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Influence of Perkinsus Marinus Infection and Oyster Health on Levels of Human-Pathogenic Vibrios in Oysters

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Influence of *Perkinsus marinus* Infection and Oyster Health on Levels of Human-Pathogenic Vibrios in Oysters

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TABLE OF CONTENTS

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LIST OF TABLES

Table Page

CHAPTER 1: Influence of *Perkinsus marinus* Infection on Levels of Human-Pathogenic Vibrios in Oysters

CHAPTER 2: Influence of Oyster Health on Levels of Human-Pathogenic Vibrios in Oysters

LIST OF FIGURES

Table Page

CHAPTER 1: Influence of *Perkinsus marinus* Infection on Levels of Human-Pathogenic Vibrios in Oysters

ABSTRACT

The eastern oyster *Crassostrea virginica* is an ecologically and commercially important species whose natural populations have been devastated by overharvesting, habitat destruction, and disease, but the rapid growth of oyster aquaculture has shown potential to restore the economic significance of this species. A key threat to the growth and sustainability of oyster aquaculture is the association of human-pathogenic *Vibrio* bacteria with product marketed for raw consumption. Two *Vibrio* species, *Vibrio vulnificus* and *Vibrio parahaemolyticus*, are the causes of the highest rates of seafood consumption-related mortality and gastrointestinal illness, respectively. Identification of the factors influencing *V. vulnificus* and *V. parahaemolyticus* prevalence and intensity in oysters is fundamental to better risk management. Within the oyster, these bacterial species interact with the same tissues as the prevalent oyster parasite, *Perkinsus marinus*, yet little is known about the effect of *P. marinus* infection on bacterial levels. Answering the fundamental question of whether *P. marinus* correlates with *V. vulnificus* and *V. parahaemolyticus* levels in oysters was the focus of this research.

Oysters were deployed in the York River, Gloucester Point, VA, where both *Vibrio* species and *P. marinus* are endemic, and were sampled at five time points when levels of both *P. marinus* and *Vibrio* species were expected to be high in oysters. Abundance of all three organisms and pathogenic strains of *V. parahaemolyticus* were determined in individual oysters using molecular methods to investigate potential correlations between parasite and bacterial abundance. Additionally, the levels of *V. vulnificus* and *V. parahaemolyticus* in relation to histopathology associated with *P. marinus* infection and other conditions were determined. The following year, manipulation of *P. marinus* disease progression, which is slowed by lower salinities and favored by higher salinities, was attempted by deploying oysters at two additional sites of different salinities to gain insight into whether the timing of *P. marinus* infection emergence directly influences *Vibrio* levels.

No correlation was observed between total abundance of *P. marinus* and either *Vibrio* species. Manipulation of *P. marinus* disease progression produced no effect on *P. marinus* emergence, so this yielded no insight into *P. marinus*-*Vibrio* interactions. Histopathological analyses did not reveal any correlations between *P. marinus* ranking, distribution, or associated tissue damage and *Vibrio* species levels. Though few in number, oysters infected by *Haplosporidium nelsoni* were characterized by higher levels of *V. vulnificus*, and oysters of peak gametogenic development had significantly higher levels of pathogenic strains of *V. parahaemolyticus*. The results with regard to *H. nelsoni* and gametogenic state warrant further study. The primary conclusion of this study is that oyster health has little influence on levels of human-pathogenic *Vibrio* species in oysters, inter-host variability in *Vibrio* levels is likely explained by other factors.

Influence of *Perkinsus marinus* Infection and Oyster Health on Levels of Human-Pathogenic Vibrios in Oysters

Global aquaculture is a growing industry with an estimated value of over $155 billion in 2013 (FAO 2015). Currently, the United States is only ranked 14th for global aquaculture production, but the US has seen steady increases in both volume and value since 2009 with oysters having the highest volume for marine shellfish production in the United States (NMFS 2015). Important regional differences exist in shellfish production in the United States but each area has its own history with oyster aquaculture.

Historically, the eastern oyster *Crassostrea virginica* played important roles in the ecology of Atlantic and Gulf Coast estuaries, providing complex habitat, an important element of benthic-pelagic coupling through its filtration, and substantial capacity for carbonate buffering (Mann & Powell 2007, Waldbusser et al. 2013). This species has also provided sustenance through wild fisheries to coastal inhabitants since pre-Columbian times, and has supported harvests that have fueled many coastal economies (Brooks 1891, Kurlansky 2006, Wennersten 1981, revised 2007, Keiner 2010). Overharvesting, habitat destruction, and, recently, diseases caused by protistan parasites have diminished the numbers and economic importance of oysters in the Atlantic Coast region, but the rapid growth of oyster aquaculture has shown the potential to restore the cultural and economic significance of this species and revitalize communities that again embrace it (Murray & Hudson 2016). This would support the continual growth of seafood production in the United States, but a key threat to the growth and sustainability of oyster aquaculture, both in the United States and worldwide, is the association of human-

pathogenic *Vibrio* bacteria with product marketed for raw consumption during the summer.

Pathogenic *Vibrio* Species Associated with Oysters

The genus *Vibrio* contains gram-negative rod-shaped bacteria that are usually motile, mesophilic, and chemoorganotrophic. They are typically 1 μm in width and 2-3 μm in length and are fermentative facultative anaerobes possessing two chromosomes (Thompson et al. 2006). The broader *Vibrionaceae* family contains a wide range of organisms, from the bioluminescent and mutualistic *V. fischeri* to the causative agent of cholera, *V. cholerae*, and their environmental range extends from freshwater to the deep sea (Thompson et al. 2006). Vibrios are among the most abundant culturable bacteria from the marine environment. They are important in degrading organic matter and linking dissolved organic carbon to higher trophic levels (Grossart et al. 2005, Turner et al. 2009). Many *Vibrio* species are part of the normal and beneficial biotic flora of aquatic animals, but some are major pathogens of a wide range of species like corals, molluscs, crustaceans, and fish, and there are twelve species of clinical significance to humans, including *V. vulnificus* and *V. parahaemolyticus* (Thompson et al. 2006).

For shellfish seafood safety, *V. vulnificus* and *V. parahaemolyticus* are the two major human pathogens of significant concern. In 2013, the American Medical Association reported the highest incidence of *Vibrio* infections to date in the United

States with a 32% increase in overall incidence, clearly demonstrating the increasing importance of these pathogens (JAMA 2014). *Vibrio* species have long been known to be associated with marine plankton, particularly zooplankton, and recently it has been suggested that plankton might serve as seasonal reservoirs (Turner et al. 2009). *Vibrio* species are seasonally influenced by temperature and salinity, but other factors like dissolved oxygen, dissolved organic carbon, pH, and turbidity may also affect the distribution of this genus (as reviewed in Thompson et al. 2006). Abundances of both *V. vulnificus* and *V. parahaemolyticus* and their relationships to temperature and salinity have been used to predict the bacterial load of these species in oysters under different environmental conditions (Motes et al. 1998, FDA 2005). Both of these bacteria occur naturally in estuarine and coastal waters and have also been isolated worldwide from beach sands (Whitman et al. 2014). These bacteria are concentrated within oysters because of the animals' filter feeding, but because *Vibrio* cells fall below the optimum size that oysters select in feeding, it is likely that association of vibrios with marine aggregates is a key to their uptake by oysters (Froelich et al. 2013, Froelich and Oliver 2013b). Of great relevance to the oyster industry is the fact that both bacteria can proliferate in oysters that have been harvested when temperatures are warm and refrigeration inadequate (Cook 1994, Cook et al. 1989).

V. vulnificus causes a number of cases of disease annually in persons with compromised immune systems and is the leading cause of seafood-associated mortality, with a 50% fatality rate produced by systemic infection and septicemia (Jones and Oliver 2009, Oliver 2006). This bacterium is responsible for over 95% of all seafood-related

deaths in the United States (Thompson et al. 2006). *V. vulnificus* primarily affects individuals with underlying chronic diseases, such as those related to alcohol abuse or infections leading to liver damage. Susceptibility is believed to be related to elevated levels of serum iron. Other risk factors associated with *V. vulnificus* infections include diabetes, low stomach acidity, cancer, HIV infection, renal and immune function abnormalities, and high-dose corticosteroid treatment (Thompson et al. 2006). Common signs of *V. vulnificus* infection include fever, nausea, and hypotension (Hlady and Klontz 1996). *V. vulnificus* can also infect via wounds, even in individuals without predisposing conditions (Thompson et al. 2006). *V. vulnificus*-related disease displays a distinct seasonality with increased risks of infection occurring from May-October due to warming temperatures (Thompson et al. 2006). Of great relevance to assessing public risk is the high variability of *V. vulnificus* levels found in oysters taken from the same location (Sokolova et al. 2005, Froelich and Oliver 2013b), the explanation for which has not been determined.

V. parahaemolyticus includes non-virulent and virulent strains with the virulent strains typically expressing thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH), coded for by the *tdh* and/or *trh* genes, respectively (Nishibuchi and Kaper 1995). Virulence in *V. parahaemolyticus* was first associated with the hemolytic abilities of some strains of the bacteria *in vitro* (Miyamoto et al. 1969), which was then linked to the possession of the *tdh* or *trh* gene (Nishibuchi and Kaper 1985, Nishibuchi et al. 1989). Environmental samples typically have a low prevalence of *tdh*- or *trh*-positive strains, but clinical samples display a much higher prevalence (Nishibuchi and Kaper

1995, Osawa et al. 1996, DePaola et al. 2000). Detection of *V. parahaemolyticus* and strains carrying the *tdh* or *trh* gene can now be accomplished routinely using a multiplex real-time PCR assay (Nordstrom et al. 2007). In *V. parahaemolyticus*, TDH and TRH are enterotoxic, cytotoxic, and hemolytic (Ljungh and Wadstrom 1982, Tang et al. 1997, Thompson et al. 2006). *V. parahaemolyticus* that can produce TDH is mostly known for causing gastroenteritis, and in the United States it is recognized as the leading cause of gastroenteritis associated with seafood consumption (Daniels et al. 2000, Scallan et al. 2011, Venkateswarlu and Nagaraj 2013). Since 1996, *V. parahaemolyticus* has been associated with wider, pandemic outbreaks, reinforcing health concerns associated with this bacterium (Chowdhury et al. 2000, Nair et al. 2007). Signs of *V. parahaemolyticus* infection include watery diarrhea, abdominal cramps, nausea, vomiting, headache, and low-grade fever.

The threats to human health and, therefore, the economic well-being of the oyster aquaculture industry and the communities dependent on it make management of *Vibrio* species an urgent priority. Identification of the factors influencing *V. vulnificus* and *V. parahaemolyticus* prevalence and intensity in oysters is fundamental to better management of the risks associated with these bacteria, especially considering there is evidence that *C. virginica* can react differently to *Vibrio* bacteria with a specificity down to the species level (Tamplin and Fisher 1989). Although advances have been made on this front (Thompson et al. 2006), gaps remain, particularly with respect to the ecology of pathogenic strains and their interactions with oyster health (FAO/WHO 2011).

Oyster Parasites

Oyster parasites are a major concern for the aquaculture industry, especially in areas like the Chesapeake Bay. Two particular parasitic diseases have been a major influence on oyster populations in the Chesapeake Bay since the 1950s. The first recorded disease to have a catastrophic impact on oyster populations was *Haplosporidium nelsoni*, known originally (and still known colloquially) as MSX, for "multinucleate sphere X". *Haplosporidium nelsoni* was initially observed in the Delaware Bay in 1957, where it caused oyster mortalities exceeding 50% (Haskin et al. 1966). It emerged in Chesapeake Bay in 1959, again leading to significant oyster mortalities (Andrews 1962). Recent studies suggest that the impact of *H. nelsoni* on the oyster populations is now waning due to increased resistance in the oysters (Carnegie and Burreson 2011). The second major oyster disease, colloquially referred to as "dermo", is caused by the protozoan *Perkinsus marinus*. While present in Chesapeake Bay oyster populations for at least half a decade preceding the emergence of *H. nelsoni* (Hewatt and Andrews 1954), its activity greatly intensified in the 1980s (Burreson and Andrews 1988), and unlike *H. nelsoni* it has continued to be a highly prevalent and pervasive disease (Burreson and Ragone Calvo 1996, Carnegie and Burreson 2012).

Currently, *P. marinus* is the dominant oyster pathogen of the East and Gulf Coasts of the US and infects nearly all oysters of market size in Virginia waters (Burreson and Ragone Calvo 1996, Carnegie and Burreson 2009, Carnegie 2013). While mortality rates

due to *P. marinus* are now probably less than 30% in most years (Carnegie, unpublished data), rates of at least moderate dermo disease can reach 50% or more (Burreson and Andrews 1988). Mortalities due to dermo are typically observed in late August through September (Burreson and Ragone Calvo 1996). Because oysters have no adaptive immunity, selective breeding has become a primary means for combating this disease in oyster industries (Ragone Calvo et al. 2003, Frank-Lawale et al. 2014).

P. marinus displays four cell forms during its life cycle. Trophozoites are uninucleate cells of 2-10 μm with eccentric nuclei, prominent nucleoli, and a single large vacuole displacing the nucleus to cell periphery (Villalba et al. 2004). This form includes the feeding stage found inside the host tissues. Trophozoites display vegetative proliferation via schizogony, with the multinucleate schizont being a second *P. marinus* cell form. The schizont is similar in size to a mature trophozoite, and can yield up to 32 daughter cells that form a "rosette-like" arrangement before separating (Villalba et al. 2004). Trophozoites can also develop into hypnospores (prezoosporangia) which are enlarged, thick-walled spherical cells. Hypnospores are the forms that develop when *P. marinus* cells are incubated in fluid thioglycollate medium (FTM), but they have also been observed in moribund hosts (Ray 1952). The hypnospore stage is tolerant of unfavorable environmental conditions. The final known form of *P. marinus* is the biflagellate zoospore stage. Zoospores tend to be ellipsoidal in shape and are released in large numbers via a discharge tube from the hypnospore (Villalba et al. 2004). All known life stages of *Perkinsus* spp. are infective, although the relative importance of each stage for transmission in natural systems is not well understood (Audemard et al. 2006).

Environmental influences like salinity and temperature are key factors in disease transmission and development with increased temperature and salinity usually increasing disease related mortalities (Chu and Volety 1997, Soudant et al. 2013). Transmission of the parasite is believed to be primarily through release from dead and decaying tissues of infected oysters into the water column, without an intermediate host, although the feces of infected oysters has also been shown to play a role in transmission and the benthos also may serve as a reservoir (Bushek et al. 2002, Villalba et al. 2004, Park et al. 2010). The ecology of the parasite outside the host is almost completely unknown.

P. marinus initially infects the gut, gill, labial palps or mantle epithelium of the oyster and then somehow penetrates the epithelium to colonize new organs, but the portal of entry is not clear (Villalba et al. 2004, Carnegie and Burreson 2012). Once inside the host, *P. marinus* causes significant pathology to stomach and intestinal epithelia of oysters, to which the oyster responds with an infiltration of hemocytes (Anderson et al. 1996). The host hemocytes use a galectin (CvGal) to recognize *Perkinsus* spp. trophozoites and then engulf/encapsulate these cells (Tasumi and Vasta 2007). The hemocytes are often unable to kill the parasite and *P. marinus* can proliferate in these cells until they rupture, releasing more parasite cells that are phagocytosed by new hemocytes to continue the cycle. Hemocyte infection allows *P. marinus* to spread throughout the host via the haemolymph (Mackin 1951). Oysters with low intensity infections of *P. marinus* tend not to display any gross disease signs, but more heavily infected individuals can be visibly watery and thin; these signs are also associated with other causes besides dermo disease, however. The occlusion of haemolymph vessels and

the impediment of circulation by accumulated hemocytes, parasite cells, and debris is believed to be the ultimate cause of death in oysters (Mackin 1951). Mortalities to the host population are an important ecological and economical effect of this pathogen, but sub-lethal infections can also have impacts on the health of the host. Evidence suggests that *Perkinsus* spp. infections favor the development of other opportunistic infections (Montes et al. 2001) and can have detrimental effects on gametogenesis and reproduction in oysters, and effects on *C. virginica* reproduction have been documented to occur when infections reach moderate intensity (Dittman et al. 2001).

Potential Intersection of Parasite and Bacteria

Within the oyster, both *Vibrio* species and *P. marinus* are found in or around the gut epithelium and hemocytes of the oyster (Harris-Young et al. 1993, Canesi et al. 2002, Carnegie and Burreson 2012, Froelich and Oliver 2013, Froelich and Oliver 2013b). This overlap in tropism could make interactions possible between the parasite and the bacteria within the oyster, perhaps through secretions of one or the other or through competition for resources or space. Little is known, however, about this potential interaction between *P. marinus* and *Vibrio* species within the *C. virginica* host system. Does the presence of *P. marinus* promote increased or decreased levels of *Vibrio* species? Do individual oyster responses modulate co-occurring species dynamics? The overarching goal of this research was to determine whether a correlation exists between *P. marinus* and *Vibrio*

species abundance during co-infection within the oyster. One motivation for this study was that this issue remains completely unresolved. Answering the fundamental question of whether *P. marinus* influences *V. vulnificus* and *V. parahaemolyticus* levels in oysters was the primary focus of this research since associations of *Vibrio* species with oysters present a global human health risk and a serious challenge to the oyster aquaculture industry. Elucidating this relationship could lead to management strategies for minimizing *Vibrio* species levels and thus risk to consumers and industry. These strategies could potentially influence oyster breeding approaches or inform human health regulators of other important factors to consider for risk assessment.

In this study, levels of *V. vulnificus* and *V. parahaemolyticus* in relation to *P. marinus* infection intensity were determined using molecular diagnostic tools. Manipulation of *P. marinus* disease progression timing was attempted to gain more insight into interactions between parasite and bacteria. Additionally, the levels of *V. vulnificus* and *V. parahaemolyticus* in relation to histopathological disruption of oyster tissue associated with *P. marinus* infection and any other pathological conditions present were determined. Histology provides important perspective on the distribution and pathological effects within host tissues, allowing for assessment of the actual disease state of the oyster and providing information on individual oyster host response.

Influence of *Perkinsus marinus* Infection on Levels of Human-Pathogenic Vibrios in Oysters

1. Introduction

Human-pathogenic *Vibrio* bacteria are increasingly a concern in oyster aquaculture but the factors governing *Vibrio* levels in individual oysters are not well understood. The within-oyster dynamics of *Vibrio* bacteria and potential interactions with protistan parasites like *P. marinus* have received little attention. It is conceivable that there could be interactions between *Vibrio* species and *P. marinus*, given that they co-occur within the oyster gut and in circulating hemocytes (Harris-Young et al. 1993, Canesi et al. 2002, Carnegie and Burreson 2012, Froelich and Oliver 2013, Froelich and Oliver 2013b). Earlier studies have addressed the question of *Perkinsus*-*Vibrio* interactions using different methods and with varying results. Sokolova et al. (2005) found that infection by *P. marinus* did not seem to predict *V. vulnificus* levels; their study, however, used a plating method that had only an 82% accuracy in *V. vulnificus* detection. In contrast, *in vitro* studies suggested oyster hemocytes exposed to *P. marinus* secretions displayed reduced vibriocidal activities against both *Vibrio* species (Tall et al. 1999, La Peyre and Volety 1999) indicating the potential for a positive relationship since more *P. marinus* secretions could conceivably lead to higher levels of *Vibrio* species within an oyster. A recent pilot study by Carnegie et al. (2013) found evidence for a negative relationship between *P. marinus* and the bacteria. Specifically, lower levels of *P. marinus* abundance detected in association with oysters using polymerase chain reaction (PCR) assays corresponded to higher levels of *V. vulnificus* and, to a lesser extent, *V.*

parahaemolyticus. The relationship of parasite to bacteria in the oyster therefore is unresolved and further study is necessary to determine what relationship, if any, may be present.

The goal of this part of my project was to determine whether there is a correlation between *P. marinus* parasitism and *Vibrio* species levels in oysters. To do this, I have chosen to rely on oysters naturally exposed to both *P. marinus* and *Vibrio* species in the York River, Virginia. Using this natural system to determine whether there might be correlations between *P. marinus* and *Vibrio* species levels may not allow the experimental control that laboratory challenges of pathogen-free oysters with *in vitro*-cultured parasite and bacterial cells would, but it is more biologically relevant for two reasons. First, oysters large enough to be marketed would be in at least their second year of either culture or growth on a natural reef. While these oysters likely would harbor parasites that recently had been acquired, we also recognize that infections overwintered from the previous year would likely be "critical" (Burreson and Ragone Calvo 1996) to levels of dermo disease and associated mortality. The integration of infections based on both earlier and more recent parasite exposure could not be reproduced easily in a laboratory setting. Second, because individual *Vibrio* cells are smaller than the size that would be selected by oysters in their feeding (generally greater than 6 μm, Newell and Langdon 1996), they would not be retained efficiently. Association with aggregates in natural systems, on the other hand, increases bacterial uptake rates (Froelich et al. 2013, Froelich and Oliver 2013b) and is probably important in influencing the degree to which oysters are exposed to *Vibrio* species This too could not be reproduced easily in the lab.

Species-specific molecular diagnostic assays allow levels of *P. marinus*, *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus*, as assessed by the presence of the *tdh* and/or *trh* genes, to be quantified. For *P. marinus*, Ray's fluid thiogycollate method (RFTM, Ray 1952) has long been a standard diagnostic tool. Whole-body-burden RFTM (Fisher and Oliver 2006) is regarded as the gold standard diagnostic method for *P. marinus*. Tissue RFTM assays using gill, mantle, and rectum are somewhat less sensitive but still regarded as superior in sensitivity to histopathology, which is considered to have low sensitivity when infections are light (OIE 2015). Neither RFTM nor histopathology is species-specific for *P. marinus*, but they are able to provide species-specific perspective on *P. marinus* infection along the Atlantic and Gulf Coasts of the US because no other *Perkinsus* parasites infect oysters in this region. PCR assays for *P. marinus* (e.g., Audemard et al. 2004) provide more genuine specificity and sensitivity that should be least comparable to RFTM assays. A recently developed quantitative PCR (qPCR) assay (Gauthier et al. 2006) was assessed and performed well in our laboratory in comparison with RFTM, histology, and a conventional PCR assay developed by Audemard et al. (2004). That PCR is specific for the *P. marinus* internal transcribed spacer (ITS) region of the ribosomal DNA and uses standards of known parasite cell density to quantify infection level in unknown tissue samples using templates/μL as a final output for standards and unknown samples. This qPCR assay was used in this chapter, although it did not quantify *P. marinus* cell density *per se* but rather template copies of a gene sequence.

For *Vibrio* species, methods of bacterial identification and enumeration typically start with an enrichment culture to select for specific *Vibrio* species from samples

(Gomez-Gil and Roque 2006). Alkaline peptone water (APW) inhibits the growth of many other bacteria, favoring *Vibrio* species, and is the United States Food and Drug Administration (FDA) recommended medium for enrichment (DePaola and Kaysner 2004), which is why it was the medium selected for this project. Following enrichment, *Vibrio* species enumeration can be accomplished through several methods including plate counts, flow cytometry, fluorescent *in situ* hybridization (FISH), colony hybridization, and qPCR (reviewed in Gomez-Gil and Roque 2006). For this project, qPCR assays were used for bacterial enumeration following serial triplicate enrichment because of the advantages of speed and specificity of these methods and because it is in agreement with FDA regulations. A qPCR assay designed by Campbell and Wright (2003) was used for *V. vulnificus* and qPCR assays designed by Nordstrom et al. (2007) were used for total *V. parahaemolyticus* and for strains containing the *tdh* and/or *trh* gene. Unlike the *P. marinus* assay, final enumeration of each bacterial species is determined through the use of FDA-recommended most-probably number (MPN) tables based on positive or negative qPCR results. MPN requires serial triplicate enrichment and estimates population density of viable microorganisms in a sample using probability. It is particularly useful in samples were the expected density is low. These FDA-recommended methods were mainly used because this would allow interested regulators to better interpret results and provided a rapid and reliable method for enumeration. However, the use of individual oysters for determination of *Vibrio* levels is not standard for the FDA when *Vibrio* detection is directed toward assessment of human health risks. In such cases, samples of 10 to 12 oysters are routinely pooled to represent a *serving* through which a consumer may typically be exposed (FDA 2005). Evaluating individual

oysters would be expected to provide much clearer perspective on correlations between *P. marinus* and *Vibrio* levels because it allows for resolution of inter-oyster variability in levels of both.

The comparison of qPCR-derived estimates of *P. marinus* and *Vibrio* species in samples of individual oysters collected in the warmest part of the year, when both parasite and bacterial tissue abundances should be peaking, represents the core of my thesis and the primary focus of this chapter. The goal of my research was to try to clarify the relationship between *P. marinus* and *Vibrio* species during co-infection. Since the approach used in this study mirrored the Carnegie et al. (2013) pilot study, I hypothesized that *P. marinus* abundance would negatively correlate with both *V. vulnificus* and *V. parahaemolyticus* likely due to host responses to the parasite.

In addition, while a more mechanistic understanding of *P. marinus*-*Vibrio*-oyster interactions is beyond the scope of my study, manipulating salinity could provide a way to obtain a more nuanced understanding of within host *P. marinus*-*Vibrio* dynamics. Salinity is a key environmental factor for disease progression of *P. marinus*, with higher salinities favoring more intense disease development (Chu and Volety 1997, Soudant et al. 2013). Thus, changing salinity regimes could provide a method to obtaining insight into a second intriguing question in this potential relationship between the parasite and bacteria. What role might disease progression play in this interaction? Does the timing of infection emergence of *P. marinus* in oysters affect the relationship it may have with the levels of human-pathogenic vibrios? Seasonal highs of *V. vulnificus* and *V. parahaemolyticus* tend to occur slightly before or during the seasonal highs of *P. marinus* in oysters (Villalba et al. 2004, Thompson et al. 2006, Audemard unpublished). Delaying

or accelerating the arrival of peak *P. marinus* intensities in oysters through salinity manipulation could conceivably relax or intensify any priority effect that may be associated with emergence of the seasonal *P. marinus* epizootic and create an environment where high numbers of parasites are interacting with the bacteria at different times or temperatures. This can be accomplished because of the aforementioned fact that *P. marinus* is influenced by salinity with lower salinities delaying disease progression and higher salinities favoring more rapid development of disease. Changing the timing of the intensification of *P. marinus*-related disease could elucidate if timing is important in the potential interaction between parasite and bacteria. Therefore, the secondary goal of this chapter was to manipulate *P. marinus* disease progression to more thoroughly analyze *P. marinus* disease timing and its interaction with *V. vulnificus* and *V. parahaemolyticus*. I hypothesized that oysters experiencing higher salinities would have larger numbers of *P. marinus* earlier in the season and that this would negatively correlate with *Vibrio* species levels.

2. Materials and Methods

2.1. Year One

2.1.1. Deployments and Field Sampling

Two-year-old diploid *C. virginica* CROSBreed (XB) oysters were obtained from the VIMS Aquaculture Genetics and Breeding Technology Center (ABC). These had been maintained continually in intertidal rack-and-bag systems at Gloucester Point on the York River since mid-August 2012, where *P. marinus* is enzootic (Burreson and Ragone Calvo 1996) and both *Vibrio* species are present (C. Audemard and K. Reece, unpublished). Sampling of forty oysters from the York River was conducted biweekly from early August to early October 2014. These were performed in two batches of twenty oysters at each sampling point, spaced 1-3 days apart, because of the limited number of individual oysters that could be processed microbiologically (see below) at one time. Sampling dates were 8/4 and 8/5; 8/17 and 8/19; 9/2 and 9/5; 9/15 and 9/16; and 9/29 and 10/2. Oysters sampled on 9/5 and 9/16 were five-year-old oysters from the same line continuously maintained in the York River.

2.1.2. Sample Processing

Collected oysters were stored at 10°C prior to processing to prevent changes in bacterial levels, with processing performed each time within 24 h of sampling. Oysters were scrubbed, measured using calipers (shell height, mm), and then shucked with a flame-sterilized oyster knife. The pallial fluid was drained and standard transverse sections (Shaw and Battle 1957) were removed from each oyster for subsequent histological processing (see Chapter 2). Remaining tissues of each oyster were weighed and an equal mass of cold phosphate-buffered saline (PBS) was added to each sample before the tissue was homogenized individually using a Janke & Kunkel Ultra-Turrax TP

18/10 S9 (IKA-Werk, Wilmington, NC) at ~55 rpm for 30 s for subsequent molecular detection and quantification of *P. marinus* and *Vibrio* species.

2.1.3. Quantification of Perkinsus marinus

2.1.3.1. DNA Extractions

DNA extractions were performed on 500 µL of oyster homogenate from each sample using a DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA) following the QIAamp® DNA Mini and Blood Mini Handbook DNA Purification from Tissues protocol, with a final elution in 200 µL of Qiagen Buffer AE. Extracted DNA was quantified using a NanoDrop 2000c (Thermo Scientific, Waltham, MA).

2.1.3.2. Standards

Stocks of oyster DNA in which *P. marinus* was not detected earlier using PCR were normalized to 200 ng/µL with Buffer AE. gBlocks® synthesized as 131-bp double-stranded fragments of *P. marinus* internal transcribed spacer (ITS) region DNA with three base differences from the original *P. marinus*-derived sequence were then diluted in 200 ng of that uninfected oyster DNA to more closely match experimental samples. This fragment was the sequence amplified for *P. marinus* detection as described in section 2.1.3.3. Standards were serially diluted (10^9 through 10^2 copies/µL) using Buffer AE.

2.1.3.3. Quantitative PCR (qPCR)

qPCR for *P. marinus* was performed using primers and probes in a TaqMan® assay developed by Gauthier et al. (2006). Reagents were added to wells in MicroAmp® Fast 96-well reaction plates (Applied Biosystems, Foster City, CA) to produce the following final concentrations in a 10-µL reaction volume: 0.2 mg/mL bovine serum albumin (BSA), TaqMan® Fast Advanced Master Mix (Thermo Scientific, Waltham, MA), forward and reverse PCR primers at 0.9 µM, and TaqMan® probe at 0.25 µM. Cycling was conducted on a 7500 Fast Real-Time PCR machine (Applied Biosystems, Foster City, CA) with an initial denaturation at 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Standards served as the positive control and a negative control of H_2O or Qiagen Buffer AE was run with each qPCR plate. The above *P. marinus* dilution series was run with each plate to create a standard curve of known values and quantify amount of ITS copies/µL in samples. qPCR output of copies/µL was converted to copies/g using the following equation:

Template copies/g = (qPCR output in copies/µL x 200 (µL of elution Buffer) x 2 (PBS 1:2 dilution))/ mass of extracted oyster sample.

2.1.4. Quantification of Vibrio *species*

2.1.4.1. Culturing and Sampling

For *V. vulnificus* and *V. parahaemolyticus*, samples were processed as described in Audemard et al. (2011) by inoculating samples into an alkaline peptone water (APW) most-probable number (MPN) triplicate series following the FDA Bacteriological Analytical Manual (1998) to select for *Vibrio* species and provide triplicate samples necessary for FDA MPN table use. For qPCR quantification, samples were taken from the MPN series as described in Audemard et al. (2011), with every triplicate group displaying even a single case of visually obvious bacterial growth among the three tubes retained for subsequent molecular analysis.

2.1.4.2. Quantitative PCR (qPCR) of Vibrio vulnificus

Detection of total *V. vulnificus* was performed in a TaqMan® assay using primers and probes developed by Campbell and Wright (2003). Reagents were added to wells in MicroAmp® Fast 96-well reaction plates at the following final concentrations in a 10-µL reaction volume: 0.4 mg/mL BSA, TaqMan® Fast Advanced Master Mix, forward and reverse primers at 0.9 µM, and TaqMan® probe at 0.25 µM. qPCR was conducted on a QuantStudio™ 6 Flex Real-Time PCR machine (ThermoFisher Scientific, Waltham, MA) with an initial denaturation at 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Each qPCR run included a positive control taken from previously determined positive environmental samples and a negative control for which H_2O was added instead of template. MPN/g values were calculated using qPCR-determined

positive and negative samples from each oyster's enrichment series and approved MPN tables (USFDA 2008).

2.1.4.3. Quantitative PCR (qPCR) of Total Vibrio parahaemolyticus

Detection of total *V. parahaemolyticus* was performed using part of the multiplex qPCR TaqMan® assay related to total *V. parahaemolyticus* quantification with primers and probes developed by Nordstrom et al. (2007). Reagents were added to wells in MicroAmp® Fast 96-well reaction plates at the following final concentrations in a 10-µL reaction volume: 0.4 mg/ml BSA, TaqMan® Fast Advanced Master Mix, forward and reverse *tlh* gene primers at 0.2 µM, forward and reverse IAC primers at 0.08 µM, *tlh* gene probe at 0.15 µM, and IAC probe at 0.15 µM. qPCR was conducted on a 7500 Fast Real-Time PCR machine following cycling conditions from Nordstrom et al. (2007) except for an initial denaturation at 95°C for 20 s. Each real-time PCR run included a positive control taken from previously determined positive environmental samples and a negative control for which H_2O was added instead of template. MPN/g values were again calculated using qPCR-determined positive and negative samples from each oyster's enrichment series and approved MPN tables (USFDA 2008).

2.1.4.4. Quantitative PCR (qPCR) of Pathogenic Vibrio parahaemolyticus *Strains*

Detection of pathogenic strains possessing the thermostable direct hemolysin (*tdh*) gene and/or the thermostable related hemolysin (*trh*) in *V. parahaemolyticus* was

performed using the multiplex qPCR primers and probes developed by Nordstrom et al. (2007). Reagents were added to wells in MicroAmp® Fast 96-well reaction plates at the following final concentrations in a 10-µL reaction volume: 0.4 mg/mL BSA, TaqMan® Fast Advanced Master Mix, forward and reverse *tdh* gene primers at 0.1 µM, forward and reverse *trh* gene primers at 0.3 µM, forward and reverse IAC primers at 0.03 µM, *tdh* gene probe at 0.08 µM, *trh* gene probe at 0.08 µM, and IAC probe at 0.15 µM. Real-time qPCR was performed either on a 7500 Fast Real-Time PCR machine or QuantStudio™ 6 Flex Real-Time PCR machine following the Nordstrom et al. (2007) cycling conditions except the initial denaturation was 95°C for 20 s. Each real-time PCR run included a positive control taken from previously determined positive environmental samples and a negative control for which H_2O was added instead of template. MPN/g values were again calculated using qPCR-determined positive and negative samples from each oyster's enrichment series and approved MPN tables (USFDA, 2008).

2.2. Year Two

2.2.1. Deployments and Field Sampling

Three groups of two-year-old oysters of the same line as Year One (but maintained in the York River since September 9th, 2013) were deployed in three different sites between April and July 2015. Sites included the York River (YR) at Gloucester Point, Virginia, characterized by salinities around 18-20; the Choptank River (CR) at Horn Point, Maryland, where salinities are typically 6-13; and Burtons Bay (BB) at

Wachapreague, Virginia, where salinities are typically > 30. One group of oysters was continually maintained in the YR for the duration of this experiment. For the CR site oysters were deployed from April 7th – July 2nd and for the BB site oysters were deployed from April 2nd – June 29th. After those ~3 months, oysters from CR and BB were returned to the YR site and maintained in rack-and-bag systems alongside those that had remained in the York River for 5 weeks before subsequent sampling in August 2015. Because salinity is known to influence *P. marinus* disease progression, these deployments of oysters at separate sites were intended to alter the time at which the seasonal *P. marinus* epizootic would reach its peak. Oysters were expected to have low levels of YR-derived *P. marinus* infections when removed from the YR site in April, as *P. marinus* levels in lower Chesapeake Bay are known to reach annual minima at that time of year (Burreson and Ragone Calvo 1996). Deployment to the low-salinity site (CR) was to retard early-season *P. marinus* proliferation and thus delay the arrival of peak *P. marinus* infection intensities. Deployment to the high-salinity site (BB) was intended to remove any restrictions on parasite proliferation that might be imposed even at the York River site by lower Chesapeake Bay salinities, theoretically allowing the parasite to reach peak intensities earlier in the season. Oysters were returned to the YR site for two reasons. One reason was to allow levels of *Vibrio* species in oysters from different sites to equilibrate to those that would be characteristic of the YR, for which there is evidence that 5 weeks is enough time for *Vibrio* species in oysters to respond to changes in surrounding salinities (Audemard et al. 2011). The second reason was to allow the temperature and salinity regime to subsequently influence all oysters from all three locations the same way to better understand the potential *P. marinus*-*Vibrio* species

dynamic and to be able to compare data between years. Fifteen oysters/group were sampled biweekly from mid-August to mid-October. Sampling dates were 8/11 and 8/13; 8/23 and 8/25; 9/7 and 9/9; 9/28 and 9/30; and 10/11 and 10/13.

2.2.2. Sample Processing

Oysters were processed following the procedures outlined in Year One.

2.2.3. Quantification of Perkinsus marinus

Procedures followed for quantification of *P. marinus* infection levels were identical to Year One except that Qiagen Buffer AE replaced H_2O as a negative control for all qPCR assays.

2.2.4. Quantification of Vibrio *species*

Procedures followed for quantification of *Vibrio* species were identical to Year One except that Qiagen Buffer AE replaced H_2O as a negative control for all qPCR assays.

2.3. Statistical Analyses

2.3.1. Significance Testing

Prevalence and abundance ranges in oyster tissues for *P. marinus* and *Vibrio* species, including pathogenic strains, were determined. All remaining statistical analyses were conducted in R version 3.2.3 (2015-12-10, R Core Team 2015) and RStudio version 0.98.493 (RStudio Team 2015). Scatterplots, Pearson's r values, and general linear models were used to investigate correlations between *P. marinus*, *Vibrio* species, and oyster height and mass. To investigate differences in bacterial levels for the extreme ends of the *P. marinus* abundance spectrum sampled, deciles were determined based on *P. marinus* abundance. Values below the first decile (lowest *P. marinus* abundance) were compared with values above the ninth decile (highest *P. marinus* abundance) using a Welch two-sample t-test for both *V. vulnificus* and total *V. parahaemolyticus*.

2.3.2. Linear Models

Data was tested for normality using a Shapiro-Wilk test and homogeneity of variance using a Fligner-Killeen test. Statistical models used were robust for deviations from normality or homogeneity of variance. Environmental data was downloaded from the Virginia Estuarine and Coastal Observing System (VECOS) for the York River at Gloucester Point to obtain continuous salinity and temperature data (http://web2.vims.edu/vecos/). Data was then condensed to daily means. Predictors used were temperature on the day of sampling, temperature the day before sampling, salinity on the day of sampling, salinity the day before sampling, oyster wet mass, oyster shell height, and interactions between temperature and salinity. Response variables were *P.*

marinus levels, *V. vulnificus* levels, and *V. parahaemolyticus* levels. Twenty-five linear models were created to explain the variability of each species in the oyster due to changing environmental conditions (Appendix A). The best model for each species for each data set was picked using Akaike information criterion (AIC). Residuals of these models were plotted to determine whether the remaining variation in *P. marinus*, after controlling for environmental factors, had any trend with regard to the remaining variation of *V. vulnificus* or *V. parahaemolyticus*.

2.3.3. Year Two

In addition to performing the tests described in the section 2.3.1 above, a linear model was created to test for differences in *P. marinus* levels between sites and dates to determine if *P. marinus* disease progression was appreciably altered by deployment to either site CR at Horn Point or site BB at Wachapreague. The response variable was log 10 *P. marinus* and the explanatory variables were site and dates. A boxplot was used to visualize *P. marinus* prevalence among sites. Based on Year One results, which indicated the twenty-five linear models created to explain the variability of each species in the oyster due to changing environmental conditions did not provide any further information, those linear models were not created for Year Two data.

2.3.4. Sampling Times and Year Differences

Differences between dates were analyzed using general linear models with log 10-transformed *P. marinus*, *V. vulnificus*, or *V. parahaemolyticus* levels as the response variables and date, temperature, and salinity as explanatory variables. For visualization, boxplots of *Vibrio* species by year were created. Differences between years for each species and salinity and temperature were analyzed using Welch two sample t-tests.

3. Results

3.1. Year One

3.1.1. Oysters

One hundred eighty-seven oysters were individually processed to quantify both *P. marinus* and the two *Vibrio* species Eleven oysters could not be assessed to a single MPN for *V. vulnificus* because all samples taken from those triplicate serial enrichments were positive, meaning the MPN enumeration could only provide a lower MPN limit. Those eleven samples were excluded from all *V. vulnificus* analyses but not from *V. parahaemolyticus* analyses. Therefore, a total of 176 oysters were statistically analyzed for *V. vulnificus* and 187 were statistically analyzed for *V. parahaemolyticus*. Oyster shell heights and tissue wet weights for each sampling time are presented in Table 1. Older oysters sampled on 9/5 and 9/16 did not have significantly different levels of *P. marinus*,

V. vulnificus, or *V. parahaemolyticus* (using Welch two sample t-tests with a Bonferroni correction) and were included in all analyses.

3.1.2. Prevalences and Range

qPCR results indicated that one hundred eighty oysters were positive for *P. marinus* (prevalence = 96.3%), and all oysters were positive for both *V. vulnificus* and *V. parahaemolyticus*. Among *V. parahaemolyticus*-positive oysters, five were positive for the *tdh* gene and eight were positive for the *trh* gene with one positive for both, for a total pathogenic *V. parahaemolyticus* prevalence of 6.4%. *P. marinus* had the highest abundance in a single oyster and a mean that was several orders of magnitude higher than either bacterial species. The highest abundance of *V. vulnificus* in an oyster was only one order of magnitude higher than *V. parahaemolyticus*. Ranges, means, and standard errors of oyster parasite and bacterial levels are presented in Table 2. Means and standard errors for each species by sampling date are presented in Table 3.

3.1.3. Distributions

Because of high variability, data was log 10 transformed and plotted. Hereafter, all analyses were performed on log 10-transformed data except pathogenic strain data. Both regression analyses of *P. marinus* versus *V. vulnificus* and *V. parahaemolyticus* suggested no correlations (p-values = 0.60 and 0.58, respectively) (Figs. 1-2). Pathogenic strains of *V. parahaemolyticus* also showed no trend with regard to *P. marinus*. A

regression analyses of *V. vulnificus* versus *V. parahaemolyticus* (Fig. 3) obtained a Pearson's r value of 0.55 indicating a positive correlation between bacteria (p-value = 1.33e-15). *V. vulnificus* and *V. parahaemolyticus* data were also plotted versus shell height and the soft tissue mass of each oyster with no significant correlations (all p-values > 0.05). *P. marinus* had no significant correlation with shell height (p-value = 0.33), but did have a significant Pearson's r value of -0.23 with oyster mass (Fig. 4) indicating a slight negative correlation (p-value = 0.0022).

3.1.4. Comparing Deciles

Oysters grouped in deciles (n = 18-19 per decile) based on *P. marinus* values showed no significant differences in means for either *Vibrio* species (p = 0.05) using a Welch two sample t-test. The trend was that oysters in the lowest *P. marinus* decile had lower means of *Vibrio* species than oysters in the highest decile (4000 MPN/g vs 16000 MPN/g in *V. parahaemolyticus,* p = 0.06; 15000 MPN/g versus 160000 MPN/g in *V. vulnificus,* p = 0.09).

3.1.5. Models

3.1.5.1. Perkinsus marinus

Two separate *P. marinus* models were created, one to fit the smaller *V. vulnificus* data set and one to fit the larger *V. parahaemolyticus* data set. The best fit model to

explain variation in *P. marinus* using the smaller *V. vulnificus* data set based on environmental data used salinity, oyster mass, and shell height. The formula for the best fit model was: log 10 *P. marinus* = (1.96 * Salinity) + (-0. 297 * Mass) + (0.0505 * Shell Height) -33.89. All predictors were significant (p = 0.05). Salinity and shell height were positive predictors and mass was a negative predictor. Adjusted R-squared = 0.2198 and p-value = 6.244e-10 (Table 4).

The model that best explained variation in *P. marinus* using the larger *V. parahaemolyticus* data set based on environmental data used salinity, oyster mass, and shell height. The formula for the best-fit model was: log 10 *P. marinus* = (1.86 * Salinity) + (-0. 291 * Mass) + (0.0511 * Shell Height) - 32.0. All predictors were significant (p = 0.05). Salinity and shell height were positive predictors and mass was a negative predictor. Adjusted R-squared = 0.211 and p-value = 4.048e-10 (Table 5). Both *P. marinus* models used the same predictors and were very similar.

3.1.5.2. Vibrio vulnificus

The model that best explained *V. vulnificus* abundance based on environmental data used temperature and salinity to explain the variation with regard to *V. vulnificus*. The best-fit model formula was: log 10 *V. vulnificus* = (0.194 * Temperature) + (0.259 * Salinity) - 6.33. Only temperature was significant (p = 0.05) and both were positive predictors. Adjusted R-squared = 0.1653 and p-value = 6.004e-08 (Table 6). Plotted residuals of this model and the *V. vulnificus* data set *P. marinus* model (not shown) revealed no significance (p-value = 0.71) indicating that once environmental factors were

removed there was still no correlation between abundance of *P. marinus* and *V. vulnificus*.

3.1.5.3. Vibrio parahaemolyticus

The model to best explain *V. parahaemolyticus* abundance based on environmental data used only temperature to explain the variation in *V. parahaemolyticus*. The best-fit model formula was: log 10 *V. parahaemolyticus* = (0.179 * Temperature) - 1.23. Temperature was a significant (p = 0.05) positive predictor. Adjusted R-squared = 0.1653 and p-value = 4.635e-09 (Table 7). Plotted residuals of this model and the *V. parahaemolyticus* data set *P. marinus* model (not shown) revealed no significance (p-value = 0.48) indicating that once environmental factors were removed there was still no correlation between *P. marinus* and *V. parahaemolyticus*.

3.2. Year Two

3.2.1. Oysters

Two hundred eighteen oysters were individually processed for quantification of *P. marinus* and both *Vibrio* species. Oyster shell heights and tissue wet weights for each site and sampling time are presented in Tables 8-10. There were significant differences in oyster shell heights and masses across all groups, with YR oysters having the largest shell

heights and oyster masses and CR oysters having the smallest shell heights and oyster masses (Table 11).

3.2.2. Prevalence and Range

Two hundred sixteen oysters were positive for *P. marinus* (prevalence = 99.1%), and all oysters were positive for *V. vulnificus* and *V. parahaemolyticus*. Fifty oysters were positive for the *V. parahaemolyticus tdh* gene and 96 oysters were positive for the *trh* gene with 44 positive for both (total pathogenic prevalence = 46.8%). *P. marinus* had the highest abundance in a single oyster and a mean that was several orders of magnitude higher than those of either bacterial species. The highest abundance of *V. vulnificus* in an oyster was only one magnitude higher than *V. parahaemolyticus*. Abundance ranges, means, and standard errors of *P. marinus* and bacterial levels are presented in Table 12. Means and standard errors for each species by sampling date are presented in Table 13.

3.2.3. Distributions

Because of high variability, data were again log 10 transformed to produce normally distributed data. Plots of *P. marinus* versus *V. vulnificus* and *V. parahaemolyticus* indicated no correlations (p-values = 0.85 and 0.16, respectively) (Figs. 5-6). Pathogenic strains of *V. parahaemolyticus* also showed no trend with regard to *P. marinus*. A regression analysis of *V. vulnificus* versus *V. parahaemolyticus* (Fig. 7) obtained a Pearson's r value of 0.50 indicating a positive correlation between the bacteria

(p-value = 3.78e-15). *V. vulnificus* and *V. parahaemolyticus* data were also plotted versus shell height and soft tissue mass of each oyster. *V. parahaemolyticus* had no significant correlations (p-value = 0.74 and 0.21, respectively). *V. vulnificus* had a significant Pearson's r value of -0.155 (Fig. 8) with oyster shell height indicating a slight negative correlation (p-value = 0.022). *V. vulnificus* also had a significant Pearson's r value of -0.216 (Fig. 9) with oyster mass indicating a slight negative correlation (p-value = 0.00135). *P. marinus* also had no significant correlation with shell height (p-value = 0.73), but it did have a significant Pearson's r value of -0.181 with oyster mass (Fig. 10) indicating a slightly negative correlation (p-value = 0.0073).

3.2.4. Comparing Deciles

Oysters grouped in deciles (n = 21/22 per decile) based on *P. marinus* values showed no significant differences in means for either *Vibrio* species (p = 0.05) using a Welch two sample t-test. The trend was for oysters in the lowest *P. marinus* decile to have a lower mean of *V. parahaemolyticus* than oysters in the highest decile (6000 MPN/g vs 16000 MPN/g, p = 0.41), but the opposite trend was true for *V. vulnificus* (50000 MPN/g vs 7000 MPN/g).

3.2.5. Site Differences

The levels of *P. marinus* were not appreciably altered by pre-assay site treatments. Sites were picked based on salinity differences and oysters were returned to

the YR to equilibrate to local conditions for over five weeks before sampling commenced. An ANOVA table indicates no significances in the state variables (Table 14). This indicates that oysters from different sites did not have any difference in *P. marinus* abundance after they were placed at the same site in the lower York River. Levels of *P. marinus* from different sites and dates are presented graphically in Figure 11.

3.3. Interannual Differences

qPCR-determined prevalence of *P. marinus* was slightly higher in Year Two than in Year One (99.1% versus 96.3%). Pathogenic strains of *V. parahaemolyticus* increased from 6.4% prevalence in Year One to 46.8% in Year Two. Also, the number of individual oysters containing both *tdh* and *trh* pathogenic genes increased from one oyster (0.5%) in Year One to 44 oysters (20.2%) in Year Two. Differences in water temperature throughout the sampling period were statistically significant ($p = 0.0048$, Welch two sample t-test) with temperatures less than half a degree higher through the study period in Year Two. The mean abundance of *V. vulnificus* for Year Two was significantly lower than Year One ($p = 0.027$) but there were was no difference in mean value of total *V. parahaemolyticus* ($p = 0.93$). For visualization, weekly samplings for *V. vulnificus* and *V. parahaemolyticus* for both years are presented (Figs. 12-13).

A linear model (Table 15, Adjusted R-squared = 0.0984, p-value = 3.089e-09) indicated that *P. marinus* was not significantly different between years but was positively correlated with sampling date, salinity, and temperature (Table 16). For *V. vulnificus*, the linear model (Table 17, Adjusted R-squared = 0.1456, p-value = 2.175e-13) also

indicated no significant differences between years but *V. vulnificus* was positively correlated with salinity and temperature (Table 18). Finally, the linear model for *V. parahaemolyticus* (Table 19, Adjusted R-squared = 0.008486, p-value = 0.1159) indicated that *V. parahaemolyticus* was not significantly different between years and was not correlated with sampling date, salinity, or temperature (Table 20). Boxplots were used for visualization (Figs. 12-13)

4. Discussion

Oysters from the same location and sampled at the same time harbor varying levels of human-pathogenic *Vibrio* species for unknown reasons (Sokolova et al. 2005). Indeed, the bacterial levels from this study spanned several orders of magnitude. This was fully captured by individually analyzing oysters for *Vibrio* species instead of using the standard pooling method. This individual treatment of oysters for quantification versus pooling applied a different approach from traditional study methods of the *Vibrio*-oyster relationship. Molecular results revealed that most oysters harbored the significant oyster pathogen *P. marinus* as well. Predictably (Cook et al. 2002), higher levels of *V. vulnificus* were correlated with higher levels of *V. parahaemolyticus* for both years. Yet there was no apparent correlation between levels of *P. marinus* and *Vibrio* species in oysters taken from the field in Virginia waters. Even comparing extreme deciles with regard to *P. marinus* abundance revealed no significant differences in *V. parahaemolyticus* and *V. vulnificus* abundance. Variability explained by temperature and

salinity, key environmental parameters for both the parasite and bacteria, was removed to investigate if the remaining variability of *P. marinus* had any correlation with the remaining variability of either *Vibrio* species, but no significant relationship was revealed. It makes sense that salinity was a predictor in the *V. vulnificus* model but not *V. parahaemolyticus* model since *V. vulnificus* thrives at more moderate salinities whereas *V. parahaemolyticus* can do well at both moderate and higher salinities (Thompson et al. 2006).

These findings agree with the study conducted by Sokolova et al. (2005) that found PCR-determined levels of *P. marinus* did not seem to predict *V. vulnificus* levels. Yet *in vitro* studies suggested oyster hemocytes exposed to *P. marinus* secretions had slower internalization and elimination of both *Vibrio* species (Tall et al. 1999, La Peyre and Volety 1999). These experiments may have been too contrived to extrapolate into more complex *in vivo* conditions. For example, it may be that oysters compensate *in vivo* by increasing the number of circulating hemocytes. Another explanation for these differences in results could be related to the oyster-*Vibrio* species interaction. Neither bacterial species is pathogenic to the oyster and both *V. parahaemolyticus* and *V. vulnificus* could be considered part of the normal oyster microbiota (Oliver 2006). These two species may simply be less affected by vibriocidal activities of the oyster hemocytes *in vivo* and therefore a decrease in that hemocyte activity, caused by *P. marinus* or other factors, would not have a noticeable effect on them.

Results also contradicted the pilot study conducted of Carnegie et al. (2013), which found an inverse correlation between levels of *P. marinus* and *V. vulnificus*. This could be because the smaller sample size of that project (n = 60) serendipitously sampled

part of a trend in the high variability of *Vibrio* species levels while this study's larger sample size (n = 405) had a better range to cover all variability that *Vibrio* species typically display. It could also be that wild oysters used in the pilot study differed from the domesticated oyster line used in this project in the *in vivo P. marinus*-*Vibro* species dynamics within their tissues. Interestingly, that pilot study had fifteen oysters (or 25% of samples) negative for *P. marinus* while this larger study with a combined sample size of n = 405 only had nine oysters (or 2.2% of samples) negative for *P. marinus*. Perhaps a study focused on *P. marinus*-free oysters and oysters with any level of *P. marinus* at the same salinity would more clearly determine why differences were found between the study here and the 2013 pilot study; however, that would not capture what is happening *in vivo* for aquaculture interests since, as this study supports, almost all oysters of market size in Virginia waters are infected with *P. marinus* (Carnegie and Burreson 2009, Carnegie 2013).

A study conducted by Green and Barnes (2010) looking at a different protozoan, *Marteilia sydneyi*, in the Sydney rock oyster *Saccostrea glomerata* found evidence that these parasites can alter the non-pathogenic microbiota, but a recent study by Pierce et al. (2016) found no consistent correlation between *P. marinus* and microbial richness in oysters. Perhaps the molecular approach in this study missed a key stage of the *P. marinus* and *Vibrio* species dynamic. Oysters were sampled every two weeks and oyster mortalities were not investigated. Oysters near death from *P. marinus*-related disease could have died in between samplings with the results that this study does not capture the extreme end of *P. marinus* related disease effects and how this interacts with *Vibrio* species. A closer look at actual disease state of oysters, using a more nuanced analysis

than qPCR-determined numbers, might answer this question. Histology would be one such way to investigate disease state rather than just total numbers and this approach is the focus of Chapter Two.

The attempt to manipulate *P. marinus* disease progression through differing salinity regimes failed. The failure of the manipulation could have been because three months at a different salinity was not enough time to appreciably impact *P. marinus*. Overwintering *P. marinus* levels play an important role in resulting summer and fall epizootics (Burreson and Ragone Calvo 1996). Perhaps shifting the timing of oyster deployments at different salinities to late fall or winter instead of late spring might more effectively impact its progression. Another potential factor influencing this failed manipulation was how different each salinity regime was. The CR site had a mean salinity of 10.3 while samples were there and the YR site had a mean salinity of 21.0 during that time. Salinity data was not available for 2015 at BB, but salinity data for 2016 in April and May at BB had a mean of 31.2. These differences are typically enough to impact *P. marinus*, so this factor alone is likely not a good explanation ((Burreson and Ragone Calvo 1996). Oysters from Year Two had similar results to oysters from Year One, supporting the initial findings that there appears to be no natural correlation between *P. marinus* and human-pathogenic *Vibrio* species. Of note is the increase in number of oysters harboring pathogenic *V. parahaemolyticus* strains and the increase of pathogenic levels in oysters from Year Two. Interestingly, total *V. parahaemolyticus* levels were not significantly higher in Year Two. It is important to note that while temperature and salinity were significantly different between years (Welch two sample t-test, p-value = 1.58e-15 and < 2.2e-16, respectively) the actual means for the sampling period were

23.8°C and 24.3°C for temperature and 20.2 and 21.0 for salinity, which are not large differences in biological terms.

As climate change continues to become a reality, the Chesapeake Bay system is expected to face many changing conditions (Najjar et al. 2010) that local oyster growers will have to contend with, especially in regards to human-pathogenic vibrios. Yet research is lacking on *Vibrio* species dynamics in oysters. Attempts at modeling or performing risk assessments of either of these bacteria appear complex and uncertain (Urquhart et al. 2014, Young et al. 2015). More research is needed to answers questions regarding inter-annual variation and to investigate other potential explanations for individual oyster level variation.

5. Tables

Table 1. Means and standard error (SE) of oyster shell height and wet mass by sampling time for Year One using the larger 187-oyster data set.

Dates	Shell Height (mm)	SE	Oyster Mass (g)	SE
Aug 4/5	87.19	1.681	8.677	0.4047
Aug 17/19	87.56	1.445	7.146	0.2648
Sep 2/5	87.05	1.697	6.432	0.3316
Sep 15/16	90.45	1.288	7.498	0.3609
Sep 29/Oct 2	89.42	1.405	7.961	0.3648

Table 2. Abundance ranges, means, and standard error (SE) of overall means for *Perkinsus marinus* (ITS region copy number), *Vibrio vulnificus*, total *Vibrio parahaemolyticus*, and pathogenic *V. parahaemolyticus* strain abundance from Year One.

Species or Gene	**Lowest**	**Highest**	**Mean**	**SE**
***P. marinus* (copies/g)**	0	7.69e+10	1.253e+09	5.11e+08
***V. vulnificus* (MPN/g)**	38	1.1e+06	3.881e+04	1.109e+04
***V. parahaemolyticus* (MPN/g)**	36	1.5e+05	8.408e+03	1.405e+03
***Tdh* (MPN/g)**	0	30	0.3722	0.2278
***Trh* (MPN/g)**	0	74	1.439	0.5697

Table 3. Sampling means and standard error (SE) of *Perkinsus marinus* (ITS region copy number), *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and pathogenic *V. parahaemolyticus* strain abundance by sampling date from Year One.

Species or Gene	**Aug 4/5 (SE)**	**Aug 17/19 (SE)**	**Sep 2/5 (SE)**	**Sep 15/16 (SE)**	**Sep 29/ Oct 2 (SE)**
***P. marinus* (copies/g)**	8.33e+06 (3.21e+06)	2.95e+07 (7.68e+06)	2.74e+09 (1.92e+09)	3.01e+09 (1.42e+09)	1.48e+08 (8.28e+07)
***V. vulnificus* (MPN/g)**	2.57e+04 (1.053+04)	5.60e+04 (3.25e+04)	2.90e+04 (6.54e+03)	8.04e+04 (4.11e+04)	2.67e+03 (5.98e+02)
***V. parahaemolyticus* (MPN/g)**	1.04e+04 (3.96e+03)	1.06e+04 (3.93e+03)	1.13e+04 (3.5e+03)	8.40e+03 (2.75e+03)	1.94e+03 (5.5e+02)
***Tdh* (MPN/g)**	1.03e+00 (1.03e+00)	9.39e-01 (7.74e-01)	0.00e+00 (0.00e+00)	0.00e+00 (0.00e+00)	7.50e-02 (7.5e-02)
***Trh* (MPN/g)**	5.86e+00 (3.05e+00)	7.69e-01 (7.69e-01)	0.00e+00 (0.00e+00)	9.23e-01 (9.23e-01)	8.25e-01 (7.52e-01)

Table 4. Summary statistics for the best fit model of *Perkinsus marinus* abundance for the smaller 176-oyster data set for *Vibrio vulnificus* comparisons. All predictors were significant. SE = standard error.

Variable	Estimate	SE	t value	p-value
Intercept	-33.89	7.363	-4.602	8.08e-06
Salinity	1.963	0.3730	5.262	4.19e-07
Weight	-0.2977	0.0721	-4.130	5.64e-05
Height	0.0505	0.0177	2.857	0.0048

Table 5. Summary statistics for the best fit model of *Perkinsus marinus* abundance for the larger 187-oyster data set for *Vibrio parahaemolyticus* comparisons. All predictors were significant. SE = standard error.

Variable	Estimate	SE	t value	p-value
Intercept	-32.04	6.991	-4.582	8.49e-06
Salinity	1.864	0.3547	5.256	4.08e-07
Weight	-0.2909	0.0705	-4.126	5.61e-05
Height	0.0512	0.0169	3.031	0.00279

Table 6. Summary statistics for the best fit model of *Vibrio vulnificus* abundance for the smaller 176-oyster data set. Only temperature was a significant predictor. SE = standard error.

Variable	Estimate	SE	t value	p-value
Intercept	-6.334	3.211	-1.972	0.0502
Temperature	0.1942	0.0329	5.897	1.9e-08
Salinity	0.2593	0.1553	1.670	0.0967

Table 7. Summary statistics for the best fit model of *Vibrio parahaemolyticus* abundance for the larger 187-oyster data set. Temperature was a significant predictor. SE = standard error.

Variable	Estimate	SE	t value	p-value
Intercept	-1.230	0.7443	-1.653	0.1
Temperature	0.1791	0.0291	6.151	4.63e-09

Table 8. Means and standard error (SE) of oyster shell height and wet mass by sampling time from the Choptank River (CR), the low salinity site, for Year Two.

Dates	Shell Height (mm)	SE	Oyster Mass (g)	SE
Aug 11/13	73.11	2.478	4.737	0.4329
Aug 23/25	69.43	2.192	4.577	0.3498
Sep 7/9	77.85	1.770	4.467	0.2600
Sep 28/30	81.20	1.689	5.790	0.4250
Oct 11/13	78.91	2.055	6.460	0.4013

Table 9. Means and standard error (SE) of oyster shell height and wet mass by sampling time from the York River (YR), the moderate salinity site, for Year Two.

Dates	Shell Height (mm)	SE	Oyster Mass (g)	SE
Aug 11/13	88.23	2.790	7.106	0.5083
Aug 23/25	84.36	4.711	7.040	1.096
Sep 7/9	85.43	2.735	7.470	0.6710
Sep 28/30	90.85	2.492	8.310	0.8965
Oct 11/13	95.73	2.393	9.831	0.5957

Table 10. Means and standard error (SE) of oyster shell height and wet mass by sampling time from Burtons Bay (BB), the high salinity site, for Year Two.

Dates	Shell Height (mm)	SE	Oyster Mass (g)	SE
Aug 11/13	81.55	2.781	5.774	0.4783
Aug 23/25	88.88	3.279	7.002	0.6157
Sep 7/9	84.84	1.791	6.512	0.3988
Sep 28/30	84.43	2.697	7.330	0.7381
Oct 11/13	84.57	2.042	7.328	0.6682

Table 11. P-values for differences in mean oyster shell height and mass among Year Two sites. BB = Burtons Bay, YR = York River, CR = Choptank River. Asterisks indicate YR had a larger mean and daggers indicate CR had a smaller mean.

Sites	Shell Height (mm)	Oyster Mass (g)
BB and YR	0.0260 *	0.0110 *
BB and CR	6.48e-08 †	3.29e-06 †
CR and YR	1.88e-11 *	5.48e-10 *

Table 12. Abundance ranges, means, and standard errors (SE) of overall means for *Perkinsus marinus* (ITS region copy number), *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and pathogenic *V. parahaemolyticus* strain abundance from Year Two.

Species or Gene	**Lowest**	**Highest**	**Mean**	**SE**
***P. marinus* (copies/g)**	0	2.551e+11	1.979e+09	1.229e+09
***V. vulnificus* (MPN/g)**	74	1.1e+6	1.149e+04	5.175e+03
***V. parahaemolyticus* (MPN/g)**	43	2.4e+5	8.62e+03	1.97e+03
***Tdh* (MPN/g)**	0	230	4.894	1.247
***Trh* (MPN/g)**	0	920	20.55	4.895

Table 13. Sampling means of *Perkinsus marinus* (ITS region copy number), *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and pathogenic *V. parahaemolyticus* strain abundance by sampling date from Year Two. SE = Standard Error.

Species or Gene	**Aug 11/13 (SE)**	**Aug 23/25 (SE)**	**Sep 7/9 (SE)**	**Sep 28/30 (SE)**	**Oct 11/13 (SE)**
***P. marinus* (copies/g)**	1.43e+08 (4.74e+07)	6.86e+09 (5.92e+09)	1.12e+09 (9.62e+08)	1.73e+09 (1.61e+09)	9.38e+07 (3.32e+07)
***V. vulnificus* (MPN/g)**	3.23e+04 (2.55e+04)	1.27e+04 (5.90e+03)	4.50e+03 (7.94e+02)	2.82e+03 (6.51e+02)	5.67e+03 (1.91e+03)
***V. parahaemolyticus* (MPN/g)**	5.72e+03 (1.28e+03)	5.24e+03 (2.38e+03)	3.77e+03 (6.17e+02)	1.67e+04 (7.35e+03)	1.13e+04 (5.53e+03)
***Tdh* (MPN/g)**	2.30e+00 (1.12e+00)	3.21e-01 (1.55e-01)	4.52e+00 (1.77e+00)	5.80e+00 (1.81e+00)	1.13e+01 (5.44e+00)
***Trh* (MPN/g)**	7.74e+00 (2,68e+00)	3.34e+00 (1.42e+00)	8.71e+00 (2.32e+00)	2.53e+01 (6.04e+00)	5.66e+01 (2.24e+01)

Table 14. Analysis of variance table investigating differences in log 10 *Perkinsus marinus* abundance and site and date. P-values indicate that there were no differences between sites and dates. df = degrees of freedom.

Variable	df	Sum of Squares	Mean of Squares	F value	p-value
Site	2	1.90	0.9487	0.4644	0.6292
Date	4	11.98	2.9953	1.4661	0.2137
Residuals	211	431.08	2.043		

Table 15. Summary statistics for the general linear model of *Perkinsus marinus* for the larger 187-oyster data set from Year One for sampling date comparisons using Year One as the baseline. All predictors were significant.

Variable	Estimate	Standard Error	t value	p-value
Intercept	-7.85187	2.32762	-3.373	0.000815
Date	0.49664	0.10331	4.807	2.17e-06
Year Two	-2.92511	0.55140	-5.305	1.87e-07
Salinity	0.32211	0.09114	3.534	0.000457
Temperature	0.28703	0.05277	5.439	9.36e-08

Table 16. Analysis of covariance table investigating differences in log 10 *Perkinsus marinus* levels and sampling date, salinity, temperature, and year using the larger 187-oyster data set from Year One. df = degrees of freedom. P-values indicate that all variables but year were significant predictors.

Variable	df	Sum of Squares	Mean of Squares	F value	p-value
Date	1	12.29	12.286	4.7698	0.0295431
Year	1	4.34	4.341	1.6854	0.1949629
Salinity	1	31.06	31.057	12.0574	0.0005722
Temperature	1	76.19	76.193	29.5805	9.358e-08
Residuals	400	1030.32	2.576		

Table 17. Summary statistics for the general linear model of *Vibrio vulnificus* for the smaller 176-oyster data set from Year One for sampling date comparisons using Year One as the baseline. Only salinity and temperature were significant predictors.

Variable	Estimate	Standard Error	t value	p-value
Intercept	0.66280	1.03073	0.643	0.52028
Date	-0.05120	0.04533	-1.130	0.259l8
Year Two	-0.32797	0.24282	-1.351	0.17758
Salinity	0.08566	0.04054	2.113	0.03526
Temperature	0.06314	0.02318	2.725	0.00673

Table 18. Analysis of covariance table investigating differences in log 10 *Vibrio vulnificus* levels and sampling date, salinity, temperature, and year using the smaller 176-oyster data set from Year One. df = degrees of freedom. P-values indicate that all variables but year were significant predictors.

Variable	df	Sum of Squares	Mean of Squares	F value	p-value
Date	1	27.593	27.5935	56.5844	3.765e-13
Year	1	1.284	1.2837	2.6325	0.105507
Salinity	1	2.107	2.1066	4.3200	0.038323
Temperature	1	3.620	3.6202	7.4238	0.006727
Residuals	389	189.696	0.4877		

Table 19. Summary statistics for the general linear model of *Vibrio parahaemolyticus* for the larger 187-oyster data set from Year One for sampling date comparisons using Year One as the baseline. There were no significant predictors.

Variable	Estimate	Standard Error	t value	p-value
Intercept	1.79803	1.00472	1.790	0.0743
Date	-0.03924	0.04459	-0.880	0.3794
Year Two	0.04970	0.23801	0.209	0.8347
Salinity	0.06422	0.03934	1.632	0.1034
Temperature	0.01688	0.02278	0.741	0.4591

Table 20. Analysis of variance table investigating differences in log 10 *Vibrio parahaemolyticus* levels and sampling date, salinity, temperature, and year using the larger 187-oyster data set from Year One. df = degrees of freedom. P-values indicate that no variables were significant predictors.

Variable	df	Sum of Squares	Mean of Squares	F value	p-value
Date	1	0.978	0.97817	2.0382	0.1542
Year	1	1.072	1.07183	2.2333	0.1359
Salinity	1	1.266	1.26570	2.6373	0.1052
Temperature	1	0.264	0.26356	0.5492	0.4591
Residuals	400	191.971	0.47993		

6. Figures

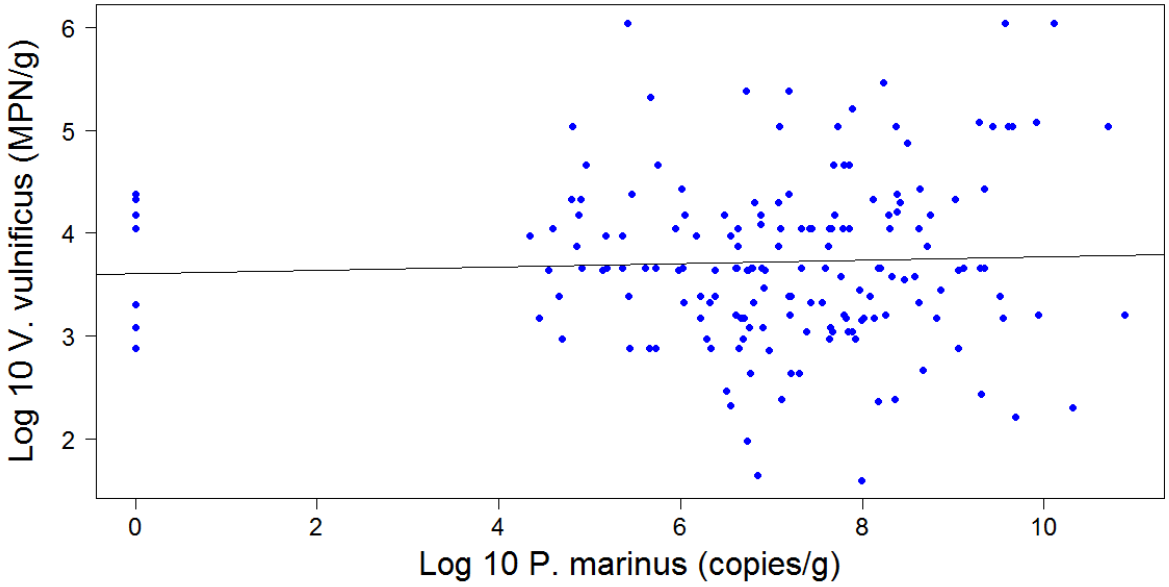

Figure 1. Log transformed *Perkinsus marinus* (ITS region copy number) and *Vibrio vulnificus* abundance using the smaller 176-oyster data set from Year One, displaying no significant correlation (Pearson's r value = 0.0403, t = 0.53263, df = 174, p-value = 0.595).

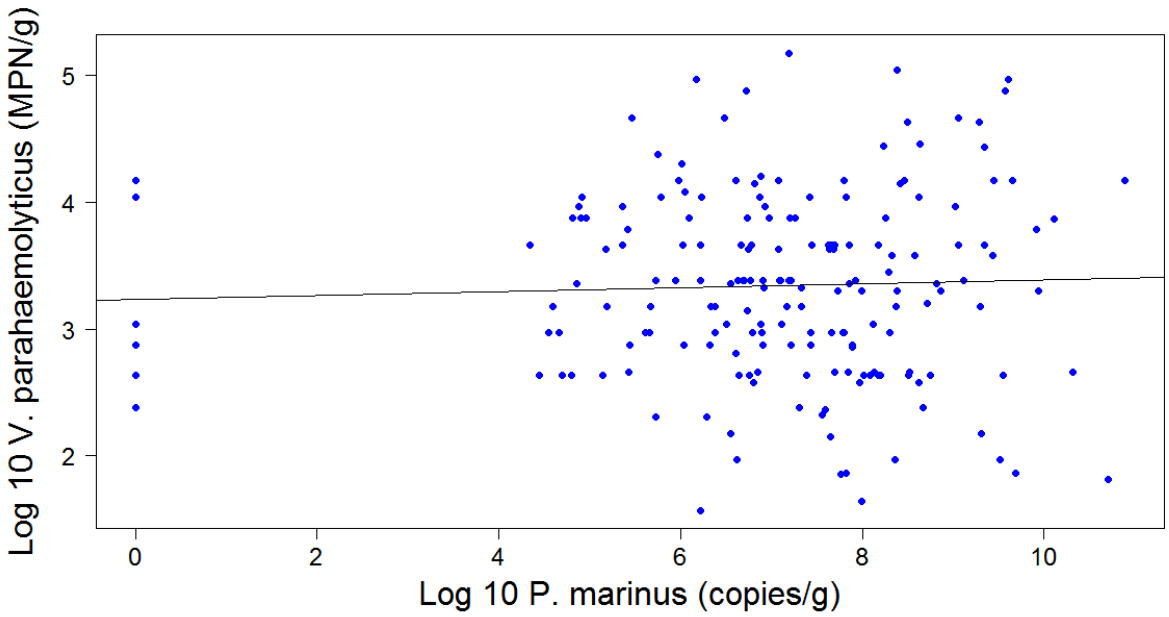

Figure 2. Log transformed *Perkinsus marinus* (ITS region copy number) and *Vibrio parahaemolyticus* abundance using the larger 187-oyster data set from Year One, displaying no significant correlation (Pearson's r value = 0.0411, t = 0.55955, df = 185, p-value = 0.5765).

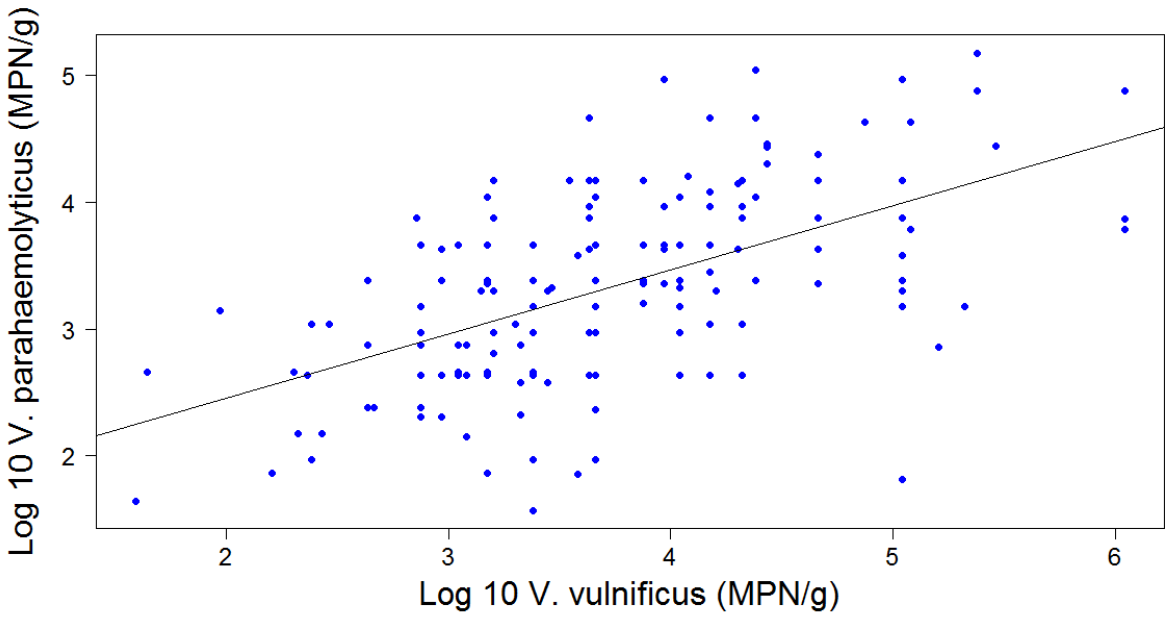

Figure 3. Log transformation of *Vibrio vulnificus* and *Vibrio parahaemolyticus* abundance from the 176-oyster data set from Year One, displaying a positive correlation (Pearson's r value = 0.55, t = 8.786, df = 174, p-value = 1.332e-15).

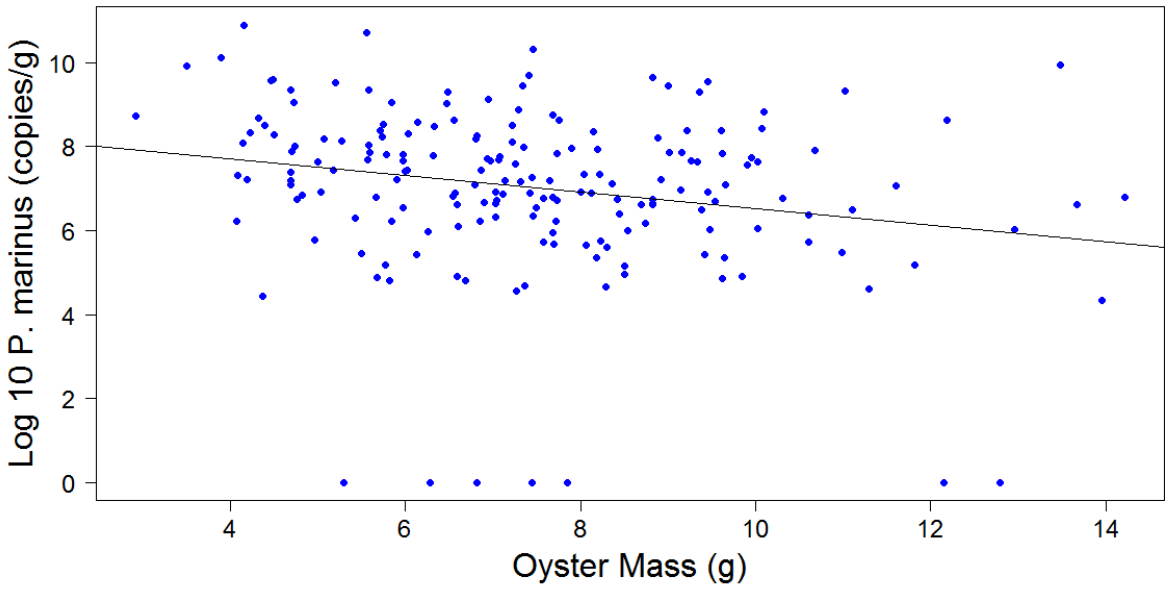

Figure 4. Log transformed *Perkinsus marinus* (ITS region copy number) abundance versus oyster mass from the 187-oyster data set from Year One, displaying a negative correlation (Pearson's r value = -0.22, t = -3.1022, df = 185, p-value = 0.0022).

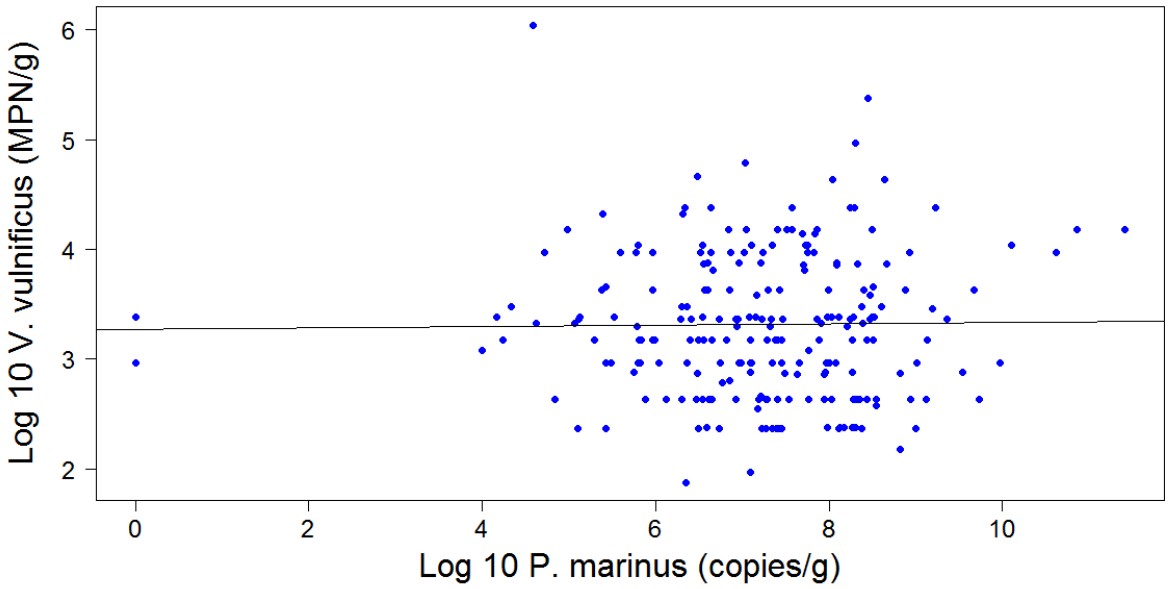

Figure 5. Log transformed *Perkinsus marinus* (ITS region copy number) and *Vibrio vulnificus* abundance from Year Two, displaying no significant correlation (Pearson's r value = 0.0129, t = 0.18943, df = 216, p-value = 0.8499).

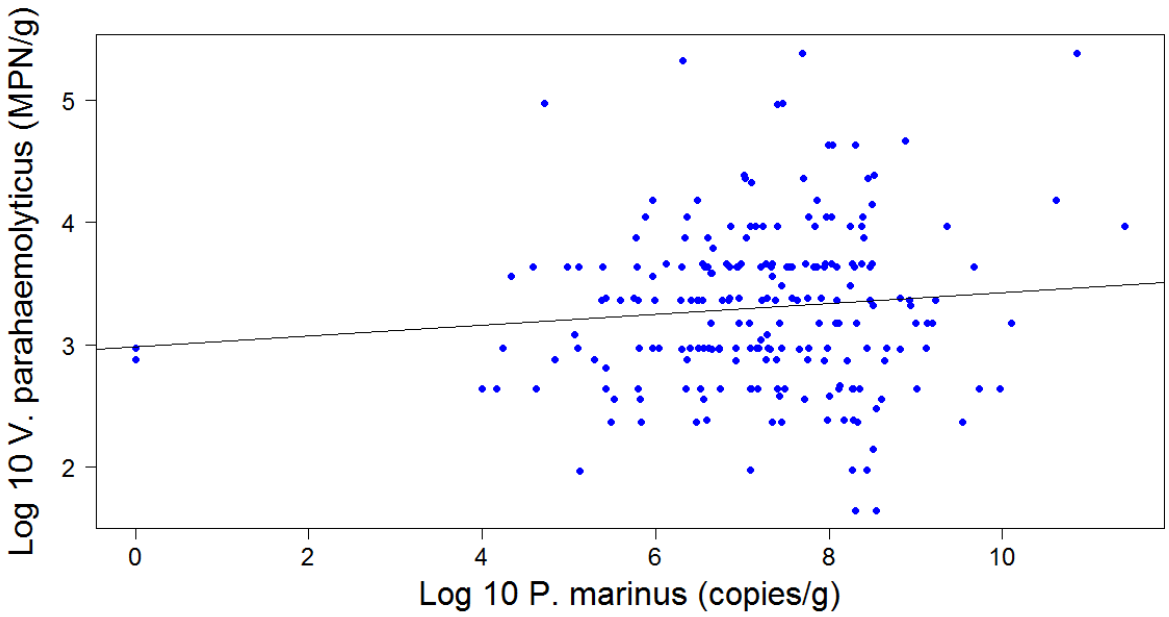

Figure 6. Log transformed *Perkinsus marinus* (ITS region copy number) and *Vibrio parahaemolyticus* abundance from Year Two, displaying no significant correlation (Pearson's r value = 0.0956, t = 1.4115, df = 216, p-value = 0.1595).

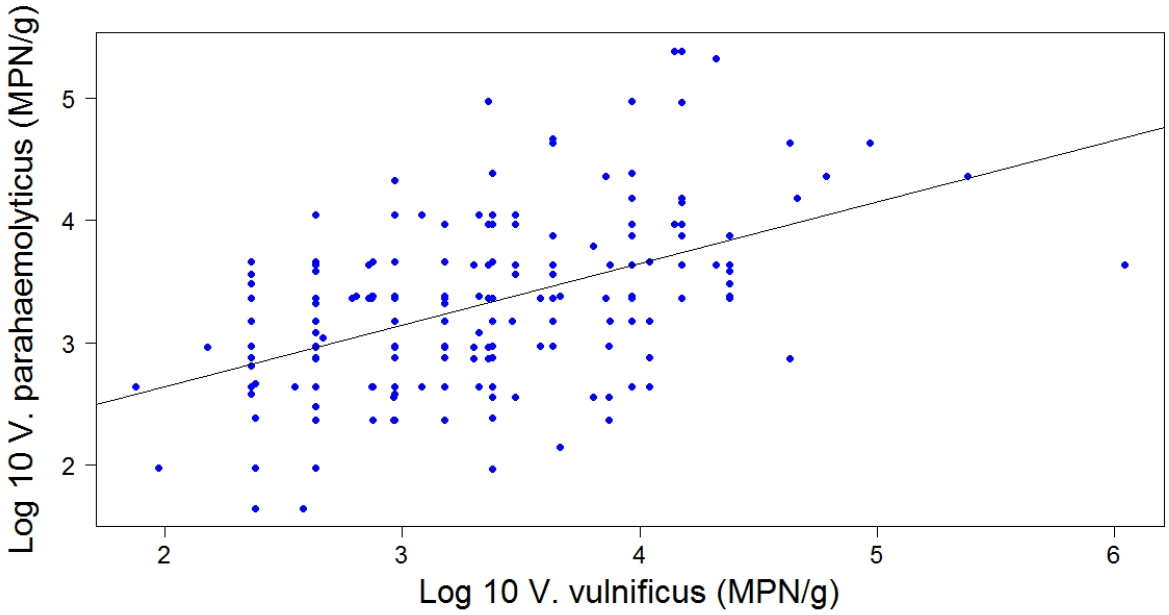

Figure 7. Log transformed *Vibrio vulnificus* and *Vibrio parahaemolyticus* abundance from Year Two, displaying a positive correlation (Pearson's r value = 0.50, t = 8.4701, df = 216, p-value = 3.775e-15).

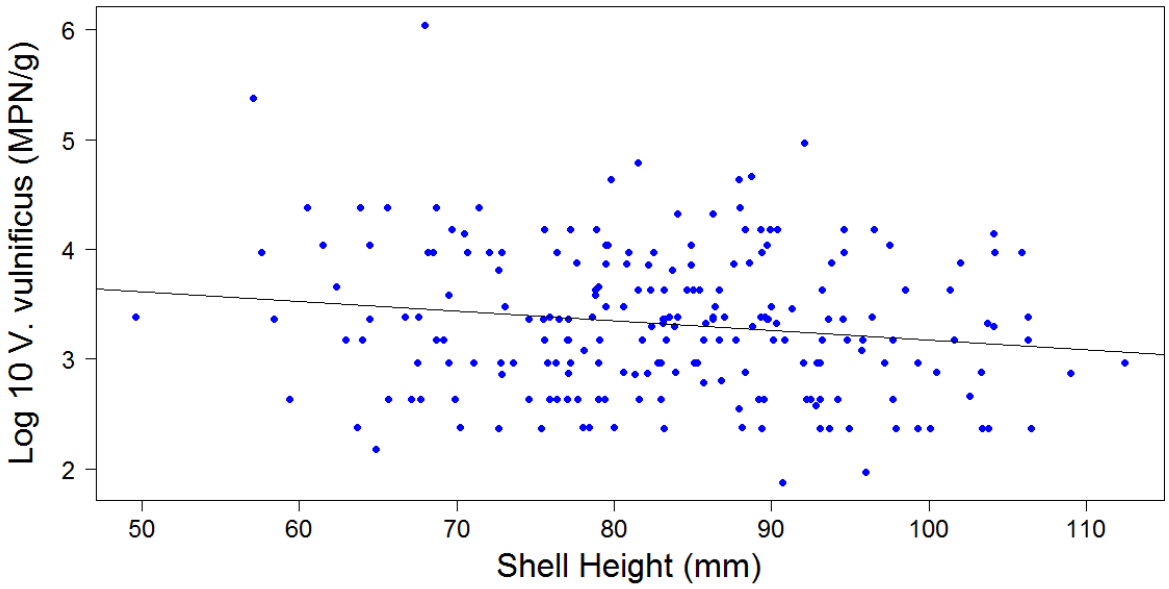

Figure 8. Shell height and log transformed *Vibrio vulnificus* abundance from Year Two, displying a negative correlation (Pearson's r value = -0.155, t = -2.3077, df = 216, p-value = 0.02196).

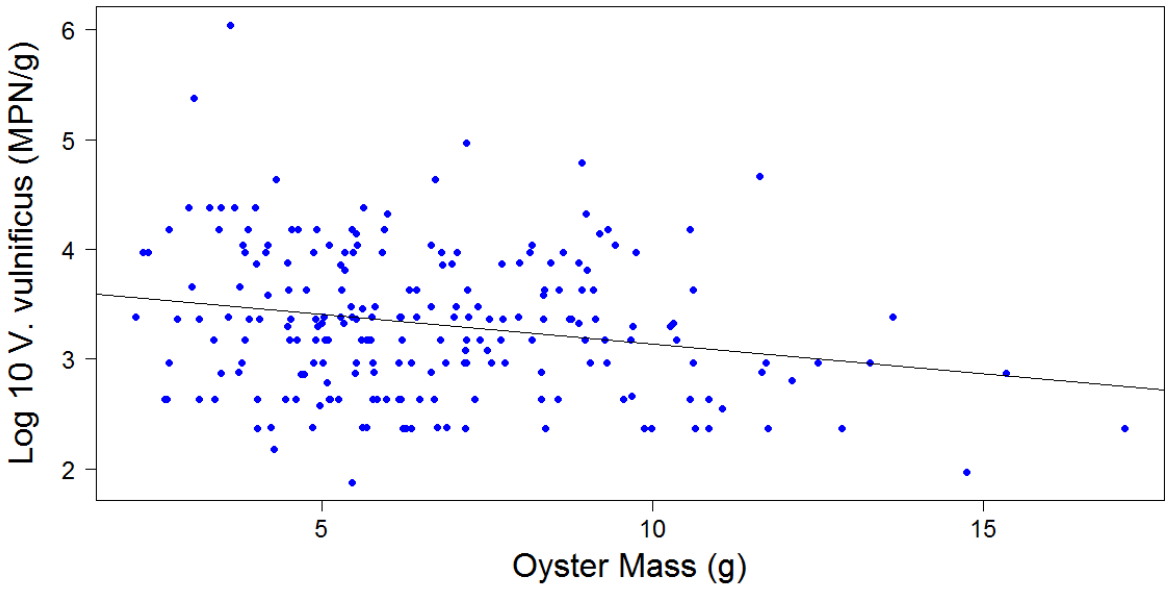

Figure 9. Oyster mass and log transformed *Vibrio vulnificus* abundance from Year Two, displaying a negative correlation (Pearson's r value = -0.216, t = -3.2477, df = 216, p-value = 0.001349).

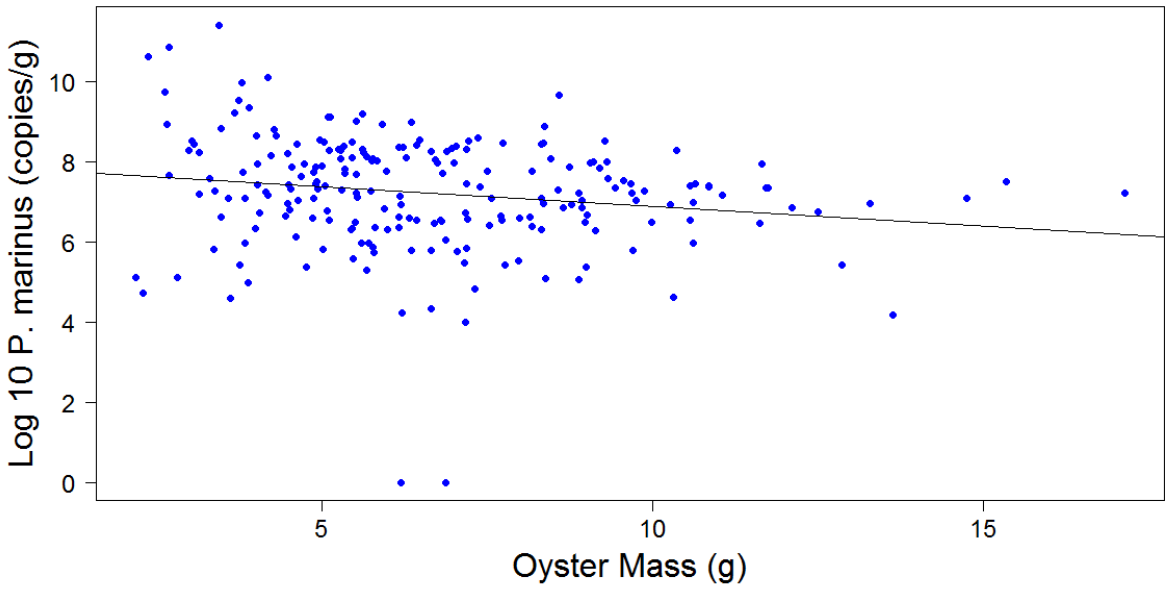

Figure 10. Oyster mass and log transformed *Perkinsus marinus* (ITS region copy number) abundance from Year Two, displaying a negative correlation (Pearson's r value = -0.181, t = -2.7087, df = 216, p-value = 0.007296).

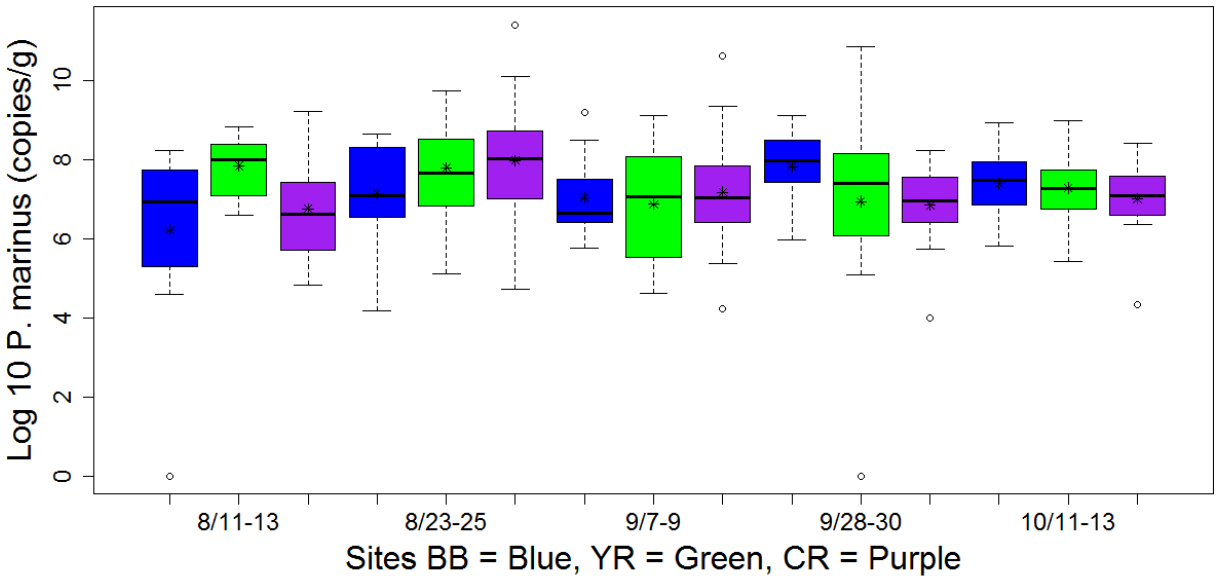

Figure 11. Log transformation of *Perkinsus marinus* (ITS region copy number) abundance from Year Two shown by sampling date and different sites of oyster deployments: from Burtons Bay to York River, York River, and Choptank River to York River. Figure indicates no significant differences between sites. Boxes show 25th and 75th percentile (IQR) with bars = medians. Upper whisker = smaller maximum value and 75th percentile + 1.5 IQR. Lower whisker = larger minimum value and 25th percentile = 1.5 IQR. Dots = values outside boxplot parameters. Blue = Burtons Bay (BB), high salinity site; Green = York River (YR), moderate salinity site; and Purple = Choptank River (CR), low salinity site. Stars indicate means.

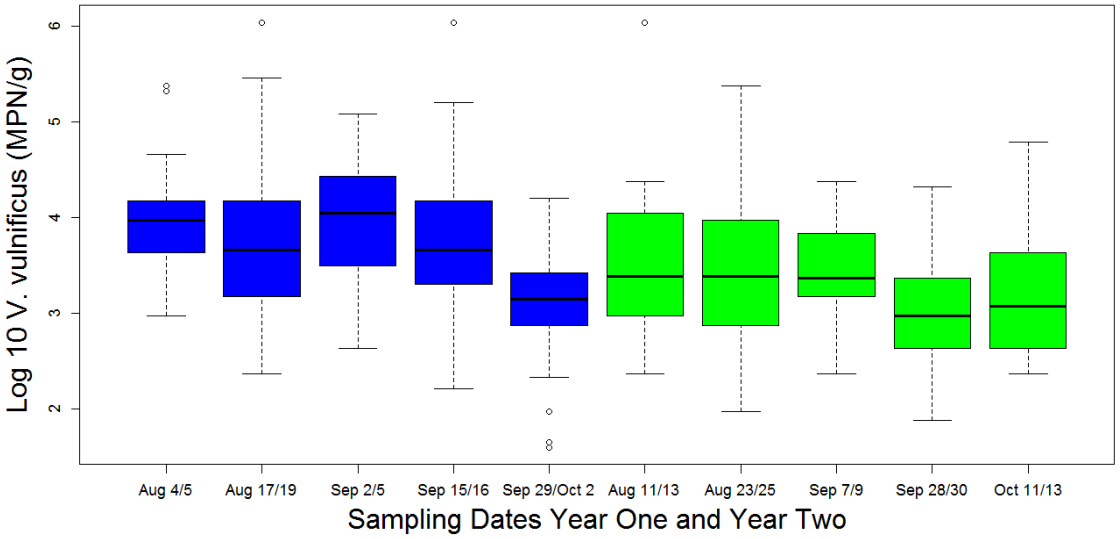

Figure 12. Log transformation of *Vibrio vulnificus* abundance from Year One (using 176-oyster data set) and Year Two, displaying an overall lower mean in Year Two. Boxes show 25th and 75th percentile (IQR) with bars = medians. Upper whisker = smaller maximum value and 75th percentile + 1.5 IQR. Lower whisker = larger minimum value and 25th percentile = 1.5 IQR. Dots = values outside boxplot parameters. Blue = Year One and Green = Year Two.

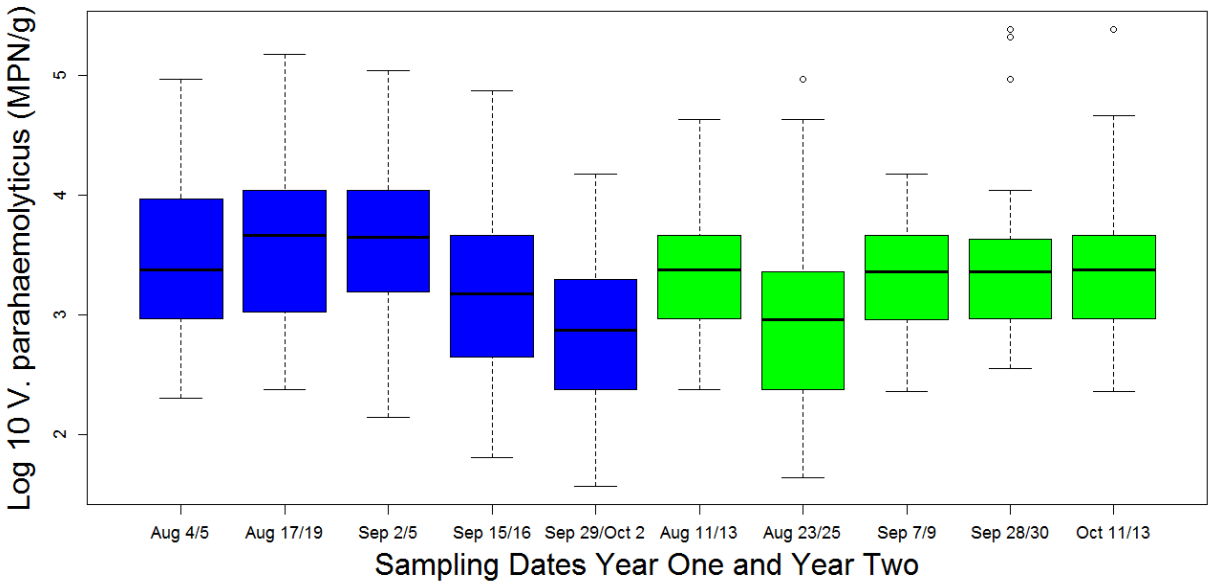

Figure 13. Log transformation of *Vibrio parahaemolyticus* abundance from Year One (using 187-oyster data set) and Year Two, displaying no significant differences. Boxes show 25th and 75th percentile (IQR) with bars = medians. Upper whisker = smaller maximum value and 75th percentile + 1.5 IQR. Lower whisker = larger minimum value and 25th percentile = 1.5 IQR. Dots = values outside boxplot parameters. Blue = Year One and Green = Year Two.

7. Appendices

Appendix A. Models and AIC values.

This appendix explains the models used to remove environmental variability in *Perkinsus marinus*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* data. Four separate groups of 25 models were created. Models labeled fit1 (1-25) were for *Perkinsus marinus* using the smaller 176-oyster data set for comparisons with *Vibrio vulnificus*. Models labeled fitVv1 (1-25) were for *V. vulnificus*. Models labeled fit1Pm (1-25) were for *P. marinus* using the larger 187-oyster data set for comparisons with *Vibrio parahaemolyticus*. Models labeled fitVp1 (1-25) were for *V. parahaemolyticus*. Examples of the 25 models of one of the four groups are presented below followed by AIC tables for each group of models.

Key to Model Code

Meaning	Model abbreviation
Data set of 176-oysters from Year One	data
Data set of 187-oysters from Year One	dataVp
Temperature the day of sampling	temp
Salinity the day of sampling	sal
Temperature the day before sampling	tempB4
Salinity the day before sampling	salB4
Mass of oyster	wgt
Shell height of oyster	hgt
Log 10 *Perkinsus marinus* qPCR levels	logPm
Log 10 *Vibrio vulnificus* qPCR levels	logVv
Log 10 *Vibrio parahaemolyticus* qPCR levels	logVp

Models of *P. marinus* for the smaller 176-oyster data set for *V. vulnificus* comparisons.

fit1= lm(logPm ~ data$temp + data$sal + data$wgt + data$temp*data$sal, data=data)
fit2= lm(logPm ~ data$tempB4 + data$salB4 + data$wgt + data$tempB4*data$salB4, data=data)
fit3= lm(logPm ~ data$temp + data$sal + data$wgt, data=data)
fit4= lm(logPm ~ data$tempB4 + data$salB4 + data$wgt, data=data)
fit5= lm(logPm ~ data$temp + data$sal, data=data)
fit6= lm(logPm ~ data$tempB4 + data$salB4, data=data)
fit7= lm(logPm ~ data$temp, data=data)
fit8= lm(logPm ~ data$tempB4, data=data)
fit9= lm(logPm ~ data$sal + data$wgt + data$temp*data$sal, data=data)
fit10= lm(logPm ~ data$salB4 + data$wgt + data$tempB4*data$salB4, data=data)
fit11= lm(logPm ~ data$wgt + data$temp*data$sal, data=data)
fit12= lm(logPm ~ data$wgt + data$tempB4*data$salB4, data=data)
fit13= lm(logPm ~ data$temp*data$sal, data=data)
fit14= lm(logPm ~ data$tempB4*data$salB4, data=data)
fit15= lm(logPm ~ data$temp + data$sal + data$temp*data$sal, data=data)
fit16= lm(logPm ~ data$tempB4 + data$salB4 + data$tempB4*data$salB4, data=data)
fit17= lm(logPm ~ data$sal + data$wgt, data=data)
fit18= lm(logPm ~ data$salB4 + data$wgt, data=data)
fit19= lm(logPm ~ data$temp + data$wgt, data=data)
fit20= lm(logPm ~ data$tempB4 + data$wgt, data=data)
fit21= lm(logPm ~ data$temp + data$sal + data$wgt + data$temp*data$sal + data$tempB4 + data$salB4 + data$tempB4*data$salB4, data=data)
fit22= lm(logPm ~ data$sal + data$wgt + data$hgt, data=data)
fit23= lm(logPm ~ data$temp + data$sal + data$wgt + data$hgt, data=data)
fit24= lm(logPm ~ data$temp + data$sal + data$wgt + data$hgt + data$temp*data$sal, data=data)
fit25= lm(logPm ~ data$tempB4 + data$salB4 + data$wgt + data$hgt, data=data)

AIC values for models of *P. marinus* for the smaller 176-oyster data set order by lowest AIC value first. df = degrees of freedom.

Model	df	AIC
fit22	5	703.4906
fit23	6	705.466
fit24	7	705.8382
fit17	4	709.6531
fit1	6	710.7483
fit9	6	710.7483
fit11	6	710.7483
fit3	5	711.6401
fit25	6	711.6728
fit21	9	713.5441
fit4	5	717.3063
fit13	5	717.4207
fit15	5	717.4207
fit5	4	718.4056
fit2	6	719.2619
fit10	6	719.2619
fit12	6	719.2619
fit18	4	724.9403
fit6	4	725.1122
fit14	5	727.0359
fit16	5	727.0359
fit19	4	737.9241
fit20	4	738.0913
fit8	3	745.8245
fit7	3	746.2268

AIC values for models of *V. vulnificus* ordered by lowest AIC value first. df = degrees of freedom.

Model	df	AIC
fitVv5	4	397.1477
fitVv7	3	397.9631
fitVv3	5	398.8081
fitVv13	5	399.0492
fitVv15	5	399.0492
fitVv19	4	399.4277
fitVv8	3	399.5868
fitVv21	9	399.6088
fitVv23	6	400.2032
fitVv9	6	400.7178
fitVv1	6	400.7178
fitVv11	6	400.7178
fitVv6	4	400.9026
fitVv20	4	401.2887
fitVv14	5	401.7292
fitVv16	5	401.7292
fitVv24	7	402.1727
fitVv4	5	402.638
fitVv12	6	403.2491
fitVv10	6	403.2491
fitVv2	6	403.2491
fitVv25	6	404.2311
fitVv18	4	422.474
fitVv17	4	427.7764
fitVv22	5	429.1585

AIC values for models of *P. marinus* for the larger 187-oyster data set order by lowest AIC value first. df = degrees of freedom.

Model	df	AIC
fit22Pm	5	741.317
fit23Pm	6	743.303
fit24Pm	7	744.131
fit17Pm	4	748.479
fit25Pm	6	748.585
fit1Pm	6	750.318
fit9Pm	6	750.318
fit11Pm	6	750.318
fit3Pm	5	750.468
fit21Pm	9	752.424
fit4Pm	5	754.528
fit13Pm	5	756.259
fit15Pm	5	756.259
fit5Pm	4	756.461
fit10Pm	6	756.508
fit12Pm	6	756.508
fit2Pm	6	756.508
fit6Pm	4	761.97
fit18Pm	4	762.91
fit14Pm	5	763.815
fit16Pm	5	763.815
fit19Pm	4	777.15
fit20Pm	4	777.415
fit8Pm	3	784.62
fit7Pm	3	784.888

AIC values for models of *V. parahaemolyticus* ordered by lowest AIC value first. df = degrees of freedom.

Model	df	AIC
fitVp7	3	383.8766
fitVp5	4	385.3544
fitVp19	4	385.6368
fitVp13	5	386.0116
fitVp15	5	386.0116
fitVp8	3	386.1946
fitVp14	5	386.5906
fitVp16	5	386.5906
fitVp3	5	387.0371
fitVp6	4	387.5502
fitVp1	6	387.7145
fitVp9	6	387.7145
fitVp11	6	387.7145
fitVp23	6	387.8767
fitVp20	4	388.106
fitVp10	6	388.1846
fitVp2	6	388.1846
fitVp12	6	388.1846
fitVp21	9	388.2555
fitVp24	7	388.8519
fitVp4	5	389.4448
fitVp25	6	390.6147
fitVp18	4	402.9829
fitVp17	4	417.9693
fitVp22	5	418.9681

Influence of Oyster Health on Levels of Human-Pathogenic Vibrios in Oysters

1. Introduction

Characterizing oyster health is a complex topic. Health is a general term that can be applied to whole bay systems, a single oyster reef, or individual oysters. The term "oyster health" is dependent on the question of interest. Ways to systematically interpret individual oyster health have been suggested at least since the beginning of the last century (Grave 1912) with many versions of a simple condition index existing with various critiques and standards suggested (e.g., Ingle 1949, Engle 1951, Lawrence and Scott 1982, Crosby and Gale 1990). More recently, these simple traditional indices have been supplemented and sometimes replaced with other approaches such as cytology and electron microscopy that have various advantages and disadvantages (Carnegie et al. 2016).

Histopathology is one such approach and there are many reasons to use histopathology in disease related studies. One advantage of histopathology is the scope of its assessment. Histopathology sits at an intermediate level between molecular work and whole organ assessment in terms of biological organization (Adams et al. 1989, Bernet et al. 1999) and captures responses to sub-lethal stress (Bernet et al. 1999). Histopathology provides important insight into the distribution and pathological effects of disease within host tissues, allowing for assessment of the actual disease state of the oyster, perspective that PCR assays cannot provide.

These advantages of histopathological assessment make it an ideal tool for gaining more nuanced perspective on oyster-*P. marinus*-*Vibrio* interactions. While the previous chapter focused on total numbers of the parasite and bacteria, i.e., the abundance or intensity within the whole sample, this component of the project sought to understand what other factors besides simple *P. marinus* numbers might influence *V. vulnificus* or *V. parahaemolyticus* presence. Standard histopathological analyses targeted at the oyster-*P. marinus*-*Vibrio* interaction represent a unique approach that will provide insight into tissue tropism of not only *P. marinus* but any other pathogens, like *H. nelsoni,* that are present. It will also capture epithelial damage, individual oyster responses, and other pathological conditions present, regardless of etiology. This also presents the opportunity to develop a standardized method to histopathologically assess oyster health, providing a more comprehensive look at oyster health than previous condition indices. Therefore, the goal of this chapter was to determine if there was any relationship between *P. marinus*-related pathologies and *Vibrio* species in oysters. Also, this study investigated if other oyster parasites or tissue conditions had a relationship with *Vibrio* species levels through the development of an oyster health rubric. The proposed oyster health rubric could be used as a general tool to provide a way to convert oyster health to a single value for the purpose of inter-study comparisons of relative oyster health or easier statistical analyses.

2. Materials and Methods

2.1. Field Sampling

Oysters samples were taken from intertidal rack-and-bag systems in the York River at Gloucester Point, Virginia, where *P. marinus*, *V. vulnificus*, and *V. parahaemolyticus* are present (see Chapter One). Sampling of forty oysters was conducted biweekly between early August and October 2014 following the procedures and dates outlined in section 2.1.1 of the previous chapter.

2.2. Sample Processing and Histology

Samples collected during Year One (see Chapter One) were used for histopathological analyses (n = 187). Transverse sections including gill, mantle, gonad, digestive gland, stomach and intestine, and associated connective tissues were fixed in Davidson's fixative (Shaw and Battle 1957) and processed using standard paraffin histological methods. Six-micron sections were stained with hematoxylin and eosin and evaluated on an Olympus BX51 light microscope. Of the oysters sampled, 115 oysters were examined using a health rubric described below. This examination involved completely scanning the histological section using 20X objective lens and using a 40X lens to investigate points of interest. Additionally, gut and intestinal epithelia and gills tips were examined using the 40X objective. Complete examination of each slide typically took 35-45 minutes. Because limited time would not allow full analyses to be

conducted on all 187 oysters, individuals determined earlier to have the highest and lowest levels of *P. marinus, V. parahaemolyticus,* and *V. vulnificus* were evaluated first to pair histology data with abundance extremes for all three, with histological analyses then progressing through individuals with more intermediate levels of each. All oysters with pathogenic strains of *V. parahaemolyticus* were evaluated histologically. Ultimately 72 oysters could not be assessed using the full health rubric. These were evaluated only for *P. marinus* and *H. nelsoni* infections and oyster gender and gonadal stage, by the VIMS Shellfish Pathology Laboratory.

2.3. Health Rubric

A health rubric consisting of 25 factors was established to evaluate oyster health. Factors focused on oyster gonadal development, parasites commonly found in *C. virginica* in this region (e.g. *P. marinus* and *H. nelsoni*, see Figures 14A and 14B, respectively), tissue-specific damage, and oyster response. An explanatory document describing each factor and how each factor was ranked was included as Appendix B. These semi-quantitative rankings capture both presence and intensity of physiologically pertinent factors (see Appendix C for relevant examples and approaches). All the ranks from all the factors were then converted to a single number for each oyster by adding the ranks of all but two factors together. The two factors excluded were oyster sex and gonadal stage, neither of which inherently indicates a disease condition. The rubric was constructed so some factors had a larger impact on the final "health" number, adding an

intrinsic weighting of significance for certain factors. For example, *P. marinus*, a "primary organism" in this rubric, has seven related inputs in the health rubric and has a theoretical maximum numeric value of 22. Those seven inputs include an overall *P. marinus* ranking as well as location rankings in digestive ducts and tubules, gill epithelium, mantle epithelium, gonad epithelium, stomach epithelium, and intestine epithelium to better evaluate *P. marinus*-related infection and distribution. In contrast, *Nematopsis* sp., a "secondary organism" in the rubric, has one related input, overall rank, in the health rubric and has a theoretical maximum numeric value of 3. The final "health" number is thus more sensitive to *P. marinus* inputs than *Nematopsis* sp. inputs. The final "health" number from the health rubric was designed to describe a single oyster's relative health with a single value that can range from 0 to 61; 0 indicates a normal healthy oyster and 61 indicates a completely diseased oyster (although an oyster "health" rank of 61 is likely only theoretical since it indicates all factors of the health rubric have reached their maximum values.

2.4. Statistical Analyses

The number of oysters harboring *P. marinus* and/or other common organisms found in oysters from Virginia waters like *H. nelsoni* or *Nematopsis* sp. was recorded based on histopathological assessments. For comparisons, bacterial data for each oyster from Chapter One was used. Welch two sample t-tests, Pearson's r correlation, linear models, analysis of variance (ANOVA), and post-hoc Tukey's honest significant difference (HSD) tests were used to investigate potential differences or relationships

among several different histological categories and transformations with a significance level set at p-value = 0.05. Where necessary, scatter plots were used for visualization.

3. Results

3.1. Oysters

One hundred eighty-seven oysters were histopathologically analyzed using either the health rubric or standard histopathological analyses for *P. marinus* and *H. nelsoni* and those data were compared to log 10 qPCR-determined levels of *V. parahaemolyticus*. Results relating to log 10 *V. vulnificus* used the smaller data set of 176 oysters for comparisons to qPCR-determined levels of the bacteria (see Chapter One). Histological analysis showed that *P. marinus* had the highest prevalence, followed by a *Nematopsis* sp. and digestive lumen ciliates (Table 21).

3.2. H. nelsoni, *and Gender and* Vibrio *Species*

Oysters infected by *H. nelsoni* (n = 18) had significantly higher *V. vulnificus* levels (p-value = 0.0038) than oysters in which this parasite was not detected (n = 169), but this was not true for total *V. parahaemolyticus* (p-value = 0.34), although the trend was a higher mean level of overall *V. parahaemolyticus* in oysters infected by *H. nelsoni*. Oysters harboring *H. nelsoni* did not contain pathogenic strains of *V. parahaemolyticus*.

Oysters with *H. nelsoni* were divided into two groups based on Big Ford Units (BFU), a measure which converts intensity and location data of *H. nelsoni* into a single score with higher scores indicating more heavily infected oysters (Ford et. al 1999, modified see Appendix B). One group had oysters with a BFU of 1 and 2 (n = 9) and the other group had oysters with a BFU of 3 and 4 (n = 9). These two BFU based groups were compared to oysters where *H. nelsoni* was not detected (Tables 22-23). The lower BFU group with *H. nelsoni* had significantly higher levels of *V. vulnificus* relative to oysters with no *H. nelsoni* detection (p-value = 0.0065) but the higher BFU group with *H. nelsoni* did not (p-value = 0.12) (Table 22). A linear model using oyster gonadal stage, oyster sex, *V. vulnificus* levels, and *V. parahaemolyticus* levels as predictors and *H. nelsoni* BFU rankings as the response variable (Table 24, Adjusted R-squared = 0.157, p-value = 1.161e-05) indicated that only *V. vulnificus* was correlated with *H. nelsoni* BFU rankings (Table 25).

Levels of *Vibrio* species in different oyster sexes were compared (Table 26). Using ANOVA, oyster sex of male (n = 42), female (n = 58), and indeterminate gender (n = 87) showed no significant differences relative to each other for total *V. vulnificus* (Table 27), but oysters in these categories did vary significantly in total *V. parahaemolyticus* (Table 28). A post-hoc Tukey's HSD test (95% CI) for total *V. parahaemolyticus* showed that males and oysters of indeterminate gender differed significantly (p-value = 0.005), but females were not significantly different from males or indeterminates. Because sex differentiation timing is related to temperature in oysters, a linear model was run including sex (male, female, and indeterminate) and temperature as explanatory variables for levels of total *V. parahaemolyticus* (Adjusted R-squared =

0.1771 and p-value = 1.952e-08). The model indicated that temperature was a significant positive predictor but oyster sex was not (Table 29).

3.3. Gonadal Stage

Numbers of oysters at each gonadal stage (inactive, developing, mature, spawning, post-spawn) at each time point are presented in Table 30. Means and standard errors of *V. vulnificus* and total *V. parahaemolyticus* for each gonadal stage are presented in Table 31. Using ANOVA, oyster gonadal stage showed significant differences among each other for *V. vulnificus* (Table 32), and for total *V. parahaemolyticus* (Table 33). For *V. vulnificus*, a post-hoc Tukey's HSD test (95% CI) showed that only mature and inactive stages differed significantly (p-value = 0.005911). For *V. parahaemolyticus*, a post-hoc Tukey's HSD test (95% CI) showed that the mature group significantly differed from the inactive and spawning group and the spawning group significantly differed from the inactive group (all p-values < 0.046).

Since oysters of mature gonadal stage had differences in levels of *Vibrio* species compared to some of the other gonadal stages, that group was further analyzed with males and females evaluated separately compared to the other gonadal stages. The sexes were not statistically different in terms of *V. vulnificus* or *V. parahaemolyticus* levels, but both male and female groups had higher levels of *V. vulnificus* (males: t = 2.36, df = 14.19, p-value = 0.033 and females: t = 2.99, df = 12.34, p-value = 0.011) compared to the inactive group. The female group was also higher compared to the spawning group (p-value = 0.047) with regard to *V. vulnificus* levels. For *V. parahaemolyticus*, again both

sexes were significantly higher in bacterial abundance than the inactive group (p-value = 0.013 and 0.012, respectively). The female group was also significantly higher compared to the developing group (p-value = 0.036). Because gonadal stage is related to temperature in oysters, separate linear models were run including gonadal stage and temperature as explanatory variables for either *V. vulnificus* or *V. parahaemolyticus*. Both models indicated that temperature was a significant positive predictor (p-value = 1.83e-07 and 8.02e-07, respectively), but oyster gonadal stage was not.

In terms of pathogenic strains (described in Chapter One), 5 of 23 oysters in the mature group harbored one or both pathogenic genes of *V. parahaemolyticus*. This means the mature group of oysters had a pathogenic strain prevalence of 21.7% compared to the overall prevalence of 6.4% for all Year One oysters. All the pathogenic strain prevalences per gonadal stage are presented in Table 34.

3.4. Perkinsus marinus *Results*

Histopathologically determined *P. marinus* levels in oysters were significantly positively correlated with qPCR results for the parasite (p-value = 1.11e-09) (presented earlier, see Chapter One), although the qPCR abundance distributions associated with the histological infection ranks overlapped considerably (Table 35). Histopathological *P. marinus* rankings were not significantly correlated with *V. vulnificus* or total *V. parahaemolyticus* levels (p-value = 0.356 and 0.164, respectively). Pathogenic strains of *V. parahaemolyticus* containing the *tdh* or *trh* gene also did not correlate with histopathological rankings of *P. marinus* either (p-value = 0.88 and 0.76, respectively). A

linear model using oyster gonadal stage, oyster sex, *V. vulnificus* levels, and total *V. parahaemolyticus* levels as predictors and *P. marinus* histopathological rankings as the response variable (Table 36, Adjusted R-squared = 0.07759, p-value = 0.005548) indicated that gonadal stage and *V. vulnificus* were correlated with *P. marinus* histopathological rankings (Table 37).

3.5. Overall Oyster Health Analysis

Using the oyster health rubric, overall oyster health rankings ranged from 0 to 31, with a median of 3 and a mean of 5.6 (standard error = 0.478). The overall "health" ranking had a significant Pearson's r value of 0.15 with log 10 *V. vulnificus* levels (p-value = 0.04503), indicating a slight positive trend (Fig. 15), but there was no significant correlation with log 10 *V. parahaemolyticus* levels (p-value = 0.3601) (Fig. 16). Pathogenic strains of *V. parahaemolyticus* containing the *tdh* or *trh* gene did not correlate with overall histopathological rankings (p-value = 0.64 and 0.31, respectively). A linear model using oyster gonadal stage, oyster sex, *V. vulnificus* levels, and *V. parahaemolyticus* levels as predictors and overall histopathological ranks as the response variable (Table 38, Adjusted R-squared = 0.1554, p-value = 1.332e-05) indicated that gonadal stage and *V. vulnificus* was correlated with overall histopathological ranks (Table 39).

4. Discussion

Oysters harboring *H. nelsoni* tended to have higher levels of *V. vulnificus* but this relationship appears complicated. Oysters with less intense infections by *H. nelsoni* had higher levels of *V. vulnificus*, but more heavily infected oysters did not have statistically different levels of *V. vulnificus* from oysters where *H. nelsoni* was not detected. These results suggest that differences in metabolic adjustment and physiological responses to varying levels of *H. nelsoni* in oysters, like clearance rate or oxygen consumption rate, could also be impacting *V. vulnificus* in some way (Barber et al. 1991). This suggests that *V. vulnificus* maybe be affected by these same physiological and metabolic changes in oysters, but would require more *V. vulnificus-H. nelsoni* directed research. The current study was not designed to specifically capture *H. nelsoni* results due to the fact that its impact in the Bay seems to be waning (Carnegie and Burreson 2011) and as such has too low a prevalence for any relationship between *V. vulnificus* and *H. nelsoni* to be easily addressed, but it does highlight the advantages of using histopathological analyses, which capture a range of pathogens in disease studies.

Gonadal stage was relevant to the abundance of both *V. vulnificus* and total *V. parahaemolyticus*, but gonadal stage correlates with temperature in oysters (Thompson et al. 1996) and once temperature was investigated as a factor there were no significant differences. For results here, the highest temperature recorded over the study period (28.6°C) was on Sept. 4th, after or during the sampling time of most of the mature (n = 21) or spawning (n = 42) oysters, whereas the coldest temperature (21.8°C) was on Sept. 27th (see Table 30). This could explain the association of *Vibrio* species and oyster with

advanced gametogenesis. There is evidence that lipophilic organic contaminates are associated with gametes in bivalves and are purged with spawning (Wilson et al. 1990, Hummel et al. 1998) and; therefore, non-spawning bivalves are routinely targeted in monitoring programs (Shigenaka and Lauenstein 1988). This could suggest another potential target to investigate a mechanism behind why oysters that are mature might harbor higher levels of bacteria. Also, the mature group might warrant further investigation because that group had a pathogenic strain prevalence of over 20% while the total pathogenic strain in all oysters was less than 7%. This is noteworthy because global environmental samples, including oysters, tend to have pathogenic strains at a prevalence of 0-6% (Kaysner et al. 1990, Cook et al. 2002, Letchumanan et al. 2014), although a higher prevalence in oysters is not unheard of in the United States (DePaola et al. 2003). For Virginia waters, there are few published data available to compare pathogenic *V. parahaemolyticus* prevalence year to year and research aimed at this goal would be worthwhile.

The metrics of the oyster health rubric related to *P. marinus* did not suggest a relationship between *P. marinus* and *V. vulnificus* or *V. parahaemolyticus*. Histopathologically, *P. marinus* and its disease effects do not appear to correlate with either *Vibrio* species. Biologically, this suggests that despite sharing similar spaces within the oyster, *P. marinus*, and its related disease effects, do not significantly impact *Vibrio*-oyster interactions. The lack of interaction indicates management of *P. marinus* by the oyster industry can be done without worrying about increasing *Vibrio* species levels in oysters. It is noteworthy that both *H. nelsoni* ranking and overall health ranking did positively correlate to *V. vulnificus* levels. It could be that the correlation with *H. nelsoni*

is also driving the correlation with the overall health ranking, although all *H. nelsoni*-related inputs in the health rubric have a theoretical maximum numeric value of 9, which is modest compared to *P. marinus* (theoretical maximum numeric value = 22). Still, after *P. marinus, H. nelsoni*-related inputs have the highest potential to influence the overall health ranking. Further use of the rubric with more oysters, especially ones positive for *H. nelsoni,* could determine if that was true. While the histopathological analyses fully captured the within host distribution of oyster parasites, distribution of the bacteria within the oyster was not measured. Since *Vibrio* species can display differing tissue distributions (Tamplin and Capers 1992), this might be a useful metric to measure in future studies.

The development of the proposed health rubric used here demonstrated the advantages of using histopathology. While the concept of recording the metrics used in the health rubric are not new (e.g., Kim et al. 2006, Kim and Powell 2007), being able to convert histopathological assessments easily into a single numeric value for ease in statistical analyses was a unique advantage this rubric provided and was utilized in this study. While categorizing qualitative data like histopathology readings can result in over simplification (Bernet et al. 1999), this health rubric attempts to minimize that impact by incorporating a broad number of factors in the final overall health ranking. The oyster health rubric could be a useful tool to enable scientists to identify differing responses in individual oysters and could allow for inter-study comparisons involving oyster health. Adaptions of this rubric could also be made for other important bivalves.

5. Tables

Table 21. Prevalence of histopathologically identified *Perkinsus marinus*, *Nematopsis* sp., digestive ciliates, gill ciliates, *Haplosporidium nelsoni*, and other organisms in oysters from Year One using the larger 187-oyster data set. *P. marinus* had the highest prevalence, followed by a *Nematopsis* sp. and digestive ciliates.

Symbiont	Prevalence
P. marinus	65.8%
***Nematopsis* sp.**	58.3%
Digestive Ciliates	17.6%
Gill Ciliates	17.1%
H. nelsoni	9.63%
Other	5.35%

Table 22. Comparisons of histopathological levels of *Haplosporidum nelsoni* and log 10 *Vibrio vulnificus* means and standard error (SE) using the smaller 176-oyster data set. BFU = Big Ford Units, see text for explanation.

	***V. vulnificus* mean (MPN/g)**	**SE**
No *H. nelsoni* detected (n = 159)	3.638	0.05846
Total *H. nelsoni* (n = 17)	4.491	0.2494
BFU rank 1 and 2 (n = 8)	4.634	0.2631
BFU rank 3 and 4 (n = 9)	4.364	0.4207

Table 23. Comparisons of histopathological levels of *Haplosporidum nelsoni* and log 10 *Vibrio parahaemolyticus* means and standard error (SE) using the larger 187-oyster data set. BFU = Big Ford Units, see text for explanation.

	***V. parahaemolyticus* mean (MPN/g)**	**SE**
No *H. nelsoni* detected (n = 169)	3.531	0.2114
Total *H. nelsoni* (n = 18)	3.318	0.05474
BFU rank 1 and 2 (n = 9)	3.373	0.3596
BFU rank 3 and 4 (n = 9)	3.688	0.2342

Table 24. Summary statistics for the general linear model of *Haplosporidium nelsoni* BFU ranks to investigate correlations. Only *Vibrio vulnificus* was a significant predictor.

Variable	Estimate	Standard Error	t value	p-value
Intercept	-2.654e-02	4.090e-01	-0.065	0.948
Inactive	1.320e-01	4.788e-01	0.276	0.783
Mature	-4.480e-02	4.646e-01	-0.096	0.932
Spawning	2.188e-01	4.351e-01	0.503	0.616
Post-Spawn	1.788e-01	4.445e-01	0.402	0.688
Indeterminate	1.164e-01	2.152e-01	0.541	0.589
Male	-1.821e-01	1.812e-01	-1.005	0.316
Vibrio vulnificus	2.421e-06	4.406e-07	5.494	1.45e-07
Vibrio parahaemolyticus	4.932e-06	3.428e-06	1.439	0.152

Table 25. Analysis of covariance table investigating correlations of *Haplosporidium nelsoni* BFU ranks to gonad, sex, and *Vibrio vulnificus* and total *Vibrio parahaemolyticus* abundance. df = degrees of freedom. P-values indicate only *Vibrio vulnificus* was significantly correlated.

Variable	**df**	**Sum of Squares**	**Mean of Squares**	**F value**	**p-value**
Gonad	4	1.431	0.3577	0.5345	0.7105
Sex	2	0.358	0.1791	0.2677	0.7655
Vibrio vulnificus	1	23.991	23.9913	35.8546	1.264e-08
Vibrio parahaemolyticus	1	1.385	1.3850	2.0698	0.1521
Residuals	167	111.744	0.6691		

Table 26. Comparisons of oyster sex and log 10 *Vibrio vulnificus* and *Vibrio parahaemolyticus* means and standard error (SE) using the smaller 176-oyster data set for *V. vulnificus* and the larger 187-oyster data set for *V. parahaemolyticus*.

	***V. vulnificus* mean (MPN/g)**	**SE**	***V. parahaemolyticus* mean (MPN/g)**	**SE**
Male	3.858	0.1505	3.582	0.1219
Female	3.806	0.0959	3.436	0.08197
Indeterminate	3.604	0.08819	3.156	0.07820

Table 27. Analysis of variance table investigating differences in log 10 *Vibrio vulnificus* abundance in different oyster sexes (male, female, and indeterminate gender) using the smaller 176-oyster data set. df = degrees of freedom. The p-value indicated that sex was not significant.

Variable	df	Sum of Squares	Mean of Squares	F value	p-value
Sex	2	2.24	1.122	1.737	0.179
Residuals	173	111.72	0.6458		

Table 28. Analysis of variance table investigating differences in log 10 total *Vibrio parahaemolyticus* abundance in different oyster sexes (male, female, and indeterminate gender) using the larger 187-oyster data set. df = degrees of freedom. The p-value indicated that sex was significant.

Variable	Df	Sum of Squares	Mean of Squares	F value	p-value
Sex	2	5.92	2.9601	5.821	0.00354
Residuals	184	93.57	0.5085		

Table 29. Summary statistics for general linear model investigating sex and temperature as predictors for *Vibrio parahaemolyticus*. Temperature was a significant positive predictor.

Variable	Estimate	Standard Error	t value	p-value
Intercept	0.0603	0.627	0.096	0.923
Indeterminate	-0.0668	0.119	-0.561	0.575
Male	0.0561	0.135	0.414	0.679
Temperature	0.134	0.0246	5.44	1.69e-07

Table 30. Number and percentage per sampling time of oysters for each gonadal stage and total gonadal stages at each time point using the larger 187-oyster data set.

	Aug 4/5	Aug 17/19	Sept 2/5	Sept 15/16	Sept 29/ Oct 2	Total
Inactive	1 (3.4%)	5 (12.8%)	7 (17.5%)	11 (28.2%)	21 (52.5%)	45 (24.1%)
Developing	1 (3.4%)	0 (0%)	1 (2.5%)	1 (2.6%)	1 (2.5%)	4 (2.1%)
Mature	11 (37.9%)	5 (12.8%)	5 (12.5%)	2 (5.1%)	0 (0%)	23 (12.3%)
Spawning	14 (48.3%)	20 (51.3%)	8 (20%)	2 (5.1%)	1 (2.5%)	45 (24.1%)
Post-Spawn	2 (6.9%)	9 (23.1%)	19 (47.5%)	23 (59%)	17 (42.5%)	70 (37.4%)

Table 31. Comparisons of oyster gonadal stage and log 10 *Vibrio vulnificus* and *Vibrio parahaemolyticus* means and standard error (SE). The smaller 176-oysers data set was used for *V. vulnificus* means and standard error and the larger 187-oyster data set was used for *V. parahaemolyticus* means and standard error.

	***V. vulnificus* mean (MPN/g)**	**SE**	***V. parahaemolyticus* mean (MPN/g)**	**SE**
Inactive	3.499	0.1075	3.037	0.1051
Developing	3.333	0.5056	3.057	0.2511
Mature	4.224	0.1848	3.825	0.1768
Spawning	3.702	0.08057	3.450	0.08181
Post-Spawn	3.742	0.1156	3.315	0.08829

Table 32. Analysis of variance table investigating differences in log 10 *Vibrio vulnificus* abundance in different oyster gonadal stages (inactive, developing, mature, spawning, post-spawn) using the smaller 176-oyster data set. df = degrees of freedom. The p-value indicated that gonadal stage was significant.

Variable	df	Sum of Squares	Mean of Squares	F value	p-value
Gonad	4	8.1	2.0245	3.27	0.013
Residuals	171	105.9	0.6191		

Table 33. Analysis of variance table investigating differences in log 10 *Vibrio parahaemolyticus* abundance in different oyster gonadal stages (inactive, developing, mature, spawning, post-spawn) using the larger 187-oyster data set. df = degrees of freedom. The p-value indicated that gonadal stage was significant

Variable	df	Sum of Squares	Mean of Squares	F value	p-value
Gonad	4	10.41	2.6026	5.317	0.000451
Residuals	182	89.08	0.4895		

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Table 34. Prevalence of pathogenic strains of *Vibrio parahaemolyticus* by gonadal stage from Year One using the larger 187-oyster data set.

Group	Prevalence	n =
Inactive	11.1%	45
Developing	0%	4
Mature	21.7%	23
Spawning	0%	45
Post-Spawn	2.8%	70

Table 35. Histopathological *Perkinsus marinus* rankings compared to qPCR-determined *P. marinus* (ITS region copy number) data from Chapter One using the larger 187-oyster data set. Minimum and maximum values of qPCR-derived *P. marinus* abundance in a sample for each histopathological rank were presented as well as means, standard errors (SE), and total number of samples.

Histopathology Rank	Minimum (copies/g)	Maximum (copies/g)	Mean (copies/g)	SE	n =
None 0	0	5.17e+08	1.861e+07	8.384e+06	64
Rare 0.5	2.18e+04	4.25e+08	5.633e+07	1.493e+07	42
Light 1	1.020e+06	1.140e+09	1.457e+08	4.62e+07	32
Light to Moderate 2	3.520e+06	2.210e+09	3.356e+08	1.848e+08	15
Moderate 3	5.920e+06	2.780e+09	8.979e+08	2.6973+08	14
Moderate to Heavy 4	1.310e+08	8.760e+09	2.691e+09	9.902e+08	11
Heavy 5	1.960e+09	7.690e+10	1.987e+10	8.824e+09	9

Table 36. Summary statistics for the general linear model of *Perkinsus marinus* histopathological rankings to investigate correlations. Only *Vibrio vulnificus* was a significant predictor.

Variable	Estimate	Standard Error	t value	p-value
Intercept	1.232e+00	6.846e-01	1.799	0.07380
Inactive	-2.453e-01	8.014e-01	-0.306	0.75991
Mature	-1.854e-01	7.777e-01	-0.238	0.81189
Spawning	-8.670e-01	7.282e-01	-1.191	0.23551
Post-Spawn	-7.000e-02	7.440e-01	-0.094	0.92516
Indeterminate	2.048e-01	3.601e-01	0.569	0.57044
Male	7.585e-02	3.032e-01	0.250	0.80277
Vibrio vulnificus	1.962e-06	7.375e-07	2.660	0.00857
Vibrio parahaemolyticus	1.967e-06	5.738e-06	0.343	0.73225

Table 37. Analysis of covariance table investigating correlations of *Perkinsus marinus* histopathological rankings to gonad, sex, and *Vibrio vulnificus* and total *Vibrio parahaemolyticus* abundances. df = degrees of freedom. P-values indicate gonad and *Vibrio vulnificus* was significantly correlated.

Variable	**df**	**Sum of Squares**	**Mean of Squares**	**F value**	**p-value**
Gonad	4	27.077	6.7692	3.6113	0.007501
Sex	2	0.429	0.2145	0.1144	0.891942
Vibrio vulnificus	1	14.861	14.8611	7.9281	0.005453
Vibrio parahaemolyticus	1	0.220	0.2202	0.1174	0.732250
Residuals	167	313.036	1.8745		

Table 38. Summary statistics for the general linear model of overall histopathological ranks to investigate correlations. Only *Vibrio vulnificus* was a significant predictor.

Variable	Estimate	Standard Error	t value	p-value
Intercept	3.613e+00	2.973e+00	1.215	0.226
Inactive	-4.180e-01	3.480e+00	-0.120	0.905
Mature	1.353e+00	3.377e+00	0.401	0.689
Spawning	-1.152e+00	3.162e+00	-0.364	0.716
Post-Spawn	1.837e+00	3.231e+00	0.569	0.570
Indeterminate	1.637e+00	1.564e+00	1.047	0.297
Male	-4.227e-01	1.317e+00	-0.321	0.749
Vibrio vulnificus	1.420e-05	3.203e-06	4.434	1.67e-05
Vibrio parahaemolyticus	1.746e-05	2.492e-05	0.701	0.484

Table 39. Analysis of variance table investigating correlations of overall histopathological ranks to gonad, sex, and *Vibrio vulnificus* and total *Vibrio parahaemolyticus* abundances. df = degrees of freedom. P-values indicate gonad and *Vibrio vulnificus* was significantly correlated.

Variable	**df**	**Sum of Squares**	**Mean of Squares**	**F value**	**p-value**
Gonad	4	595.3	148.82	4.2107	0.002835
Sex	2	19.7	9.86	0.2791	0.756842
Vibrio vulnificus	1	788.5	788.52	22.3101	4.893e-06
Vibrio parahaemolyticus	1	17.4	17.36	0.4913	0.484341
Residuals	167	5902.4	35.34		

6. Figures

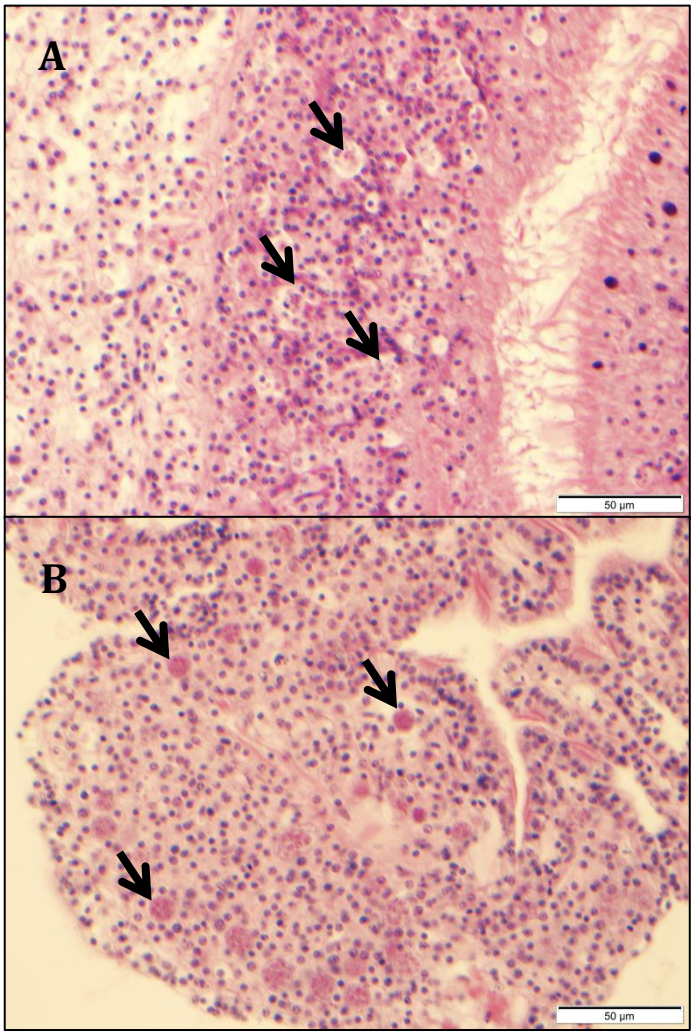

Figure 14. Common pathogens of oysters on histological sections of *Crassostrea virginica*. (A) Moderate infection (health rubric rank = 3) of *Perkinsus marinus* in oyster digestive epithelium. Arrows indicate several of many clusters of *P. marinus* cells. (B) A heavy infection (health rubric rank = 4) of *Haplosporidium nelsoni* in oyster gill, with arrows indicating several of many *H. nelsoni* plasmodia present. Scale bars = 50 µm.

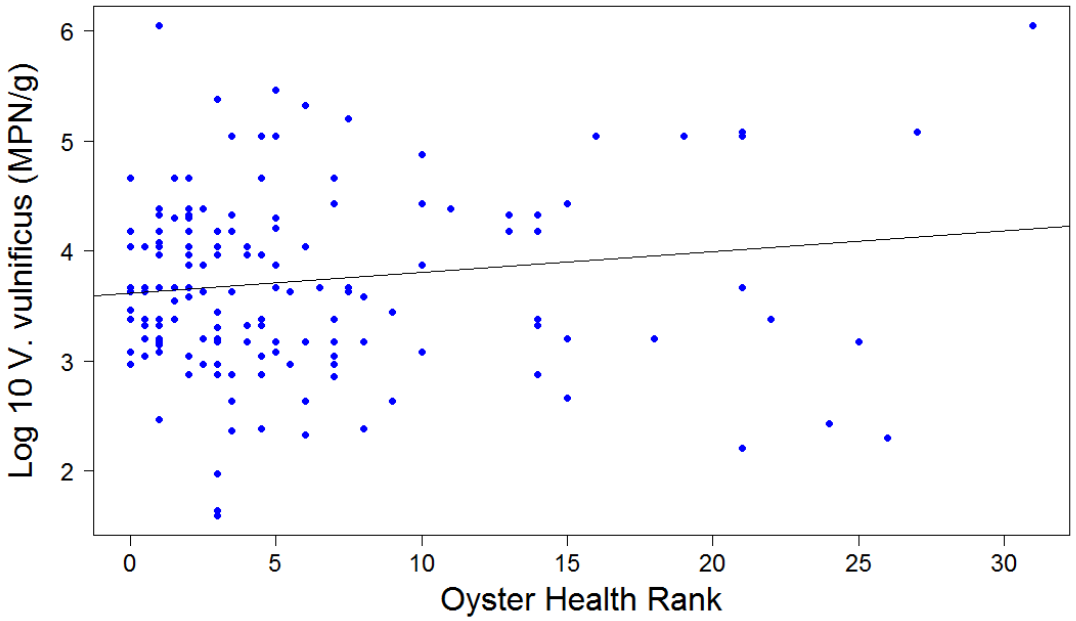

Figure 15. Overall oyster health ranks and log 10 *Vibrio vulnificus* abundance using the smaller 176-oyster data set from Year One, displaying a significant positive correlation (Pearson's r = 0.151, t = 2.019, df = 174, p-value = 0.04503).

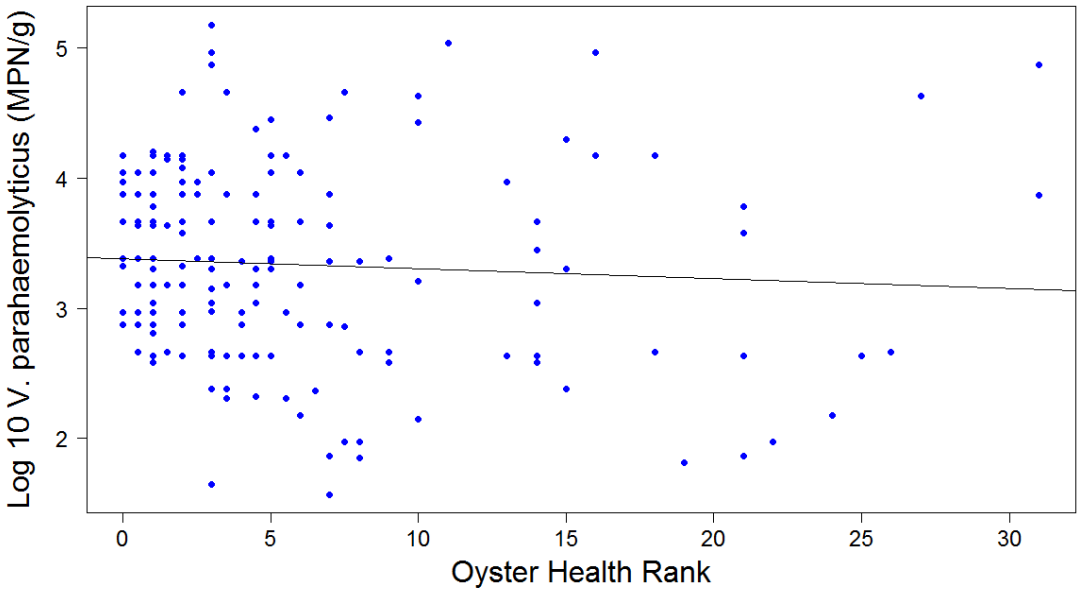

Figure 16. Overall oyster health ranks and log 10 total *Vibrio parahaemolyticus* abundance using the smaller 176-oyster data set from Year One, displaying no significant correlation (Pearson's r = -0.0673, t = -0.917, df = 185, p-value = 0.3601).

7. Appendices

Appendix B

OYSTER CHARACTERTISTICS

Oyster Sex

M=Male
F=Female
H=Hermaphrodite
I=Indeterminate gender

Gonadal Stage

I=Inactive
D=Developing, ED=early, LD=late
M=Mature
S=Spawning
PS= Post-Spawning

PRIMARY ORGANISMS

***Perkinsus marinus* Overall (0-5)**

N=None, 0
R=Rare, 0.5
L=Light, 1
LM=Light-Moderate, 2
M=Moderate, 3
MH-Moderate-Heavy, 4
H=Heavy, 5

(NOTE: rankings defined as R = 1-10 cells or clusters of cells; L = 11-30 cells or clusters of cells, L-M = 31-49 cells or clusters of cells; M = 50 or more clusters of cells representing significant digestive epithelial colonization but with few cells obvious in the rest of the visceral mass; MH = beyond an M in that *P. marinus* is clearly colonizing hemolymph spaces of the connective tissues but to a great degree; H = parasite abundant in the digestive epithelia and throughout the other tissues and organs. Rating method originally developed by R. Crockett and L. Ragone Calvo, VIMS Shellfish Pathology Laboratory)

***Perkinsus marinus* in Epithelial Organs (0-3)**
N=None, 0
P=Present, 1
C=Common, 2
A=Abundant, 3
(NOTE: rankings defined as P = at least one, C = 10-30, A = >30. This ranking applies separately to each section of **Gill ep., Mantle ep., Gonad, Stomach ep.,** and **Intestine ep**.)

***Perkinsus marinus* in Digestive ducts and tubules (0-2)**
N=None, 0
P=Present, 1
C=Common, 2
(NOTE: rankings defined as P = at least one, C = >15)

***Haplosporidium nelsoni* Intensity (0-4)**
N=None, 0
R=Rare, 1
L=Light, 2
M=Moderate, 3
H=Heavy, 4
(NOTE: classification based on Ford & Haskin (1982), pg 124, but combining "very light" and "light" into one category.)

***Haplosporidium nelsoni* Location**
E=Epithelial
S=Sub-epithelial/local
G=General
(NOTE: Classification based on Ford & Haskin (1982), pg 124)

***Haplosporidium nelsoni* BFU (0-4)**
(NOTE: Combining data from *H. nelsoni* Intensity and *H. nelsoni* Location to rank parasite levels based on Ford et al. (1999), pg 477.)

***Haplosporidium nelsoni* sporulation (0-1)**
Presence/absence ranking.

SECONDARY ORGANISMS
***Haplosporidium costale* (0-4)**
N=None, 0
R=Rare, 1
L=Light, 2
M=Moderate, 3
H=Heavy, 4
(NOTE: classification based on Ford & Haskin (1982), pg 124 for *H. nelsoni* but combining "very light" and "light" into one category.)

***Nematopsis* (0-3)**
N=None, 0
R=Rare, 1
C=Common, 2
A=Abundant, 3
(NOTE: With classifications as follows: R = 1-5 cells, C = 6-20 cells, A = >20 cells, based on R. Crockett and C. Dungan, pers. comm.)
Rickettsia-like organisms (0-3)
N=None, 0
R=Rare, 1
C=Common, 2
A=Abundant, 3
(NOTE: With classifications as follows: R = 1-4, C = 5-10, A = >10, based on C. Dungan and R. Crockett, pers. comm.)

Ciliates in gut (0-3)
N=None, 0
R=Rare, 1
C=Common, 2
A=Abundant, 3
(NOTE: With classifications as follows: R = 1-5 cells, C = 6-20 cells, A = >20 cells, based on C. Dungan and R. Crockett, pers. comm. This ranking applies separately to **Ciliates in gills** as well.)

Bucephalus (0-1)
Presence/Absence ranking

OYSTER ASPECTS (regardless of etiology)
Hemocytosis Intensity (0-2)
N=Normal, 0
L=Light, 1
H=Heavy, 2

Ceroid Intensity (0-2)
N=Normal, 0
L=Light, 1
H=Heavy, 2
(NOTE: 1-5 ceroid per field is considered “Light”.)

Digestive Ep. Damage (0-3)
N=None, 0
L=Light, 1
M=Moderate, 2
H=Heavy, 3
(NOTE: Classifications different from organism rankings. Rankings are defined as follows: N = normal, L = disruption/erosion present focally, M = disruption/erosion present multifocally but with normal structure still present in places, H = disruption/erosion severe and widespread. This ranking applies separately to each section of **Digestive Ep. Damage, Gill Ep. Damage, Dig. Tubule Damage,** and **CT Damage**)

Appendix C

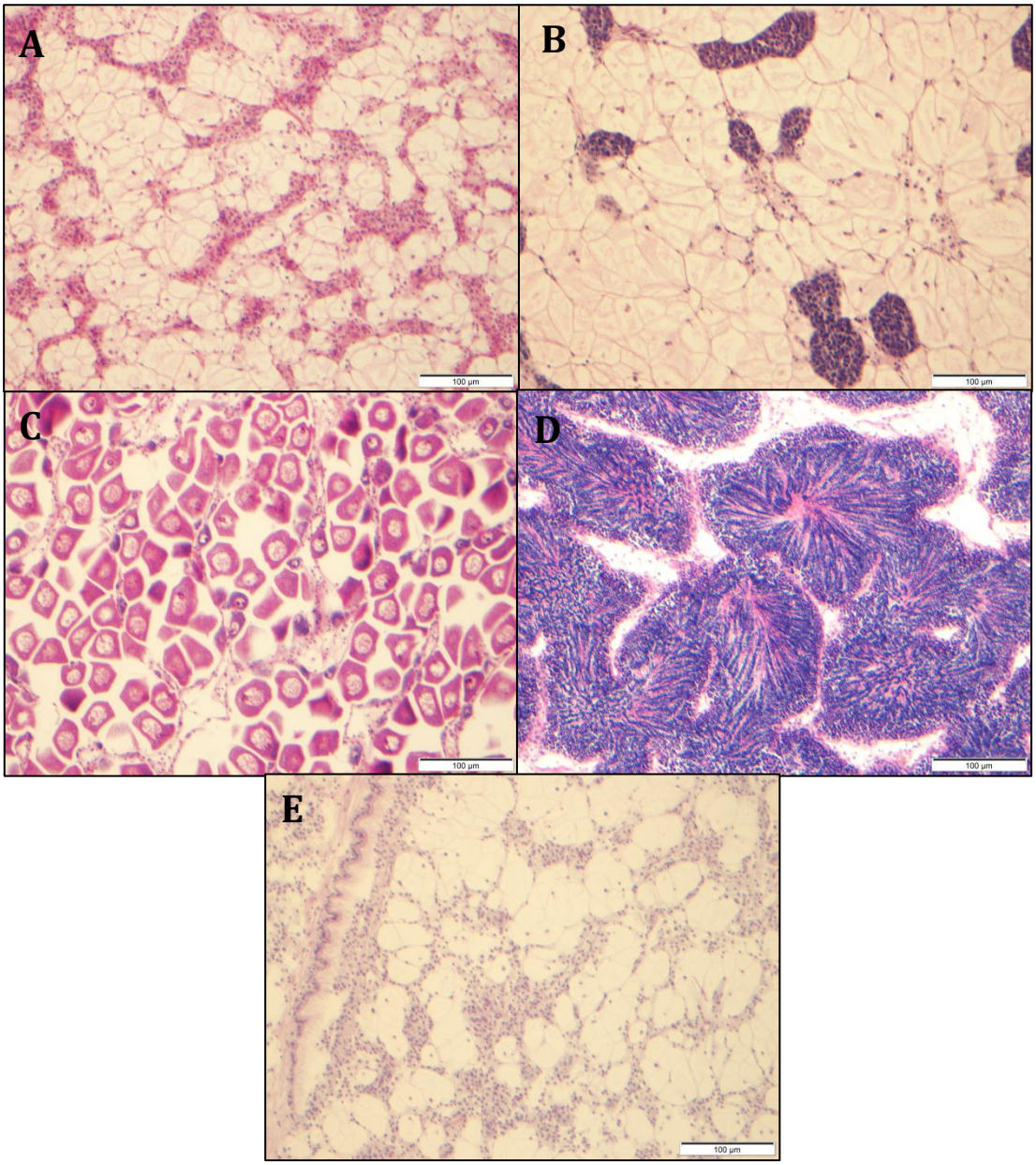

Figure A. Gonadal development of oysters on histological sections of *Crassostrea virginica*. (A) Inactive oyster gonad. Undifferentiated follicles and connective tissue are apparent. (B) Developing oyster gonad. Connective tissue still apparent but the follicles are beginning to develop. (C) Mature female oyster. There is little to no connective tissue and eggs have become separated from germinal tissue. (D) Mature male oyster. There is little to no connective tissue and sperm flagella are bundled. (E) Post-spawn oyster. Connective tissue is apparent and hemocytes have infiltrated the gonadal and gonoduct regions. All scale bars = 100 μm.

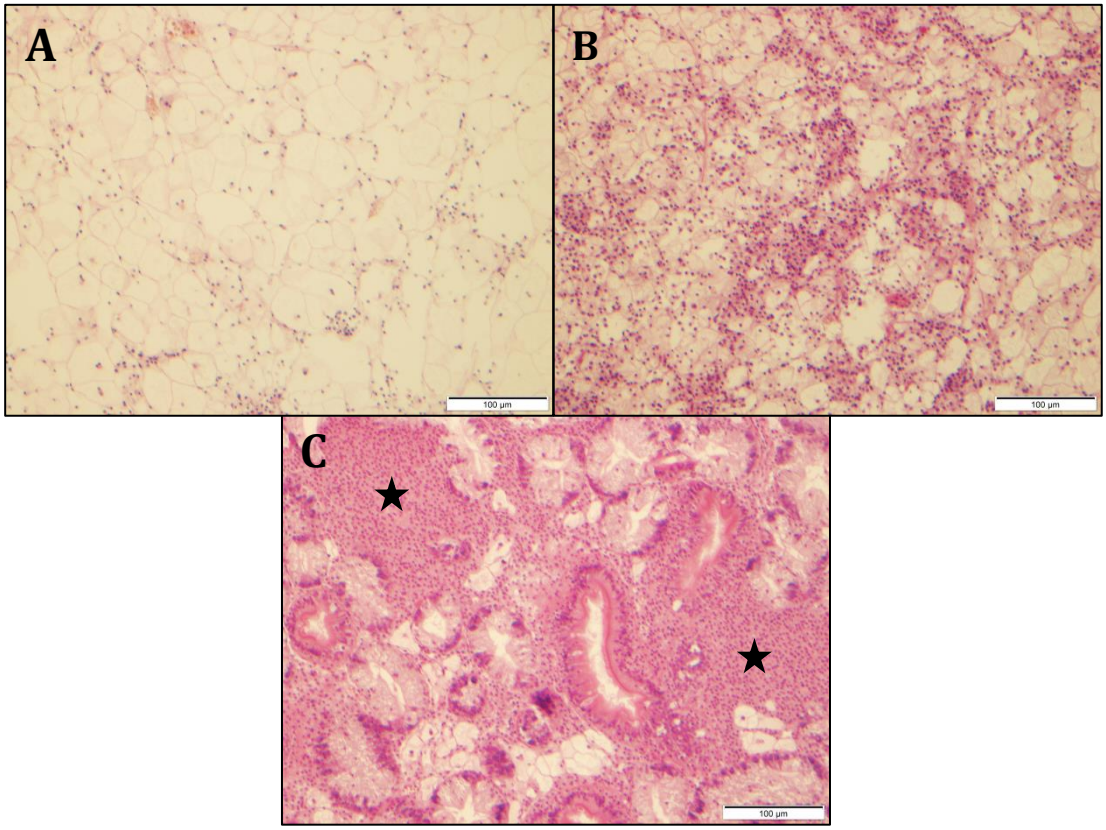

Figure B. Hemocytosis of oysters on histological sections of *Crassostrea virginica*. (A) Normal hemocyte activity in oyster connective tissue (health rubric rank = 0). (B) Light hemocyte activity in oyster connective tissue (health rubric rank = 1). (C) Heavy hemocyte activity around oyster digestive glands (health rubric rank = 2). Stars located in center of masses of hemocytes. All scale bars = 100 µm.

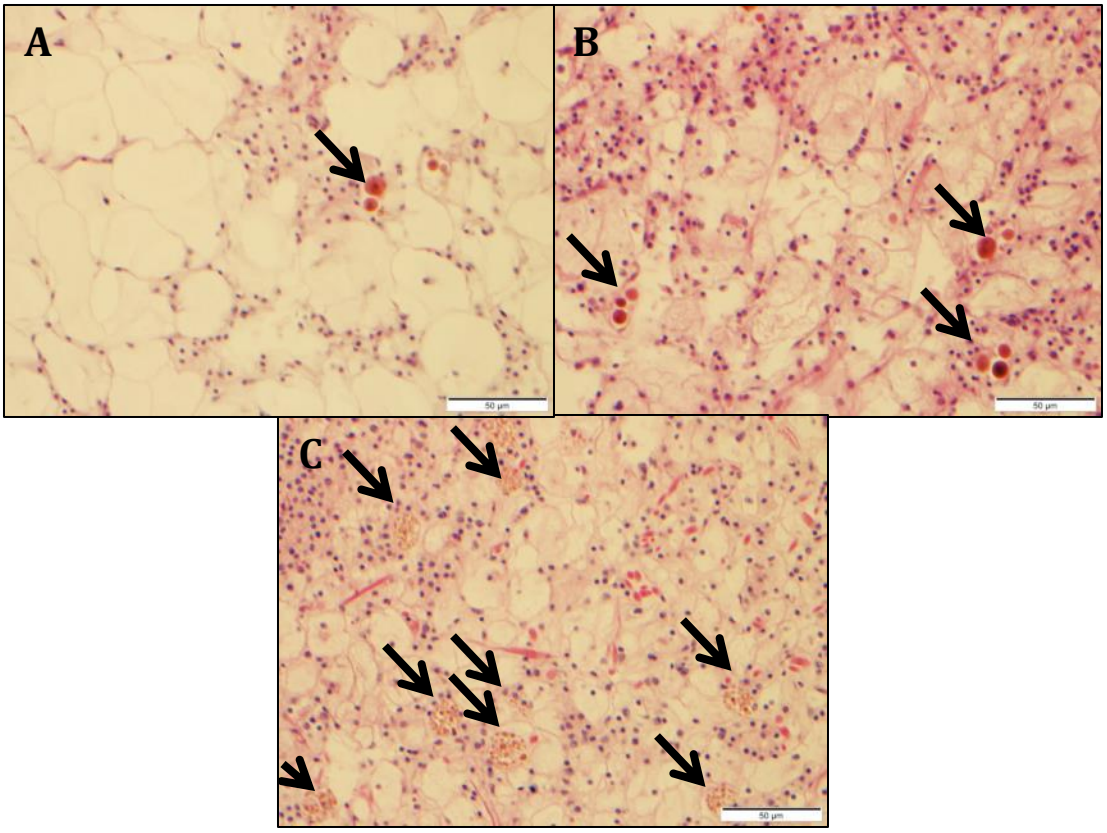

Figure C. Ceroid in oysters on histological sections of *Crassostrea virginica*. (A) Normal diffuse ceroid accumulation in oyster connective tissue (health rubric rank = 0). (B) Light ceroid accumulation in oyster connective tissue (health rubric rank = 1). (C) Heavy ceroid accumulation in oyster connective tissue (health rubric rank = 2). All arrows indicate ceroid. All scale bars = 50 μm.

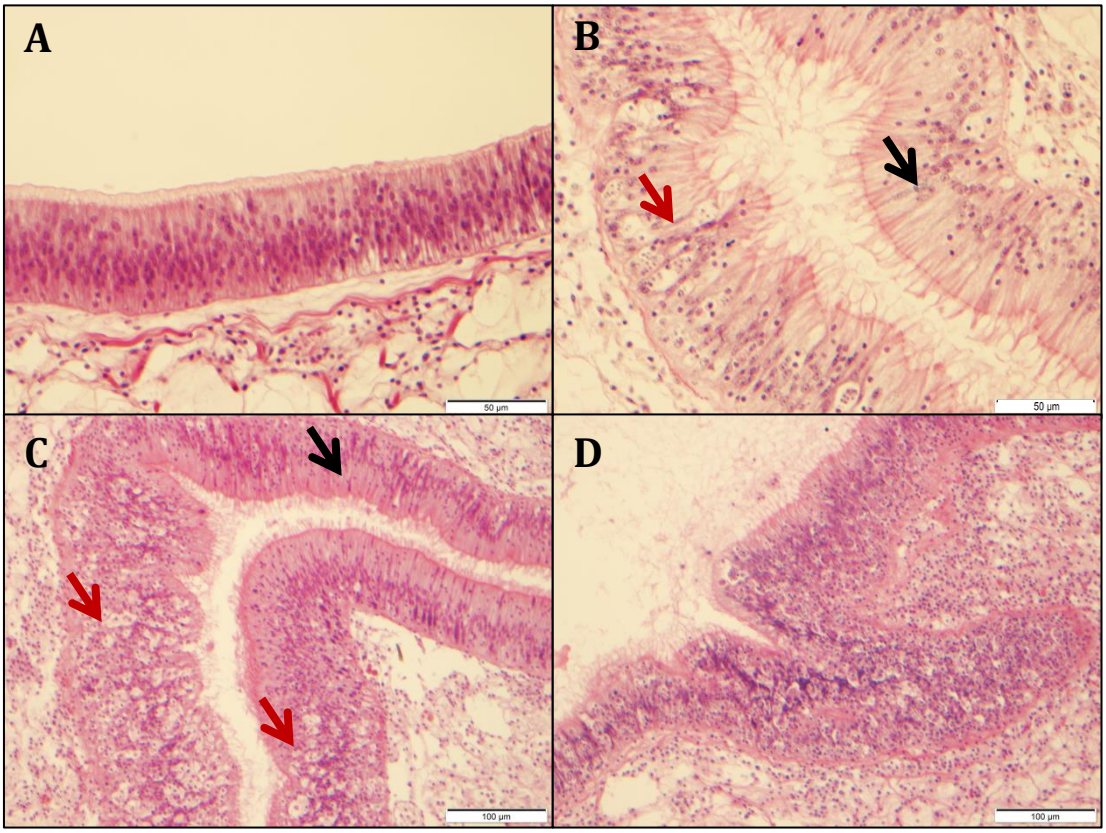

Figure D. Digestive epithelia in oysters on histological sections of *Crassostrea virginica*. (A) Normal epithelium in an oyster (health rubric rank = 0). Scale bar = 50 µm. (B) Local disruption of epithelium. Red arrow indicates region of disrupted epithelium and black arrow indicates region of normal epithelium (health rubric rank = 1). Scale bar = 50 µm. (C) Multifocal disruption of epithelium. Red arrows indicate regions of disrupted epithelia and black arrow indicates region of normal epithelium (health rubric rank = 2). Scale bar = 100 µm. (D) Severe and widespread disruption of epithelium (health rubric rank = 3). Scale bar = 100 µm. *Perkinsus marinus* is the etiological agent in all cases of disruption. Note the change in scale bar in panel C and D.

SUMMARY

The overall objective of this study was to resolve whether a correlation may exist between *P. marinus* infection, oyster health, and reproductive status more generally and levels of two human-pathogenic *Vibrio* species, *Vibrio vulnificus* and *Vibrio parahaemolyticus*, in *C. virginica*. First, quantitative PCR (qPCR)-generated data were used to compare *P. marinus* infection intensity and the abundance and *V. vulnificus* and *V. parahaemolyticus* in individual oysters. Second, qPCR data on *V. vulnificus* and *V. parahaemolyticus* were compared against histopathological measures including infection by *H. nelsoni* as well as the reproductive status of the oyster. This was again performed on individual oysters, and the use of individual oysters rather than pooled samples of ten or twelve was used to better capture individual variability and determining oyster health status and was a significant innovation of my research. Finally, manipulation of *P. marinus* disease progression by deployment at sites of lower and higher salinity than the York River was attempted to gain more insight into whether the timing of the presence of abundant intense *P. marinus* infections directly influences *Vibrio* levels.

Results demonstrated no clear correlation between total qPCR-determined levels of *P. marinus* and either *V. vulnificus* or *V. parahaemolyticus*. Oysters contained varying levels of all three species providing plenty of potential interactions, but no correlations were found. No correlations were found with pathogenic strains of *V. parahaemolyticus* either. Histopathological analyses did not reveal any correlations between *P. marinus*

ranking, distribution, or tissue damage and *Vibrio* species levels. Histopathology did reveal that oysters containing *H. nelsoni* had higher levels of *V. vulnificus* but sample size was too low to investigate this result further. The *P. marinus* disease progression manipulation was not successful, so no further insight into the oyster-*P. marinus*-*Vibrio* interactions was provided. Oysters with advanced gametogenesis appeared to have higher levels of both *V. vulnificus* and *V. parahaemolyticus*, but that was likely related to the correlation of oyster reproduction and warmer water temperatures. Still gametogenically advanced oysters were interesting because this group had significantly higher levels of pathogenic strains of *V. parahaemolyticus.*

This study contradicted the pilot study conducted by Carnegie et al. (2013) which found an inverse correlation with *V. vulnificus*. Inconsistencies in results were likely due to the differences in sample size between studies (n = 60 versus n = 405), the naturally high variability in *Vibrio* species levels in oysters and the differences in origins of oysters used in each study. This study also did not support two *in vitro* studies that suggested oyster exposed to *P. marinus* secretions could have higher levels of both *Vibrio* species (Tall et al. 1999, La Peyre and Volety 1999). Conversely, this study is in agreement with the results from Sokolova et al. (2005) which focused on *V. vulnificus* and found no evidence for a relationship between *P. marinus* and that bacterial species.

Overall, this study presents evidence that there is no naturally occurring interaction between the prevalent oyster parasite, *P. marinus*, and the human-pathogenic bacterial species, *V. vulnificus* and *V. parahaemolyticus*. As climate change continues to influence estuarine systems, identifying dynamics governing the oyster-*Vibro* species interactions will become increasingly important. This study suggests two other potential

factors to be investigated to explain the high variation of *Vibrio* species levels found in oysters. First, a closer look at the other well-known oyster parasite, *H. nelsoni*, appears to be justified when investigating *V. vulnificus* variability. Second, gonadal development of the oyster could be playing a role in *V. parahaemolyticus* pathogenic strain prevalence. However, this study shows that *P. marinus* parasitism can be ruled out as an influence on human-pathogenic *Vibrio* species in oysters.

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