ABSTRACT

Title of Thesis: COMPARATIVE GROWTH RATES OF *Vibrio parahaemolyticus* SEQUENCE TYPE (ST) 36 AND NON-ST36 STRAINS IN LIVE OYSTERS AND IN CULTURE MEDIA

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Vibrio parahaemolyticus is a pathogenic marine bacterium that can cause seafood-related gastroenteritis. Infections originating from cooler waters in the northeast U.S. are typically rare, but recently these regions have shown an increase in infections attributed to the ecological introduction of pathogenic sequence type (ST) 36 strains, which are endemic to the cooler waters of the Pacific Northwest. A 2005 risk assessment performed by the Food and Drug Administration (FDA) modeled post-harvest growth of *V. parahaemolyticus* in oysters as a function of air temperature and length of time oysters remain unrefrigerated. This model, while useful, has raised questions about strain growth differences in oyster tissue and whether invasive pathogenic strains exhibit different growth rates than non-ST36 strains, particularly at

lower temperatures. To investigate this, ST36 and non-ST36 strain growth was observed in broth culture over 72 hours at 15°C and growth rates of strains compared. To investigate growth in live oysters, eastern oysters (*Crassostrea virginica)* were injected with ST36 strains and non-ST36 strains and growth rates were measured using most probable number (MPN) enumeration. *V. parahaemolyticus* presence was confirmed using polymerase chain reaction (PCR) by targeting the thermolabile hemolysin gene (*tlh*), thermostable direct hemolysin (*tdh*), *tdh*-related hemolysin (*trh*), and a pathogenesis-related protein (*prp*). Growth rates of ST36 strains were compared against the FDA model and several other datasets of *V. parahaemolyticus* growth in naturally inoculated oysters harvested from Washington State (*Crassostrea gigas)* and the Chesapeake Bay (*C. virginica)*. Our data indicated that growth rates from most oyster studies fall within the mean of the FDA model, but with slightly higher growth at lower temperatures for ST36 strains injected into live oysters. In broth culture, growth rates were not correlated with sequence type for the strains tested. These data suggest further investigations of ST36 growth capability in oysters at temperatures previously thought unsuitably low for vibrio growth are warranted.

COMPARATIVE GROWTH RATES OF *Vibrio parahaemolyticus* SEQUENCE TYPE (ST) 36 AND NON-ST36 STRAINS IN LIVE OYSTERS AND IN CULTURE MEDIA

by

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Dedication

I want to dedicate this thesis to my husband Andrew and my daughter Penelope. Andrew, you have always supported me in whatever I set my sights on and loved me through good times and bad, and none of this would have been possible without your support. Penny, you can't read this yet, but I hope that someday you will. Initially I was scared at the thought of having a baby while still pursuing a graduate degree. However, I am so glad that you came when you did. Watching you learn and grow for the last year has been the most inspiring thing, and you make me want to be better in everything that I do. Bringing you into the world while in school, working full time, and in the midst of a terrifying global pandemic is something I will never forget, but I'm so happy you exist, and you'll always inspire me.

I dedicate this thesis to my parents for providing me support, encouragement, and the opportunity to go to college and end up where I am today. Additionally, I want to also dedicate this to my many family members in mine and my husband's family and my friends, who I appreciate immensely.

Lastly, I dedicate this thesis to my late grandfather Thomas Nees. When I first started studying vibrio, he suffered a terrifying vibrio infection from getting pinched by a crab. He had to endure shock trauma, surgeries, and a lot of care to get his health back. He never once lost his sense of humor in that time, and he enjoyed chatting with me about vibrio and was so interested in the work that I do. As an avid sailor, he loved the work that I did and I wish he was still around to see me graduate and work with NOAA, continuing the vibrio work that he loved to talk to me about.

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List of abbreviations

Vp- *Vibrio parahaemolyticus*

APW- Alkaline Peptone Water

CDC- Centers for Disease Control and Prevention

COL- Cooperative Oxford Laboratory

CSS- Consolidated Safety Services Incorporated

FDA- Food and Drug Administration

MPN- Most Probable Number

NCCOS- National Centers for Coastal Ocean Science

NOAA- National Oceanic and Atmospheric Administration

NOS- National Ocean Service

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

Tdh- thermostable direct hemolysin

Tlh- Thermolabile hemolysin

Trh- Tdh-related hemolysin

Prp- Pathogenesis related protein

ST36- Sequence Type 36

DNA- Deoxyribonucleic Acid

MLST- Multilocus Sequence Typing

PNW- Pacific Northwest

NE- Northeast

Chapter I: Introduction

Vibrio parahaemolyticus (Vp) is a species of genus *Vibrio* that is capable of causing gastroenteritis from consumption of raw or undercooked seafood (1). The Centers for Disease Control and Prevention estimates that Vp causes around 45,000 cases of vibriosis each year in the United States (2). While infections are often self-limiting in nature, outbreaks can lead to significant negative impacts to the aquaculture industry including oyster growers, restaurants, and consumers. Vp is commonly found in the brackish water column in warmer months and is naturally found in the gut contents of eastern oysters, *Crassostrea virginica* (3). Because it naturally occurs in marine ecosystems, eradication of the bacteria is not possible and controlling infection is instead focused on mitigating risk.

Vibrio bacteria, both Vp and *V. cholerae*, are the only pathogenic marine bacteria that have spread globally in a pandemic manner (4). Previously, the only incidence of transcontinental migration by a Vp strain was the spread of the ST3 O3:K6 pandemic clonal complex from India to nearly every major continent in the world (4-9). However, in 2012, the Northeast U.S. and Spain experienced an increased number of infections that were attributed to the migration of sequence type 36 (ST36) (serotype O4:K12) strains that are endemic to the cooler waters of the U.S. Pacific Northwest (10, 11). These strains have been characterized by multilocus sequence typing (MLST) analysis of seven housekeeping genes and are remarkably similar in terms of chromosomal content (12). Additionally, these ST36 strains deemed the Pacific Northwest Complex are suspected to have a lower infective dose than other strains, potentially making them more virulent (13). Genomic investigation

of the strains isolated from the North American East Coast suggests that while the Pacific Northwest strains had been introduced to other areas of the country prior to 1995, a diversification event in 1995 led to newer lineage of ST36 strains (14). Investigations of the ST36 lineages also indicate that this modern lineage was introduced to the East Coast multiple times before becoming a resident sequence type of the region and that this modern lineage has undergone multiple diversification events since the introduction and incorporation into resident strain populations of the U.S. East Coast (14). After their 2012 introduction, these strains contributed to outbreaks in these regions and subsequently sustained residency in local waters, continuing to infect oyster consumers (15, 16). More recently, it's been reported that several ST36 strains of the Pacific Northwest lineage have migrated into Lima, Peru between the years 2011 and 2016, adding to the questions about whether or not these strains have pandemic potential similar to the aforementioned ST3 O3:K6 complex (17).

In the mid-Atlantic region, the oyster aquaculture industry is consistently growing. For example, the latest reports of total revenue of the eastern oyster fishery in Maryland indicate a 380% increase from 2007 to 2016 (18). The oyster industry in the Chesapeake Bay region supports the local economy, as well as clearer water and a healthier ecosystem. Vp infections impact aquaculture industry revenue and burden the healthcare system, with healthcare costs estimated at around $20 million per year (19). To mitigate illnesses caused by Vp, the U.S. Food and Drug Administration (FDA) released a risk assessment in 2005 that modeled Vp growth in live oysters post-harvest as a function of air temperature at the time of harvest and the length of

time oysters remain unrefrigerated (20). Subsequently, other agencies such as the National Oceanic and Atmospheric Administration's National Centers for Coastal Ocean Science have used this FDA model to create models for specific regions (https://products.coastalscience.noaa.gov/vibrioforecast/). These models assist the aquaculture industry, including oyster growers, by conveying information to assist with understanding Vp growth in their product based on time of harvest, air temperature at time of harvest, and different cooling strategies used. However, there is a lack of sufficient data on growth rates on the non-native ST36 Vp strains that are causing infections and seemingly out-compete local strains, especially at lower temperatures that are typically suboptimal for Vp growth. These strains are native to cooler waters of the Pacific Northwest, and it is expected that they may grow well at temperatures typically considered too low for optimal Vibrio growth (<15℃). Furthermore, this risk assessment study was performed prior to the 2012 outbreaks in which these strains took up residence in East Coast waters, which could potentially leave gaps in knowledge of Vibrio growth in scenarios beyond what was done in the risk assessment.

States that have historically had Vp infections, or states where illness is reasonably likely to occur based on environmental metrics, are required to have control plans that are overseen by the states in coordination with FDA. The National Shellfish Sanitation program keeps record of these rules and regulations implemented by these authorities (21). These control plans require harvesters to adhere to harvest time restrictions during warmer months and require harvested product to be cooled to 10℃ within a certain amount of time. These control plans are incredibly beneficial in

maintaining lower temperatures in harvested oysters to control growth of Vibrio species, and because of this, it would be additionally beneficial to recognize the behavior of these invasive strains at lower temperatures.

The goals of this study were to 1) calculate the growth rates of ST36 and non ST36 Vp strains in live *C. virginica* oyster tissue harvested from MD, USA; 2) calculate growth rates for naturally inoculated *C. gigas* oysters harvested from WA, USA; 3) compare growth rate data obtained from these experiments to previously published growth rate data of Vp in *C. virginica* oysters and the 2005 FDA model; 4) calculate growth rates of ST36 and non ST36 strains in culture media. The data obtained from this work will assist in evaluating the strength of the FDA model predictions, as well as help health organizations and the aquaculture industry better understand the potential risks of harvest practices in the presence of invasive strains that persistently cause infections throughout the U.S. east coast.

The hypotheses of this study are as follows:

- ST36 strains will likely show faster growth at all temperatures when injected into live oysters.
- ST36 strains growth may be faster at lower temperatures due to them being cold adapted because of their lineage originating from the Pacific Northwest. Their growth may be faster at higher temperatures because of their pathogenic tendencies in humans, and their affinity for human body temperature.

- Environmental strains will show significantly slower growth at lower temperatures compared to ST36 strains, but will likely fall within predictions of the FDA 2005 risk assessment model.
- ST36 strains may exceed the growth expectations of the FDA 2005 risk assessment model for post-harvest Vp growth, due to evolutionary changes that have likely happened since the conception of the model.

Chapter II: Review of the literature

2.1 *Vibrio* bacteria

Vibrio bacteria are ubiquitous marine bacteria that are capable of causing human infection. There are over 100 recognized species at this time, with nearly a dozen having history of infecting humans. Vibrio are halophilic gram-negative rod-shaped bacteria that don't produce spores. Their shape is slightly curved, like a comma, and they are motile with most species possessing a monotrichous polar flagella (22). Vibrio were first discovered in 1854 when the cholera disease made its way to Florence, where Filippo Pacini discovered the causative agent as a comma shaped bacillus and described these as "vibrio", as the word in Latin means "to shake", indicating the high activity of the bacteria when observed under the microscope (23). There are three main species of interest of Vibrio within the context of human health. While dozens of Vibrio species act as opportunistic pathogens, the ones that are most commonly implicated in human illness are *Vibrio cholerae, Vibrio vulnificus,* and *Vibrio parahaemolyticus* , hereon referred to as Vp (24, 25).

V. cholerae is well known as the causative bacterium of cholera disease. The first identified pandemic of cholera is said to have begun in India in 1817, and there have been outbreaks and pandemics of cholera ever since (26). While infections from this species are rare in developed nations like the United States, there are still outbreaks of cholera in developing countries that don't have consistent access to clean water (25). *V. vulnificus* is more limited in distribution around the globe, but is a major contributor to vibrio illness called vibriosis and deaths attributed to the Vibrio genus. *V. vulnificus* is responsible for the majority of seafood related deaths and

causes infections globally (27). *V. vulnificus* is also naturally occurring and is endemic to areas like the Chesapeake Bay, where it is extremely prevalent in the brackish waters during the warm summer months (28).

Vp causes the majority of seafood related vibriosis infections in the United States (29). While it is less often fatal, there is possibility of resulting septicemia from gastroenteritis infections. Often, infections are self-limiting in nature but the impacts to healthcare systems, restaurants, and the aquaculture industry can be significant. Vp has also been shown to cause wound infections, though these infections aren't as prevalent as infections from consumption of contaminated seafood or seawater. Howard et al. 1993 found that various species of vibrio other than cholerae, including Vp and *V. vulnificus,* were capable of both soft tissue infections and primary bacteremia (30). Vp will be discussed in greater detail below.

2.2. *Vibrio parahaemolyticus*

2.2.1 *V. parahaemolyticus* overview and general ecology

Vp falls in line with other vibrio with its halophilic nature and affinity for warmer brackish water. Vp have been shown to prefer an average salinity of 17ppt and temperatures above 60° F, or around 15° C, and is more influenced by temperature changes than salinity (31). Davis et al. (2017) did find negative associations between Vp levels and high salinity, though this relationship was modulated by turbidity and low temperatures (32). Additionally, high salinity has been used as a depuration technique for shellfish containing Vp; Larsen et al. (2015)

found that by day 3 of high salinity exposure (35 psu), levels of Vp showed significant reduction (33).

Vp is ubiquitous and found globally in coastal environments, such as in the Pacific Northwest (34), the Mid-Atlantic (15, 35), the NE United States (36), the Gulf of Mexico and the northwest coast of Mexico (37, 38), Japan (1), Taiwan (39). Europe (8, 9, 40), South America (17), and even Alaska (41). Specifically, Vp has been detected in Chesapeake Bay waters for decades (42). Additionally, Vp is relatively stable at a range of temperatures. Studies have shown that exponential phase cultures of Vp could be stored at -20° C and still be recoverable; Vp was shown to maintain the integrity of cellular membranes even at colder temperatures by changing their physiological state, and additionally sustained metabolic activity in this state (43). Vp is additionally shown to be positively correlated with chlorophyll-a levels, so ongoing studies are currently investigating whether or not HABs and other algal events can cause increases in Vp levels in the water column (44). However, some data has shown no relationship between blooms and levels of Vp, so this relationship is not entirely clear (45). Vp are naturally a part of the microbiome of several species of oysters, including *Crassostrea gigas* and *C. virginica*, and can accumulate in oyster tissues. Additionally, levels of Vp aren't constant from oyster to oyster; Klein et al. (2017) found that some oysters contained 100-fold higher levels of presumed pathogenic Vp than neighboring oysters (46). While Vp infections typically happen in warmer months, it's expected that increased water temperatures due to climate change will affect Vp levels and possibly extend the season in which they are typically problematic for humans (47).

2.2.2. Growth characteristics and survival of *V. parahaemolyticus*

One explanation for the overall abundance of Vp globally is the ability for the bacterium to rapidly proliferate, often with short generation times. Certain growth conditions have been found to support a 10 minute generation time at higher temperatures (35°C). (48). Vp have been found to be prevalent in coastal waters during summer months. Colwell et al. (1984) found Vp cycling based on seasons in the Chesapeake Bay (49). Vibrio naturally grow and proliferate inside of oysters. While Vp can be prevalent in the water column, Vp can accumulate in oysters and increase during the process of harvest and distribution to retailers (50). Various studies have been done to investigate growth and survival rates of Vp as a means to protect consumers. Gooch et al. (2002) found an increase of 1.7 log CFU/g (50-fold) in live oysters that were stored at 26° C post-harvest after only 10 hours (50).

Additionally, studies have shown that Vp can survive at low temperatures, such as a finding from Johnson et al. (1973) indicating survival of Vp at 4° C after higher initial incubation temperature (51). A large majority of studies done have been on homogenates and raw or cooked seafood, such as a study by Bradshaw et al. 1974 that found that vibrio levels leveled off growth at 12.8° C (52). Matches et al. (1971) investigated reduction of Vp at refrigeration temperatures in fish homogenate and found that Vp still survived, though showed reduced numbers, at 0.6° C (53). More recently, there has been some research done on cytotoxicity of Vibrio on human intestinal cells based on salt concentration in media. Whitaker et al. (2020) found increased cytotoxicity by Vp grown in 1% salt medium compared to 3%, suggesting that salinity might have an effect on the pathogenic nature of Vp (54). Vp have been

shown to be sensitive to acid stress, with high sensitivity at a pH below 6.0 (55). Though Vp cells can be sensitive to environmental stressors, they can also bounce back from stressful environments such as high acid or low temperatures; Jiang et al. (1996) found starved Vp cells were revived with nalidixic acid and increased temperatures and were then capable of growth after a non-culturable state (56).

2.2.3. *V. parahaemolyticus* virulence and detection

Scientists have been molecularly detecting the presence of Vp both environmental and clinical samples for decades. Detection at the species level for Vp is typically done by polymerase chain reaction (PCR) targeting the thermolabile hemolysin coding gene, denoted *tlh* (57).This protein has the ability to lyse erythrocytes by phospholipase activity (58). Typically, the presence of this gene does not indicate the ability of the isolate to cause human infection.

There are two genes that are used as markers for potential virulence in Vp, thermostable direct hemolysin (*tdh*) and tdh-related hemolysin (*trh*). While most Vp infections result from strains that have either one or both of these genes, about 10% of clinical strains don't have either (59). *Tdh* has the capability of forming pores in the membranes of erythrocytes that can lead to severe diarrhea from loss of water through the cellular pores (60). *Trh* was discovered after the discovery of tdh, and was found to be incredibly similar with 70% homology to tdh, hence the name tdh-related hemolysin. *Trh* is heat labile and can cause fluxing ionic levels similar to effects caused by *tdh* (61). The ability to detect the trh and tdh genes is useful in identifying possible pathogenic strains, which are typically detected in 0.3 – 3% of total Vp detections (62). Methods developed by researchers at the FDA have been able to

detect pathogenic Vp much better than earlier methods with combinations of culture testing and MPN analysis coupled with molecular analysis. There is a possibility of false-positives in virulence testing, due to close homology of the tdh gene for Vp and *V. hollisae*, but researchers have noted that the detection of non-pathogenic Vp and high abundance of tdh-containing *V. hollisae* is not a likely occurrence (62). Similarly, Nordstrom et al. 2007 also detected the trh gene in some cultures of *V. alginolyticus* (62). While some of these genes may not always be unique to Vp, the combination of *tlh*, *trh*, and *tdh* in molecular testing usually can provide a robust diagnostic for detecting potentially harmful Vp.

One drawback to current testing methods is that they are generally culture based, and Vp is capable of entering a viable but non-culturable (VBNC) state. Johnston et al. (2002) found that Vp entering a VBNC state could be revived after physiologically transforming into coccoid cells, but typically would not be captured in culture based methods of detection (43).

Most environmental isolates don't test positive for either of these virulence genes. However, this is highly variable; Parveen et al. (2020) found that DE Bay oysters had much higher levels (8-fold) of *trh*+/*tdh*+ Vp when compared to Chesapeake Bay oysters (63). Nevertheless, again in the Chesapeake Bay, 1-2% of isolates obtained from *C. virginica* during a relay study tested positive for *trh* and *tdh*, genes even though most clinical cases (over 90%) show at least positive tdh when cultured (64).

2.3. *V. parahaemolyticus* serotyping and sequence typing

2.3.1. MLST sequence typing

Multilocus Sequence Typing, or MLST, is a method of typing bacterial species using specific genes of interest unique to that species (65). Typically, this consists of using the sequences of internal fragments of house-keeping genes, which are genes that are universally conserved across all species genomes. 450-500 base pairs are sequenced for each gene and single nucleotide differences between sequences are assigned different allele number identifiers. This typing is said to be able to detect point mutations or recombination. MLST analysis can also be done via PCR of the house-keeping genes when there is DNA present but no ability to culture the organism, like from clinical samples. This method was revolutionary for molecular science and epidemiology, as it allows the ability to use sequence data to compare between laboratories and databases. Sequence typing is commonly used as a way to characterize bacteria and compare relatedness of a strain to other known strains, as well as track mutations. Because this data is also sequence based, there is continuity between different laboratories performing analyses because of the precise nature of the sequencing process. MLST data can easily be accessed on websites like http://pubmlst.org/.

A comprehensive seven-gene MLST profile was created for Vp by researchers in 2008 (12). This analysis examines three genes from chromosome I and 4 genes from chromosome II. A less extensive method analyzing four genes from chromosome I was previously used to investigate pandemic strains from the O3:K6 serotype (discussed in the subsection below). However, this was not extensive enough

to get a clear picture of phylogenetic diversity within the Vp species. The house-keeping genes analyzed include RecA protein (*recA*), DNA polymerase III alpha subunit (*dnaE*), DNA gyrase subunit B (*gyrB*), threonine 3-dehydrogenase (*dtdS*), transhydrogenase alpha subunit (*pntA*), dihydro-orotase (*pyrC*), and tryptophanase (*tnaA*). *RecA, dnaE,* and *gyrB* are located on chromosome I whereas the remaining are located on chromosome II. Clonal complexes are commonly referred to in Vibrio research, and indicate strains that share six of the seven total allelic profiles for the house-keeping genes (12).

2.3.2. Pandemic serotypes and outbreaks

The first outbreak of illness attributed to Vp happened in October of 1950 in Japan from consumption of shirasu, or dried sardines; 272 individuals became sick with gastroenteritis and 20 people died as a result of the outbreak (66). Following this outbreak, Vp continued to cause sporadic infections globally from a variety of strains identified with serotyping. Serotyping has been a historic method for analyzing Vibrio prior to advancements in sequencing, including MLST analysis. Serotyping involves a slide agglutination test using O, H, and K antisera (67). Vp serotypes are identified by combinations of different O and K antigens (68). The combination of antigens from this test indicate the serotype of the Vp strain.

The pandemic Vp serotype O3:K6 of was first reported in 1994 from hospitalized patients in Calcutta, India and from travelers arriving in Japan (69). In 1996, the O3:K6 serotype was found to cause 50-80% of all Vp infections during an outbreak in Calcutta, India (39). This serotype spread across the globe and became a pandemic clonal complex, causing outbreaks in other areas such as Taiwan, where

O3:K6 was responsible for 61 – 71% of outbreaks between 1996 – 1999 (39). O3:K6 and the subsequent outbreaks put Vp in a broader spotlight in terms of global epidemiology and the risks associated with commonly encountered marine bacteria.

While O3:K6 is a concerning serotype, it is not the only group of Vp that is capable of outbreaks and infections. For instance, an outbreak in Maryland, USA from crab products in 1971 was attributed to a range of serotypes including O4:K11 (majority of cases), O3:K30, and O2:K28 (35). In Washington state, O4:K12 was attributed to infections (70). Furthermore, strain and sequence typing using whole genome sequencing and MLST methods have drastically changed how pandemic and outbreak causing strains are categorized and tracked. Sequence typing allows researchers to examine the relatedness between strains and sequence types. For example, pandemic Vp strains identified in China between 1997 – 2007 were comprised of 22 serotypes, and one sequence type denoted ST3 showed great diversity of serotypes within the sequence type (71). This was expected to be due to the ability of Vp to exchange antigens via horizontal gene transfer, which is a common mechanism that bacteria use to share genes. Closely related sequence types are grouped into clonal complexes, such as ST3 being a part of clonal complex (CC) 3. Sequence types of interest in the NE USA include ST631, which is an emerging sequence type causing sporadic infection, and ST36 has caused extensive infections and will be discussed in greater detail below.

2.3.3 Sequence type 36 (ST36) *V. parahaemolyticus*

While the pandemic strains belonging to the O3:K6 serotype caused the most extensive outbreaks in US history (72), infections and outbreaks in the Pacific Northwest (PNW) US were found to be genetically different from the pandemic strains after 1989 (13, 73). Prior to 2012, infections in the NE USA attributed to Vp were rare, sporadic, and overall not on the radar of oyster growers and consumers. This was generally due to the colder waters in the NE and Vp's affinity for warmer water. ST36 emerged in the NE US in May-June 2012 when 28 people fell ill after consuming contaminated oysters (74). Between 2010 to 2016, ST36 were found to be the cause of 50% of Vp infections for the NE US, specifically from ME, NH, MA, and CT (75). From 2012-2013, ST36 strains represented 28% of Vp infections in Maryland (15). Additionally, this sequence type was concurrently discovered to be the causative agent of an outbreak in Galicia, Spain where 51 individuals suffered illness after consuming shrimp (10). This was the first type this sequence type had ever been detected in Europe, and additionally was the first outbreak in Europe from Vp carrying both the *tdh* and *trh* virulence genes. While the mode of transmission of ST36 strains across the globe is still unknown, there is speculation that it could be due to ballast water transport and the ability for microorganisms to survive in ballast water and be transported large distances (76). Ruiz et al (2000) investigated ballast water from ships entering ports in the Chesapeake Bay had significant amounts of pathogenic *V. cholerae*, and some cells were viable upon arrival (77).

Identifying possible ST36 isolates of Vp is done in several ways. ST36 strains typically exhibit the O4:K12 serotype, which can be a first clue when running

diagnostics. Full MLST can be done on sequence data, but this can sometimes be cost prohibitive. While typically the seven housekeeping genes are needed to fully profile the sequence type of a Vp strain, there has been some research indicating that using a four allele approach is relatively accurate in determining of ST36 strains (78). This research by Whistler et al. 2015 found that a combination of the *tlh*, *trh*, and *tdh* gene combined with a separate marker, noted the pathogenesis related protein (*prp*), was able to correctly identify the presence of ST36 strains in all strains tested (43 strains). Additionally, the *prp* gene wasn't identified in any of the tested environmental isolates that weren't ST36. The ability to run these four loci in a multiplex PCR assay is very valuable for detecting possible pathogenic strains.

2.4. Impacts and management of *V. parahaemolyticus*

2.4.1. Economic impacts of *V. parahaemolyticus*

While most focus in the United States is on human illness, Vp does have the ability to infect and cause illness in other species. Notable, Vp can infect shrimp and cause acute hepatopancreatic necrosis disease (AHPND), which can be devastating for economies reliant on shrimp exports (79). In the US, it doesn't typically impact other species to a point that becomes problematic. Still, the biggest impacts of Vp are the healthcare costs associated with infections, the impacts to oyster growers for costs related to Vibrio regulations, and the impacts to consumers who fall ill from consuming undercooked seafood.

Estimates for the costs associated with Vp infections range based on exposure, whether it's direct (contact or accidental ingestion of seawater) or indirect via gastrointestinal illness. Direct exposure to Vp carries a cost estimate of $1.5 million,

whereas indirect gastrointestinal exposure costs $21 million (5). However, Vp illnesses often go unreported due to the self-limiting nature of the infection. This could mean there are impacts that cannot be fully extrapolated because there isn't sufficient data to investigate hypotheses or questions, like the possible impacts to consumers when they are ill and lose wages or suffer other consequences from their infections.

Recently, some research effort has focused on how regulatory actions due to Vibrio impact the aquaculture industry in an economic context. This is an expanding research topic that is relatively novel, but surveys of Washington State oyster growers and restaurants found that traceable cases carry a $61,880 burden, and prevention measures average $0.45 for a dozen oysters landed (80). This data is significant due to the fact that much burden is placed on the oyster grower, but the regulations in place are vital to protecting consumers from increased prevalence of vibriosis. This type of research has been completed in the Pacific Northwest, but conclusions can not necessarily be extrapolated to apply to other regions.

2.4.2. *V. parahaemolyticus* management and regulation

Four outbreaks that occurred in the US between 1997 and 1998 sickened 700 people across multiple states. This included an outbreak in the Pacific Northwest attributed to O4:K12 (29). The other outbreaks in these years were in Texas, the Northeast Atlantic, and New York (20). Historically and currently, seafood is regulated for safety by the Food and Drug Administration (FDA). The FDA is a federal representative to the Interstate Shellfish Sanitation Conference (ISSC), and the National Shellfish Sanitation Program (NSSP) is a regulatory body that brings

together federal, state, and industry to evaluate compliances and provide guidelines for safe harvesting (81). After these aforementioned outbreaks, the FDA proposed a risk assessment in 1999 to determine public health impacts from Vp exposure from raw oyster consumption (20). This risk assessment has become the basis for Vibrio management as a way to protect consumers from falling ill from consumption of raw or undercooked seafood. The assessment modeled pathogenic Vp per serving of oyster in six major oyster harvesting areas including Louisiana, the Gulf coast excluding Louisiana, Mid and NE Atlantic, the PNW (both dredged and tidal), and compared these regions against the four seasons (20). Using these predictions, states have created control plans to prevent excessive illnesses. Some of the strictest control plans are in the PNW region, specifically Washington State, where O4:K12 ST36 strains likely originated (14, 82).

Chapter III: Methodology of the study

3.1. Summary of methodology

Growth rate observations for ST36 and non-ST36 Vp strains were done by measuring cell concentrations over time of four ST36 strains and three non-ST36 strains grown in live oysters. Additional ST36 and local strains were investigated for culture trials. For culture media trials, strains were individually inoculated into broth media and 600nm optical density measurements were taken over the course of 3 days at 15°C. For live oyster trials, oysters were injected with a combination of strains (all four ST36 strains or all three non-ST36 strains), incubated for 10 hours at temperatures 15, 20, 25, and 30 °C, and Vp cell concentrations enumerated using a three tube most probable number (MPN) method according to the FDA bacteriological analytical manual (BAM). MPN results were confirmed with PCR and gel electrophoresis analysis. Growth rates of ST36 and non ST36 strains were calculated based on exponential growth phases in their growth curves and then statistically compared.

3.2. Preparing ST36 and non-ST36 inoculums for oyster injection

For the non ST36 strain trial, three environmentally isolated strains of Vp were used: 43, 930, and 204 (Table 1). The strains were inoculated onto T_1N_2 agar plates (2% NaCl, 1% pancreatic digest of casein, and 2% agarose w/v) and incubated at approximately 22°C, for 24 hours $\pm$ 4 hours. Two ml of LB (Miller) (Sigma Aldrich, MO, USA) broth was inoculated with a loop-full of each strain and incubated at 25°C in a static incubator for 24 hours $\pm$ 4 hours. Following incubation, 20mL of LB (Miller) broth was inoculated with 133µL of each strain to form a

cocktail of all three cultures. This cocktail culture was incubated at 30°C shaking (100 rpm) for two hours. Following shaking incubation, the optical density (OD) (600nm) was measured to estimate the bacterial concentration. One mL of culture was washed twice by centrifuging at 8,000 rcf for one minute, discarding the supernatant, and re-suspending the pellet in 1mL phosphate buffer solution (PBS, 085% NaCl, 0.058% NaH_2PO_4, 0.25% Na_2HPO_4 w/v). Ten-fold serial dilutions were performed from 10^6 – 10^8 (estimation made based on OD measurement) through 10^{-1} by inoculating 800µL of washed culture into 7.2mL PBS for each dilution. The 10^4 dilution was set aside as the inoculum. Replicate T_1N_2 agar spread plates were prepared for the 10^3, 10^2, and 10^1 dilutions by transferring 200µL of the diluted culture onto the plate and spreading the culture with a flame-sterilized metal spreader. Spread plates were incubated at room temperature upside down for 24 $\pm$ 4 hours and then CFUs counted to calculate the precise concentration of the inoculum. Four ST36 strains (PHL-3, PHL-4, 9701173, and 12315 [Table 1]) were used to prepare the inoculum using the same protocol as described for non ST36 strains. To create both cocktails, l, 100µL of each strain was added to 20mL of LB (Miller) broth.

Table 1. Strain, Sequence Type, Genotype, Source, Year, and Location for all isolates used in this study. Vp strains used in this study for both culture and live oyster methods. Presence of the following genes is denoted by + when present, - when absent; thermostable direct hemolysin-related hemolysin (*trh*), thermostable-direct hemolysin (*tdh*), and pathogenesis related protein (*prp*).

Strain ID	Sequence type (ST)	*tlh* +/-	*trh* +/-	*tdh* +/-	*prp* +/-	Source	Isolation year	State
PHL-3[a]	36	+	+	+	+	Human Stool	2012	WA
PHL-4[a]	36	+	+	+	+	Human Stool	2012	WA
EN9701173[a]	36	+	+	+	+	Human Stool	1997	WA
12315[a]	36	+	+	+	+	Human Stool	2006	WA
43[b]	322	+	-	-	-	Oyster	2007	WA
204[b]	3	+	+	-	-	Oyster	2007	WA
930[b]	3	+	-	+	-	Oyster	2007	WA
2012V-1076[c]	36	+	+	+	+	Human Stool	2012	MO
2012V-1103[c]	36	+	+	+	+	Human Stool	2012	WA
2012V-1108[c]	36	+	+	+	+	Human Stool	2012	MA
2012V-1109[c]	36	+	+	+	+	Human Stool	2012	MA
2012V-1131[c]	36	+	+	+	+	Human Stool	2012	CA
2012V-1134[c]	36	+	+	+	+	Human Stool	2012	CA
CDC_K4639[c]	36	+	+	+	+	Human Stool	2012	NY

[a] Isolated by the Washington Department of Health

[b] Provided by the NOAA Northwest Fisheries Science Center and Washington Department of Health, Seattle, WA

[c] Provided by the FDA, Division of Seafood Science and Technology, Gulf Coast Seafood Laboratory, Dauphin Island, AL

3.3. Oyster inoculation and incubation

C. virginica oysters were purchased from Captain's Ketch Seafood in Easton, MD. All oysters were Choptank Sweets oysters sourced from Marinetics Inc. from the Choptank River (MD, USA) and were uniform in size (2-3 inches). Oysters were acclimated from refrigeration temperatures to ambient water temperatures over the course of 7 days prior to experiments. Oysters were acclimated to 18°C for two hours prior to dosing. The oysters were rinsed and scrubbed with room temperature potable tap water and allowed to dry before processing. Using a 1/16" drill bit fitted to a power drill, each oyster was drilled on the right valve approximately 1⁄4 distance from the hinge and slightly to the left in order to inject the gut region. This technique was first tested on several oysters using tissue dye as the inoculum and the oysters dissected to indicate that the inoculum would reach the gut tissue. One control group was tested at time point 0 for all trials, where 4 oysters uninoculated were processed to ensure there were no prior background levels of Vp present (use of winter harvested oysters prevented background levels). Once drilled, each oyster was injected with 100uL of an estimated 10^4 concentration of the Vibrio strain cocktail, using a separate 1 mL 27 gage syringe for each group of 4 oysters. After injection, the drilling site was sealed with critoseal tube sealant (Leica, Wetzlar, Germany) and each oyster placed into a sterile plastic bag, left open, and placed level on a tray. Oysters were transferred to an incubator set to the desired temperature of the experimental trial being performed (15°C, 20°C, 25°C, 30°C) for the duration of the experiment. Temperatures were chosen based on favorability of Vp for higher temperatures and control plans based on lower temperatures for oyster storage.

3.4. Oyster processing and MPN protocol

At each time point (0, 3, 7, and 10 hours), three groups of four oysters were removed from the incubator and left on the bench prior to processing within one hour. Oysters were shucked on a sterile metal tray with sterilized aluminum foil. Four oysters were opened using a sterile oyster shucking knife inserted into the side of each oyster and the entire animal, including the adductor muscle and mantle fluid, was transferred into a sterile tared blender jar. An equal amount of PBS (weight/volume) was added to the blender jar and blended on high for 90 seconds. A three tube most probable number (MPN) protocol as indicated in the FDA Bacteriological Analytical Manual (BAM) was followed for enrichment of oyster homogenate in alkaline peptone water (APW) (Thermo Fisher Scientific, MA, USA) and MPN tubes incubated at 35°C 18-24 hours (83) (Figure 1). Following incubation, MPN tubes were observed for turbidity and one mL of each MPN tube culture was transferred to microcentrifuge tubes. The microtubes were boiled in a dry bath at 100°C for 10 minutes and stored at -80°C for PCR analysis.

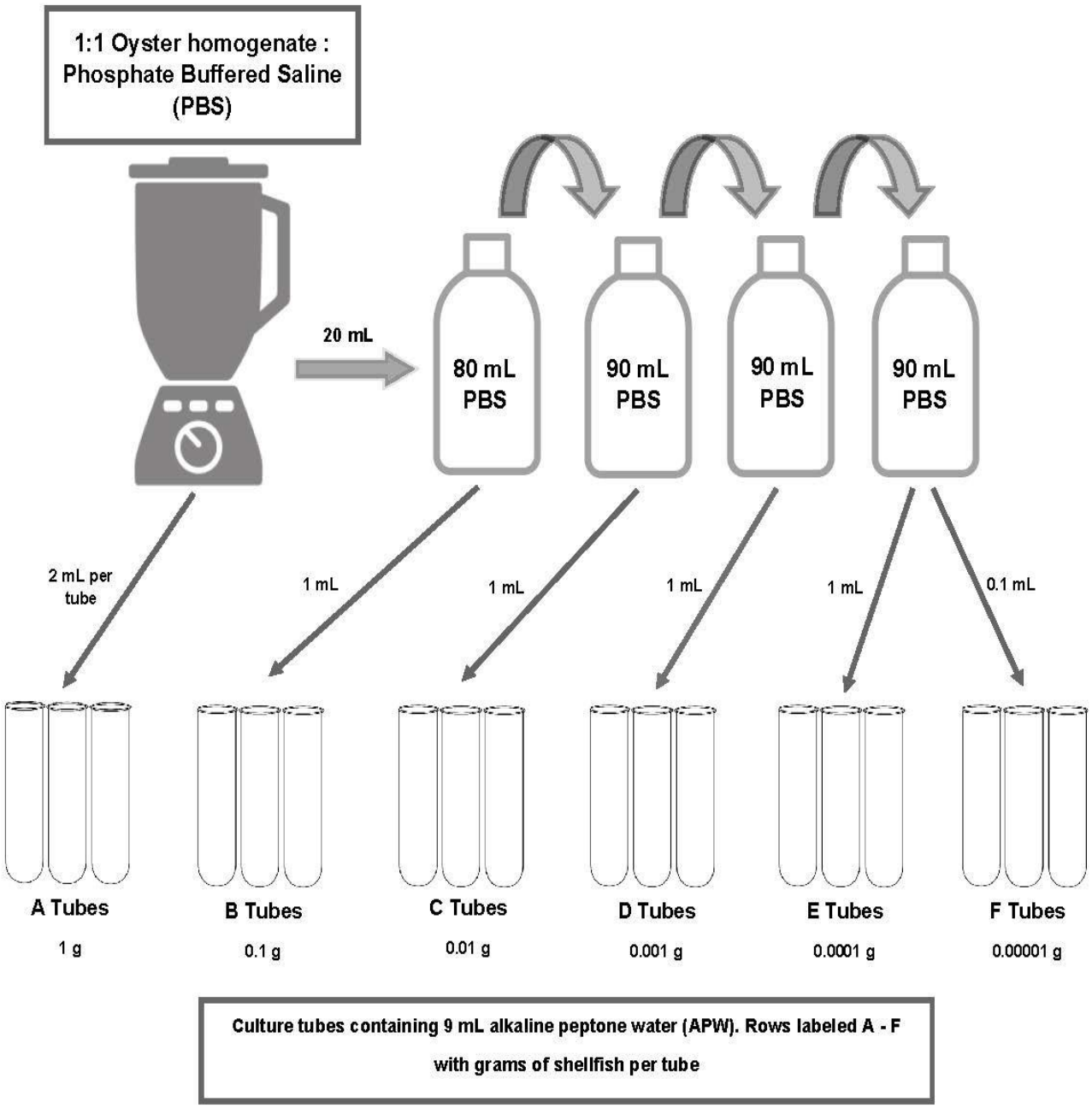

Figure 1. MPN protocol. Visualization of homogenization and 3-tube MPN method adapted from the FDA BAM and used for this study (83).

3.5. PCR parameters and gel electrophoresis for detection of *V. parahaemolyticus*

A multiplex PCR was performed on boiled MPN samples targeting 4 genes: *tlh, trh, tdh,* and *prp* (Table 2). PCR reactions were carried out using, per sample, 1µL of each primer, 5µL of 5x flexi-buffer (Promega, WI, USA), 1.5µL $MgCl_2$, 2µL dNTP solution (Epicentre, WI, USA), 7.3µL of nuclease-free PCR grade water, 0.2µL GoTaq polymerase (Promega, WI , USA), and 1µL of DNA template (boiled MPN culture) for a 25µL reaction volume. Cycling parameters included an initial denaturation at 94°C for 60 seconds, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 60 seconds, and extension at 72°C for 60 seconds, with a final extension step of 72°C for 10 minutes (1 cycle). Amplification products were stored at -20°C until further analysis. PCR products examining presence/absence of all four target genes were examined using Invitrogen double-comb ethidium bromide E-gels (Invitrogen, CA, USA). Amplicon size was confirmed with comparison to a 1 kb plus molecular weight marker.

Table 2. Primer sequences, amplicon sizes, and sources for all PCR primers used in this study. The ST36prp primer set was only used in conjunction with all other primers for identifying presence of ST36 strains.

Gene/locus	Primer direction/name	Sequences (5' to 3')	Amplicon Size (bp)	Source
tlh	F2	AGAACTTCATCTTGATGACACTGC	401	(78)
	R	GCTAC-TTTCTAGCATTTTCTCTGC		(84)
tdh	F	GTAAAGGTCTCTGACTTTTGGAC	269	(84)
	R	TGGATAGAACCTTCATCTTCACC		
trh	F	CATAACA-AACATATGCCCATTTCCG	500	(84)
	R	TTGGCTTCGATATTTTCAGTATCT		
ST36*prp*	F2	TGCGGAATCTGATCTTTATCCTC	1,028	(78)
	R2	AACTGTTG-GGTCTTCGTCTAACC		

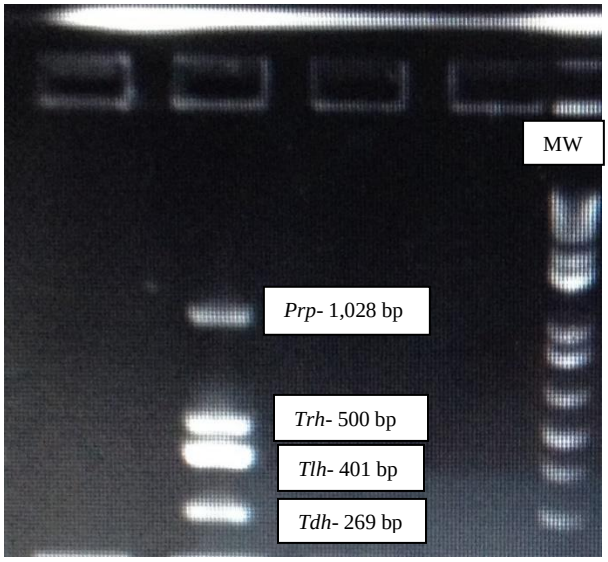

Figure 2. Example of amplicons using gel electrophoresis. Prp, trh, tlh, and tdh amplicons shown on a 2% agarose ethidium bromide gel compared to a 1kb plus molecular weight marker denoted by MW. This illustrates the amplicon sizes being examined, as listed in Table 2.

3.6. *V. parahaemolyticus* ST36 and environmental strain growth in LB broth medium

Growth rates (log10/hour) were observed for all strains used in the live oyster trials as well as additional ST36 strains obtained from the FDA Gulf Coast Seafood Laboratory in Dauphin Island, AL (Table 1). All strains were grown from frozen glycerol stocks (OPS diagnostics, NJ, USA) on LB (Miller) agar plates at 20℃ for 24 $\pm$ 4 hours. One inoculating loop of each strain was transferred into individual conical tubes containing 5 ml of LB broth and incubated at 30°C shaking (100rpm) for two hours. Assuming each strain culture was approximately 10^8 CFU/ml, each strain was diluted to 10^4 by 10-fold serial dilutions in LB broth. 500 µl of each strain was

inoculated into 4.5 ml of LB broth for the final inoculum. One hundred fifty µl of each strain was inoculated in triplicates into a black-walled microplate, avoiding the outer 2 wells of each row horizontally and vertically to negate microplate edge effect throughout incubation. The microplate was transferred to a Biotek Synergy2 microplate reader (Biotek, VT, USA) that was located in a low temperature incubator set to 15° C. This method of low temperature incubation was tested in the instrument prior to the experiment using a smart button temperature logger (ACR Systems, FL, USA) placed into the microplate reservoir and consistent 15°C temperature readings verified over the course of 72 hours. The microplate reader measured optical density (600nm) over the course of 72 hours at 30-minute intervals with 5 second shaking prior to each reading.

3.7. Natural infection study on *Crassostrea gigas* oysters

C. gigas oysters were harvested by Taylor Shellfish, from Totten Inlet in Shelton, WA. All oysters were of a uniform size, between 3 and 4 in. Oysters were placed in sterilized trays and covered with aluminum foil and subsequently incubated at temperatures 15, 20, and 25°C for 24 hours. Three groups of six oysters were removed and processed at 0, 5, 10, and 24-hour time-points. Oysters were processed using the same FDA three-tube MPN method described previously, and Vp estimated with PCR analysis of *tlh, tdh*, and *trh* genes (Figure 1, Table 2).

3.8. Statistical analysis

For live oyster trials, Vp cells per gram of oyster tissue were calculated using the FDA BAM method for most probable number estimations (83). MPN data were

log transformed and a regression analysis was performed to assess growth rates (log10/ hour). The three replicates at each measurement for oyster trials were averaged and standard error calculated to ensure there were no statistically significant differences between replicates.

For broth study trials, optical density data was downloaded from the Biotek plate reader and sorted into separate datasets per strain. The Growthcurver package was used to calculate various metrics including lag time and growth rate per minute for each strain (85). Growthcurver uses the following logistical equation for calculating the intrinsic growth rate (*r*) per well over time (*t*):

$$Nt = \frac{K}{1 + (\frac{K - N0}{N0})\, e^{-rt}}$$

where N_0 is the population at the start of the growth trial and the carrying capacity is indicated by *K*. Growth rates per hour of each replicate of each strain were calculated using Growthcurver and then significance analyzed using a one-way ANOVA and a least square means procedure using Tukey's adjustment for multiple comparisons. Growth curves for each strain can be found in Appendix I (Figure S1).

Chapter IV: Results of the study

4.1. Growth rates in live oysters

The ST36 strain cocktail injected into live oysters exhibited a faster growth rate per hour (log10/hour) (0.091) at 15 °C incubation compared to non ST36 strains (0.043) (Table 3, Figure 3). Furthermore, the ST36 strain cocktail showed a faster growth rate per hour at 15°C than all other trials compared for this study, including the natural infection of *C. gigas* oysters (0.05), previous studies done on naturally inoculated *C. virginica* in MD waters in 2005 and 2006 (0.054, 0.022 respectively), and the 2005 FDA model predictions for Vp growth at 15°C (0.0381) (Table 3, Figure 3) (20, 86). All oyster trials showed expected patterns in growth rate per hour where lower temperature incubation led to slower growth rates, except in the ST36 oyster trial in which the growth rate per hour at 15°C was slightly higher than at 20°C, but followed the expected growth rate trajectory at temperatures above 20°C (Figure 3). Standard error could not be calculated for growth rates in live oyster tissue due to the nature of the study, in which pooled oysters at each time point measurement were a new sample that could not be statistically connected to a certain pool of oysters during the previous measurements. However, standard error was evaluated for the replicates averaged for each measurement time point. Growth curves of CFU/g calculated by MPN are shown in figures S2 and S3 (Supplementary materials in Appendix I). All aforementioned live oyster studies were averaged as a way to compare collective results against the 2005 FDA model predictions, and the results of this comparison

indicate that the FDA model falls within the calculated mean and standard error of the averaged studies (Figure 4).

Table 3. Calculated growth rates (log10/hour) of ST36, non ST36, naturally inoculated *C. gigas* trials from this study, and other studies referenced for comparison. Growth rates were calculated for temperature 15°, 20°, 25°, and 30°C except for the naturally inoculated *C. gigas* trial which included incubation temperatures of 15°, 20°, and 25°C.

Trial/Study	Incubation Temperature (°C)	Growth rate (log10/hour)	Source
ST36 injection	15	0.091	This Study
	20	0.062	
	25	0.188	
	30	0.263	
Non ST36 injection	15	0.043	This Study
	20	0.057	
	25	0.3	
	30	0.3524	
C. gigas natural uptake	15	0.05	This Study
	20	0.08	
	25	0.12	
C. virginica natural uptake 2005	15	0.054	(86)
	20	0.107	
	25	0.28	
	30	0.264	
C. virginica natural uptake 2006	15	0.022	(86)
	20	0.058	
	25	0.177	
	30	0.175	
2005 FDA model	15	0.0381	(20)
	20	0.088	
	25	0.1579	
	30	0.2485	

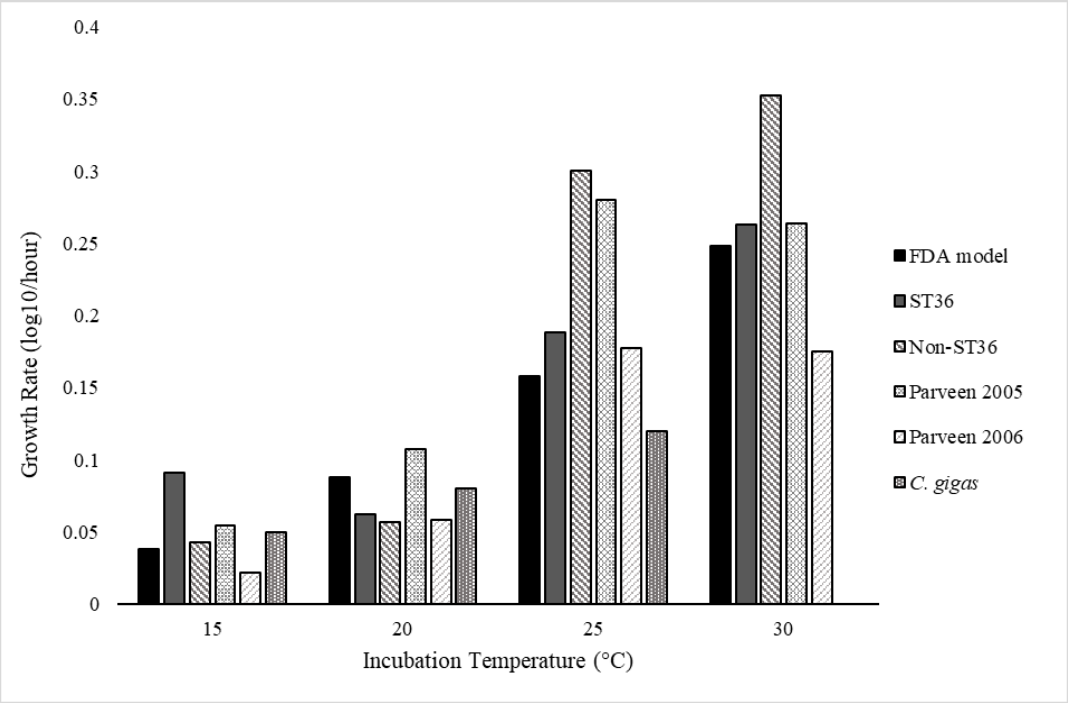

Figure 3. Growth rate per hour of Vp in live oysters. Growth rate (log10/hour) of injected Vp ST36 and non-ST36 strains in *C. virginica* oyster tissue at temperatures 15-30°C, two growth rate studies previously conducted on naturally inoculated *C. virginica* Chesapeake Bay oysters (86), a study conducted on naturally inoculated *C. gigas* in the Pacific Northwest at temperatures 15-25°C, and the 2005 FDA risk assessment model (20).

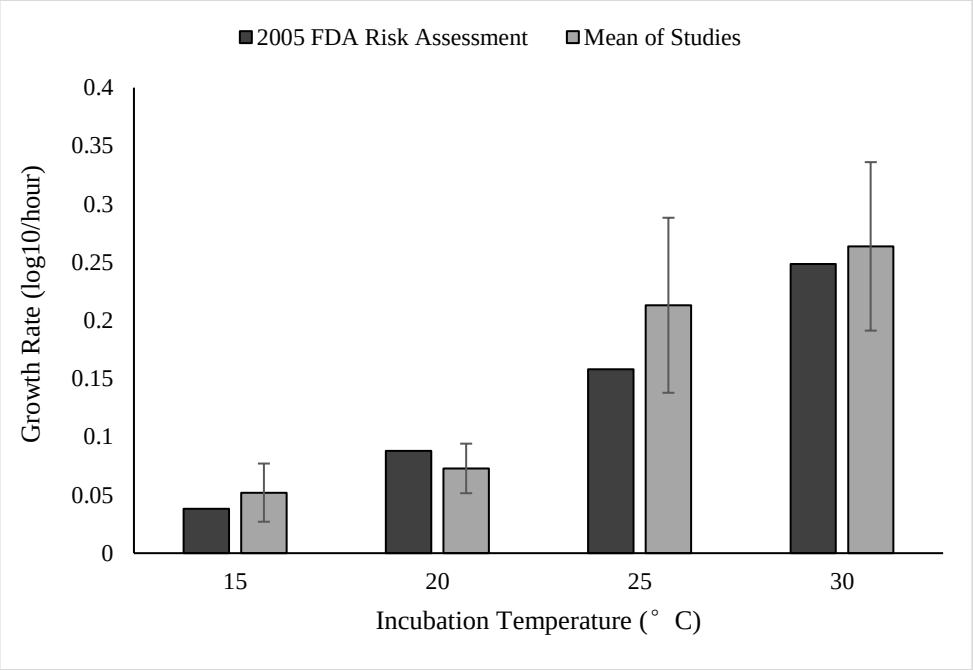

Figure 4. The mean of oyster studies compared to the FDA model. The 2005 FDA risk assessment model depicting growth rate (log10/hour) at temperatures 15-30°C and the mean of all previously referenced studies (20) (Figure 2).

4.2. Growth rates in broth culture

Growth rates in culture were calculated for from growth curves of individual strains, including the same strains used for the oyster injection trials as well as additional ST36 strains and one local strain isolated from wild oyster tissue in MD (Table 1, Figure 5). Average growth rates per hour for all strains at 15 °C were; 12315 (0.163), EN9701173 (0.141), PHL-3 (0.181), PHL-4 (0.188, 2012V-1076 (0.167), 2012V-1103 (0.161), 2012V-1108 (0.155), 2012V-1109 (0.168), 2012V-1131 (0.169), 2012V-1134 (0.161), CDC_K4639 (0.151), 204 (0.171), 43 (0.149), and 930 (0.157). A one-way ANOVA and Tukey's procedure correcting for multiple

comparisons indicate that growth rates were not significantly different among strains for both ST36 and non-ST36.

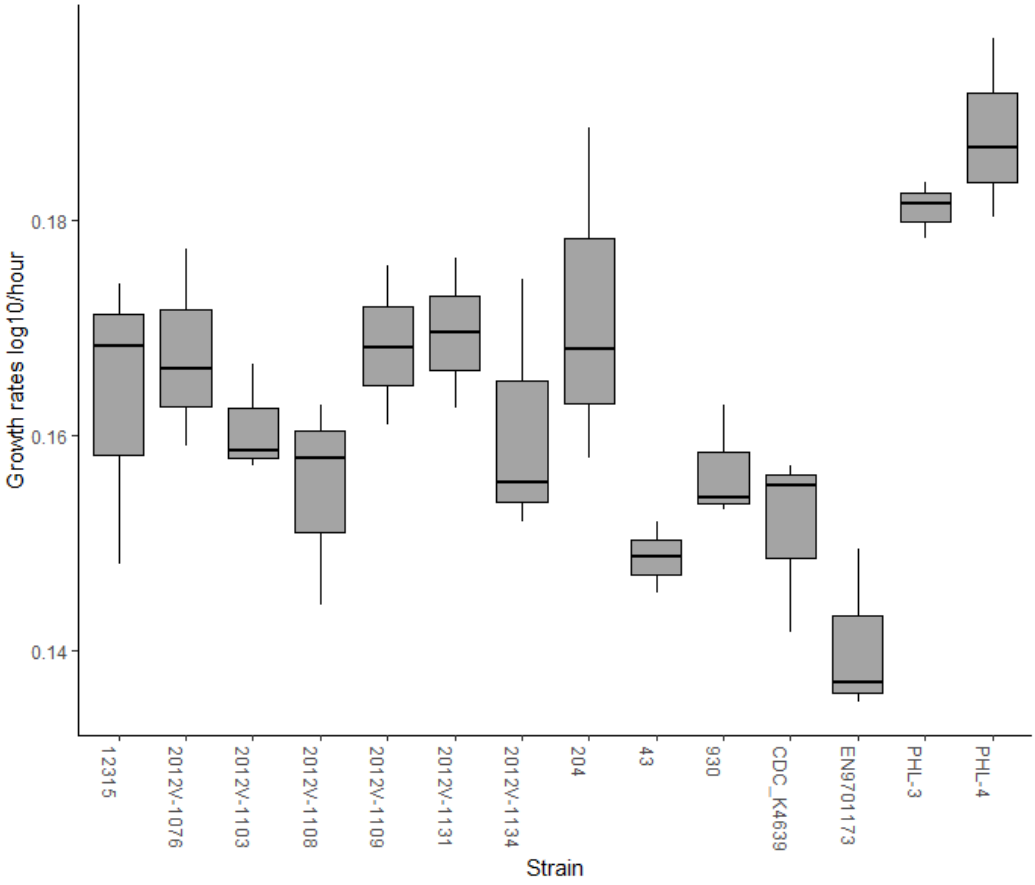

Figure 5. Growth rate per hour of strains grown in broth culture. Growth rates of each strain per hour were calculated using the Growthcurver package in R (85). Calculated growth rates include the median indicated by horizontal black lines within the boxes, and range of values per strain replicates indicated by the vertical lines per box.

4.3. Compared growth rates between broth culture and live oyster injection

Growth rates at 15℃ for ST36 and non-ST36 strains differed when grown in broth versus the rates observed in live oysters. For ST36 strains injected into oysters, the observed growth rate at 15 ℃ was only slightly lower than what was observed in broth culture trials. The difference between live oyster and broth culture growth rates was more pronounced for non-ST36 strains used in this study, where the observed growth rate for the non-ST36 strains injected into oysters was nearly half of the growth rates seen for these strains in broth culture.

Chapter V: Discussion and conclusions

5.1. Discussion

The main purpose of this study was to investigate growth rate differences exhibited by ST36 and non ST36 Vp strains both inside of live oyster tissue as well as in broth cultures, as there is currently no available data on the growth characteristics of these sequence type strains. When Vp were injected into oysters, the ST36 strains exhibited faster growth (log10/hour) in live oyster tissue than non ST36 at the same temperature, 15°C (0.091 and 0.043, respectively) (Figure 3). Furthermore, the ST36 trial showed faster Vp growth at 15°C compared to the 2005 FDA risk assessment model, the naturally inoculated study on wild harvested *C. gigas* in WA, USA, and two other previously published studies on naturally inoculated *C. virginica* oysters in MD, USA (Figure 3). This phenomenon was only noted at 15°C, with other trials showing faster growth at other temperatures compared to the ST36 injection trial.

Interestingly, the ST36 trial indicated a faster growth rate at 15°C than at 20°C. Although the ST36 strains exhibited faster growth than non-ST36 strains, it is currently unknown if this would result in increased Vibrio related illnesses when compared to FDA seafood safety model predictions due to uncertainty of infective doses. Some pathogenic Vp strains, including ST36 strains, are suspected of lower infective doses (87).

Inoculation of oysters with specific strains by natural uptake was a method that was considered for this study in lieu of injection. There have been successful studies done on Vibrio growth in oysters using this method (88-90). However, this method of tank inoculation was tried for this study resulting in significant variability between replicate groups of pooled oysters and individual oysters during trials (unpublished data). This type of variability is common, as it's known that Vibrio concentrations in oysters can vary greatly oyster to oyster. This is also evident when comparing the two studies done on naturally inoculated oysters by Parveen et al. in 2005 and 2006; the same methods were used for both of these years, but as shown in figure 2, the growth rates observed varied at the same temperatures between years (86). For this reason, methods similar to those used by Kaysner et al. were employed to ensure less variability between replicates and to provide a more precise growth rate (91).

The results of the broth culture study indicate that strain growth rates per hour are slightly variable but generally consistent and do not appear to be related to a strain being ST36, at least for the strains used in this study (Figure 5). While all strains used in the oyster injection studies were isolated in WA, USA, the strains tested in broth

culture came from varying states including WA, MO, MA, CA, and NY (Table 1). Because this work was completed on oysters harvested from the Mid-Atlantic, a next step in investigating growth rate differences at lower temperatures is to isolate and sequence type strains from *C. virginica* tissue in the Mid-Atlantic region. This would allow for a more comprehensive library of strains and growth characteristics that could be further compared to other locations and sequence types, including further comparison to more ST36 strains, especially clinical and environmental ST36 isolates from the Mid-Atlantic region.

When considering the year that each strain used in the broth study was isolated, most were isolated between years 2006-2012, with one strain isolated prior to 2000 (strain EN9701173, 1997, Table 1). Throughout all trials of both the broth and live oyster experiments, a trend was noticed where this strain typically showed slower growth than other strains used; there was a significant difference between growth rate between EN9701173 and PHL4 (p value <0.0001). PHL4 was not significantly faster growing than the other strains, but was overall the fastest growing leading to a significant different between these two strains, the fastest and slowest growers. While the EN9701173 strain was included in the strain dose cocktail for the live oyster experiments, this strain was likely outcompeted by the other three strains that showed faster growth at lower temperatures.

Genetic analysis of the Vibrio genus has indicated that many species have gained more genes than they have lost over the course of their evolutionary history. For instance, several species of Vibrio have shown increase in genes related to metabolism, allowing them to be one of the most successful and fastest replicating

marine bacteria (92). Lin et al. (2018) suggests that this evolutionary history is evident of Vibrio's ability to gain and lose genes as needed to remain competitive and inhabit new niches (92). As previously mentioned, a recently discovered genetic diversification event in 1995 led to a divergent population of ST36 strains that invaded the U.S. East Coast (14). The newer ST36 lineage that resides in the East Coast is genetically similar to the old Pacific Northwest lineage, but is still considered distinct. Additionally, investigation of the ST36 clades evolution over its migrations indicates that the strains that now reside in the East Coast show signs of genomic simplification, or smaller genomes, than that of the old Pacific Northwest complex and even those that were isolated from Spain in 2012 (14). There has additionally been some research done on bacteria and gene loss, indicating that some bacteria that have greater gene loss, and therefore smaller genomes, show faster rates of mutation and evolution (93). There are many speculations that can be made as to why the East Coast lineage has shown greater gene loss when vibrio in general have gained more genes over their history. The results of these studies indicate that it is possible that the ST36 East Coast strains have been able to mutate faster with smaller genomes, in which the gene loss could have been advantageous to its survival in a new environment. More research investigating this lineage and genes gained/loss over their migration would be beneficial to understanding these strains that are now endemic to East Coast waters, especially coupling these genetic factors with growth activity.

Strain growth studies have been done by other researchers in the past. Miles et al. was an original study looking at growth in a broth culture using isolates from

patients with gastroenteritis, using the fastest growing strain of four strains available to create a mathematical model predicting growth (94). This study by Miles did not consider pathogenicity or genotype which is almost always incorporated in more recent studies. Additionally, work done by Gooch et al. found that the study done by Miles overestimated growth rates of Vp by four-fold, which is why this study combined both a broth culture study as well as live oyster component (50). Yoon et al. found *tdh*- (non-pathogenic) grew faster than *tdh*-containing "pathogenic" strains in every medium tested, which included broth culture and Korean oyster slurry (95). This study also noted that this phenomenon was less notable as the study temperature increased, indicating that at lower temperatures the non-pathogenic strains typically had faster growth rates (95). Our study found no significance between pathogenic and non-pathogenic in broth when generalized, but the opposite effect in live oysters where the pathogenic ST36 strains had faster growth rates than non-pathogenic, or *tdh*- strains, at the lowest temperature tested (15℃).

Something of importance to note is that when all of the studies referenced in this paper are averaged at each challenge temperature 15-30°C, the mean of the studies falls within the expected growth rates modeled by the 2005 FDA risk assessment (Figure 2). When collectively investigating the various studies including the ST36 and non ST36 injection trials from this study, the FDA model is still performing adequately. This is a positive outlook on the performance of the FDA model and the ability for this model to provide meaningful guidance to the aquaculture industry in terms of Vibrio growth in live oysters. While this model is very useful, there is still apparent variability in Vibrio growth rates in oysters

depending on oyster species and location based on the compilation and comparisons of the experiments referenced in this study. Continuing to investigate Vp growth in live shellfish is a way to further the industry's understanding of vibrio growth tendencies and investigate possible anomalies that stray from the model expectations. For example, the ST36 strains used for the oyster tissue experiment in this study were the strains that were available at the time, but as more strains are isolated and become available, it would be beneficial to investigate growth characteristics of more strains inside of live oyster tissue to further investigate the lower temperature (15°C) phenomenon noted in this study. Additionally, because these pooled ST36 strains showed faster growth in oyster tissue at 15°C, similar research on more ST36 strains is warranted to further understand if this is a result that is indicative of this sequence type in general. This method of investigation in live oysters would also prove useful concerning other sequence types, such as ST631 strains that are emerging as another lineage of interest in North America (75). The FDA risk assessment assumes that growth rates for pathogenic and non-pathogenic strains of Vp are similar. However, this assessment was conducted over 15 years ago and before genetic events that have led to distinct populations of pathogenic strains (20). As strains continue to evolve, more studies are needed to confirm whether or not pathogenic strains have adapted to faster growth at lower temperatures.

Due to the fact that ST36 strains have been presumed to be the collective cause of increased Vp infections in the Northeast United States, it is useful to understand how their growth may differ from other sequence types (74). The main vector of infection by ST36 strains is raw or undercooked seafood, so the results

presented in this study have implications for the aquaculture industry in terms of preventing illness and reiterating the importance of keeping product at a temperature low enough to prevent vibrio growth. Furthermore, ST36 strains are continuing to mutate and cause increasing illness throughout the world (16). Additionally, other sequence types are becoming more prominent and may undergo similar genetic events that could lead to more resilient bacteria that have capabilities of increasing rates of infection. This study indicates a need to continue research on how growth rates differ by strain, and over time, and how strains and sequence types of interest survive and proliferate inside the live oysters.

5.2. Conclusions, significance, and future studies

A major finding of this study is that there may be something that influences Vp growth in oyster tissue that wasn't captured in the broth trial experiments. This is evidenced by the significant growth differences noticed in the cocktail injected into live oysters versus the relatively stable growth rates between sequence types used for this study. Future studies utilizing more of these strains in live oysters will be able to confirm this phenomenon in a more concrete manner.

Some of our hypotheses were supported by this work. Notably, ST36 strains did show faster growth injected into live oysters, although this only happened at 15 and 20° C rather than all temperatures. Environmental strains did show slower growth at lower temperatures when compared to ST36 strains in live oysters and they did fall within the scope of the FDA model. Additionally, natural inoculation studies on *C. gigas* in WA state did show faster growth rates compared to studies done in the

Mid-Atlantic. ST36 strains additionally exceeded the FDA model assumptions, except for at 20° C. Thus, most of the hypotheses were supported, but this study additionally opened up room for further questions regarding growth rates and sequence typing. It is possible that isolation year and location may play a more significant role for survival and growth of Vp. strains than sequence type. More data is needed to investigate that hypothesis, and further studies would greatly benefit the scientific community, regulatory officials, and the aquaculture industry in continuing to provide meaningful science for preventing illness. This study provides further validation for the 2005 FDA risk assessment nearly 20 years later, which is promising data for use of this model in other modeling and management efforts. However, further work with more strains and sequence types should be done to continue validation of the model considering the evolution of Vibrio and an ever-changing climate.

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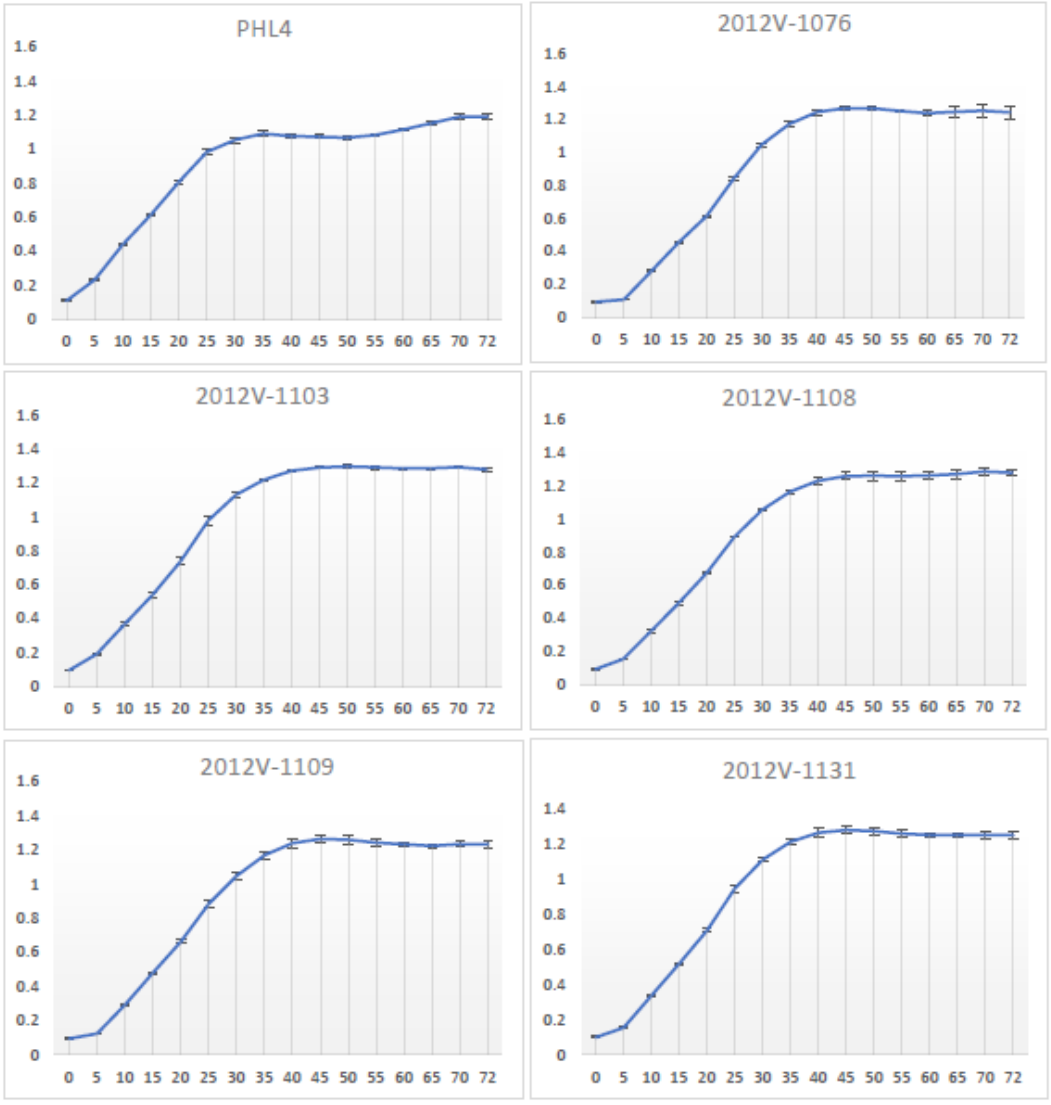
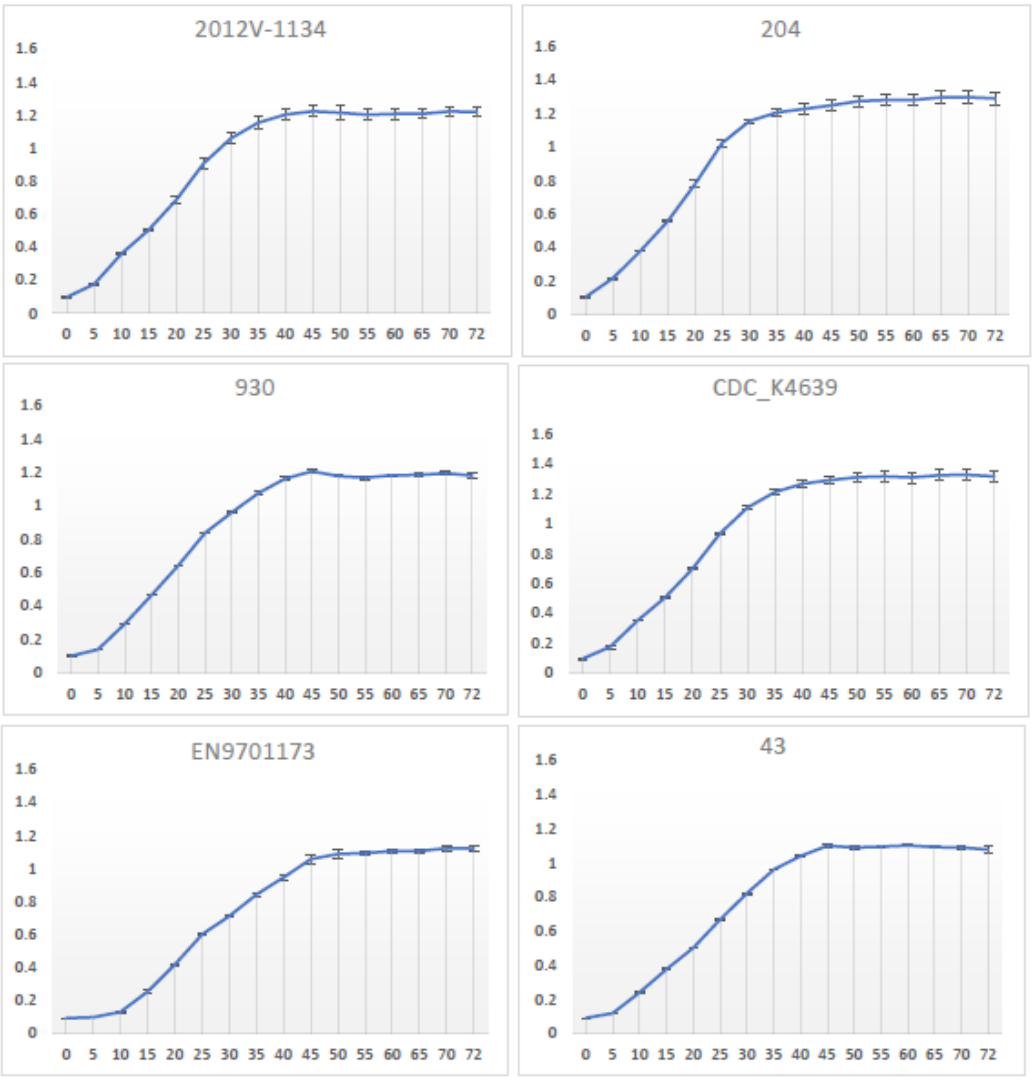
Appendix I. Supplemental figures

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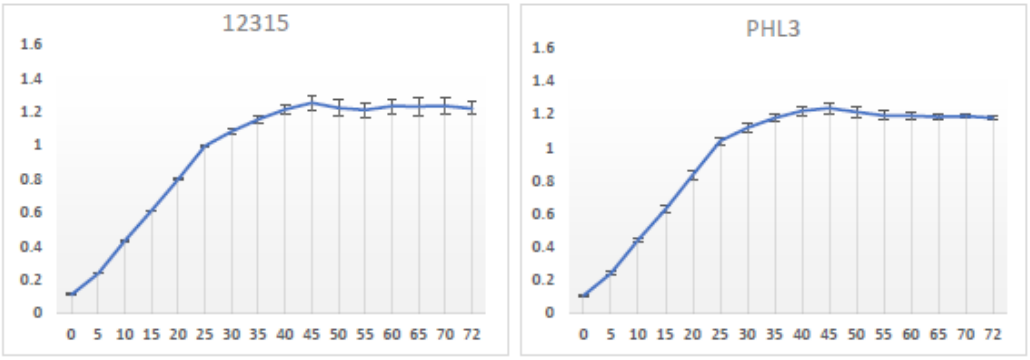

Figure S1. Growth curves of strains in broth culture.

Mean growth curves of all strains in LB (Miller) broth incubated at 15°C and error bars indicating standard error for each measurement. Optical density (600nm) measurements are indicated on the y-axes and time (in hours) indicated on the x-axes.

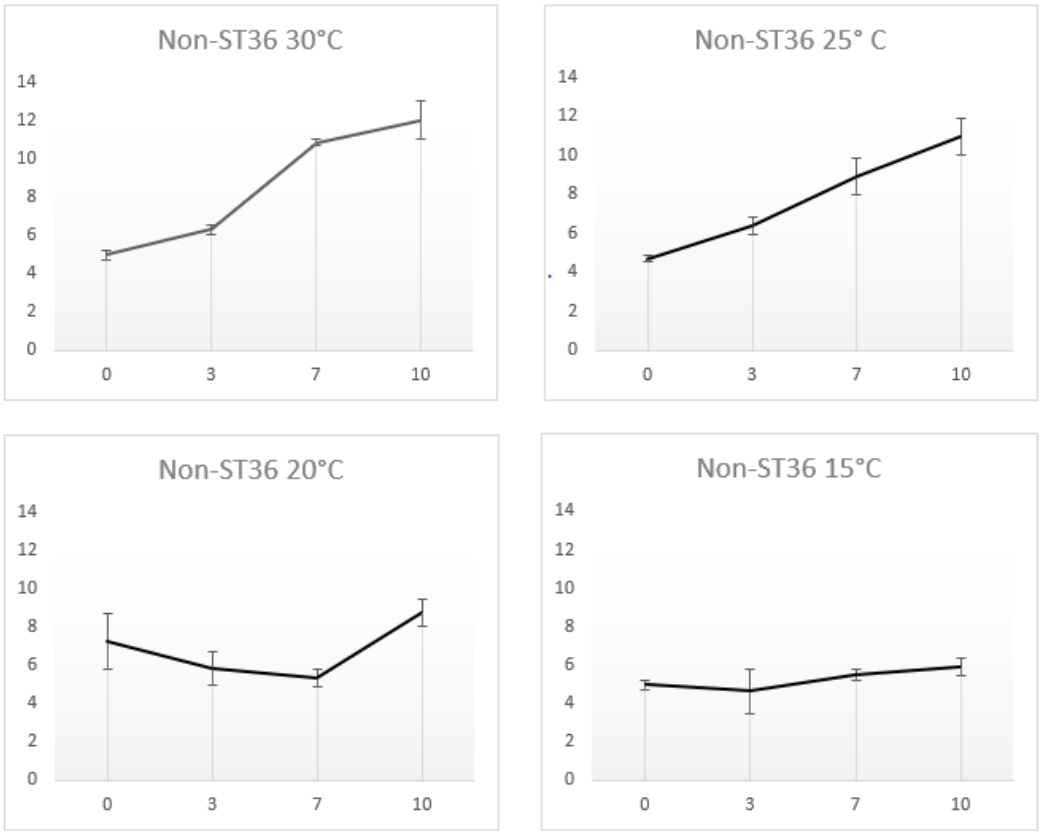

Figure S2. Non-ST36 strains growth in oysters per temperature trial.

MPN/gram calculations per time-point for all temperature trials for non-ST36 strains injected into live oysters. Log (ln) MPN/gram are indicated on the y-axes and time (in hours) of measurement are indicated on the x-axes.

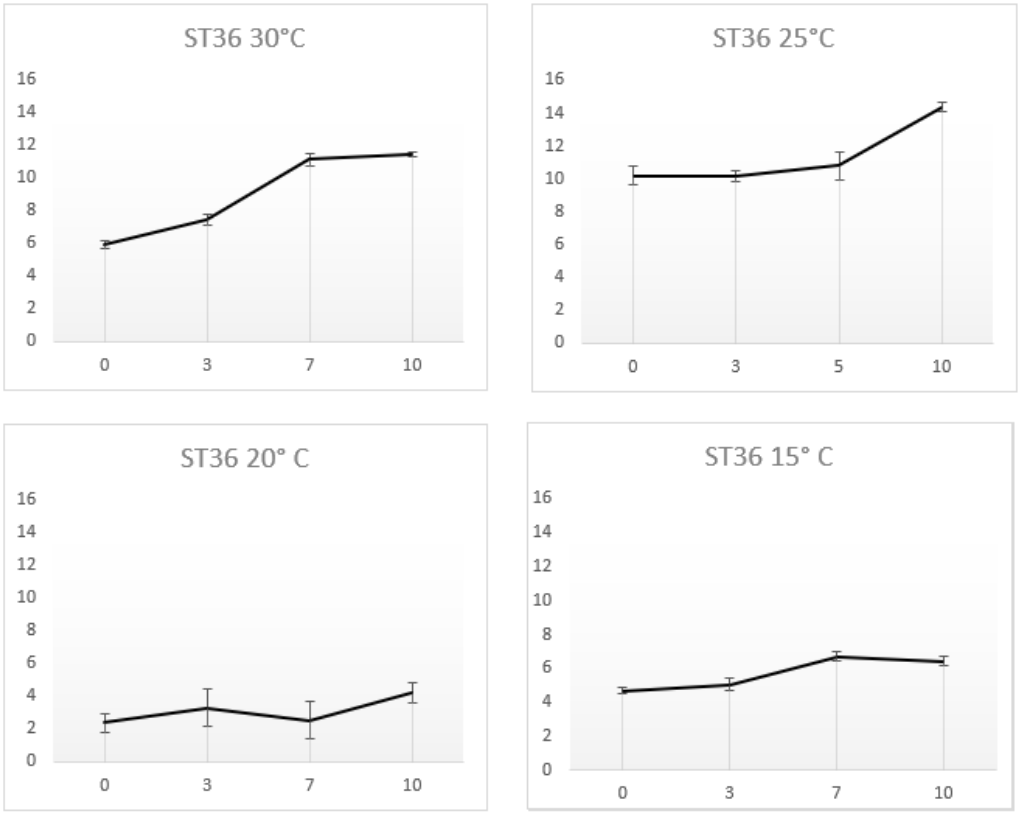

Figure S3. ST36 strains grown in oysters per temperature trial.

MPN/gram calculations per time-point for all temperature trials for ST36 strains injected into live oysters. Log (ln) MPN/gram are indicated on the y-axes and time (in hours) of measurement are indicated on the x-axes.