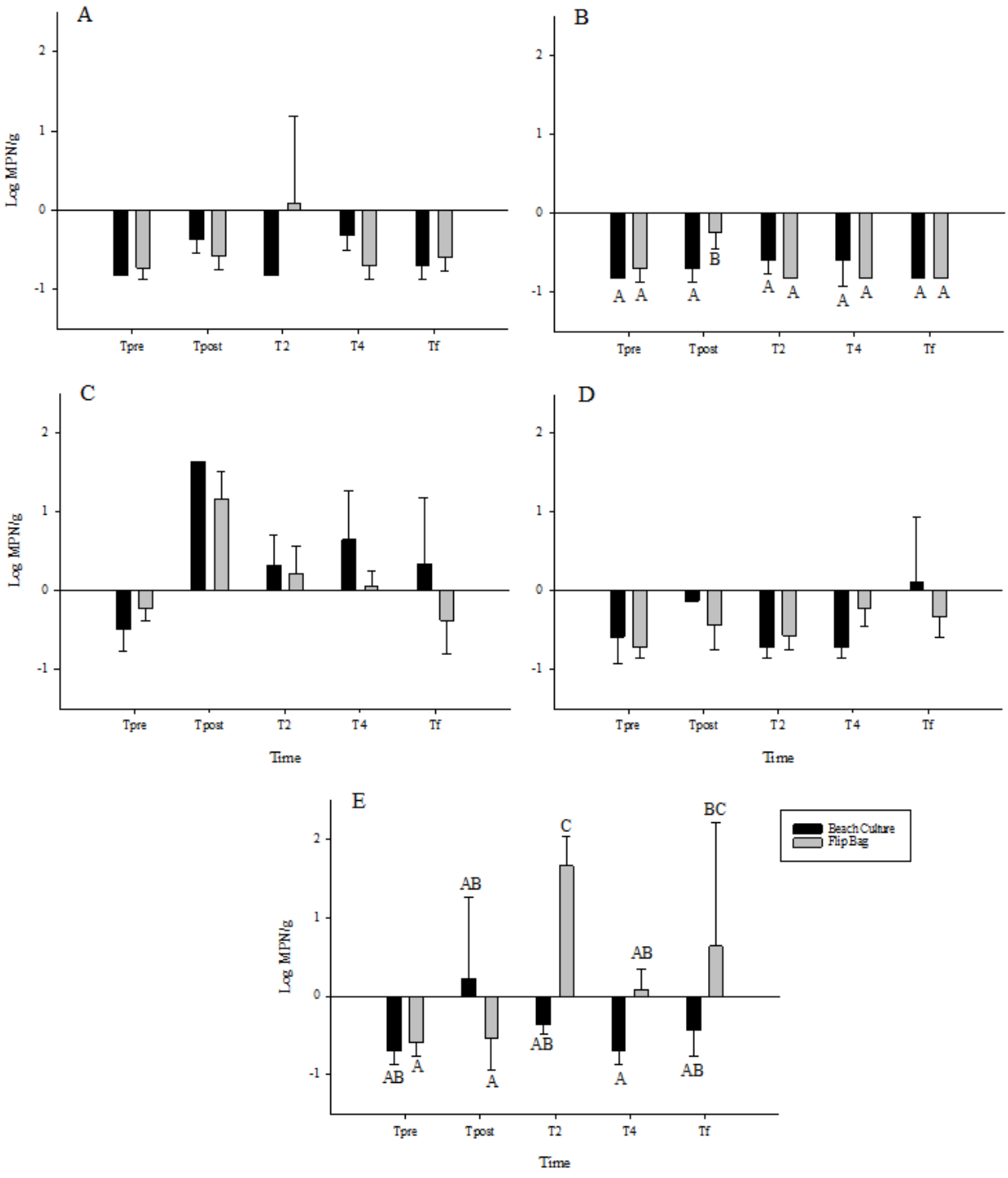

Figure 2.6. Effect of culture method and sampling time on mean log-transformed total *tdh+ V. parahaemolyticus* levels in oysters from Trial A (A; July 16-17, 2019), Trial B (B; July 19-20, 2019), Trial C (C; July 21-22, 2020), Trial D (D; July 31-August 1, 2020), and Trial E (E; August 4-5, 2020) prior to tidal desiccation (T_{pre}), following maximum air exposure (T_{post}), 2-h following resubmersion from the incoming tide (T_2), 4-h following resubmersion (T_4), and 24-h

following T_{pre} collection (T_f). Bars represent standard error. Letters represent significant differences in *trh+ V. parahaemolyticus* levels, as determined by the post hoc comparison test.

3.5. Vibrio vulnificus

Due to the large number of samples (57.3%) of *V. vulnificus* found below the limit of detection (0.3 MPN/g), statistical analysis was not conducted for *V. vulnificus* in oysters. For *V. vulnificus* (*Vv*) in oysters from beach culture samples, levels increased following tidal desiccation (T_{post}) from T_{pre} levels in Trial A (2.75 ± 0.55 log MPN/g), Trial B (0.19 ± 0.15 log MPN/g), and Trial C (0.13 ± 0.66 log MPN/g) with no change observed in Trials D and E (Figure 2.7). For levels in oysters from flip bag samples, increases following tidal desiccation were seen in Trial A (0.25 ± 0.81 log MPN/g), Trial B (0.43 ± 0.47 log MPN/g), and Trial D (0.97 ± 0.71 log MPN/g). In Trial E, a decrease in levels following T_{post} (0.45 ± 0.44 log MPN/g) was observed. No change was observed for Trial C.

Following T_2, levels of *Vv* in oysters from beach culture samples decreased from T_{post} samples in Trial A (1.31 ± 0.55 log MPN/g), Trial B (0.52 ± 0.15 log MPN/g), and a small decrease in Trial C (0.03 ± 0.16 log MPN/g) (Figure 2.7). No change was observed in levels in Trial D and a small increase was observed in Trial E (0.13 ± 0.10 log MPN/g). For oysters from flip bag samples, levels from T_2 decreased from T_{post} levels in Trial A (2.02 ± 0.81 log MPN/g), Trial B (1.21 ± 0.47 log MPN/g), Trial D (0.87 ± 0.71 log MPN/g), and Trial E (0.93 ± 0.44 log MPN/g). In Trial C, an increase in levels was observed following T_2 (0.36 ± 0.76 log MPN/g).

Following T_4, levels of *Vv* in oysters from beach culture oyster decreased from T_{post} samples in Trial A (2.56 ± 0.55 log MPN/g) and Trial B (0.52 ± 0.15 log MPN/g) (Figure 2.7). While an increase was observed in Trial C (0.20 ± 0.16 log MPN/g), no change was observed in levels for Trials D and E. For oysters from flip bag samples, levels following T_4 decreased from

T_{post} levels in Trial A (2.98 ± 0.81 log MPN/g), Trial B (1.11 ± 0.47 log MPN/g), Trial D (0.87 ± 0.71 log MPN/g), and Trial E (0.93 ± 0.44 log MPN/g). In Trial C, an increase in levels in T_4 samples (1.49 ± 0.76 log MPN/g) was observed.

Approximately 24-hours following the collection of pre-exposure samples (T_f), levels of *Vv* in oysters from beach culture samples decreased from post-exposure (T_{post}) samples in Trial A (2.75 ± 0.55 log MPN/g), Trial B (0.52 ± 0.15 log MPN/g), and Trial C (0.13 ± 0.16 log MPN/g). In Trials D and E, small increases in levels (0.20 ± 0.06 log MPN/g and 0.10 ± 0.10 log MPN/g) were observed. For levels in oysters from flip bag samples, levels at T_f decreased from T_{post} levels in Trial A (1.74 ± 0.81 log MPN/g), Trial D (0.74 ± 0.71 log MPN/g), and Trial E (0.93 ± 0.44 log MPN/g). An increase in levels was observed in Trials B and C (0.11 ± 0.47 log MPN/g and 0.20 ± 0.76 log MPN/g, respectively).

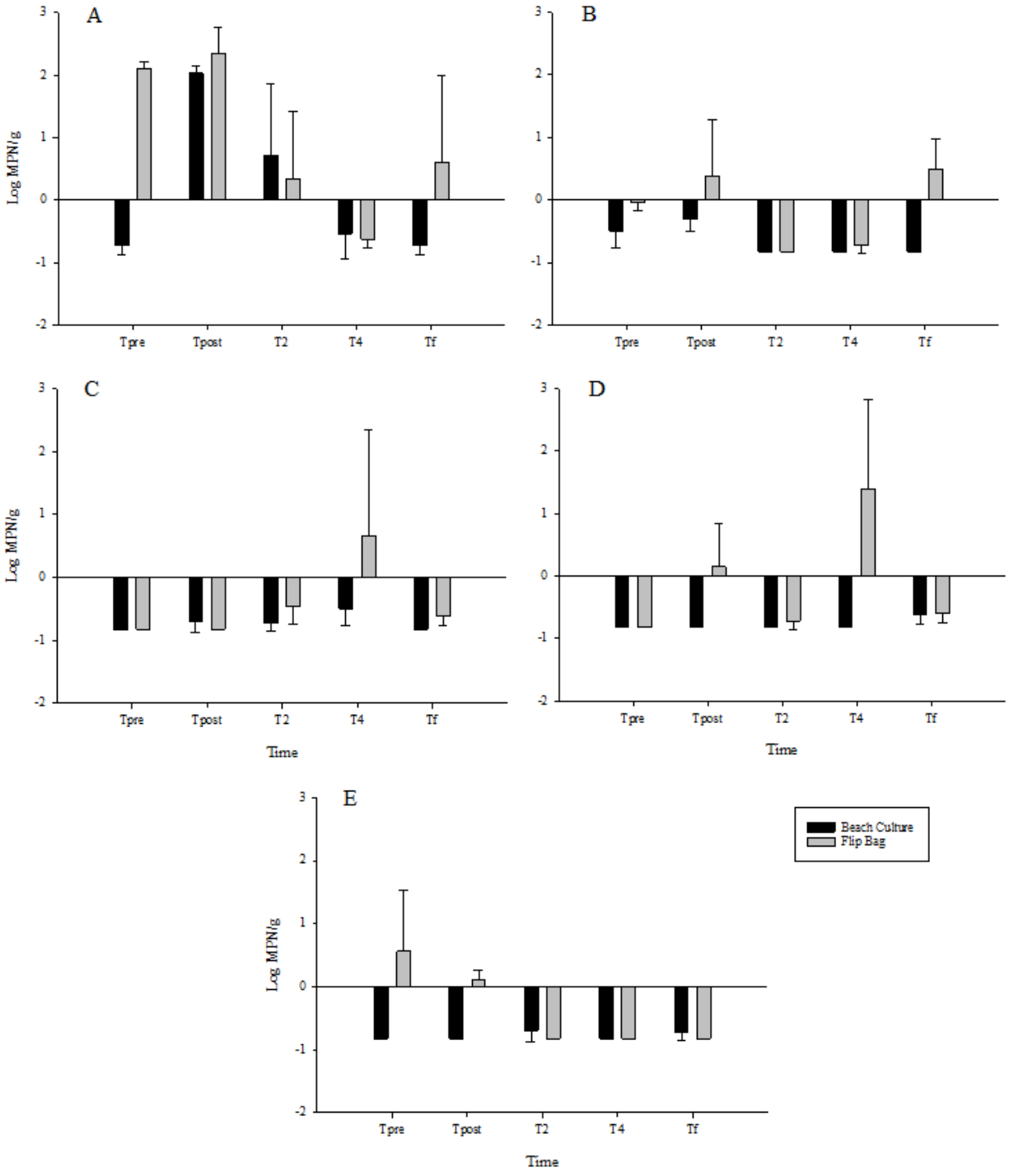

Figure 2.7. Effect of culture method and sampling time on mean log-transformed total *V. vulnificus* levels in oysters from Trial A (A; July 16-17, 2019), Trial B (B; July 19-20, 2019), Trial C (C; July 21-22, 2020), Trial D (D; July 31-August 1, 2020), and Trial E (E; August 4-5, 2020) prior to tidal desiccation (T_{pre}), following maximum air exposure (T_{post}), 2-h following

resubmersion from the incoming tide (T_2), 4-h following resubmersion (T_4), and 24-h following T_{pre} collection (T_f). Bars represent standard error.

3.6. Single factors and interactions within trials

For total *Vp*, In Trial A and C, a significant effect was observed for both culture method ($p \leq 0.06$) and time ($p \leq 0.04$) on total *Vp* levels in oysters, but there was no interaction between these factors (Figure 2.4, $p \geq 0.65$). For culture methods, regardless of time, mean levels of total *Vp* in oysters from flip bags (2.00 log MPN/g and 1.53 log MPN/g, respectively) were significantly higher than mean levels from beach culture samples (1.35 log MPN/g and 0.42 log MPN/g, respectively). In Trial B, a significant effect was observed for the interaction of culture method and time on total *Vp* levels in oysters (Figure 2.4, $p \leq 0.03$). Levels of total *Vp* in oysters from flip bags were significantly ($p \leq 0.09$) higher than levels from beach culture samples at time points T_{pre}, T_{post}, T_4, and T_f and there were no significant differences at T_2 ($p \geq 0.16$). In Trial D and E, there was no significant effect observed for culture method ($p \geq 0.47$), sampling time ($p \geq 0.19$), nor their interaction ($p \geq 0.14$) on levels of total *Vp*.

For *trh+ Vp*, In Trials A, C, and D, a significant effect was observed for both culture method ($p \leq 0.01$) and time ($p \leq 0.01$) on levels in oysters, but there was no interaction between these factors (Figure 2.5, $p \geq 0.17$). For culture methods, regardless of time, mean levels of *trh+ Vp* in oysters from flip bags (1.34, 2.78, and 1.35 log MPN/g, respectively) were significantly higher than mean levels from beach culture samples (0.38, 1.90, and 0.37 log MPN/g, respectively). In Trials B and E, a significant effect was observed for the interaction of culture method and time on *trh+ Vp* levels in oysters (Figure 2.5, $p \leq 0.06$). For Trial B, levels of *trh+ Vp* in oysters from flip bags were significantly ($p \leq 0.08$) higher than levels from beach culture samples at time points T_{pre}, T_{post}, T_4, and T_f and there were no significant differences at T_2

($p \geq 0.12$). In Trial E, levels of *trh+ Vp* in oysters from flip bags were significantly ($p \leq 0.02$) higher than levels from beach culture samples at time points T_{pre}, T_2, T_4, and T_f and there were no significant differences at T_{post} ($p \geq 0.69$).

For *tdh+ Vp,* In Trial C, a significant effect was observed for both culture method ($p \leq 0.09$) and time ($p \leq 0.01$) on levels in oysters, but there was no interaction between these factors (Figure 2.6, $p \geq 0.49$). For culture methods, regardless of time, mean levels of *tdh+ Vp* in oysters from beach culture samples (0.49 log MPN/g) were significantly higher than mean levels from flip bag samples (0.16 log MPN/g). In Trials B and E, a significant effect was observed for the interaction of culture method and time on *tdh+ Vp* levels in oysters (Figure 2.6, $p \leq 0.06$). In Trial B, levels of *tdh+ Vp* in oysters from flip bags were significantly ($p \leq 0.01$) higher than levels from beach culture samples at time point T_{post} and there were no significant differences at the remaining time points ($p \geq 0.15$). In Trial E, levels of *tdh+ Vp* in oysters from flip bags were significantly ($p \leq 0.01$) higher than levels from beach culture samples at time point T_2 and there were no significant differences in the remaining time points ($p \geq 0.11$). In Trials A and D, there was no significant effect observed for culture method ($p \geq 0.53$), sampling time ($p \geq 0.13$), nor their interaction ($p \geq 0.32$) on levels of *tdh+ Vp* (Figure 2.6 5).

4. Discussion

The first objective of the study was to test the effect of desiccation on *Vibrio* spp. levels. Farm raised oysters in the Pacific Northwest are subjected to daily tidal cycles that exposes them to warm air temperatures and direct sunlight that can rapidly increase *Vibrio* spp. levels within oysters (*5, 9*). In the current study, following maximum air exposure, *Vibrio* spp. levels generally increased from pre-exposure levels within oysters. This is similar to previous studies which demonstrated a significant increase in total and pathogenic *V. parahaemolyticus* in oysters

exposed to air temperatures of 21 °C to 28 °C, although no significant increase in *V. vulnificus* levels (*14*). These elevated levels were observed to return to those not significantly different from levels found in initially harvested samples.

Total *V. parahaemolyticus* (*Vp*), pathogenic *V. parahaemolyticus* (*trh*+ and *tdh*+), and *V. vulnificus* (*Vv*) levels in oysters were monitored over the course of a single tidal cycle to determine how levels may fluctuate over time. Following exposure during low tide, total *Vp* increased in almost all cases, and in half the cases this was a significant increase. Only in beach culture oysters in Trial D was a decrease observed; flip bag oysters only increased 0.20 log in total *Vp* levels following T_{post} during this trial as well. This difference may be due to the air exposure in Trial D happening the earliest in the day of any trial, limiting the amount of time oysters spent exposed to the warmest air temperatures of the day. These results are consistent with previous studies that observed increases in total *Vp* levels following intertidal air exposure in the region could occur (*14*, *26*), though this study finds that this was not consistently the case.

Following 2-hours of resubmersion by the incoming tide (T_2), levels of total *Vp* decreased from T_{post} levels in all trials across both culture methods (significantly in three cases) but increased in Trial E's flip bag samples at this (and subsequent) timepoints. The cause of this increase in flip bags in this one trial may be attributed to influences such as strong currents, wave action or other disturbances that affected the behavior of the oysters in the flip bags. In the future, accounting for this with data loggers that are able to measure an influence of wave action could explain this pattern.

Following 4-hours of resubmersion by the incoming tide (T_4), levels of total *Vp* decreased from T_{post} levels in beach culture oysters in four of five trials (one significantly), with the exception of Trial C which increased. This increase may be explained by Trial C having

significantly higher water temperatures over time compared with all other trials (*6*, *7*). After 4-hours of resubmersion in flip bags, decreases in levels from T_{post} to T_4 were observed in four of five trials (three significantly) aside from Trial E, which increased.

Following 24-hours in the tidal cycle after T_{pre} samples were collected (T_f), total *Vp* levels in all trials for beach culture oysters decreased from T_{post} (three significantly). These results are consistent with previous studies in the region that observed a decrease in levels following a full tidal cycle (*14, 26*). In contrast, total *Vp* levels in flip bag oysters only saw a decrease in two of the five trials and an increase in the remaining three trials.

In two trials (D, E), culture method, sample time, nor their interaction had any significant effect on total *Vp* levels. In another two other trials (A, C), both culture method and time had a significant effect, with flip bags having higher mean levels of total *Vp* than beach culture. Similarly, in Trial B, at four of five time points, levels of total *Vp* were higher in flip bags than beach culture (with no difference at the fifth timepoint).

Despite the trends, these results suggest a large amount of variation within samples and among trials making generalizations difficult. For example, while total *Vp* tended to increase during tidal exposure, in many trials this was not a significant increase and in one case levels decreased over this time. At the 2-hour and 4-hour timepoints, levels tended to decrease but this varied by trial and the patterns were not consistent across culture methods. Similarly, the effect of culture method depended heavily upon trial, though when there was an effect, flip bag oysters tended to have higher levels, for example, at T_f, levels of total *Vp* had decreased from T_{post} in all trials for beach culture oysters, but not in the majority of trials for flip bag oysters.

For levels of *trh+ V. parahaemolyticus*, (*trh+ Vp*), following exposure during low tide in beach culture oysters, levels increased in four of five trials (two significantly), but decreased in

Trial B. While the cause of this decrease cannot be explained by variables measured, *Vibrio* spp. levels stayed below T_{post} levels, for the remainder of this trial. In flip bag oysters, levels increased in four of five trials afte T_{post} (two significantly), aside from Trial E which decreased. The cause of this decrease during exposure to warm air temperatures cannot be explained by variables measured. In summary, despite variation among trials, air exposure during low tide created conditions that often led to an increase in *trh+ Vp,* which is consistent with previous studies that observed increases in pathogenic *V. parahaemolyticus* levels following intertidal air exposure in the region (*14*).

For the T_2 time point, levels of *trh+ Vp* decreased in four of five trials (one significantly) in beach culture oysters. In Trial D, levels following resubmersion (in all subsequent time points) in both culture methods rose and stayed above T_{post} levels. This trend might be explained by Trial D having occurred during the earliest sampling time of all trials and therefore subjecting oysters to exposure during the coolest part of the day and resubmersed as air temperatures increased. In the flip bag trials, levels of *trh+ Vp* decreased in three out of five cases (significantly in two) with increases observed in Trial E. In this trial for flip bag oysters, levels of *trh+ Vp* stayed significantly higher than T_{pre} and T_{post} in all resubmersion time points. This trend was comparable to total *Vp* levels in the same trial and may be attributed to other influences or disturbances affecting filtration rate.

Following the T_4 time point, levels of *trh+ Vp* in beach culture oysters were observed to unexpected minimally increase in three of five trials (Trials C, D, and E). The cause of this trend cannot be explained by variables measured but shows the variability in *Vibrio* spp. levels among trials in the study. In contrast, levels of beach culture oysters in Trial A significantly decreased from T_{post} levels. In flip bag oysters, levels decreased in three of five trials (one significantly).

Trials D and E stayed elevated, seemingly having no effect of resubmersion over the course of the tidal cycle.

At time point T_f, levels of *trh+ Vp* decreased in beach culture oysters in four of the five trials, although none of these decreases were significant from T_{post}. Levels in Trial D continued to stay elevated in both culture methods. Levels of *trh+ Vp* in flip bag oysters decreased in only two of the five trials. Those trials (B, D, and E) showing increases at this time point could possibly be explained by the natural variability of *trh+ Vp* populations in oysters over the course of a full tidal cycle.

In Trials B and E, a significant effect for the interaction of culture method and sampling time was observed on levels of *trh+ Vp* where both trials showed that flip bag oysters had significantly higher levels in four of the five time points. In the other three trials (A, C, and D), both culture method and time had a significant effect (with no interaction), and mean levels of *trh+ Vp* were higher in flip bag oysters compared with beach culture oysters.

Despite this trend, generalizations are still difficult to make as variation within samples and among trials was still prevalent. For example, even though levels tended to increase following maximum air exposure, it was not a significant increase in a majority of the trials and in two cases, levels decreased. Inconsistent patterns in levels following resubmersion was also viewed with two trials showing little to no decreases in levels. It is important to note that levels of *trh+ Vp* tended to show as higher in flip bag oysters compared to beach culture oysters in the individual trials.

Levels of pathogenic *tdh+ V. parahaemolyticus* (*tdh+ Vp)* were generally low and in about 1/3 of the cases were non-detects, and the results should be considered with these overall

low levels in mind. Following low tide exposure, levels of *tdh+ Vp* increased in all cases, and in half of the cases the increase was significant.

After T_2 in the tidal cycle, levels of *tdh+ Vp* decreased in beach culture oysters in four of five cases (two significantly) except in Trial B. The majority of samples in this trial detected no levels of *Vp tdh+*. In flip bag oysters, three of the five cases (two significantly) resulted in decreasing levels. Levels in Trials A and E both had increased following 2-hours of resubmersion. In both of these trials, there were a high number of samples that failed to detect levels of *tdh+ Vp*.

Following T_4, levels of *tdh+ Vp* in beach culture oysters decreased in three of the five cases (two significantly). The two trials (A and B) that resulted in increases both failed to detect levels in the majority of their samples. In flip bag oysters, levels of *Vp tdh+* decreased in three of the five cases (two significantly) with Trials D and E having increasing levels following the 4-hours of resubmersion.

After the T_f time point, levels of *tdh+ Vp* in beach culture oysters decreased in four of five cases (two significantly), with the exception of Trial D which actually increased. This can be explained by the high number of samples failing to detect *tdh+ Vp*. In flip bag oysters, three of five trials resulted in decreases following T_f (two significantly) with Trials D and E continuing to have increased levels compared with T_{post} levels.

In two trials (A and D), culture method, sample time, nor their interaction had any significant effect on *tdh+ Vp* levels. In trials B and E, both culture method and sample time had a significant effect, with flip bag oysters having higher mean levels of *tdh+ Vp*. However, in Trial C, mean levels of *tdh+ Vp* were found to be higher in beach culture oysters in four of five time points – the only instance in this study where levels were higher in beach culture than flip bags.

These results suggest variation of *Vp tdh+* among the trials and low limits of detection overall. While levels did increase during tidal exposure in all trials, this was not a significant increase in many trials. At 2-hours and 4-hours of resubmersion, levels tended to decrease, but this varied by trial with no consistent pattern.

When examining samples for levels of *Vibrio vulnificus* (*Vv*) in oysters from both culture methods, the species was not detected in the majority cases. Because of this, trends are difficult to discern as those samples that did detect levels of *Vv* resulted in a near significant change overall for the trial despite not being high levels compared to other *Vibrio* spp. targets. When *Vv* was detected, as it was following T_4 in flip bag oysters, these changes appear to be alarming, despite staying relatively low at both T_2 and T_4. *Vv* has been found at low frequencies in waters for the west coast (*18*) and according to the Washington State Health Department (*37*), there have been no reports of *Vv* infection attributed to humans coming in contact with Washington marine waters or from eating shellfish in Washington state, two of the most common causes of *Vv* infection (*27, 15*). Comparable to *tdh+ Vp*, the low amount of detection within trials shows the lack of conclusive patterns.

Taken in total, these results underscore the importance of variation among trials. There were very few, if any, cases where a result was consistent for all trials. For example, while mean levels of total *Vp* and pathogenic *trh+ Vp* in oysters tended to increase following tidal exposure and decrease following resubmersion, this was not always the case. This suggests that caution should be exercised in drawing conclusions from limited trials. Furthermore, the causes of this variation warrant further examination to determine what factors drive this variability. Certainly, the potential for high levels of these *Vibrio* spp. exists and needs to be taken into consideration but, should not be presumed.

Following resubmersion, while levels of total *Vp* and *trh+ Vp* decreased in most of the trials through 2 and 4-hours of resubmersion, supporting results found in previous studies (*14, 26*), the decreases were not significant in most of these cases. Overall, while levels of *Vibrio* spp. generally decreased during resubmersion, the lack of trends noticed in increases of levels following maximum air exposure makes it difficult to determine proper recovery times or make generalizations about how resubmersion affects potentially elevated levels. With high variation making generalizations across trials difficult, it is suggested that this be further studied.

Additionally, while total *Vp* and pathogenic *trh+ Vp* were common and regularly observed in this study, pathogenic *tdh+ V. parahaemolyticus* and *V. vulnificus* levels were low in this study and in large part, not detected in many samples. These low levels make interpretations of patterns difficult but suggest that these were not of significant concern during this study.

In terms of different culture methods, in most of the trials and sample times there was no significant difference in levels of *Vibrio* spp. between beach culture and flip bag oysters, though levels tended to be higher in flip bags. When there was a significant effect of culture method, typically levels were found to be significantly higher in oysters coming from flip bags rather than those grown out in beach culture. It was also noticed that oysters were able to recover from elevated *Vibrio* spp. levels in more cases than oysters from flip bags These trends could be due at least in part to a number of factors including potential changes in the behavior of oysters within flip bags, relative to beach culture. For example, wave action and the movement of oysters within the floating bags during high tide might lead oysters to stop filtering more often than those on the direct bottom. In this study, the flip bags were stocked at very low densities which might have affected the oysters' response to wave action; future studies should mimic typical commercial stocking densities to reduce this potential variable. These suggested causes are

speculative, but the causes of the apparent differences in the levels of *Vibrio* spp. between these culture methods should be better understood.

As farmers and public health officials strive to ensure that consumers receive a market product with minimal risk, understanding the effects of tidal desiccation on *Vibrio* spp. levels and how they fluctuate following resubmersion is of high importance. In this study, two culture methods on a commercial shellfish farm were studied over the course of a tidal cycle. While variation among trials was substantial, the data generated supports the potential for increases following tidal desiccation and observing resubmersion times of no less than 4-hours following tidal desiccation before harvesting with intent for sale on the half-shell market.

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Chapter III. Summary for the Industry

Background

Oyster farmers routinely use different culture methods, such as bottom culture and off-bottom culture, and pre-harvest techniques to consistently produce oysters that are of the highest quality. When farmers decide which culture methods, practices, and location are the best fit for them, they also must know the inherent risks involved, specifically surrounding public health concerns about potential increases in bacterial levels within the oysters.

Vibrio bacteria are commonly found in marine and estuarine waters, can be concentrated within oysters during filter feeding, and may multiply when filter feeding is interrupted**.** Certain species of vibrio are known to cause infections in humans, often occurring through the consumption of raw or undercooked shellfish. Typically, farmers ensure that they are keeping this risk to a minimum by following strict protocols and regulations during and after harvest, maintaining their oysters at certain cold temperatures within specific time periods. Control strategies aimed at upholding the safety surrounding shellfish consumption are implemented through the National Shellfish Sanitation Program (NSSP). The NSSP creates guidelines that regulate the harvesting, processing, and shipping of shellfish and continue to change in order to address public health issues. Typically, farmers ensure that they are keeping this risk to a minimum by following strict protocols and regulations during and after harvest, maintaining their oysters at certain cold temperatures within specific time periods. These protocols are outlined in the NSSP, with specific regulations defined by each participating state.

Air-drying and Resubmersion

Recent research has explored how oyster farmers might influence the risk from vibrio bacteria through different culture methods and handling practices prior to harvest. In some areas,

farmers air-dry their oysters by taking them out of the water, to control biofouling by preventing unwanted organisms from establishing on the oysters' shells and the gear used to raise them. This air-drying interrupts the oysters' filter feeding activity, and also exposes them to warmer air temperatures, allowing vibrio bacteria to proliferate within the oysters. These practices can increase vibrio levels and subsequently increase the risk from consumption of these oysters. Based on recent studies, farmers can mitigate this increased public health risk by ensuring that the oysters are resubmersed for designated periods of time following practices of handling and air-drying. Resubmersion allows for filter feeding to resume and has been proven to effectively reduce increased levels of vibrio bacteria over time. After these designated periods of resubmersion, levels of vibrio bacteria will have returned to background levels. The farmers then must harvest within an appropriate "time-temperature window" to be sold on the half-shell market for raw consumption. Required resubmersion times are specified in state regulations. These requirements may also vary within states dependent upon factors such as culture gear type used and handling types applied. For example, farmers in Alabama must observe either a 7 or 14 day resubmersion period, based on culture gear type, before they can harvest their oysters for sale intended for raw consumption.

Bottom Culture and Flip Bags

In many parts of the Pacific Northwest, the Pacific oyster (*Crassostrea gigas*) is raised in the intertidal zone using a bottom culture method, known as "beach culturing", which allows oysters to grow-out directly on the substrate as naturally as possible. In this region, farmers observe a tidal cycle that can expose entire tidal flats for several hours per day. This tidal cycle exposes their crop to warm air temperatures and direct sunlight and can increase the risk posed by vibrio bacteria. Farmers make use of the extreme tidal cycles by preparing for intertidal

harvesting, where oysters are gathered into large containers to be harvested later once the tide returns, submerging the oysters, and allowing for large vessels to collect and transport them to processing plants. Currently, Washington state regulations require farmers to observe 4-hours of resubmersion before they can remove oysters from the water to harvest. Recently, a new culture method known as tumble bag, or flip bag farming, has become popular and more commonly used. This method exposes oysters to variables, such as sunlight and wave action, in different ways from on bottom methods that could potentially affect how vibrio levels fluctuate within them. We sought to determine the effects of these culture practices and natural tidal exposure on the levels of vibrio bacteria in farmed oysters raised on the Puget Sound tidal flats.

Testing Effects of Tidal Exposure

In this study, vibrio levels in Pacific oysters raised using beach culture and flip bags on a commercial shellfish farm in Samish Bay, Washington (Figure 3.1) were compared. Trials were performed in July of 2019 and between July and August of 2020 for a total of five trials all together. Tumble/flip bag culture, oysters were stocked into mesh bags and hung on the same lines as bags used for commercial production (Figure 3.2). These bags had buoys attached to them to aid in collection, but otherwise were handled as traditionally done to grow oysters using this method. Bottom culture, or beach culture, oysters were gathered near the beach, among those used for commercial harvest (at the same tidal height as the flip bags).

For each trial, oysters were collected at five different time points: before exposure to air, at maximum air exposure and before tidal levels resubmersed the oysters, 2-hours after resubmersion, 4-hours after resubmersion, and 24-hours from the first collection time point. This would allow us to evaluate the effects of tidal exposure on vibrio levels within harvest-ready oysters, and how they fluctuated following resubmersion from the incoming high tide. Vibrio

levels observed prior to air exposure were compared to levels following maximum exposure to determine if the period of natural air-drying resulted in elevated vibrio levels. The vibrio levels at time points following maximum air exposure could then be compared to observe how they fluctuated following resubmersion. Vibrio levels within the two culture methods were also compared to observe if an increase in levels, or recovering from these elevated levels, differed.

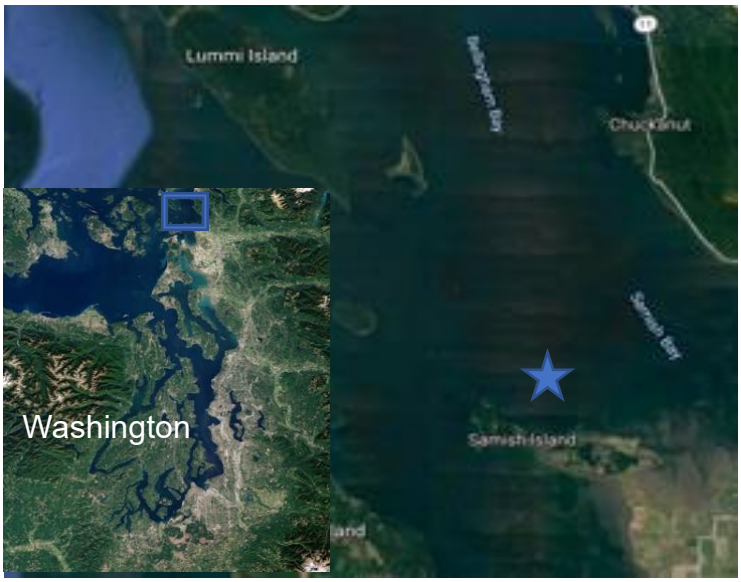

Figure 3.1. Map of the field site used during the study within the Puget Sound and Pacific Northwest region.

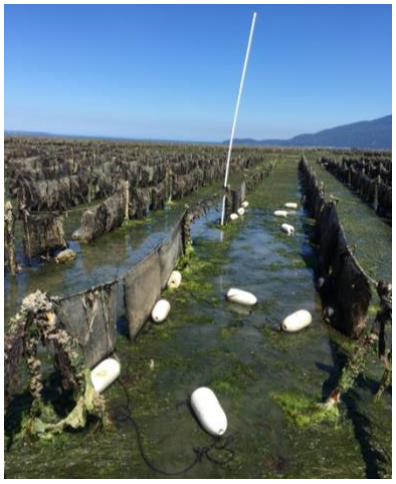

Figure 3.2. A) Commercial flip bag's used for sampling during tidal desiccation, marked by white buoys. B) Flip bags submersed by the incoming tide, marked by white buoys.

Summary

Among the species of vibrio bacteria found in Washington state estuarine waters, we tested oysters for levels of total *Vibrio parahaemolyticus* (*Vp*), pathogenic strains of *V. parahaemolyticus* (*tdh*+/*trh*+) (*tdh*+ *Vp* and *trh*+ *Vp*, respectively), and *Vibrio vulnificus* (*Vv*). Among them, levels of total *Vp* were found in the highest densities (Figure 3.3). For Trial B, we observed significant differences between the two culture methods and sampling time, due to total *Vp* levels being significantly higher in flip bag oysters compared to beach culture oysters overall and in four out of five time points. Through the five trials, total *Vp* levels generally increased following maximum air exposure, excluding beach culture oysters in one trial (D) where levels decreased. In Trials A, B, and D, for both culture methods, levels of total *Vp* returned, or were less than those recorded prior to air exposure, at 2-hours or 4-hours of resubmersion. In the remaining trials, levels fluctuated throughout the tidal cycle with no obvious trends noticed.

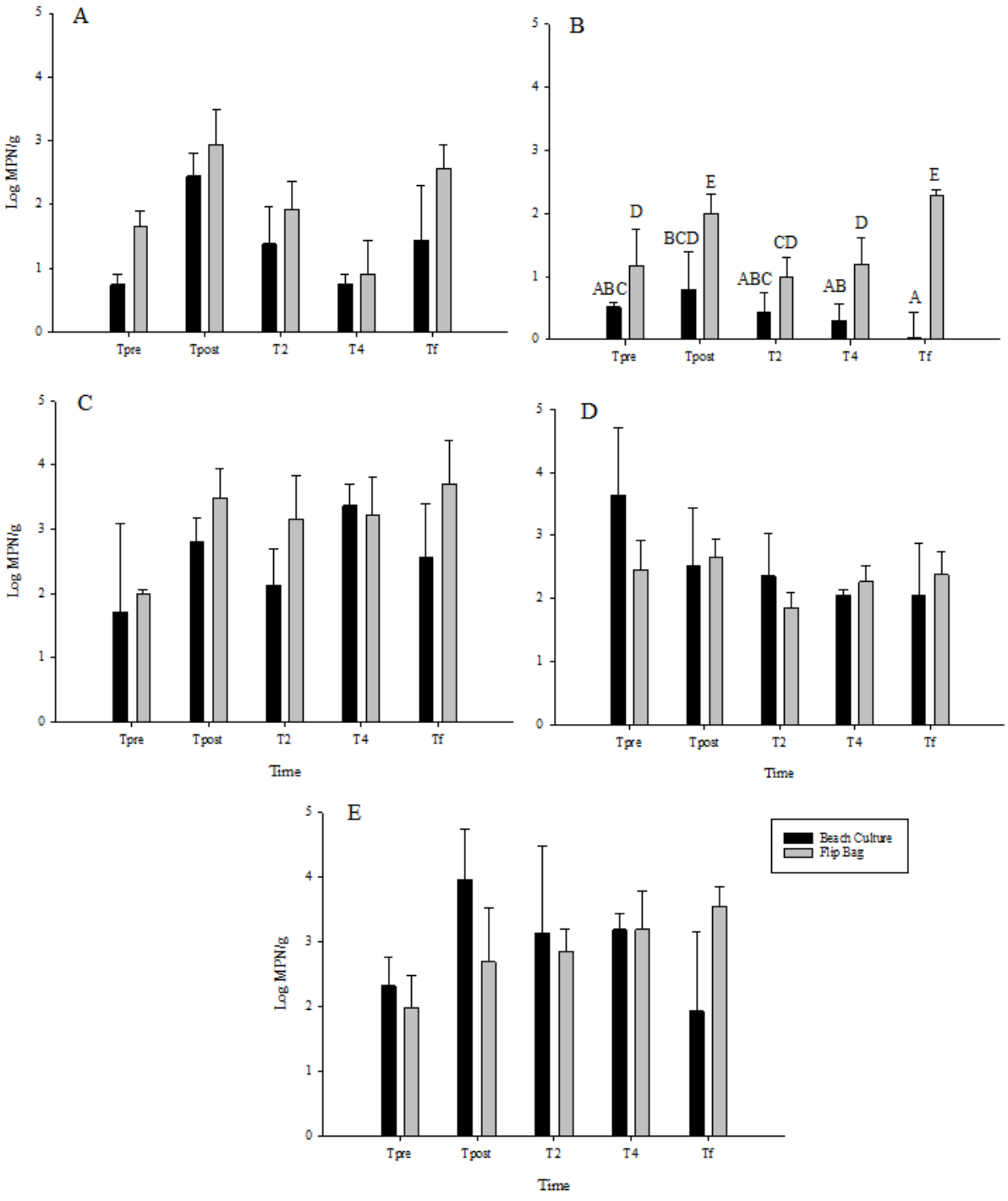

Figure 3.3. Levels of *Vibrio parahaemolyticus* in oysters over time in Trial A (A; July 16-17, 2019), Trial B (B; July 19-20, 2019), Trial C (C; July 21-22, 2020), Trial D (D; July 31-August 1, 2020), and Trial E (E; August 4-5, 2020). On the Y-axis, levels of vibrio are reported as Log MPN/g values. On the X-axis, sampling times are represented in chronological order as prior to tidal desiccation (T_{pre}), following maximum air exposure (T_{post}), 2-h following resubmersion

from the incoming tide (T_2), 4-h following resubmersion (T_4), and 24-h following T_{pre} collection (T_f). Bars represent standard error. Letters represent significant differences in vibrio levels.

Levels of *trh+ Vp* in oysters increased, such as levels of total *Vp,* following maximum air exposure, with four out of five trials resulting in increases for both culture methods. Following these increases and subsequent resubmersion from the incoming tide, there was more variation seen in *trh+ Vp,* resulting in levels decreasing in only half of trials at 4-hours of resubmersion. It is important to note that in trials (B and E) where significant differences between culture methods and time were viewed, levels of *trh+ Vp* in flip bag oysters tended to be higher than those in beach culture oysters. In testing oysters for levels of *tdh+ Vp* and *Vv*, a high number of samples failed to detect either species. Therefore, the low levels made for difficult trends to follow and proved to lack many conclusive patterns and were considered not of an increased concern for the duration of the study.

Conclusions

This study examined the effects of tidal exposure on vibrio bacteria levels within harvest ready oysters grown-out using two different culture methods. We first examined how vibrio bacteria levels fluctuated following tidal desiccation, then following resubmersion from the incoming tide, while also comparing the two culture methods. The following conclusions were gathered from the data collected.

- After exposure to elevated air temperatures during low tide, levels of vibrio bacteria increased in most cases. While these increases were not always statistically significant, they do indicate a trend in increasing levels following maximum exposure.
- After 4-hours of resubmersion from the incoming tide, vibrio bacteria levels decreased in a majority of cases where levels increased following maximum exposure.

- Levels of *tdh+ Vp* and *Vv* were not detected in many samples and were low in many of the others, suggesting that the public health concern was relatively low for these species throughout the study. This is consistent with existing work and epidemiology for the area.
- In general, levels of vibrio bacteria were higher in oysters grown-out in the flip bag culture method compared to those with the beach culture method and in in cases where there were significant differences between culture methods, levels of vibrio bacteria were higher in flip bag oysters compared to beach culture oysters in all cases excluding one.
- Beach cultured oysters were able to recover from elevated vibrio levels in more cases than flip bag oysters.

Given the results of this study, preliminary trends were observed that showed some differences between the two culture methods. Notably, vibrio bacteria levels decreased in most cases in both culture methods. While these data are not strong enough to support different regulatory requirements for each culture method, the trends suggest that further study of the two methods, as well as factors and variables that may be causing differences between them, is warranted.